ABSTRACT

Title of Document: TAXONOMY AND ECOPHYSIOLOGY OF *PSEUDO-NITZSCHIA* IN THE CHESAPEAKE BAY

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Pseudo-nitzschia is a diatom genus known to produce the neurotoxin, domoic acid (DA), which causes Amnesic Shellfish Poisoning (ASP) and Domoic Acid Poisoning (DAP). Field studies were conducted in the Chesapeake Bay to determine which species were present, their toxicity and their spatial and temporal distribution. Strains were isolated from the Chesapeake Bay region and growth and toxin content were studied. The effect of rapid increases in light at low temperatures on toxin production physiology was investigated.

Toxic *Pseudo-nitzschia* is present in the Chesapeake Bay; however, abundance and toxin production are highly variable. Six species of *Pseudo-nitzschia* were identified: *P. pungens*, *P. calliantha*, *P. subpacifica*, *P. cuspidata*, *P. fraudulenta* and *P. multiseries*. The most abundant species was *P. calliantha*. *Pseudo-nitzschia* abundances were associated with low temperature (2-21°C) and high salinity (6-32) and were highest in winter and spring. Compared to other diatom species, *Pseudo-nitzschia* abundances were low, rarely present above 1000 cells mL^{-1} and they did not occur as monospecific blooms. Low *Pseudo-nitzschia* abundances and low, irregular domoic acid concentrations may partially explain the lack of documented toxic events in the Chesapeake Bay.

Growth rate and toxin content of strains of *Pseudo*-nitzschia exposed to different nitrogen sources and irradiances varied significantly, even among strains of the same species isolated from the same water sample. Strain-level differences were responsible for most of the variability in growth rate and toxin content. Sequences of the internal transcribed spacer (ITS) and large subunit (LSU) rRNA matched morphological species definitions, but offered no explanation for the physiological variability. Populations of *Pseudo-nitzschia* in the mid-Atlantic coastal zone appear to be comprised of numerous ecotypes that require sorting in the future.

The hypothesis that DA is produced as an energy modulation strategy when the light and dark reactions of photosynthesis are decoupled was tested by exposing exponentially growing *P. multiseries* to a rapid increase in irradiance at a low temperature. High light and low temperature conditions increased nitrate (NO_3^-) uptake, nitrite (NO_2^-) and ammonia (NH_4^+) release and decreased DA production by the cells. These results could have important implications for natural populations of *Pseudo-nitzschia* at times of low temperature and high light fluctuations, such as during spring blooms and upwelling events.

This thesis answered several questions about *Pseudo-nitzschia* populations in the Chesapeake Bay area and their ecophysiology, but raised many more. Physiological adaptations and biogeography of *Pseudo-nitzschia* and DA content of Chesapeake bivalves should be studied further to contribute to the development of predictive models for *Pseudo-nitzschia* bloom formation and toxin production.

TAXONOMY AND ECOPHYSIOLOGY OF *PSEUDO-NITZSCHIA* IN THE CHESAPEAKE BAY

By

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Specific Contributions

Chapter 1: Introduction

Chapter 2: Distribution, abundance and domoic acid analysis of the toxic diatom genus *Pseudo-nitzschia*

Dr. Elisabeth Gantt assisted with electron microscopy of field samples.
Drs. David Caron and Astrid Schnetzer assisted with toxin analysis.
Walt Butler assisted with sample collection.
Dr. David Kimmel assisted with statistical analysis.

Chapter 3: Intra- and interspecies differences in *Pseudo-nitzschia* growth and toxicity while utilizing different nitrogen sources

Dr. Elisabeth Gantt took electron micrographs and assisted with electron microscopy.
Drs. David Caron and Astrid Schnetzer assisted with toxin analysis.
Dr. Stephen Bates allowed use of his *Pseudo-nitzschia multiseries* culture.
Holly Bowers performed the genetic analysis and provided phylogenetic trees.

Chapter 4: The effect of a rapid increase in irradiance on domoic acid production and nitrate uptake by *Pseudo-nitzschia multiseries*

Dr. Patricia Glibert assisted in planning the experiments.
Jeff Alexander and Dan Gustafson assisted in performing experiments.
Dr. Carrie Solomon assisted in NH_4 analysis.
Lois Lane, Sara Rhodes and Jen O'Keefe provided NO_3^-, NO_2^-, NH_4^+ and CHN analysis.

Chapter 5: Summary

Abbreviations

ANOVA	Analysis of variance
ASP	Amnesic shellfish poisoning
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMAA	β-methylamino-L-alanine
CE	Capillary electrophoresis
CTD	Conductivity temperature depth
DA	Domoic acid
DAP	Domoic acid poisoning
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EU	European Union
FADH	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FISH	Fluorescence in-situ hybridization
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GAP	Glyceraldehyde 3-phosphate
ITS	Internal transcribed spacer
HAB	Harmful algae bloom
HNLC	High nutrient low chlorophyll
HPL	Horn Point Laboratory
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography with detection by mass spectroscopy
LOD	Limit of detection
LOQ	Limit of quantitation
LSU	Large subunit
MD DNR	Maryland Department of Natural Resources
MPAA	Microsporin amino acids
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PAR	Photosynthetically available radiation
PCR	Polymerase chain reaction
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
SSU	small subunit
TEM	Transmission electron microscope
TLC	Thin layer chromatography
USEPA	United States Environmental Protection Agency
UV	Ultraviolet

CHAPTER 1: Introduction

Since the first incident of Amnesic Shellfish Poisoning (ASP) in Prince Edward Island, Canada in 1987 (Bates et al. 1989, Perl et al. 1990), *Pseudo-nitzschia* and domoic acid (DA) have been the subject of intense research by ecologists, physiologists and chemists. *Pseudo-nitzschia* is a pennate diatom of global importance; it has been recorded from nearly every major marine and estuarine environment and DA has been found in the tissue or feces of organisms in multiple trophic levels in the oceans. Despite twenty years of study and the relatively simple taxonomy, life cycle (when compared to other species of harmful algae) and structure of DA, there is still much to learn about the taxonomic relationships, toxin production and food web interactions within the genus *Pseudo-nitzschia*. The following is a summary of *Pseudo-nitzschia* taxonomy, physiology, ecology and DA chemistry from the initial description of the genus to the present.

Taxonomy, Biogeography and Natural History

Taxonomy

The genus *Pseudo-nitzschia* was originally defined by Peragallo and Peragallo (1900) from the genus *Nitzschia* and has been subjected to many taxonomic changes over the last century based on frustule morphology (Fig. 1.1). Fifty years after being defined, *Pseudo-nitzschia* was reduced to a section of the genus *Nitzschia* on the basis of its raphe and motility (Hustedt 1958). By 1965 there were 18 species and two subspecies in the *Pseudo-nitzschia* group: *seriata, seriata* f. *obtusa*, *pseudoseriata, pungens, pungens* f. *multiseries, fraudulenta,*

subfraudulenta, subpacifica, heimii, turgidula, prolongatoides, turgiduloides, lineola, barkleyi, inflatula, cuspidata, actydrophila, granii, subcurvata and *delicatula* (Hasle 1964, 1965). *Nitzschia lineola* and *N. barkleyi* were combined into *N. lineola* (Hasle 1965). In 1971, a new species, *N. pungiformis,* was described (Hasle 1971). *Nitzschia delicatula* became *N. pseudodelicatissima* in 1976 (Hasle 1976) and *N. actydrophila* reverted back to the older name *N. delicatissima* (Heiden & Kolbe 1928, Hasle 1965). Eventually, *Pseudo-nitzschia* was again separated from *Nitzschia* as a distinct genus by Hasle (1994) based on morphological characters and later supported by analysis of the 18S ribosomal RNA (rRNA; Douglas et al. 1994). The basic features of *Pseudo-nitzschia* spp. as defined by Hasle (1994) are as follows: 1) weakly silicified, 2) shallow, flattened or smoothly curved valve, 3) extremely eccentric raphe not elevated above valve, 4) no poroids on raphe canal walls, 5) no conopea and 6) presence of non-poroid silica strip at junction between valve face and distal mantle.

Shortly after *Pseudo-nitzschia* was reinstated as a genus, *pungens* f. *multiseries* was raised in rank, creating the species *P. multiseries* (Hasle 1995). *Nitzschia pseudoseriata* became *P. australis*, using an older species name and the new genus name (Frenguelli 1939, Hasle 1965, Rivera 1985). Many more new species have since been defined: *P. sinica* (Qi et al. 1994), *P. multistriata* (Takano 1995), *P. micropora* (Priisholm et al. 2002), *P. americana*, *P. brasiliana, P. linea* (Lundholm et al. 2002b) and *P. galaxiae* (Lundholm & Moestrup 2002). Lundholm et al. (2003) redefined *P. pseudodelicatissima* into three species: *P. calliantha, P. pseudodelicatissima* and *P. caciantha* and redefined *P. cuspidata*.

P. delicatissima was redefined and split into two additional species: *P. dolorosa* and *P. decipiens* (Lundholm et al. 2006). Currently, there are 31 species of *Pseudo-nitzschia*.

Not all of these species produce the toxin DA, which makes species identification very important. This is typically done via electron microscopy. Individual species of *Pseudo-nitzschia* can be identified on the basis of striae and fibulae density, poroid structure, presence or absence of a central interspace and valve shape, length and width (Hasle & Syvertsen 1997). The genus can be identified with light microscopy by the characteristic step chain pattern. Cell length and width can also be measured via light microscopy. *Pseudo-nitzschia* is divided into two groups based on valve width: the *seriata* group (> 3μm) and the *delicatissima* group (< 3μm) (Hasle 1965, Hasle & Syvertsen 1997).

Traditional microalgal taxonomy has been heavily reliant on microscopy and has been revised as improvements in technology have enabled researchers to resolve increasingly smaller detail. In diatoms, this microscope-based taxonomy has meant that species were defined based on details in the frustule (Fig. 1.1). More recently, molecular techniques are adding to morphological species definitions, being used to confirm existing species and defining new species (Lundholm et al. 2002a, Lundholm & Moestrup 2002, Lundholm et al. 2003, Lundholm et al. 2006). The focus of most molecular work in *Pseudo-nitzschia* has been the ribosomal RNA. Sequence data can be used to construct a phylogeny or parsimony tree to describe relatedness between species or between strains (Orsini et al. 2002, Orsini et al. 2004, Cerino et al. 2005).

Analysis of molecular data reveals another level of diversity in natural populations not detectable through morphological methods alone and could reveal ecotypes or cryptic species. Using microsatellite markers, Evans et al. (2005) found 98% clonal diversity in isolates from the North Sea. Orsini et al. (2004) found 5 distinct lineages within *P. delicatissima* in the Gulf of Naples before a bloom, while during a bloom all strains collected belonged to the same clade. These dynamics could be evidence of cryptic species or ecotypes in *P. delicatissima*. Genetic analysis of laboratory cultures show strains of *P. delicatissima* belong to two different clades (Lundholm et al. 2006). Molecular data, in combination with mating experiments, shows the presence of reproductively isolated groups within *P. delicatissima* and *P. pseudodelicatissima* (Amato et al. 2007). Molecular methods are a new way to investigate evolution and ecology in natural *Pseudo-nitzschia* populations.

Biogeography

Pseudo-nitzschia is a cosmopolitan genus; however, some tropical and polar species exist as well as coastal and oceanic species (Hasle 1965, Skov et al. 1999, Hasle 2002; Fig. 1.2). Many species of *Pseudo-nitzschia* are found over a wide range of salinity and temperature (*P. pungens*) while other species are restricted to a narrow environmental regime (*P. prolongatoides* and *P. turgiduloides*). *Pseudo-nitzschia pungens*, *P. heimii, P. inflatula*, *P. pseudodelicatissima* and *P. fraudulenta* can be found in coastal and oceanic, tropical and temperate waters while *P. brasiliana*, *P. caciantha, P. decipiens, P.*

micropora and *P. sinica* have only thus far been found in tropical waters. *Pseudo-nitzschia obtusa* can be found primarily in arctic coastal regions and *P. turgiduloides* and *P. prolongatoides* are restricted to the Antarctic region alone. *Pseudo-nitzschia turgidula* and *P. granii* are limited to cold waters. There has been no report of *P. seriata* from the southern hemisphere while *P. subcurvata* has only been reported in the southern hemisphere (Skov et al. 1999). *Pseudo-nitzschia americana, P. calliantha, P. cuspidata, P. delicatissima* and *P. linea* are found in tropical and temperate coastal waters. *Pseudo-nitzschia australis*, *P. galaxiae,* and *P. multiseries* are found in coastal temperate regions. *Pseudo-nitzschia subfraudulenta* is a coastal warm water species while *P. subpacifica* is an oceanic warm water species. *Pseudo-nitzschia dolorosa* has been found only in upwelling regions. *Pseudo-nitzschia lineola* has been reported in the open ocean and coastal regions in temperate and polar areas. *Pseudo-nitzschia multistriata* has been reported mostly in the tropical and temperate Pacific. The accuracy of a map showing *Pseudo-nitzschia* biogeography depends on correct species identification and thorough examination of global waters. Some of the rarer species could actually be more common or have a wider range than currently believed.

Approximately 12 *Pseudo-nitzschia* species are documented DA producers (Table 1.1). On the west coast of the United States, the major DA producers are *P. australis*, *P. multiseries* and *P.* cf. *pseudodelicatissima* (could be *P. cuspidata*; Adams et al. 2000, Stehr et al. 2002, Lundholm et al. 2003, Bates & Trainer 2006). *Pseudo-nitzschia pseudodelicatissima*, *P. seriata* and *P. calliantha*

have caused DA contamination in shellfish in Atlantic Canada (Bates et al. 1998, Bates & Trainer 2006). In Europe, the toxigenic species are *P. seriata*, *P. australis* and *P. multiseries* (Bates & Trainer 2006). In New Zealand *P. australis* is the main source of DA (Rhodes et al. 1998b).

Molecular studies of microsatellite markers reveal few differences between *P. pungens* isolates from the North Sea and Atlantic Canada, suggesting minimal barriers to gene flow (Evans et al. 2005, Lundholm & Moestrup 2006). Microsatellite markers in *P. multiseries* from Pacific and Canadian Atlantic isolates show substantial differences and hence barriers to gene flow between the two regions (Evans et al. 2004, Lundholm & Moestrup 2006). Molecular methods of examining diversity should be applied to a larger investigation of *Pseudo-nitzschia* biogeography in order to describe global population dynamics and genetic relatedness between populations in different regions.

Life History

Pseudo-nitzschia, like many pennate diatoms, can reproduce sexually (Geitler 1935). Clonal cultures of *Pseudo-nitzschia* will gradually decrease in cell size over time and eventually die if they do not undergo sexual reproduction. This is due to vegetative cell division and splitting of the frustule between two daughter cells. The halves of the frustule fit together like a glass Petri dish, with one side slightly smaller than the other. The daughter cell that receives the smaller of the two frustules will grow a new second frustule inside the first. This cell will be smaller than the initial parent cell. In this way, the average

dimensions of the cell gradually decrease until they become so small the culture can no longer survive. However, if cells undergo sexual reproduction, cell size is restored.

A *Pseudo-nitzschia* cell will become sexualized when cell length has decreased below a threshold size, known as the first cardinal point, which in *P. multiseries,* is approximately 63% of the length of largest cells (Bates & Davidovich 2002). Sexual reproduction must occur before the cells reach a minimum length, which in *P. multiseries*, is approximately 30 μm (Bates & Davidovich 2002). In *P. delicatissima*, this size range is from 19-80 μm (Amato et al. 2005). During this size window, cultures of *Pseudo-nitzschia* can be mixed together to stimulate sexual reproduction. *Pseudo-nitzschia* is dioecious, meaning that male and female gametes are produced by separate clones and intraclonal mating is rare or absent. These "sexes" are referred to as "+" and "-" in *Pseudo-nitzschia*. While no monoecious clones of *Pseudo-nitzschia* have been reported, mating between two clones of the same sex has been observed, suggesting that a single culture could switch sex under some conditions which have not yet been investigated, or more than one mating type exists (Davidovich & Bates 1998).

The sexual cycle differs between pennate and centric diatoms (Drebes 1977, Round et al. 1990). Centrics are characterized by oogamous reproduction involving the formation of flagellated male gametes and non-motile female gametes. The first paper reporting sexual reproduction in *P. multiseries* caused considerable debate and criticism for claiming that *Pseudo-nitzschia*, a pennate diatom, had oogamous reproduction (Subba Rao et al. 1991). Observation of

flagellated gametes in cultures of *P. multiseries* was consistent with fungal contamination and was considered anomalous (Rosowski et al. 1992).

Mating in *Pseudo-nitzschia* can be achieved simply by mixing clones of the same species, but opposite sex. Clones must be in good physiological condition to undergo sexual reproduction. This means that clones must be mixed during exponential growth phase, which can be anywhere from 3-6 d after inoculation of a batch culture. Clones must receive a sufficient amount of light during a 24 h period. A photoperiod length up to 16:8 L:D, the maximum studied, will increase gamete and auxospore production (Davidovich & Bates 1998, Hiltz et al. 2000). These results suggest that parent cells must be healthy and photosynthesizing to produce energy for sexual reproduction.

Sexual reproduction has been described in *P. multiseries, P. pseudodelicatissima, P. calliantha* (Davidovich & Bates 1998), *P. subcurvata* (Fryxell et al. 1991) and *P. delicatissima* (Amato et al. 2005). Despite some differences in the amount of time necessary to complete sexual reproduction, the mating process is similar in all *Pseudo-nitzschia* species tested. The first step in sexual reproduction is parental pairing between cells of the opposite sex. Two cells will pair valve to valve, lying parallel with close alignment of the cells. The next stage is gametogenesis. The paired cells divide meiotically and the cell contents divide along the apical plane to form spherical gametes, two per cell. These gametes are identical in appearance and non-flagellated, but the behavior of the gametes differs between sexes. One cell produces two active gametes (- male) and the other two passive gametes (+ female). The frustules of both cells open,

permitting the active gametes to enter and fuse with the passive gametes. This is not always successful in both pairs of gametes or in all pairing of parent cells. When it is successful, this fusion takes only 1-2 min. After gamete fusion, the resulting zygote expands to form larger auxospores inside which the initial cell is formed. The entire process, from gamete production to formation of initial cells takes 2-4 d.

This process has not been documented in nature for several reasons. Calculations suggest that three years may pass before a cell becomes sexualized (Davidovich & Bates 1998). The entire mating process itself only takes 2-4 d and when mating occurs in the laboratory there are few auxospores compared to vegetative cells. Thus, paired cells and auxospores would be rare in natural populations. Furthermore, it is likely that the coupling between parent cells is weak enough that sampling and preservation techniques can disrupt them: placing mixtures of clonal cultures on an orbital shaker at 170 rpm prevented or reduced sexual reproduction (Gordon 2001, Bates & Davidovich 2002). It is unknown how well gametes and auxospores survive sampling and preservation, if at all.

Preliminary work has shown an interesting relationship between epibiont bacteria and *Pseudo-nitzschia* sexual reproduction. Some axenic clones of *P. multiseries* would not undergo sexual reproduction until bacteria were reintroduced (Thompson 2000). Other mixtures of axenic clones did undergo sexual reproduction; however, it is possible that there was bacterial contamination. Further work must be done to determine the role of bacteria in sexual reproduction in this species.

There is also some evidence that a type of "pheromone" or other chemical is being produced by sexually active *Pseudo-nitzschia*. Filtrates of sexually reproducing clones induce higher gamete production in other clones (Haché 2000). These results suggest that a chemical is produced that improves gamete production and thus would synchronize gamete production in already sexualized cells, but not necessarily aid in location of other sexualized cells.

Sexual reproduction in *Pseudo-nitzschia* is important for DA production. Clonal cultures of *Pseudo-nitzschia* decrease in size over time, as described previously, and also lose their ability to produce DA (Bates 1998). Offspring of *P. multiseries* clones that lose their ability to produce DA can be toxic, sometimes even more toxic than their parents were initially (Bates et al. 1999). Sibling clones can have significant variability in DA production, which could be accounted for by genetics or by the presence of different types and numbers of epibiont bacteria.

Timing and frequency of sexual reproduction in *Pseudo-nitzschia* has important implications for the genetic structure of populations. Sexual reproduction is an important source of genetic variability. While direct evidence of this effect has not been documented in natural populations, cell size has been found to abruptly increase seasonally (D'Alelio et al. 2006). Understanding sexual reproduction in the field is an essential part of describing *Pseudo-nitzschia* population dynamics.

Ecology and Bloom Dynamics

Blooms of *Pseudo-nitzschia* happen relatively frequently, in some regions seasonally, and in a wide variety of locations. In culture, *Pseudo-nitzschia* spp. can grow in salinities as low as 6 and as high as 48 and at temperatures as low as 5°C and as high as 30°C with a broad range for optimum growth (Miller & Kamykowski 1986, Jackson et al. 1992, Lundholm et al. 1997, Cho et al. 2001, Thessen et al. 2005). However, different species in natural populations can demonstrate distinct correlations with environmental characteristics, which suggests seasonal succession of species or regional specificity (Fryxell et al. 1997). In the South China Sea, *P. pungens* peaks in April, May and June, *P. multistriata* is present only in spring and *P. sinica* and *P. subpacifica* are found in late fall and early winter (Qi et al. 1994). In addition, *P. pungens* in the colder (1-10°C) waters of the North China Sea is present only in winter and spring while *P. pungens* in the warmer (21-29°C) East and South China Seas is present year round revealing two ecotypes (Zou et al. 1993). On the west coast of the United States, *P. pungens* is abundant in the summer and autumn as well as *P. fraudulenta*, *P. subpacifica* and *P. heimii*. *Pseudo-nitzschia multiseries* is abundant in the autumn and winter while *P. delicatissima* is abundant in the spring and summer. *Pseudo-nitzschia pseudodelicatissima*, *P. seriata* and *P. australis* are common in the autumn (Fryxell et al. 1997). Many species may coexist, but different growth and loss rates can lead to complex bloom dynamics and seasonal succession.

Pseudo-nitzschia blooms can be stimulated by nutrients from two sources: upwelling or mixing events and riverine inputs. Both sources stimulate *Pseudo-nitzschia* blooms at concentrations of 8 – 22 μM NO_3^-, 2.4 – 35 μM Si, 0.2 – 2 μM PO_4^{3-} (Dortch et al. 1997, Scholin et al. 2000, Trainer et al. 2000, Loureiro et al. 2005), but in different temperature and salinity regimes. *Pseudo-nitzschia* abundances and domoic acid concentrations on the west coast of the United States are associated with low temperature, high salinity and high nutrient conditions typical of upwelling (Villac 1996, Trainer et al. 2000, Trainer et al. 2002). Similarly, upwelling regions off the coast of Portugal contain high concentrations of *Pseudo-nitzschia*, which are used as upwelling indicators during spring and summer (Moita 2001, Loureiro et al. 2005).

Riverine inputs have stimulated toxic *Pseudo-nitzschia* blooms in many regions and are characterized by lower salinities and higher temperatures than upwelling zones (Bird & Wright 1989, Smith et al. 1990, Horner & Postel 1993, Dortch et al. 1997, Trainer et al. 1998, Scholin et al. 2000, Spatharis et al. 2007). A distinction between nutrients in upwelling and river plumes is that riverine inputs are likely the result of anthropogenic nutrient loading. Sedimentological data show an increase in *Pseudo-nitzschia* abundance in the Mississippi River plume since 1950 suggesting a response to eutrophication (Parsons et al. 2002). However, in addition to an increase in nitrogen and phosphorus, nutrient ratios in Mississippi River water have also changed, i.e., a decreasing Si:N ratio which is favorable for *Pseudo-nitzschia* in culture (Turner & Rabalais 1991, Sommer 1994). Other river systems have also affected *Pseudo-nitzschia* abundances.

When the mouth of the Yellow River in China was artificially redirected in 1976, the location of the *Pseudo-nitzschia* bloom abruptly changed location to follow the river plume (Zou et al. 1993). *Pseudo-nitzschia* abundance in the plume of the Yangtze River is positively correlated to NO_3^- and PO_4^{3-} concentrations (Zou et al. 1993). In the South China Sea, *Pseudo-nitzschia* abundances respond to increased land runoff after rainfall (Qi et al. 1994). An analysis of *P. delicatissima* and *P. pseudodelicatissima* dynamics and environmental parameters in the Bay of Fundy, Canada show the importance of NO_3^- and NO_2^- concentrations to abundance of these species (Kaczmarska et al. 2007). These coastal studies show a response to riverine nutrients, changing nutrient ratios and eutrophication.

Much of the seasonal variability in *Pseudo-nitzschia* abundance can be explained by regular shifts in wind, light, temperature and river flow. In the northern Gulf of Mexico, *Pseudo-nitzschia* abundance peaks in spring, corresponding to the average maximum in river flow with another small peak in fall during wind events that mix the stratified water column (Dortch et al. 1997). Many *Pseudo-nitzschia* blooms occur in the spring and fall, when irradiance is relatively low (Parsons et al. 1998, Mercado et al. 2005). In culture, *P. multiseries* can out compete other phytoplankton species at low irradiance with a short photoperiod (Sommer 1994). However, low light may contribute to the demise of autumn blooms (Bates et al. 1998). Day length can affect growth rates, cell yield, toxin production and influence which species of *Pseudo-nitzschia* becomes dominant (Fehling et al. 2005).

Local meteorological phenomenon, such as winds and heavy rainfall events, can stimulate *Pseudo-nitzschia* blooms. Wind events can be especially important for transporting toxic blooms inland from upwelling sites offshore (Trainer et al. 2000, Trainer et al. 2002) or providing mixing necessary to bring nutrients into the photic zone (Lund-Hansen & Vang 2004). Heavy rainfall after a drought can cause a dramatic increase in *Pseudo-nitzschia* abundances in the river outflow, such as in Eastern Canada in 1987 (Bates et al. 1998).

Larger scale changes in weather such as the El Niño Southern Oscillation can affect *Pseudo-nitzschia* abundances by controlling upwelling near the west coast of the United States. During weak ENSO years, upwelling is high and therefore so are *Pseudo-nitzschia* abundances (Fryxell et al. 1997). However, *Pseudo-nitzschia* can still take advantage of other favorable events, such as increased runoff after rainfall, during strong ENSO years and bloom. Both 1991 and 1998, years with large toxic events on the west coast of the United States, were strong ENSO years.

The decline of *Pseudo-nitzschia* blooms is less studied than initiation. Parasitic fungi may play an important role in the demise of *Pseudo-nitzschia* blooms (Bates et al. 1998). Parasitic oomycetes and chytrids have infected *P. multiseries* and *P. pungens* in eastern Prince Edward Island, Canada. Additionally, fungal parasites have been observed in cells during bloom decline in coastal Washington, USA (Horner et al. 1996) and an unexpected decrease in *P. multiseries* abundance in the Skagerrak between 1991 and 1993 was suspected to be caused by parasitic fungi (Hasle et al. 1996). Viruses are known to infect

marine diatoms (Nagasaki et al. 2004, Nagasaki et al. 2005), but no studies exist on viral infections in *Pseudo-nitzschia* and the genus may be immune (Caron pers. comm.; Coats pers. comm.). High pH resulting from dense blooms could also lead to bloom decline. Laboratory cultures of several *Pseudo-nitzschia* species could not continue exponential growth at pH from 8.7 to 9.3 (Lundholm et al. 2004). The exact mechanisms of *Pseudo-nitzschia* bloom decline are uncertain and could be caused by multiple factors.

Domoic Acid Production as an Adaptation

The reason *Pseudo-nitzschia* produces toxin is largely unknown, as is true for toxin production by most harmful algal bloom (HAB) species. An obvious hypothesis for the production of a potentially toxic, energy-demanding secondary metabolite is grazer deterrence (Turner et al. 1998). Harmful algal species and their toxins have a varied effect on metazoan and protistan grazers (Turner & Tester 1997, Turner et al. 1998). DA has anti-helminthic and insecticidal properties (Takemoto & Daigo 1958, Maeda et al. 1984) but, at levels found in nature, does not seem to affect crustacean zooplankton or other marine invertebrates that feed on *Pseudo-nitzschia*. Krill, *Euphausia pacifica*, feed on toxic and non-toxic *Pseudo-nitzschia* in the laboratory and in the field (Bargu et al. 2003, Bargu & Silver 2003); however, grazing on non-toxic *P. pungens* decreased in the presence of added dissolved DA suggesting a direct affect on the filtering appendages (Bargu et al. 2006). Toxic *Pseudo-nitzschia* has no detrimental effect on ingestion rates or egg hatching success in the copepods

Calanus glacialis, Temora longicornis and *Acartia clausi* (Windust 1992, Maneiro et al. 2005). DA does not slow grazing, reduce egg hatching success and production or increase mortality in *Acartia tonsa* or *T. longicornis* (Lincoln et al. 2001). Sea scallops, (*Placopecten magellanicus*), California mussels (*Mytilus californianus*) and blue mussels (*Mytilus edulis*) can feed on toxic *Pseudo-nitzschia* with no sign of illness (Wohlgeschaffen et al. 1992, Jones et al. 1995b, Douglas et al. 1997) but the Pacific oyster (*Crassostrea gigas*) had an immune response to toxic *P. multiseries* and experienced respiratory acidosis from shell closure (Jones et al. 1995a,b). *Mytilus edulis* injected with 1 – 500 ng DA g^{-1} body weight exhibited no neurotoxic, immunotoxic or genotoxic effects (Dizer et al. 2001). These studies, with the exception of the *C. gigas* study, argue against DA as a feeding deterrent: neither crustacean zooplankton nor bivalve molluscs decrease their grazing in the presence of DA.

Some organisms stop or reduce their grazing on *Pseudo-nitzschia* because of the size and/or shape of the cells, such as the appendicularian *Oikopleura dioica* (Tonnesson et al. 2005). The eastern oyster *Crassostrea virginica* will feed on toxic cells at the same rate as non-toxic cells, but grazes other types of diatoms at higher rates (Roelke 1993, Thessen et al. 2002). Smaller *C. virginica* (~40 mm) were completely unable to graze on *Pseudo-nitzschia* suggesting a size and/or shape effect (Thessen et al. in prep). Mesozooplankton grazers, such as copepods, require an increased handling time for large, chain-forming diatoms, and may not graze on *Pseudo-nitzschia* especially if a preferred prey type is also available (Smetacek et al. 2002, Olson et al. 2006).

A second hypothesis is that DA is produced to act as an allelopathic agent, increasing the ability of *Pseudo-nitzschia* to compete for light or nutrients by killing or inhibiting its microbial competitors (Bates 1998). This is a possibility during large monospecific *Pseudo-nitzschia* blooms if high levels of DA are released into the surrounding medium. Bio-assays showed inhibited growth in some bacterial species, but not others. One study found inhibition of all bacterial growth (Stewart et al. 1998) while the other found no inhibition (Windust 1992) despite using the same species, *E. coli,* as a reference. Algal assays testing growth with and without DA exposure showed that DA had no effect on the diatoms *Skeletonema costatum* and *Chaetoceros gracilis* (Windust 1992). A toxic culture of *P. multiseries* had no allelopathic effects on *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina* (Lundholm et al. 2005). Additions of DA alone caused no allelopathic effect on *C. ericina*, *E. gymnastica*, *Karenia mikimotoi*, *H. triquetra*, *Heterosigma akashiwo*, *Prorocentrum minimum*, *P. micans*, *Pyramimonas propulsa* and *R. marina* (Lundholm et al. 2005). The lack of detrimental effects of DA on the growth of many species of bacteria and microalgae argues against the evolution of DA as an allelopathic chemical.

A third hypothesis is that DA protects *Pseudo-nitzschia* cells from ultraviolet light. DA absorbs in the UV range and photodegrades (Bates et al. 2003, Bouillon et al. 2006). Its ring structure is similar to, albeit much smaller than, microsporin-like amino acids (MPAA) used in many marine organisms to protect cells from UV light (e.g., Karentz et al. 1991). Toxic *P. multiseries* is resistant to the effects of

UV light while non-toxic *P. fraudulenta* and *P. pungens* show decreased growth in UV treatments (Hargraves et al. 1993). Further, cellular DA concentrations were lower in the UV treatments. However, rather than suggesting that the DA was photodegrading and protecting the cell, lower DA levels were interpreted as a shift of energy away from DA production and toward the production of a UV protectant. DA has also been suggested as important in increasing photosynthetic potential through the use of UV. A field study found that a planktonic community dominated by *Pseudo-nitzschia* spp. was able to use the entire UVA spectrum to enhance carbon fixation suggesting that DA could aid in photosynthesis by capture of additional wavelengths; however, the UVB spectrum inhibited carbon fixation (Mengelt & Prézelin 2005). *Pseudo-nitzschia multiseries* cells have been found to fluoresce blue when excited by UV light, a response typical of some proteins; however, MPAAs are not known to fluoresce. There are 4 photoisomers of DA, but their spectral properties are unknown (Wright et al. 1990).

A fourth hypothesis is that DA is produced as a means of modulating energy flow within the cell (Pan et al. 1998). DA production begins when cell division stops and primary metabolism slows, such as during stationary phase of growth in a batch culture. Under these conditions, the photosynthetic apparatus is still harvesting light energy, which now must be consumed by secondary metabolism, like DA synthesis. Another way to disrupt energy flow in the cell is to decouple the light and dark reactions of photosynthesis by exposing cells to rapid increases in light under low temperatures (Lomas et al. 2000). Cells harvest more light energy than can be immediately consumed by the dark reactions. Low temperature

and fluctuating light conditions often occur in nature during the spring and fall and during upwelling events, which are also times of high *Pseudo-nitzschia* abundance and toxicity (Dortch et al. 1997, Trainer et al. 2000). If DA is produced to balance energy flow during times of decoupled photosynthesis and growth during stationary phase in culture, then a similar decoupling in nature could result in excess DA production.

Other hypotheses have not been investigated as thoroughly. The low N content of DA argues against its use as a nitrogen storage molecule (Bates et al. 1991), but the possibility has not been investigated. While salinity has an effect on DA production, there is no evidence that DA is produced to cope with osmotic stress (Jackson et al. 1992, Doucette et al. in press). The most recent hypothesis states that domoic acid is produced under low iron stress or high copper stress to chelate those metals (Rue & Bruland 2001, Maldonado et al. 2002, Wells et al. 2005).

Production of a glutamate-derived neurotoxin is common among plants, especially the algae (Laycock et al. 1989). Molecules like L-glutamic and L-aspartic acid are ubiquitous among algae while γ-amino-n-butyric acid and β-alanine are common in algae and higher plants (Curtis & Watkins 1961). DA is unique in that it is the most potent of the algal derived neurotoxins (Laycock et al. 1989). Another potent neurotoxin produced by a plant is β-methylamino-L-alanine or BMAA (Vega & Bell 1967). It is found in the seeds of the cycad, the oldest living seed plant and the evolutionary link between angiosperms and gymnosperms (Brenner et al. 2003). Much like DA, BMAA is a non-protein amino acid that acts

as an agonist of glutamate receptors (Copani et al. 1990, Manzoni et al. 1991) and causes convulsions and neural damage in primates (Spencer et al. 1987). BMAA is suspected of causing Guam's dementia, a neurological disorder similar to Parkinson's and Alzheimer's disease, which affected the Chamorro people of Guam who consumed both the seeds of the cycad and flying foxes (bats) that consumed cycad seeds (Whiting 1963, Banack & Cox 2003). However, this link between cycads and Guam's dementia is still controversial. Physiological experiments demonstrate a similarity between effects of the algal toxin DA and the cycad toxin BMAA (Spencer et al. 1987, Jeffery et al. 2004). These studies do not explain why either are produced. One could argue that BMAAs are produced for protection of the seeds. Many seeds have germination inhibitors that must leach away before germination, as after an intense rain (Partridge & Wilson 1990). Studies show that BMAA affects hypocotyl shortening, cotyledon expansion and root and shoot growth in *Arabidopsis* seedlings (Brenner et al. 2000). All of this suggests that glutamate receptors and glutamate agonists, besides any possible defensive role, could be an important family of cell signal molecules in plants.

Iron hypothesis

Recent work has shown DA production in response to Fe limitation, supporting a new hypothesis that *Pseudo-nitzschia* produces DA to act as a chelator under Fe limitation or Cu toxicity (Maldonado et al. 2002, Wells et al. 2005). However, there is no conclusive evidence to show DA production during Fe limitation is different from DA production during P or Si limitation.

Work by Bates et al. (2000) showed that a strain of *P. multiseries* (CLN-1) in batch culture produced less DA when grown in low concentrations of Fe. Their conclusion states that it is unlikely DA is produced as an Fe acquisition strategy used under Fe-limiting conditions because the cell needs Fe to produce DA. Fe is a key component in multiple enzymes for DA synthesis (nitrate reductase, glutamine synthetase and aconitase) as well as the photosynthetic apparatus. Cells grown in low Fe medium showed decreased chlorophyll content. In their experiments, Fe limitation inhibits DA production. However, DA does bind Fe and Cu and structurally resembles a siderophore, a chemical used by terrestrial plants to bind Fe in the soil (Rue & Bruland 2001). Studies show that DA can bind Fe and Cu sufficiently to affect their bioavailability in the ocean; however, other acidic cellular components can also bind Fe, like citrate and glucuronate (Bates et al. 2000, Rue & Bruland 2001).

Another study by Maldonado et al. (2002) seems to show the opposite pattern, with low Fe cultures producing more DA than high Fe cultures. How can these two experiments be reconciled? One major difference is that the Bates et al. (2000) study used higher Fe concentrations in their media. There was no effect of Fe on specific growth rate, whereas in the Maldonado et al. (2002) study, low Fe cultures grew slower than high Fe cultures. Since growth rate has been shown to affect DA production (Pan et al. 1996a), the increased DA in the low Fe treatment could be the result of the low growth rate instead of a direct Fe effect. Another difference between the two studies is time scale. The Bates et al. (2000) study looked at the effect of Fe in a batch culture over several days whereas the

Maldonado et al. (2002) study looked at the effect of Fe in exponentially growing cultures over 12 h. Maldonado et al. (2002) also use a voltammetric method to measure the concentration and stability of Fe complexes, rather than directly measuring DA, to increase sensitivity. Their claim, that actively growing *Pseudo-nitzschia* immediately release DA into the medium to chelate Fe, would have been more definitive if they had also checked DA release in silicate- or phosphate-limited cultures with the very sensitive voltammetric method.

The Maldonado et al. (2002) study proposes that DA is produced in actively growing cells and immediately released by active transport under low Fe conditions to compete for Fe in coastal environments, whereas macronutrient limitation causes an increase in intracellular DA when the cells stop dividing which is released when cell membrane integrity is compromised. Interactions between Fe and macronutrient limitation could explain the variability in coastal *Pseudo-nitzschia* blooms. When macronutrients become limiting before Fe, the result may be high intracellular concentrations of DA and thus highly toxic blooms. When Fe limitation occurs before macronutrient limitation, low or non-toxic blooms may occur since the DA that is produced is excreted. DA-enhanced Fe uptake was much slower than Fe uptake in other coastal diatoms when corrected for surface area, arguing against the ability of DA to make *Pseudo-nitzschia* a more efficient Fe competitor (Maldonado & Price 1996, Maldonado et al. 2002). However, specific growth rates of Fe-deficient cultures were higher than reported specific growth rates for other diatom species at similar Fe concentrations (Sunda & Huntsman 1995, Maldonado & Price 1996, Maldonado

et al. 2002). In another study, Fe uptake in *Pseudo-nitzschia* was higher than in other coastal diatoms, but was not corrected for surface area (Wells et al. 2005). In bioassay experiments, low Fe treatments had enhanced DA production (Wells et al. 2005).

Cu also has an effect on DA production and growth rate (Maldonado et al. 2002, Rhodes et al. 2004). Maldonado et al. (2002) hypothesize that DA is produced to bind Cu to reduce toxic effects on the cell. However, it has also been shown that some diatoms need Cu for Fe uptake (Peers et al. 2005). Some eukaryotes that possess a high-affinity Fe III uptake system use a Cu-containing oxidase (MCO) to oxidize Fe II (Stearman et al. 1996). Sequence analysis of the *Thalassiosira pseudonana* genome reveals a gene with homology to MCO genes of other known MCO users (Armbrust et al. 2004). Culture experiments show that oceanic diatoms have low growth rates in low Cu concentrations even if given sufficient Fe. In bioassay experiments, the addition of Cu resulted in a higher biomass than in unamended controls and in treatments amended with Fe alone (Peers et al. 2005). Further, DA production is stimulated under Cu-limiting and Cu-toxic concentrations (Maldonado et al. 2002, Rhodes et al. 2004, Peers et al. 2005).

Fe addition experiments in High Nutrient Low Chlorophyll (HNLC) regions of the ocean stimulate growth of diatoms, primarily *Pseudo-nitzschia* spp. (De Baar et al. 2005, Leblanc et al. 2005, Tsuda et al. 2005). *Pseudo-nitzschia* can survive at low abundances in these regions and are therefore able to respond to an artificial enrichment. However, the *Pseudo-nitzschia* responding to Fe

enrichment in these experiments has been either non-toxic or untested (A. Marchetti pers. comm.) The Fe III binding constant of DA is much too low to compete with other potential chelators in the water column unless DA is present at very high concentrations, which have not been documented in these areas (Rue & Bruland 2001, Wells et al. 2005).

While the culture experiments on Fe-limitation and DA production are compelling, most studies to date have used only one strain of each species tested (Bates et al. 2000, Maldonado et al. 2002, Wells et al. 2005). Multiple strains could reveal a highly variable DA response similar to other studies looking at the affects of bacteria, pH or nitrogen source on DA production (Bates et al. 1995, Lundholm et al. 2004, Kaczmarska et al. 2005, Chapter 3 of this thesis).

Domoic Acid

Domoic Acid Chemistry and Detection

The chemistry and toxicology of DA has recently been reviewed (Jeffery et al. 2004). Domoic acid (MW 311) is a water-soluble, heat stable analogue of the amino acid glutamate (Hatfield et al. 1995, Leira et al. 1998). It was first isolated from *Chondria armata* and named after the Japanese word for seaweed – *domoi* (Mos 2001). DA is toxic to vertebrates because it binds to neurons twenty times more powerfully than ordinary neurotransmitters (Teitelbaum et al. 1990), resulting in a massive depolarization of the neuron. The subsequent increase of intracellular Ca^{2+} causes swelling and cell death. This happens in the hippocampus, the part of the brain associated with memory.

There are many methods of detection of DA, which are described in detail in Quilliam (2003). The current global standard for detection of many algal toxins in shellfish is the mouse bioassay (Fernandez et al. 2003, Todd 2003). This involves preparing an extract of the tissue to be tested and injecting it into a test mouse. If the mouse exhibits symptoms, a toxin is considered present. However, the limit of detection of the mouse bioassay for ASP toxin is 40 mg DA kg^{-1} shellfish, above the regulatory limit of 20 mg DA kg^{-1} shellfish. High Performance Liquid Chromatography (HPLC) with UV detection is the most commonly used chemical analytical method for DA detection due to its simplicity, speed and reproducibility. However, while the limit of detection is low enough to be used for routine monitoring of shellfish, it is not low enough for work with seawater and plankton. Thus, HPLC methods based on derivatization of fluorescence reagents were developed (Pocklington et al. 1990). Other chromatographic-based assays for DA include thin-layer chromatography (TLC), capillary electrophoresis (CE) and liquid chromatography with detection by mass spectroscopy (LC-MS). When establishing the presence of DA in a new area or in a new species, comparing chromatographic peaks to a DA standard is not conclusive. Spectroscopic data such as ultra-violet or mass spectra are necessary to confirm identification.

Receptor based assays and immunoassays are relatively new. Receptor based assays utilize the specificity of a toxin for a particular action site and measure activity of the toxin rather than discrete structural components. The receptor binding assay for DA involves measuring the competition between a known amount of labeled kainic acid and DA in the sample to be tested for

glutamate receptors in brain tissue (VanDolah et al. 1997). The newest detection method is an immunoassay or ELISA, which is based on competition of DA in the sample with a DA-conjugated protein for anti-DA antibodies (Garthwaite et al. 1998). Receptor-based assays and immunoassays have the advantage of giving low limits of detection, but can be costly.

In many early studies on DA and *Pseudo-nitzschia,* samples were taken quickly and stored frozen until analysis within two weeks because of potential DA degradation (e.g., Bates et al. 1991). Light, temperature and pH were all factors believed to affect DA stability. Over time, studies have shown that DA is not as unstable as first thought. Ambient temperatures, artificial light and repeated chilling and warming do not degrade DA in saline solution (Johannessen 2000); however, DA does degrade under acidic conditions (Quilliam et al. 1989).

Once DA is released from the cell, as with any other molecule, it can be degraded or remain intact. Intact adsorption onto suspended particles removes only a minor fraction of dissolved DA from the water column (Lail et al. 2007). Bacterial degradation is a logical pathway, but the ability to grow on or degrade DA is rare among marine sediment and water bacteria (Stewart et al. 1998). DA is inhibitory to resting cells and growing cultures of some bacteria (Stewart et al. 1998). However, bacteria isolated from molluscs (*M. edulis* and *M. arenaria*) that depurate DA very quickly have the ability to grow on and degrade DA (Stewart et al. 1998). It is currently unknown if DA can serve as an energy or carbon source for eukaryotes known to use dissolved free amino acids.

Another degradation pathway is photolysis. DA can be transformed into non-toxic isomers and degraded by exposure to light in the UV range (Wright et al. 1990, Bates et al. 2003, Bouillon et al. 2006). The rate of degradation of DA exposed to sunlight in natural seawater is 0.017 to 0.035 d^{-1}, enough to be a significant sink (Bouillon et al. 2006). However, light penetration in seawater can be limited, especially in turbid coastal areas. Low light might increase the possibility that dissolved DA could remain in the water column after a bloom has dissipated. Particulate DA could also become buried in sediments.

Domoic Acid Biosynthesis

The DA molecule is created from glutamate and an isoprenoid pyrophosphate both originating from acetate, but derived by different pathways (Fig. 1.3; Laycock et al. 1989, Douglas et al. 1992, Pan et al. 1998, Ramsey et al. 1998). Research suggests the origin of the isoprenoid pyrophosphate is through the glyceraldehyde 3-phosphate (GAP) -pyruvate pathway while glutamate is a product of the Citric Acid cycle (Lichtenthaler et al. 1997, Ramsey et al. 1998). DA synthesis requires a substantial amount of energy and therefore competes with primary metabolism. Therefore, less ATP is available for DA biosynthesis during nutrient uptake and growth. Conversely, during Si limitation in *P. multiseries,* cellular levels of ATP increase and can then be used for DA synthesis (Pan et al. 1996 a,b).

Identifying and characterizing genes that control DA biosynthesis is an active research topic. Results from phylogenetic studies comparing different

species of *Pseudo-nitzschia*, *Nitzschia navis-varingica* and *Amphora coffeaeformis* (two DA-producing species that do not belong to *Pseudo-nitzschia*) suggest that either the ability to produce DA evolved independently several times, genes have been laterally transferred or multiple losses of species have occurred (Janson & Hayes 2006). Two approaches are being used to find DA genes (Bates & Trainer 2006). The first approach involves using a cDNA microarray to screen for genes with expression patterns that correlate with DA production (Boissonneault 2004). This method revealed 12 transcripts that were upregulated during DA production. The second approach is using subtraction techniques when comparing Si replete and Si deplete cultures to identify genes involved in DA production. No definitive DA production genes have been discovered, but the current whole genome sequencing of *P. multiseries* (by E. V. Armbrust, B. Jenkins and S. Bates) will greatly assist in this search.

Toxin Production Physiology

The physiology of DA production has been reviewed elsewhere (Bates 1998). It is commonly known that growth phase, nutrients, temperature, irradiance and bacteria play a role in DA production. Many studies show *Pseudo-nitzschia* cultures produce little DA until cell division has stopped. In batch cultures, DA production often starts at the onset of stationary phase and DA content of the cells peaks about one week later. Some cultures produce DA during late exponential phase, possibly because this is a period of transition when some cells have stopped growing and are producing DA while other cells are still

dividing. However, one culture of *P.* cf. *pseudodelicatissima* from the Northern Gulf of Mexico produced the highest levels of DA during early exponential growth (Pan et al. 2001).

In continuous culture, toxin content increases when growth is slowed by decreasing the dilution rate (Pan et al. 1996a). This growth effect means that many factors that slow growth would also indirectly increase toxin production. Studies showing that an increased pH will increase toxin production also show that growth rate decreases under these circumstances, making it difficult to know the effect of pH alone (Lundholm et al. 2004). Cultures grown on urea have higher DA production, but also have a slower growth rate (Armstrong Howard et al. 2006). The increase in toxin production when growth slows must be taken into account when investigating factors that affect DA production.

Nutrient limitation is widely used to induce DA production in culture, with Si and P limitation commonly used. Initially, it was hypothesized that DA production was specifically linked to Si limitation in *P. multiseries*, but *Pseudo-nitzschia* cultures will produce toxin when growth is limited by Si or other factors in the presence of replete nitrogen and light. Cells do not produce DA under nitrogen limitation (Bates et al. 1991). Toxic levels of NH_4^+ and Cu also limit growth and subsequently increase DA production (Bates et al. 1993a).

Low temperature has been found to have a negative affect on cell division and DA production in some strains (Lewis et al. 1993); however, toxic mussels have been harvested from ice-covered water, implying that *Pseudo-nitzschia* can produce DA at near 0°C (Smith et al. 1993). A culture of *P. seriata* produced

more DA at 4°C than 15°C which could reflect cold water adaptation (Lundholm et al. 1994). Metabolism typically decreases with lower temperatures, but many enzymes involved in the production of DA have differing temperature optima. For example, RUBISCO, the enzyme that fixes atmospheric carbon, operates optimally at a higher temperature (Li et al. 1984, Smith & Platt 1985, Descolas-Gros & de Billy 1987) than nitrate reductase, which transforms NO_3^- into NO_2^- (Packard et al. 1971, Kristiansen 1983, Dohler 1991, Lomas & Glibert 2000). Both fixed carbon and reduced nitrogen are required for DA synthesis. Temperature has an obvious effect on DA production by regulating the speed of multiple enzyme reactions within the cell. No studies have examined the effects of rapid changes in temperature on DA production, although rapid temperature changes can uncouple the light and dark reactions of photosynthesis.

Irradiance is, of course, a very important control on DA production since it provides the energy necessary for biosynthesis. Irradiances below 100 μmol photons $m^{-2}sec^{-1}$ can lead to decreased DA production, a trait that has consequences for mass culture of toxic *Pseudo-nitzschia* (Whyte et al. 1995). Self-shading in large culture vessels can reduce DA production compared to small cultures in which self-shading is less important (Whyte et al. 1995). *P. multiseries* can produce DA under constant light (Villac et al. 1993a), but no experiments have been performed to examine the effect of extremely short photoperiods on DA production (Bates 1998). In *P. seriata*, cultures exposed to a long photoperiod (18:8 L:D) had higher total toxin production than those exposed to a short photoperiod (9:15 L:D) (Fehling et al. 2005). The effects of rapid

changes in irradiance on DA production have not been studied, although rapid shifts may temporarily uncouple the light and dark reactions of photosynthesis (Lomas & Glibert 1999).

As noted previously, bacteria play a role in DA production, with the exact mechanisms unknown. Thus far there is no evidence that bacteria can produce DA autonomously (Bates et al. 2004). Axenic cultures of *P. multiseries* can produce DA but at much lower levels than non-axenic cultures. The variability between DA production in different cultures of the same strain may be partially explained by differences in bacterial flora (Kaczmarska et al. 2005). Bacteria have been observed on the frustule and free-living in culture media. It has been suggested that the bacteria are using organic matter released from the diatom (because their location on the frustule, the raphe and cingulum, are locations where organic matter could be released) while providing the diatom with a co-factor, such as a precursor that enables DA production under physiologically stressful conditions. Addition of amino acids, like proline, stimulated growth in axenic *Pseudo-nitzschia* cultures (Stewart et al. 1997), which supported suggestions that bacteria may produce specific amino acids that stimulate growth of the diatom, i.e., *Pseudo-nitzschia* and its attached bacteria live symbiotically (Stewart et al. 1997). However, another hypothesis states that DA is produced to chelate trace metals in competition with bacteria that produce their own chelator, gluconic acid, as the addition of gluconic acid to a *Pseudo-nitzschia* culture will increase DA production (Osada & Stewart 1997).

Toxicology, Food Webs and Monitoring

Domoic Acid Toxicology

Tests in animal models have repeatedly documented DA effects in adults and developing animals. Intraperitoneal injections up to 1.25 mg DA $kg^{-1}d^{-1}$ cause no signs of toxicity in adult female rats, but doses of 1.75 mg DA $kg^{-1}d^{-1}$ induced abortions (Khera et al. 1994). Doses of 2 mg DA $kg^{-1}d^{-1}$ can cause death in pregnant rats. Lactating females given doses of 1 mg DA $kg^{-1}d^{-1}$ transferred the toxin to their young up to 24 h after exposure, but not enough to cause acute symptoms (Maucher & Ramsdell 2005). Research shows that DA lingers in breast milk longer than in blood plasma and that neonatal rats are much more susceptible to DA than adults (Xi et al. 1997, Doucette et al. 2000, Maucher & Ramsdell 2005). This is probably because in adults, DA poorly penetrates the blood-brain barrier, but in fetuses and newborns, this barrier is incomplete (Mayer 2000). A study comparing the effects of DA on young adult versus old adult rat brains shows equal sensitivity to initial exposure and reduced sensitivity to a second exposure in the younger brains (Kerr et al. 2002). Oral doses have less effect than injection of DA in animals and almost all DA ingested is excreted in feces. This suggests that DA is poorly absorbed from the gut (Iverson et al. 1989). Other studies with rats and cynomolgus monkeys show that DA is well distributed in body water and rapidly excreted, as expected for a hydrophilic compound, and implies that renal function is important for DA removal (Truelove & Iverson 1994). Numerous studies show death, brain damage, reduced learning

and memory ability in rats and primates exposed to DA in the laboratory (Jeffery et al. 2004).

Fish have long been considered immune to the effects of DA. However, injections of DA can produce neurological symptoms suggesting that fish are susceptible to DA at doses similar to rats and monkeys (Lefebvre et al. 2001). Fish exposed to DA in the field showed high levels of DA in their gut, but DA levels were 1000 X lower in the brain tissue, suggesting that DA uptake from the gut is low. The complete absence of neurological symptoms in fish given oral doses of DA provides more evidence that little DA is taken up through the gut and even less passes the blood-brain barrier. Despite proven susceptibility to injections of DA, fish exposed to DA in the field may not display symptoms.

A study of toxicity in fish eggs injected with DA showed effects even at small doses (Tiedeken et al. 2005). Injections of 0.4 mg DA kg^{-1} reduced hatching success by 40% and injections of 1.2 mg DA kg^{-1} reduced hatching success by 50%. In the eggs that lived, embryos convulsed at 2 days post fertilization. All surviving eggs injected with 4 mg DA kg^{-1} had no response to touch at 4 days post fertilization and at 5 days post fertilization had constant rapid pectoral fin movements possibly analogous to the scratching observed in laboratory rodents exposed to DA. Not only are fish susceptible to DA at similar doses as mammals, they are also susceptible to developmental toxicity.

Amnesic Shellfish Poisoning

Pseudo-nitzschia was not recognized as a toxic diatom until the first documented incident of ASP occurred in Prince Edward Island, Canada in 1987 when residents ate DA contaminated mussels (*Mytilus edulis*) from Cardigan Bay estuaries (Bates et al. 1989, Wright et al. 1989). Out of 250 reported illnesses, 107 met the case definition for ASP (Perl et al. 1990). Common symptoms were vomiting, abdominal cramps, diarrhea, incapacitating headache and loss of short-term memory (Perl et al. 1990). Nineteen people were hospitalized, twelve requiring intensive care because of seizures, coma, severe lung congestion, and unstable blood pressure (Perl et al. 1990). Some of the twelve intensive care patients showed additional serious neurological problems including inability to speak, irritability and uncontrollable facial movements (Perl et al. 1990). Four people died, three in the hospital and one three months after apparent recovery (Perl et al. 1990, Teitelbaum et al. 1990). Brain tissue from three of the four dead patients revealed severe cell damage, especially in the hippocampus and amygdala (Perl et al. 1990, Teitelbaum et al. 1990). Of those patients that lived, the more severely affected experienced memory deficits as much as five years after DA consumption (Todd 1993). One patient who suffered short term memory loss also developed epilepsy one year after exposure (Cendes et al. 1995).

The causative organism was found to be *P. multiseries*, which was blooming in Cardigan Bay at the time of the outbreak (November to December) and declined shortly thereafter (Subba Rao et al. 1988, Bates et al. 1989). The

identity of the toxin as DA was confirmed by proton nuclear magnetic resonance spectra in mussel tissue, cultured *P. multiseries* and plankton samples from Cardigan Bay (Bates et al. 1989, Wright et al. 1989). A positive correlation was found between the concentrations of *P. multiseries* and DA in plankton samples (Bates et al. 1989). No DA was found in cultures of other diatom species, 10 isolated from Cardigan Bay and 12 obtained from a culture collection. Small amounts of DA were found in a local macroalga, *Chondria baileyana* (Bates et al. 1989).

Prior to the 1987 ASP event, DA had not been detected in shellfish (Wright et al. 1989). Mussels from Cardigan Bay and patients' uneaten mussels were initially tested for PSP toxins using the mouse bioassay; however, the test mice exhibited involuntary scratching of their shoulders with their hind legs, a symptom atypical of PSP (Perl et al. 1990). Mussels were also tested for dangerous bacteria, viruses and chemical residues; none were found. Metabolites, including DA, were extracted from whole mussels and DA was identified using HPLC, high-voltage paper electrophoresis, ion-exchange chromatography and ultraviolet, infrared and mass spectroscopy (Wright et al. 1989). Dissected mussels contained the most toxin in the digestive gland (Wright et al. 1989).

No new cases of ASP have been reported since 1987. However, DA has been detected in shellfish (Villac et al. 1993b, Wekell et al. 1994, Rhodes et al. 1998a, Vale & Sampayo 2001) and a small outbreak of "food poisoning" in Oregon in 1992 possibly could have been the result of DA in razor clams consumed by locals (Todd 1993, Wright 1998). Effects of single or multiple

exposures to levels of DA too low to cause outward symptoms are unknown; low levels of DA could have negative effects without causing an obvious toxic event. Preliminary work shows a possible risk to mental development of infants whose breastfeeding mothers eat contaminated shellfish and children who eat contaminated shellfish themselves (Grattan et al. 2002).

Only DA levels above 40 μg g^{-1} wet weight of mussel meat will show symptoms in test mice (Todd 1993). During the 1987 event, seemingly unaffected individuals consumed 0.2-0.3 mg kg^{-1}, persons with mild symptoms had consumed 0.9-2.0 mg kg^{-1} and the most serious cases had consumed 1.9-4.2 mg kg^{-1}. After the 1987 ASP event, the Canadian government enacted a 20 μg DA g^{-1} mussel flesh action limit, which when exceeded, would authorize closure of the shellfish bed (Waldichuk 1989). This action limit has also been adopted by the European Union, New Zealand, United States and Australia.

Domoic Acid Poisoning

The first documented case of domoic acid poisoning (DAP) involved brown pelicans *Pelicanus occidentalis* and Brandt's cormorants *Phalacrocorax penicillatus* in 1991 in Monterey Bay (Fritz et al. 1992, Work et al. 1993a,b). The birds exhibited strange behaviors indicative of central nervous system disorder, such as scratching, head weaving, wryneck, clenched toes and loss of righting reflex. Histological examination of brain tissue from dead birds revealed lesions similar to those found in the human brains during the 1987 Canadian ASP event (Scholin et al. 2000). Plankton samples collected in Monterey Bay at the time of

the incident showed phytoplankton assemblages to be almost unialgal *P. australis* at 4 X 10^4 cells L^{-1} maximum concentration. *Pseudo-nitzschia australis* frustules were found in the birds' stomachs at 1.5 X 10^6 recognizable *P. australis* fragments g^{-1} wet w and in the stomachs of the birds' prey, the northern anchovy *Engraulis mordax* (Fritz et al. 1992). This incident showed that a second species of *Pseudo-nitzschia* could produce DA at sufficient levels to cause a toxic event and that planktivorous fin fish can be vectors of DA to higher trophic levels (Garrison et al. 1992).

In 1996, 150 brown pelicans *Pelecanus occidentalis* died at the tip of the Baja Peninsula, Mexico from feeding on mackerel *Scomber japonicus* contaminated with DA from an unknown *Pseudo-nitzschia* sp. (Sierra-Beltran et al. 1997). The birds' stomachs were empty, indicating recent vomiting, but smears from the digestive tract revealed *Pseudo-nitzschia* frustules. Viscera from pelicans and mackerel tested positive for DA by HPLC. Another incident in the Gulf of California in 1997 killed 766 common loons *Gavia immer* and 182 sea mammals belonging to 4 different species including the common dolphin (Sierra-Beltran et al. 1998). Microscopic analysis found *P. australis* frustules in the stomachs of the common dolphin *Delphinus capensis* and the sardine *Sardinops sagax* found inside the dolphin stomachs. Histological examination of dolphin brain tissue showed distinct lesions.

Another DAP event occurred in Monterey Bay in 1998, this time affecting over 400 California sea lions *Zalophus californianus* in addition to marine birds (Scholin et al. 2000, Gulland et al. 2002). Again *E. mordax* was acting as a

vector, delivering DA from a local *P. australis* bloom (maximum concentration ~1.3 X 10^5 cells L^{-1}) that had responded to high Si levels, indicative of terrestrial run-off (Scholin et al. 2000). DA was found in *E. mordax*, sea lion body fluids and plankton samples, but not in mussels, *Mytilus edulis*. Of the 400 sea lions affected, only 81 were found alive and transported to The Marine Mammal Center where 48 died despite treatment (Scholin et al. 2000, Gulland et al. 2002). All affected animals displayed neurological symptoms: seizures, head weaving, ataxia, unresponsiveness and abnormal scratching. Five females had fetuses detectable by ultrasound, but no detectable fetal heartbeats (Gulland et al. 2002). Histological examination of brain tissue from the 48 dead sea lions revealed lesions in the hippocampus. This event recurred in 2000, with 184 sea lion strandings (Gulland et al. 2002). Between the two events, 129 animals recovered and were released. Eleven re-stranded within four months giving a re-stranding rate of 9%, 0.5% higher than re-stranding rates for sea lions rehabilitated for other reasons (Gulland et al. 2002). Two of these animals had an atrophied hippocampus and were euthanized. Eight animals appeared normal after a week of treatment and re-released (Gulland et al. 2002).

Symptoms of ASP and DAP have many similarities consistent with the pharmacology of DA and the effects of its biochemical analog kainic acid and glutamic acid in animal models (Perl et al. 1990, Hampson & Manalo 1998, Schrader & Langlois 2001). The distinctive characteristic of DA poisoning appears to be permanent damage to the hippocampus, which can be detected even after DA has been eliminated from the body (Cendes et al. 1995, Gulland et al.

2002). Long term memory effects and/or chronic siezures have been documented in affected humans (Cendes et al. 1995), but only seizures have been documented in animals (Gulland et al. 2002).

Food Web Interactions

As a diatom, *Pseudo-nitzschia* is an important primary producer at the base of the food web. It is consumed directly by a wide variety of organisms from heterotrophic dinoflagellates to planktivorous fish (Table 1.2). It can form dense blooms and be an important source of food for these primary consumers, thereby introducing DA into higher trophic levels. As a hydrophilic molecule, it does not bioaccumulate. Instead, DA is concentrated in the digestive system with little transfer to surrounding tissues and can be quickly eliminated from the body. The toxin is moved through the food chain during blooms when primary consumers with guts full of *Pseudo-nitzschia* are eaten by secondary consumers. Eventually DA is depurated, but depuration rates can vary, from hours in the blue mussel (*M. edulis*), Mediterranean cockle (*Acanthocardia tuberculatum*), and greenshell mussel (*Perna canaliculus*), to several days in the mussel *M. galloprovincialis* (Novaczek et al. 1992, Wohlgeschaffen et al. 1992, Mackenzie et al. 1993, Vale & Sampayo 2002). Three bivalves that are very slow to depurate are the razor clam *Siliqua patula* (> 86 days), the scallop *Placopecten magellanicus* (> 14 days) and the scallop *Pecten maximus* (~ 416 days; Wohlgeschaffen et al. 1992, Horner et al. 1993, Douglas et al. 1997, Blanco et al. 2002).

Differential DA accumulation is an important factor in commercial species which are often not eaten whole. Most scallops, for example, are dissected and only the adductor muscle is eaten, which is the tissue containing the least amount of DA (Douglas et al. 1997, Blanco et al. 2002). Many commercially harvested animals, such as crabs, fish and cephalopods, retain most of the DA in their viscera, not typically consumed by humans (Horner & Postel 1993, Costa et al. 2003, Costa & Garrido 2004, Costa et al. 2004, Costa et al. 2005). However, studies do show trace amounts of DA in consumable tissues. Animal feeds that consist of whole fish captured during *Pseudo-nitzschia* blooms can be contaminated and sicken animals far from coasts or toxic blooms (Naar et al. 2002). However, DA in rainbow trout (*Oncorhynchus mykiss*) feed containing fish meal made from contaminated anchovies did not affect fish health nor lead to contaminated trout (Hardy et al. 1995). Hence, differential distribution of DA in vector tissues can be important for processing of commercially harvested species.

Packaging and handling procedures have an effect on which tissues are toxic. During storage, for example, DA can transfer from the digestive system into surrounding tissues (Smith et al. 2006). Freezing and thawing can affect distribution of DA within crab tissues (Hatfield et al. 1995, Costa et al. 2003). Storage in pickling brine or frozen storage can cause DA to leach into the surrounding medium (Leira et al. 1998). Removal or flushing of the digestive tract or hepatopancreas can decrease DA in bivalves (Leira et al. 1998, Campbell et al. 2003). Boiling of toxic animals before ingestion can also reduce DA body burdens by causing the toxin to leach into the boiling media (Costa et al. 2003).

Many different types of organisms can have DA in their tissues; however, not all of these organisms are filter feeders or their predators. Scavengers and deposit feeders have also been found to contain DA (Goldberg 2003). Scavengers could become contaminated by eating DA contaminated remains. Deposit feeders could become contaminated by consuming flocs of *Pseudo-nitzschia* that sink to the benthos at the end of a bloom (Goldberg 2003). Some carnivores, like the swimming crab *Polybius henslowii*, can contain high levels of DA but there have been no recorded incidents of poisoning in their predator, the yellow-legged gull (*Larus cachinnans*) that feeds on them almost exclusively (Alvarez 1968, Munilla 1997, Costa et al. 2003). There may be a limit in the number of trophic transfers over which DA can still be present at high enough concentration to cause a toxic event: to date all recorded DAP and ASP events involved only three trophic levels, *Pseudo-nitzschia*, a bivalve or a planktivorous fish and a bivalve or fish predator.

Monitoring

Due to its variable toxicity and cosmopolitan distribution, *Pseudo-nitzschia* poses a unique management challenge worldwide. Monitoring via traditional microscopic (generally light) techniques for cell enumeration and identification can be too time consuming for regions with immediate ASP or DAP threats. Since the presence of *Pseudo-nitzschia* does not guarantee the presence of DA, abundance data alone are rarely a sufficient basis for management decisions. In addition, consumer illness is not from DA in plankton or in water; a

seafood vector must be involved. This means that an effective monitoring program will include *Pseudo-nitzschia* identification and enumeration, DA quantification and testing of potential vectors.

Traditional plankton sampling techniques are typically useful for *Pseudo-nitzschia* monitoring; however, there are situations when this sampling method may be inadequate. Physical and biological processes are known to concentrate *Pseudo-nitzschia* into subsurface layers from several meters (Ryan et al. 2005) to less than a meter thick (Rines et al. 2002). These cells can be transported long distances and inoculate unexpected blooms at the surface (Bates & Trainer 2006). *Pseudo-nitzschia* can also be missed when intermingled in colonies of *Chaetoceros socialis* (Rines et al. 2002) instead of free in the water column. Oceanographic characteristics of a region should be taken into account when developing a sampling program so that any heterogenous distributions of *Pseudo-nitzschia* in the water column can be defined.

Several days may pass from the time a water sample is collected to identification of *Pseudo-nitzschia* species via electron microscopy (generally not available to most routine monitoring programs). This can be too long and inadequate to separate toxic from non-toxic strains. Molecular probes have come into use to increase speed of identification and resolution between genetically distinct strains. Molecular probes are designed to adhere to a specific set of molecules associated with a particular algal group and can be used as a basis for detecting these groups in natural samples. There are three types of molecular probes: lectin-binding, antibodies or nucleic acid.

Lectin-binding probes have been used to discriminate between species of *Pseudo-nitzschia* with limited success. Trials with New Zealand derived cultures were able to discriminate between 6 of 7 *Pseudo-nitzschia* species tested; *P. delicatissima* and *P. pseudodelicatissima* could not be distinguished (Rhodes 1998a). All 7 species tested from Spain and all 3 species tested from Korea were successfully identified using lectin binding patterns, but there were differences among strains of the same species (Fraga et al. 1998, Cho et al. 1999). Lectin binding patterns can differ between strains of the same species depending on origin and possibly physiological condition and therefore are not broadly useful as a monitoring tool.

Antibodies can be designed to bind to molecules (antigens) like glycoproteins, peptides, N-containing carbohydrates or toxins. Antibodies have been developed and used for identification of *P. pungens* and *P. multiseries*, but immunoassays are much more commonly used to detect DA in shellfish and water samples (Bates et al. 1993b, Vrieling et al. 1996, Rhodes et al. 1998a).

DNA probes for detecting species have been targeted at small subunit (SSU), large subunit (LSU) and internal transcribed spacer (ITS) regions of rRNA. There are several types of rRNA targeted probes: whole-cell hybridization (labeling of intact cells), fluorescent in-situ hybridization (FISH), sandwich hybridization (measuring DNA in cell homogenate) and PCR methods (PCR replication of targeted genome). New Zealand is the first country to use molecular probes for routine harmful algal bloom (HAB) monitoring and risk assessment (Rhodes et al. 1998a). Design of these probes depends on obtaining rRNA sequences with

desired specificity. This can be a challenge for *Pseudo-nitzschia*, a group with high intra- and interspecific variability. Whole cell fluorescent LSU targeted probes have been developed for identifying *P. australis*, *P. pungens*, *P. multiseries*, *P. heimii*, *P. fraudulenta*, *P. delicatissima*, *P. pseudodelicatissima* and *P. americana* in cultures originating from the west coast of the United States (Miller & Scholin 1996). These probes were then used to develop whole cell and sandwich hybridization methods for detection of species in natural samples at near real-time (Scholin et al. 1997, Miller & Scholin 1998). Probes for *P. multiseries*, *P. pungens*, *P. fraudulenta* and *P. delicatissima* have been used successfully in the Gulf of Mexico, US west coast, the Wadden Sea and Korean waters (Miller & Scholin 1996, Vrieling et al. 1996, Miller & Scholin 1998, Parsons et al. 1999, Cho et al. 2002). However, not all rRNA probes work in all regions. The probe designed to work on *P. pseudodelicatissima* from the west coast of the United States did not work on *P. pseudodelicatissima* in samples from the Gulf of Mexico (Parsons et al. 1999). This could be because of high genetic variability within the *P. pseudodelicatissima* group, which was later divided into additional species based on morphological and molecular analysis (Lundholm et al. 2003). Designing *Pseudo-nitzschia* species-specific probes in some regions, like the Chesapeake Bay, are particularly challenging because of high variability within morphologically defined species (H. Bowers pers. comm). In some regions, like the Gulf of Naples, genus-specific probes based on clone libraries have been used to successfully separate species, with multiple probes used to identify *P. delicatissima* and *P. pseudodelicatissima*, two morphologically

defined species that probably include cryptic species (McDonald et al. 2007). RNA-based detection methods are particularly useful when strain differences and cryptic species are important.

After determining the abundance and species of *Pseudo-nitzschia* present, further management decisions can be made. Some countries use "trigger levels" of abundance to initiate shellfish sampling. In New Zealand, when *Pseudo-nitzschia* is > 50% of total phytoplankton, a concentration of 5 X 10^4 cells L^{-1} will trigger shellfish sampling. When *Pseudo-nitzschia* is < 50% of total phytoplankton, a concentration of 10^5 cell L^{-1} will trigger shellfish sampling. DA is quantified by HPLC and UV detection. If shellfish contain more than 20 mg kg^{-1} DA, harvesting is stopped until three consecutive samples spaced out over at least 2 weeks have < 20 mg DA kg^{-1} (Todd 2003). Countries with limits for DA in shellfish are Canada, USA, New Zealand, Chile, Peru and EU member states. The limit is 20 mg kg^{-1} edible meat. A limit of 30 mg DA kg^{-1} in cooked viscera of Dungeness crab has been set by the FDA in the United States. In order to minimize economic impact of DA contamination, the EU allows harvest of scallops with whole body burdens of DA between 20 and 250 mg kg^{-1} if they are sold after total removal of the hepatopancreas (Fernandez et al. 2003).

Summary

Twenty years ago, an outbreak of food poisoning in Canada made *Pseudo-nitzschia* the first known toxic diatom. Since that time, our increased

understanding of DA and *Pseudo-nitzschia* has prevented additional ASP outbreaks and led to new ideas about diatom physiology. Molecular methods are promising new innovations in *Pseudo-nitzschia* taxonomy, biogeography and functional diversity. New DA quantification methods are decreasing analytical limits of detection and enabling detailed physiological studies. Management plans have been developed using these technologies to prevent ASP outbreaks with minimal economic loss. However, DAP events have occurred multiple times and show no sign of being controlled. Despite 20 years of research, further work is needed to fully understand *Pseudo-nitzschia* taxonomy, ecology and physiology.

Research Questions and Approaches

Detailed regional studies are an important source of information on *Pseudo-nitzschia* ecology and distribution. The Chesapeake Bay is one of the largest estuaries in the United States, which hosts a large human and water fowl population and multiple fisheries. Many types of harmful algae can be found in the Chesapeake, including *Pseudo-nitzschia*. Very little research has been conducted on mid-Atlantic estuarine *Pseudo-nitzschia* despite evidence of toxicity in regional isolates. Monitoring of potentially harmful algae by Maryland state government does not include examination by electron microscopy or toxin analysis. The first objective of this thesis was a regional survey of *Pseudo-nitzschia* species and DA in the Chesapeake Bay to fill a gap in knowledge and improve harmful algae monitoring in the area (Chapter 2).

Physiological studies testing many strains are important for assessing intra- and interspecies variation, genetic variation and functional diversity. Many *Pseudo-nitzschia* studies test only one strain and extrapolate results to all species and strains. Functional diversity between strains in growth rate, nutrient uptake and toxin production can vary widely. This functional diversity could indicate the presence of cryptic species or ecotypes within a regional population. Understanding this variability is an important part of applying culture studies to natural populations and describing *Pseudo-nitzschia* ecology. Thus the second objective was characterization of inter- and intraspecies (strain) differences in growth rate and toxicity of *Pseudo-nitzschia* (Chapter 3).

An important, yet poorly understood part of *Pseudo-nitzschia* physiology is DA production. *Pseudo-nitzschia* is found globally, but is common and toxic during periods of low temperature and fluctuating irradiance such as spring, fall and in upwelling zones. Could *Pseudo-nitzschia* reduce nitrate as an adaptation to cold water environments similar to other diatoms found under similar conditions? How would this increase in NO_3^- reduction and change in photosynthetic energy flow affect DA production? An increased understanding of toxin production physiology would not only aid in the prediction and mitigation of toxic blooms, but add to knowledge of diatom biology and cellular processes. The third objective of this thesis was to test two hypotheses: that nitrogen metabolism in *Pseudo-nitzschia* can be used as an adaptation to rapid light fluctuations in a cold environment and that this adaptation affects DA production (Chapter 4).

A survey of *Pseudo-nitzschia* species and DA in the field, an assessment of intra- and interspecies differences and an investigation of DA production during periods of decoupled photosynthesis and growth are an important part of understanding the taxonomy and ecophysiology of *Pseudo-nitzschia* in an estuarine environment such as the Chesapeake Bay.

Table 1.1 Diatoms with documented domoic acid production

Species	Reference
Amphora coffeaeformis	**Shimizu et al. 1989**
Nitzschia navis-varingica	**Kotaki et al. 2000**
Pseudo-nitzschia australis	**Fritz et al. 1992**
P. calliantha	**Martin et al. 1990**
P. cuspidata	**Bill et al. 2005**
P. delicatissima	**Smith et al. 1991**
P. fraudulenta	**Rhodes et al. 1998b**
P. galaxiae	**Cerino et al. 2005**
P. multiseries	**Bates et al. 1989**
P. multistriata	**Rhodes et al. 2000**
P. psuedodelicatissima	**Lundholm et al. 1997**
P. pungens	**Rhodes et al. 1996**
P. seriata	**Lundholm et al. 1994**
P. turgidula	**Rhodes et al. 1996**

Table 1.2: Species known to ingest *Pseudo-nitzschia* and/or become contaminated with domoic acid

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Protists				
Dinoflagellate	*Gyrodinium* sp.	Monterey Bay, CA	frustules in fecal pellets	Buck et al. 2005
Dinoflagellate	*Noctiluca scintillans*	NW Spain	cells in food vacuoles	Escalera et al. 2007
Dinoflagellate	*Protoperidinium* spp.	Oslofjord, Norway	abundances of *Protoperidinium* spp. tracked abundances of *Pseudo-nitzschia*	Kjært et al. 2000
Echinoderms				
Sand dollar	*Dendraster excentricus*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Mollusks				
Olive snail	*Olivella biplicata*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Channeled basket whelk	*Nassarius fossatus*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Mussel	*Mytilus californianus*	San Diego, CA	DA in tissue	Busse et al. 2006
Mussel	*Mytilus galloprovincialis*	Riá de Muros, Spain	DA in tissue	Blanco et al. 2002
Blue mussel	*Mytilus edulis*	New Brunswick, Canada; Nantucket Shoals; Georges Bank; Washington coast; Japan and Aviero Lagoon, Portugal	DA in tissue, fed on *Pseudo-nitzschia* in the lab and accumulated DA	Haya et al. 1991, Wohlgeschaffen et al. 1992, Douglas et al. 1997, Horner and Postel 1993, Kawatsu and Hamano 2000, Vale and Sampayo 2001

Table 1.2 cont.

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Mollusks				
Greenshell mussel	*Perna canaliculus*	New Zealand	fed on *Pseudo-nitzschia* in lab and accumulated DA	Mackenzie et al. 1993
Horse mussel	*Modiolus modiolus*	Atlantic Canada	DA in tissue	Gilgan et al. 1990
Rock mussels		Portugal	DA in tissue	Vale and Sampayo 2001
Mediterranean cockle	*Acanthocardia tuberculatum*	Algarve, Portugal	DA in tissue	Vale and Sampayo 2001
Common cockle	*Cerastoderma edule*	Mondego estuary and Aveiro Lagood, Portugal	DA in tissue	Vale and Sampayo 2001
King scallops	*Pecten maximus*	Scotland	DA in tissue	Smith et al. 2006, Campbell et al. 2003
Scallop	*Placopecten magellanicus*	New Brunswick, Canada	DA in tissue, fed on *Pseudo-nitzschia* in the lab and accumulated DA	Haya et al. 1991, Wohlgeschaffen et al. 1992, Douglas et al. 1997
Sea scallop		Eastport, Maine	DA in digestive gland	Shumway 1989
Soft-shell clams	*Mya arenaria*	New Brunswick, Canada; Atlantic Canada	DA in tissue	Haya et al. 1991, Gilgan et al. 1990
Razor clams	*Siliqua patula*	California/Washington/ Oregon coasts	DA in tissue	Horner et al. 1993, Horner and Postel 1993, Wekell et al. 1994
Razor clam	*Ensis* spp.	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo 2002
Clam	*Ruditapes decussata*	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo 2002

Table 1.2 cont.

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Mollusks				
Carpet shell	*Venerupis pullastra*	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo 2002
European flat oyster	*Ostrea edulis*	Aveiro Lagoon, Portugal	DA in tissue	Vale and Sampayo 2002
Pacific Oyster	*Crassostrea gigas*	Puget Sound, WA	DA in tissue	Horner and Postel 1993
Common octopus	*Octopus vulgaris*	Portugese coast	DA in gut, digestive gland, brachial heart	Costa et al. 2004
Squid	*Loligo opalescens*	Monterey Bay, CA	DA is tissues, frustules in stomach	Bargu et al. 2007
Common cuttlefish	*Sepia officinalis*	Portugese coast	DA in digestive gland	Costa et al. 2005
Annelids				
Polychaete worm	*Poebius meseres*	Monterey Bay, CA	frustules found in gut	Uttal and Buck 1996, Buck et al. 1992
Echiurans				
Fat innkeeper worm	*Urechis caupo*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Crustaceans				
Sand crab	*Emerita analoga*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Swimming crab	*Polybius henslowii*	Portugese coast	DA in viscera, tissue	Costa et al. 2003
Blue band hermit crab	*Pagurus samuelis*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Blue crab	*Callinectes sapidus*	US Pacific Coast	DA in tissue	Altwein et al. 1995

Table 1.2 cont.

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Crustaceans				
Rock crab	*Cancer pagurus*	US Pacific Coast	DA in tissue	Altwein et al. 1995
Dungeness crab	*Cancer magister*	California/ Oregon/ Washington coasts	DA in tissue	Horner and Postel 1993, Wekell et al. 1994
Stone crab	*Menippe adina*	US Pacific Coast	DA in tissue	Altwein et al. 1995
Ghost shrimp	*Callianassa californiensis*	Monterey Bay, CA	DA in tissue	Goldberg 2003
Copepod	*Acartia tonsa*		grazed on and accumulated DA in the lab	Lincoln et al. 2001
Copepod	*Acartia clausi*		grazed on *Pseudo-nitzschia* in the lab, accumulated DA in tissues	Maneiro et al. 2005
Copepod	*Temora longicornis*		grazed on and accumulated DA in the lab	Lincoln et al. 2001, Windust 1992
Copepod	*Calanus glacialis*		grazed on *Pseudo-nitzschia* in the lab	Windust 1992
Krill	*Euphausia pacifica*	Monterey Bay, CA	frustules in gut and DA in tissue	Bargu et al. 2002, Bargu and Silver 2003
Spiny lobster	*Palinurus elephas*	US Pacific Coast	DA in tissue	Altwein et al. 1995
Gooseneck barnacles	*Pollicipes pollicipes*	Portugal	DA in tissue	Vale and Sampayo 2002
Fishes				
Northern Anchovey	*Engraulis mordax*	Monterey Bay, CA	frustules and DA in gut, DA in tissues	Fritz et al. 1992, Work et al. 1993a, Lefebvre et al. 1999, Lefebvre et al. 2002, Scholin et al. 2000, Wekell et al. 1994

Table 1.2 cont.

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Fishes				
European anchovy	*Engraulis encrasicolus*	Portugal	DA in tissue	Vale and Sampayo 2002
Mackerel	*Scomber japonicus*	Cabo San Lucas, Mexico; Monterey Bay, CA, San Diego, CA	frustules and DA in gut	Sierra-Beltran et al. 1997, Lefebvre et al. 2002, Busse et al. 2006
Jack mackerel	*Trachurus symmetricus*	San Diego, CA	DA in viscera	Busse et al. 2006
Herring		Monterey Bay, CA	frustules and DA in gut	Naar et al. 2002
Sardine	*Sardinops sagax*	Baja California peninsula	frustules and DA in gut	Sierra-Beltran et al. 1998, Lefebvre et al. 2002
Atlantic sardine	*Sardina pilchardus*	Portugese and Namibian coast	frustules and DA in gut, DA in tissues	Costa and Garrido 2004, Vale and Sampayo 2002, Doucette pers. comm.
Pacific sanddab	*Citharichthys sordidus*	Monterey Bay, CA, San Diego, CA,	DA in viscera and tissue	Lefebvre et al. 2002, Busse et al. 2006, Goldberg 2003
Albacore	*Thunnas alalunga*	Monterey Bay, CA	DA in viscera	Lefebvre et al. 2002
Jack smelt	*Atherinopsis californiensis*	Monterey Bay, CA	DA in viscera	Lefebvre et al. 2002
Longspine combfish	*Zaniolepis latipinnus*	San Diego, CA	DA in viscera	Busse et al. 2006

Table 1.2 cont.

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Birds				
Brown pelican	*Pelecanus occidentalis*	Monterey Bay, CA; Cabo San Lucas, Mexico	frustules and DA in gut, death, neurological disorder	Fritz et al. 1992, Work et al. 1993ab, Sierra-Beltran et al. 1997
Brandt's cormorant	*Phalacrocorax penicillatus*	Monterey Bay, CA	frustules and DA in gut, death, neurological disorder	Fritz et al. 1992, Work et al. 1993ab
Double-crested cormorant	*Phalacrocorax auritus*	Monterey Bay, CA	frustules and DA in gut, death, neurological disorder	Fritz et al. 1992, Work et al. 1993ab
Pelagic cormorant	*Phalacrocorax pelagicus*	Monterey Bay, CA	frustules and DA in gut, death, neurological disorder	Fritz et al. 1992, Work et al. 1993ab
Western gull	*Larus occidentalis*	Monterey Bay, CA	frustules and DA in gut, death, neurological disorder	Fritz et al. 1992, Work et al. 1993ab
King penguin	*Aptenodytes forsteri*	Kentucky Zoo	death, frustules and DA in their food	Naar et al. 2002
Common loon	*Gavia immer*	Baja California peninsula	death	Sierra-Beltran et al. 1998
Mammals				
Common dolphin	*Delphinus capensis*	Baja California peninsula	frustules in gut, DA in tissues, brain lesions, death	Sierra-Beltran et al. 1998
Humpback whale	*Megaptera novaeangliae*	Monterey Bay, CA	frustules and DA in feces	Lefebvre et al. 2002
Blue whale	*Balaenoptera musculus*	Monterey Bay, CA	frustules and DA in feces	Lefebvre et al. 2002
Right Whale	*Eubalaena glacialis*	North Atlantic	DA present in body	Bates and Trainer 2006
California Sea lion	*Zalophus californianus*	Monterey Bay, CA	death, neurological disorder, frustules and DA in feces, DA in body fluids	Scholin et al. 2000, Gulland et al. 2002

Table 1.2 cont.

Organism		Location	Evidence	Reference
Common Name	Scientific Name			
Mammals				
Southern Sea Otter	*Enhydra lutris nereis*	California coast	DA in urine	Kreuder et al. 2003
Human	*Homo sapiens*	Prince Edward Island, Canada	death, neurological disorder, DA in food, brain lesions	Perl et al. 1990, Teitelbaum et al. 1990, Cendes et al. 1995

Figure 1.1 Diagram showing diatom frustule morphology: A) Broad girdle view and valve view. B) Cross-sectional view of *Pseudo-nitzschia* spp. and *Nitzschia* spp. for comparison. Note the differences in the raphe. Created using information from Hasle and Syvertsen (1996).

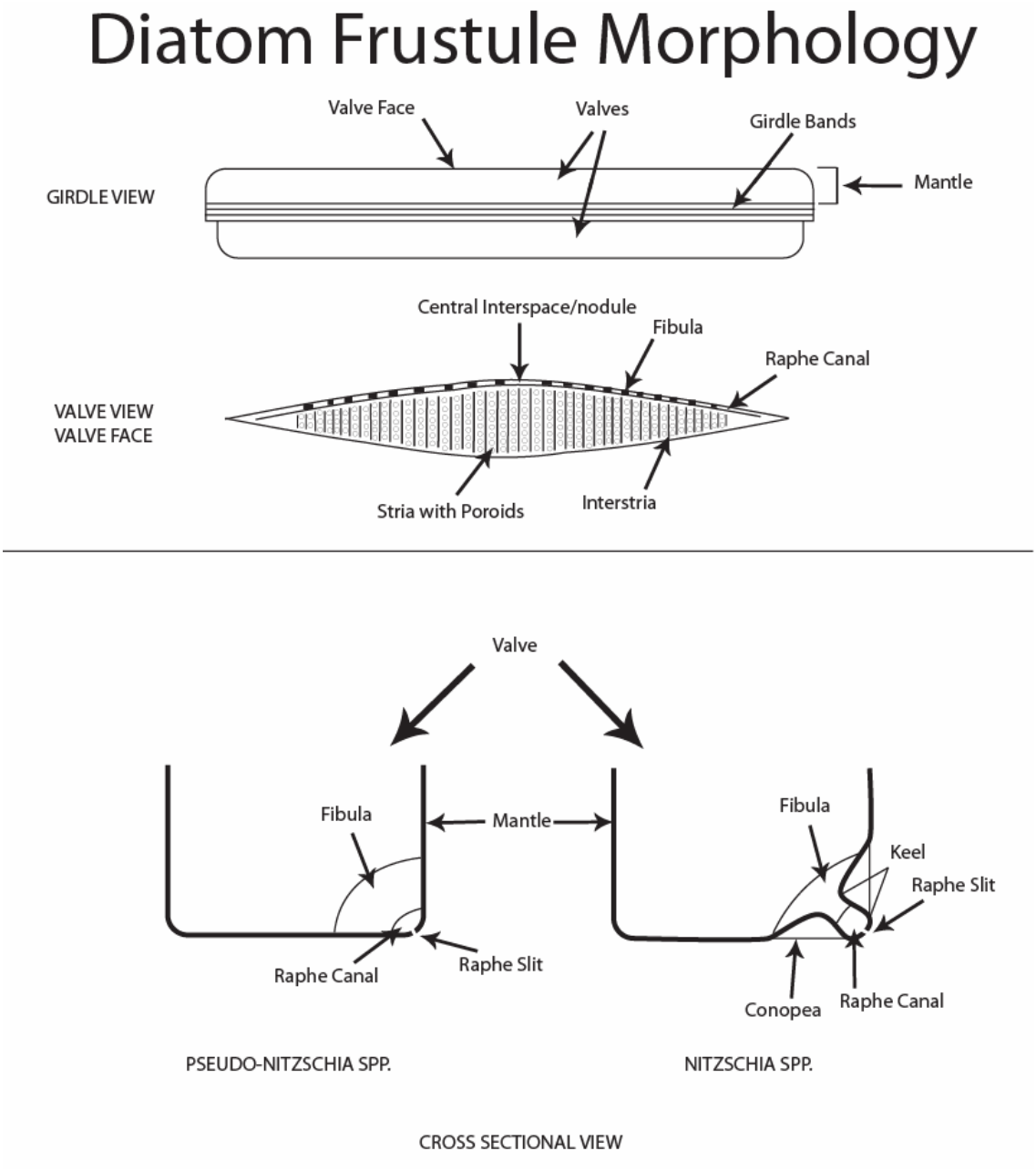


Figure 1.2 World map showing the global distribution of *Pseudo-nitzschia* spp. Symbols outlined in blue indicate the report was made before a major taxonomic revision of the species. References used to create map are listed in Appendix A.

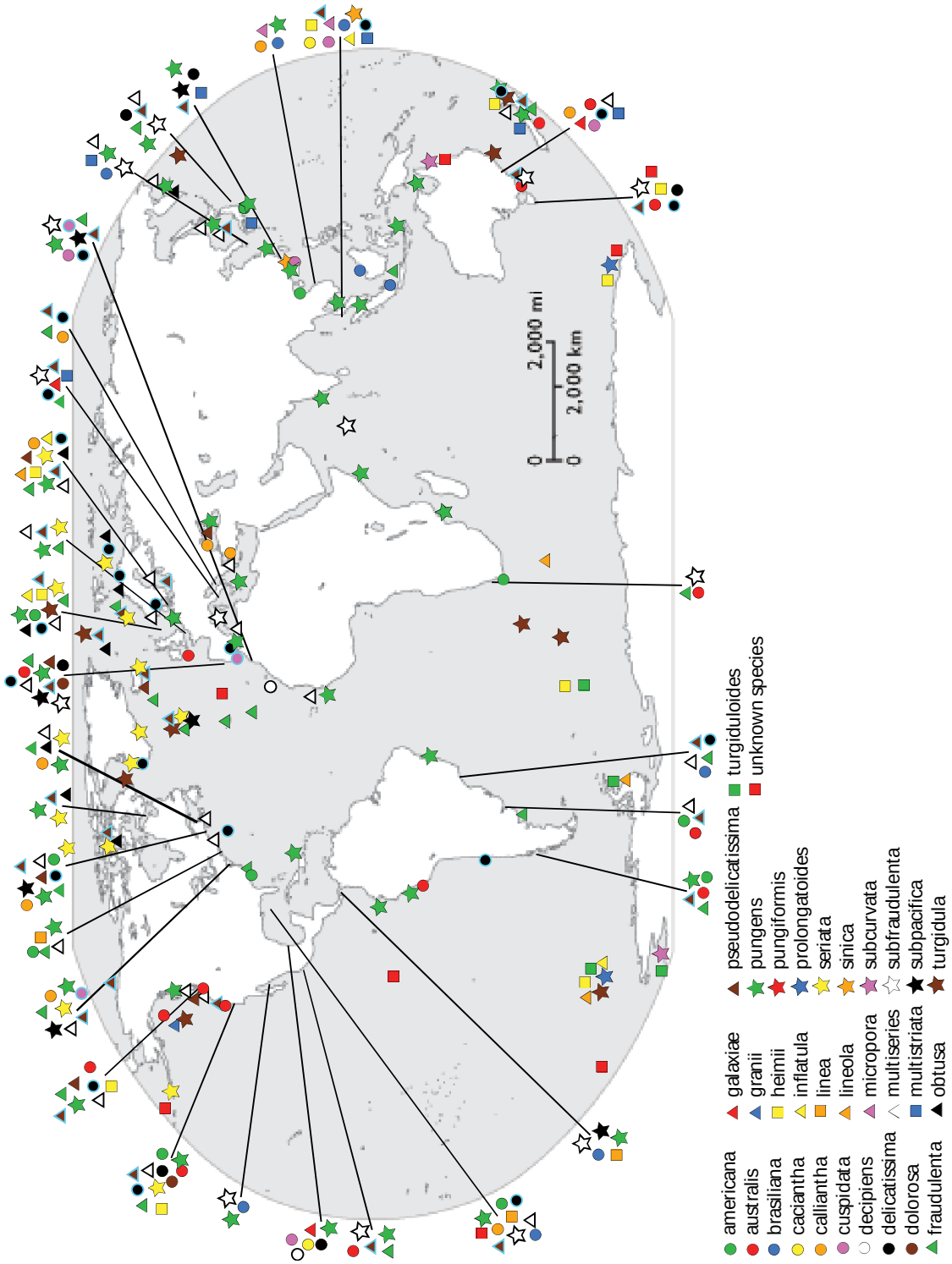
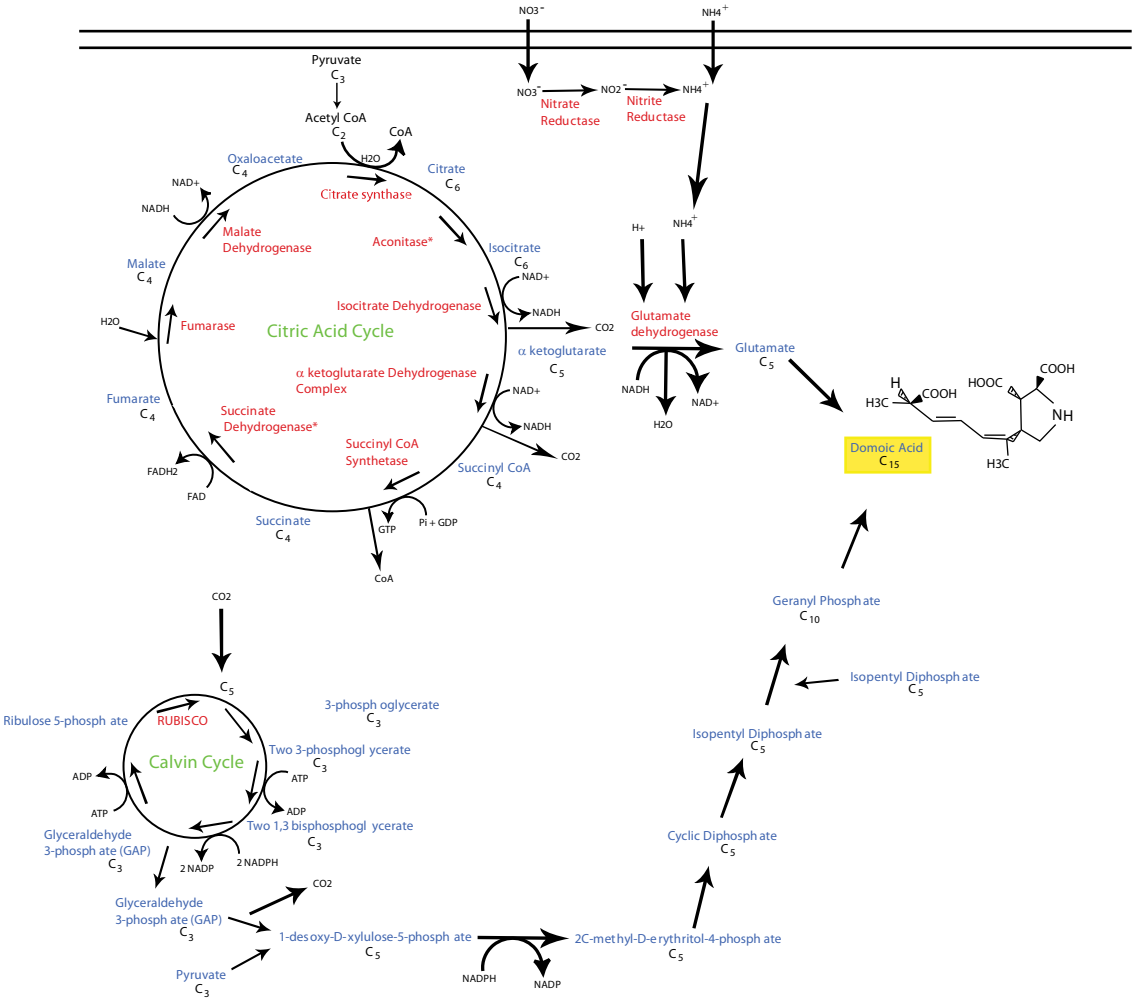


Figure 1.3 Diagram showing DA biosynthesis inside the cell. Enzymes are in red. Products are in blue. The * indicates the enzyme contains Fe. Created using information from Pillay (2006), Douglas et al. (1994), Laycock et al. (1989), Ramsey et al. (1998) and Stryer (1995).



Chapter 2: Distribution, abundance and domoic acid analysis of the toxic diatom genus *Pseudo-nitzschia* from the Chesapeake Bay

Abstract

Very little research has been conducted on mid-Atlantic estuarine populations of the diatom *Pseudo-nitzschia* despite recent evidence of toxicity in regional isolates. We collected field samples from the Chesapeake Bay region from 2002 to 2006 for *Pseudo-nitzschia* enumeration and toxin analyses. Abundances of *Pseudo-nitzschia* were highest in the winter and spring at ~10^3 cells mL^{-1}. Domoic acid was detectable in 46% of samples tested, but concentrations were generally low, ranging from 4 to 1037 pg DA mL^{-1}. Although *Pseudo-nitzschia* populations were observed year round when salinity was ≥ 5, populations were highest from February to May when temperatures were low (2-15°C) and salinity relatively high (≥ 10). Six species of *Pseudo-nitzschia* were identified via transmission electron microscopy of the samples: *P. pungens* (Grunow ex P. T. Cleve) Hasle, *P. calliantha*, *P. subpacifica* (Hasle) Hasle, *P. cuspidata* (Hasle) Hasle emend. Lundholm, Moestrup et Hasle, *P. fraudulenta* and *P. multiseries*. *P. calliantha* was the most common and not previously reported from the Chesapeake Bay. Of these species, *P. pungens*, *P. calliantha*, *P. cuspidata*, *P. fraudulenta* and *P. multiseries* are known to produce DA.

Introduction

Pseudo-nitzschia is a diatom genus known to produce the excitatory neurotoxin, domoic acid (DA), causing Amnesic Shellfish Poisoning (ASP) in humans and Domoic Acid Poisoning (DAP) in marine mammals and birds. *Pseudo-nitzschia* blooms are common in coastal waters, but were recognized as

being potentially toxic only 20 years ago (Bates et al. 1989). Although *Pseudo-nitzschia* is found world-wide, toxic events have been reported from only a few geographic regions, specifically: Atlantic Canada and the west coast of North America (Perl et al. 1990, Fritz et al. 1992, Sierra-Beltrán et al. 1998, Scholin et al. 2000). There have been numerous reports of domoic acid (DA) in shellfish above the regulatory limit of 20 μg g^{-1} from the Atlantic and the Pacific since 1987. It is not known if this is because toxic *Pseudo-nitzschia* are becoming more abundant, conditions are becoming more conducive to DA production, or if DA was previously unrecognized or underreported in some regions.

Eight species of *Pseudo-nitzschia* have previously been identified in the Chesapeake Bay by light and electron microscopy (Hasle 1965, Marshall 1980, 1994, Marshall et al. 2005). In reports and databases from the Maryland Department of Natural Resources (MD DNR) phytoplankton monitoring program (where samples are analyzed by light microscopy only), *Pseudo-nitzschia* species are reported as either *P. seriata* or *P. pungens* (W. Butler, pers. comm.). *Pseudo-nitzschia* species in the Chesapeake were generally assumed to be non-toxic, and as a result there has been relatively little research on this genus in this region. However, data now demonstrate that regional isolates do produce domoic acid (Thessen et al. 2003).

Potentially toxic algae should be monitored in estuaries which have complex plankton dynamics and are subject to long term change and episodic events, even if these species have not previously caused a toxic event in the area. Seasonal variation in Chesapeake Bay planktonic communities is driven by

salinity, nutrients and light availability (Fisher et al. 1999, Roman et al. 2005). Interannual variation is in part a result of changes in freshwater discharge (Roman et al. 2005, Zhang et al. 2006). Both time scales of variation can be affected in the long term by eutrophication (Kemp et al. 2005) and climate change (Najjar et al. 2000) and by episodic events like hurricanes (Miller et al. 2006a).

Under the current climate regime and eutrophic conditions the chlorophyll *a* maximum in the Chesapeake occurs in spring (Harding 1994, Zhang et al. 2006). In the mesohaline and oligohaline region of the Bay this spring chlorophyll *a* peak represents an increase in diatoms, particularly *Skeletonema potamos, S. costatum, Cerataulina pelagica* and *Dactyliosolen fragilissimus* (Marshall et al. 2005). The spring diatom bloom can start as early as mid winter and continue into late spring with chlorophytes, cyanobacteria and cryptomonads increasing in abundance as the diatoms decline (Marshall et al. 2005). Eutrophication and climate forcing can change the spring bloom in the Chesapeake (Harding 1994, Miller & Harding 2007).

The Chesapeake Bay region's large human population, multiple fisheries and aquatic bird populations (Robbins & Bystrak 1977, Houde et al. 1999, Kemp et al. 2005) make it potentially vulnerable to ASP and DAP. It is important to document the occurrence of *Pseudo-nitzschia*, its spatial and temporal distribution and toxicity in order to assess the potential for toxic events in the region and where or when they might occur. We determined *Pseudo-nitzschia* species distribution, abundance, toxicity and relationship to season, salinity and temperature. These data are used to assess the potential for a toxic event in the

Chesapeake Bay area, to present a better understanding of the ecology of *Pseudo-nitzschia* species in the Chesapeake Bay region and contribute to the knowledge of *Pseudo-nitzschia* biogeography.

Materials and Methods

Water samples were collected throughout the Chesapeake Bay and selected locations along the Delmarva Atlantic Coast during the fall, winter and spring from 2002 to 2007 for *Pseudo-nitzschia* enumeration, species identification and toxin analysis (Fig. 2.1). Samples were collected from the surface and below the pycnocline, if present. Most samples were collected by MD DNR as part of their routine monitoring program. Additional samples were collected by A. E. T. or D. K. S. (HPL; Table 2.1). Average freshwater flow per month was calculated from data collected by the United States Geological Survey at the Connewingo Dam on the Susquehanna River, a large tributary to the Chesapeake Bay (www.usgs.gov).

MD DNR routinely monitors water quality and phytoplankton throughout the year (www.chesapeakebay.net). During fall, winter and spring (2004-2007) additional samples were collected specifically for *Pseudo-nitzschia* enumeration, species identification and DA analysis. During summer, when historical MD DNR data has shown *Pseudo-nitzschia* species to be low in abundance, specific samples for *Pseudo-nitzschia* analyses were not collected. We relied on data from the MD DNR database for summer *Pseudo-nitzschia* abundance. MD DNR collected water samples from all depths by pump for preservation in acid Lugol's

and enumeration with the Utermohl method (Hasle 1978). Salinity and temperature were measured with a Hydrolab sonde (Hach Company, Loveland, Colorado). Our laboratory collected surface samples with a bucket and samples at depth with a 10 L Niskin bottle either from a dock or small boat. Salinity and temperature data were collected with a CTD or a YSI 63.

The samples collected specifically for *Pseudo-nitzschia* analysis (100 mL; collected by MD DNR or the authors) were fixed with 0.5% gluteraldehyde (final concentration) for enumeration to genera via epifluorescence microscopy (Nikon Eclipse E800, excitation 450-490 nm, dichromatic beam splitter 500 nm, barrier filter 515 nm, Nikon filter set EF-4 B-2A; Guillard & Sieracki 2005) and morphological identification to species via transmission electron microscopy (TEM). Our limit of detection with epifluorescence was 4 cells mL^{-1}.

In 2003-2007, samples for DA analysis (300 mL) were collected and filtered through Whatman GF/F filters within a few hours. Both filter and filtrate were stored at -80°C in the dark. Only samples that were found to contain *Pseudo-nitzschia* spp. via epifluorescent microscopy were processed for domoic acid via ELISA. Only samples that were shown to contain particulate DA were processed for dissolved DA. DA was quantified via an ASP cELISA test kit from Biosense Laboratories capable of processing a maximum of 36 samples per kit (Bergen, Norway; Garthwaite et al. 1998). Each assay has a limit of quantitation (LOQ) and a limit of detection (LOD = 1/3 LOQ). Values below the LOQ and above the LOD are considered positive for DA, but semi-quantitative.

Samples with *Pseudo-nitzschia* abundances above 100 cells mL^{-1} were processed for TEM. Frustules were too sparse for identification at lower abundances. For morphological identification, we used the following method from Lundholm et al. (2002a). A 10 mL aliquot was removed from the preserved sample and digested using 2 mL of 30% H_2SO_4 and 10 mL of a saturated $KMnO_4$ solution in a 100 mL Erlenmeyer flask. The flask was covered with parafilm and swirled periodically throughout the digestion. After 24 hours, a saturated oxalic acid solution was added until the sample became transparent. Samples were rinsed 3-4 times with distilled, deionized water and pelleted via centrifugation. The pellet was resuspended in 1 mL of water. One drop was placed on a 200 μm mesh Copper grid with a formvar film. TEM was used to identify species of *Pseudo-nitzschia* based on frustule morphology by taking measurements of images on photographic negatives (Skov et al. 1999; Lundholm et al. 2002a,b; Lundholm & Moestrup 2002; Hasle & Lundholm 2005).

Temperature, salinity, freshwater flow and *Pseudo-nitzschia* abundance data from 2002 to 2006 were analyzed by Spearman's rank correlation at the 5% level (SPLUS®, version 6, Insightful Corporation, Seattle, WA, USA). Data was analyzed by season and year.

Results

We identified six species of *Pseudo-nitzschia* via TEM in field samples collected from 31 stations in Maryland, Virginia and Delaware waters: *P. calliantha, P. fraudulenta, P. pungens, P. subpacifica, P. cuspidata* and *P.*

multiseries (Table 2.1). The most common was *P. calliantha* which occurred at 15 out of 31 stations ranging in salinity from 32 on the Atlantic coast of Maryland (Station 29) to ~10 in the upstream portion of the Choptank River (Station 15; Table 2.1). The second most abundant taxon was *P. fraudulenta* which occurred at five out of 31 stations from the Atlantic coast of Maryland (Station 30, salinity 32) to the mesohaline Bay (Station 2, salinity 15; Table 2.1). *Pseudo-nitzschia pungens* was also detected on the Atlantic Coast of Maryland and Delaware (Stations 25 and 29, salinity 32) and in the mesohaline portion of the Chesapeake Bay (Station 3, salinity 15; Table 2.1). *Pseudo-nitzschia multiseries* was detected on the Atlantic Coast of Maryland (Station 30, salinity 32) and in the Choptank River (Station 16, salinity 20; Table 2.1). *Pseudo-nitzschia cuspidata* and *P. subpacifica* were both found near Tangier Sound in the mesohaline portion of the Chesapeake Bay (Stations 9 and 10, salinity 15-20; Table 2.1).

Samples were collected during fall, winter and spring from 31 stations in three states and found *Pseudo-nitzschia* spp. present at all but three stations however, not in a dense, monospecific bloom. In general, abundances were highest in early to mid-spring, corresponding to the annual diatom bloom (Malone et al. 1988). Maximum *Pseudo-nitzschia* abundance was 6254 cells mL^{-1} in the Pocomoke Sound in April of 2004 (Fig. 2.2; Station 20). *Pseudo-nitzschia* abundances and species varied among stations (Table 2.1). Some stations (e.g. 5) had detectable *Pseudo-nitzschia* via epifluorescence microscopy, but had less than the 100 cells mL^{-1} needed for morphological identification via TEM. The routinely sampled stations where *Pseudo-nitzschia* was present most frequently

were 14 (63%) at the mouth of the Patuxent River, 9 (56%) in Tangier Sound, 17 (55%) at the mouth of the Choptank River, 4 (32%) in the middle of the Chesapeake Bay and 7 (30%) near the mouth of the Potomac River (Table 2.1; Figure 2.1).

Although many of our samples contained *Pseudo-nitzschia*, most did not contain detectable and quantifiable DA (Fig. 2.3). DA concentrations in field samples that tested positive ranged from 3.9 pg DA mL^{-1} in a Maryland coastal bay in November of 2004 (station 31) to 1037.2 pg DA mL^{-1} on the Atlantic Coast of Maryland in March of 2004 (station 29). Sixty-two percent of the toxin samples processed from station 4 in mid-Chesapeake Bay were positive for DA as were 41% of the samples processed from station 9 in Tangier Sound and 17% of the samples processed from station 3 in the upper Chesapeake Bay (Table 2.1; Fig 2.1). Species present in samples with detectable DA were *P. calliantha*, *P. fraudulenta*, *P. subpacifica* and *P. pungens*.

Salinity and temperature varied seasonally at all stations except 1, where salinity was 0 year round (Figs. 2.4, 2.5, 2.6, 2.7, 2.8). Temperature dipped below 5°C in winter with a minimum in January or February and surpassed 25°C in summer with a peak in July or August. Salinity varied widely between stations from station 1 with a salinity of 0 to station 23 with salinity above 30. Winter was characterized by a period of relatively low temperature (≤ 5°C) and relatively high salinity (> 10) at stations where percent frequency of *Pseudo-nitzschia* was highest.

According to data collected by MD DNR, summer months had the lowest *Pseudo-nitzschia* abundances. Only 25% of samples collected by MD DNR during summer months from 1984 to 1994 contained any *Pseudo-nitzschia* and only 3% of those samples had concentrations ≥ 1000 cells mL^{-1}. From September to January, *Pseudo-nitzschia* abundances gradually increased (Fig. 2.9). Abundances were highest from February to May (≥ 1000 cells mL^{-1}) with the highest value recorded in April. DA concentrations were highest from December to March with the highest value recorded in March (Fig. 2.9).

Spearman's rank correlation shows a statistically significant association of *Pseudo-nitzschia* abundances with lower temperature and higher salinity during spring and fall and during the years 2002, 2004 and 2006. High *Pseudo-nitzschia* abundances were correlated with higher salinity in winter and lower temperatures in 2005. *Pseudo-nitzschia* abundances were not significantly correlated with salinity or temperature in 2003 or 2007 (Table 2.2). Seasonally, flow did not significantly correlate with *Pseudo-nitzschia* abundance. In 2002 and 2007 flow was negatively correlated with abundance while in 2004 and 2005 the opposite was true. In 2003 and 2006 there was no significant relationship (Table 2.2).

Discussion

Pseudo-nitzschia is a potentially toxic diatom that occurs in the Chesapeake Bay and its abundance responds to temperature and salinity. These results are quite similar to previous distributions noted in other systems. The association of high *Pseudo-nitzschia* abundances with low temperatures and high

salinity is similar to the pattern for *P. calliantha* in the Adriatic Sea (Caroppo et al. 2005) and *Pseudo-nitzschia* spp. on the West Coast of the United States (Trainer et al. 2000). *Pseudo-nitzschia* spp. in our study area were not found at salinity below 5 and temperature above 21°C (Fig. 2.10). This is consistent with laboratory growth experiments showing a lower salinity at ~6 (Thessen et al. 2005). Out of 533 samples collected, 132 (25%) fell outside these salinity (5) and temperature (21°C) limits. None of these samples contained *Pseudo-nitzschia* spp. However, out of the other 401 samples collected above 5 salinity and below 21°C, only 32% contained *Pseudo-nitzschia* spp. These results argue for a relatively broad tolerance of both salinity and temperature for *Pseudo-nitzschia* in the field. The statistical data (Table 2.2) indicate that low temperature and high salinity will favor high *Pseudo-nitzschia* abundances, but the generally low (<0.5 or >-0.5) correlation coefficients (ρ) suggest a weak dependence on these environmental variables.

The temperature and salinity limits on *Pseudo-nitzschia* presence can be seen geographically in Chesapeake Bay circulation patterns. The percent frequency of *Pseudo-nitzschia* occurrence at regularly sampled stations along the mainstem of the Chesapeake Bay increased with salinity toward the mouth of the estuary (Table 2.1; Figure 2.1). *Pseudo-nitzschia* abundances are higher on the saltier eastern shore of the Chesapeake than the western shore (Boicourt et al. 1999; Fig. 2.2). Annual variation in *Pseudo*-nitzschia abundance can partially be explained by temperature and salinity dynamics at the individual stations (Figs.

2.4, 2.5, 2.6, 2.7, 2.8) with high abundances occurring when and where temperatures are relatively low and salinity is relatively high.

The statistical relationships between flow and *Pseudo-nitzschia* abundance indicate a more complicated relationship between these two factors than between abundance and temperature or salinity. The negative correlation in 2002 reflects the correlation between abundance and salinity, since lower freshwater flow means higher salinity in the Chesapeake. The positive correlations in 2004 and 2005 suggest a relationship between abundance and nutrient loading from the Susquehanna River since *Pseudo-nitzschia* is known to respond to increased nutrients (Dortch et al. 1997). There is no significant relationship between flow and abundance in 2003 or 2006 (Table 2.2). Those are also years in which the range of flow did not exceed 1700 m^3sec^{-1} in consecutive months unlike the other years when the range of flow was as much as 2100 to 3100 m^3sec^{-1} (Fig. 2.11).

In general, *Pseudo-nitzschia* abundance is greater near the mouth of a tributary and declines upriver (Fig. 2.2). However, this relationship was not observed in the Choptank River (Stations 15, 6, 16 and 17) where the maximum *Pseudo-nitzschia* abundance at station 15 (intuitively, the station with lower salinity because it is farther upriver) is higher than the next downstream station 6 (Fig. 2.2). Since station 15 was sampled from 2002 to 2004 and station 6 was sampled from 2004 to 2007 we believe the data reflect salinity differences between the two sampling periods. A severe drought occurred during the 2002-2004 sampling period, so freshwater flow was less and therefore salinity was higher at station 15 from 2002-2004 than at station 6 from 2004-2007 (Reaugh et

al. 2007; Figs. 2.6 and 2.11). These data demonstrate the high potential for variability in the Chesapeake *Pseudo-nitzschia* population from one year to the next and the effect of precipitation and thus salinity on natural *Pseudo-nitzschia* populations.

Our survey found *P. calliantha*, a new species previously unreported in the Chesapeake Bay since its description from a redefinition of *P. pseudodelicatissima* (Lundholm et al. 2003). Therefore, reports of *P. pseudodelicatissima* in the Chesapeake Bay before 2003 could have been *P. calliantha* (Marshall 1994). Unfortunately, there are no micrographs available to support this hypothesis. Other previously reported species include *P. delicatissima, P. pungens, P. seriata, P. fraudulenta, P. subpacifica, P. cuspidata* and *P. multiseries* (Hasle 1965, Marshall 1980, 1994, Marshall et al. 2005).

DA detection in field water samples throughout the Chesapeake Bay was generally low; however, some of the same species reported here contain higher DA elsewhere (Bates et al. 1989, Martin et al. 1990, Rhodes et al. 1998b). Reasons for these differences include genetic differences (strain differences) and differing environmental controls. A comprehensive genetic characterization of populations in regions that frequently have high DA producing *Pseudo-nitzschia* and regions that have low or non-toxic *Pseudo-nitzschia* would provide a valuable comparison. Further, once elucidated genetically, laboratory experiments with varying environmental conditions on these genotypes might provide insights into functional differences in the strains (Chapter 3, this thesis).

Environmental control of DA content in the Chesapeake has several possibilities. The turbid waters of the Chesapeake Bay could block sufficient light energy for DA synthesis or the salinity regime in the Chesapeake could affect DA production. During spring and winter, when *Pseudo-nitzschia* are most likely to be abundant, irradiances in the mixed layer are below 100 μmol photons m^{-2} sec^{-1} necessary for maximum DA production (Bates 1998, Chapter 5 this thesis). Laboratory cultures of one strain of *P. multiseries* produced maximum levels of DA in salinities above 20 (Doucette et al. in press) which, in our study, are present only near the mouth of the Chesapeake. It is noteworthy that most ASP or DAP episodes have occurred in oceanic or near-oceanic environments (i.e., Prince Edward Island, California coast) and the highest DA levels per mL observed in our study occurred in higher salinity areas. These data suggest that *Pseudo-nitzschia* abundances and toxicity could increase in the Chesapeake Bay during periods of low rainfall and increased salinity.

Climate change projections for the mid-Atlantic include an increase in temperature and freshwater discharge (Najjar 1999, Najjar et al. 2000). The increased freshwater flow is predicted to decrease salinity by as much as 27.5% near the mouth of the Susquehanna River and shift the current isohalines down bay by as much as 55 km (Gibson & Najjar 2000). While diatoms are favored during periods of high flow and low residence times (Paerl et al. 2006), a decrease in salinity and increase in temperature could reduce *Pseudo-nitzschia* abundances in the Chesapeake. However, due to gravitational circulation (Boicourt et al. 1999), an increase in freshwater flow on the surface would cause saltier oceanic

water, and presumably *Pseudo-nitzschia*, to be transported farther up estuary at depth.

The lack of a documented toxic event in the Chesapeake argues against making *Pseudo-nitzschia* a monitoring priority; however, having historical data on abundance and toxicity could prove valuable in the future. Current environmental conditions in the Chesapeake Bay area appear to be unfavorable to toxic *Pseudo-nitzschia* blooms, but these conditions may not remain constant due to cultural eutrophication and climate change. In addition, there is a possibility that toxic events or sublethal effects have occurred but are undocumented. Preliminary work suggests that subacute doses of domoic acid in infants and children have an adverse affect on mental development and memory, suggesting that doses too low to cause acute symptoms can still have an effect (Grattan et al. 2002). In addition, many of the over-wintering water fowl in the Chesapeake are seaducks, which are known to consume marine invertebrates, potential DA vectors (Perry et al. 2004). A rare or small toxic event involving these birds could be easily missed and go unreported (Shumway et al. 2003).

Our study did not include an assessment of DA accumulation and persistence in shellfish exposed to *Pseudo-nitzschia* blooms (in field or laboratory), but that would be a logical next step since humans and animals have not become ill from DA in water or algae but are intoxicated through a shellfish or fish vector (Perl et al. 1990, Fritz et al. 1992, Scholin et al. 2000). Additionally, our data suggest that more saline coastal bay environments, like the Maryland, Virginia and Delaware coastal bays, a series of coastal lagoons

between the Chesapeake and the Atlantic, may be at greater risk for DA-producing *Pseudo-nitzschia* blooms and potential shellfish contamination.

Chesapeake Bay *Pseudo-nitzschia* populations are dynamic, consisting of several species and exhibiting seasonal patterns and variation in their abundance from year to year that can be partially explained by changes in temperature and salinity. Some of this *Pseudo-nitzschia* produces DA although not at levels high enough to cause a toxic event. There have been no documented ASP or DAP events in the Chesapeake to date, but because of the dependence of *Pseudo-nitzschia* abundances and toxicity on changing environmental conditions, current monitoring data could be useful in the future.

Table 2.1 *Pseudo-nitzschia* (Pn) and domoic acid sampling in the Chesapeake Bay region.

Station[a]	Years Sampled	Collected By	n_1[b]	Samples with Pn	Species present	n_2[c]	Samples with DA
1	September 2004-May 2007	MD DNR	50	0	none	0	0
2	September 2004-May 2007	MD DNR	50	3	*P. calliantha, P. fraudulenta*	2	2*
3	September 2004-May 2007	MD DNR	50	9	*P. pungens, P. calliantha*	7	2*
4	September 2004-May 2007	MD DNR	50	16	*P. calliantha*	14	9*
5	September 2004-May 2007	MD DNR	50	7	ND[d]	5	5*
6	September 2004-May 2007	MD DNR	50	2	ND	2	0
7	September 2004-May 2007	MD DNR	50	15	*P. calliantha*	8	0
8	September 2004-May 2006	MD DNR	32	0	none	0	0
9	September 2004-May 2007	MD DNR	50	28	*P. calliantha, P. subpacifica*	22	9*
10	April 2005	MD DNR	1	1	*P. cuspidata*	1	0
11	April 2005	MD DNR	1	1	*P. calliantha*	1	SL[e]
12	February 2002-June 2004	HPL	23	0	none	0	0
13	February 2002-June 2004	HPL	26	5	ND	1	1
14	February 2002-June 2004	HPL	24	15	*P. calliantha*	3	3*
15	February 2002-June 2004	HPL	45	5	*P. calliantha*	0	0
16	February 2002-June 2004	HPL	47	13	*P. calliantha, P. multiseries*	3	1
17	February 2002-June 2004	HPL	42	23	*P. calliantha, P. fraudulenta*	2	2*
18	October 2003	HPL	1	1	*P. fraudulenta*	1	0

Table 2.1 cont.

Station[a]	Years Sampled	Collected By	n_1[b]	Samples with Pn	Species present	n_2[c]	Samples with DA
19	**November 2004**	**HPL**	**1**	**1**	***P. calliantha***	**1**	**0**
20	**April 2004**	**MD DNR**	**1**	**1**	**ND**	**1**	**1**
21	**October 2003**	**HPL**	**1**	**1**	***P. calliantha***	**1**	**0**
22	**November 2004**	**HPL**	**1**	**1**	***P. calliantha***	**1**	**1**
23	**November 2004**	**HPL**	**1**	**1**	***P. calliantha***	**1**	**SL**
24	**February 2006**	**HPL**	**1**	**1**	**ND**	**1**	**1***
25	**February 2006**	**HPL**	**1**	**1**	***P. pungens***	**1**	**1***
26	**February 2006**	**HPL**	**1**	**1**	**ND**	**1**	**0**
27	**February 2006**	**HPL**	**1**	**1**	**ND**	**1**	**1***
28	**February 2006**	**HPL**	**1**	**1**	***P. fraudulenta***	**1**	**1***
29	**March 2003**	**HPL**	**1**	**1**	***P. calliantha, P. pungens***	**1**	**1**
30	**March 2003**	**HPL**	**1**	**1**	***P. multiseries, P. fraudulenta***	**1**	**SL**
31	**November 2004**	**HPL**	**1**	**1**	**ND**	**1**	**1**

[a]Station locations are given in Figure 2.1.
[b]n_1 is the total number of samples taken at that station.
[c]n_2 is the total number of samples analyzed for DA. Only samples with *Pseudo-nitzschia* species abundances detectable with epifluorescent microscopy (limit of detection 4 cells mL^{-1}) were tested for domoic acid.
[d]ND indicates that none of the samples from that station contained *Pseudo-nitzschia* abundances above the 100 cells mL^{-1} needed for morphological identification via TEM.
[e]SL indicates a sample lost during toxin analysis.
*Indicates stations with samples containing detectable domoic acid below the limit of quantitation

Table 2.2 Spearman's rank coefficient (ρ) for *Pseudo-nitzschia* species abundance and temperature (temp, °C), salinity or flow (m^3s). Data were grouped and analyzed by season and year.

	temp		salinity		flow	
	ρ	**p value**	**ρ**	**p value**	**ρ**	**p value**
winter	**-0.0045**	**0.9554**	**0.4108***	**0.0000**	**0.0683**	**0.4032**
spring	**-0.1743***	**0.0067**	**0.2692***	**0.0000**	**-0.0766**	**0.2343**
fall	**-0.1864***	**0.0309**	**0.1787***	**0.0386**	**0.1284**	**0.2310**
2002	**-0.6094***	**0.0002**	**0.3601***	**0.0265**	**-0.4312***	**0.0078**
2003	**-0.0733**	**0.6385**	**0.2446**	**0.1175**	**0.2548**	**0.1029**
2004	**-0.2309***	**0.0401**	**0.3194***	**0.0045**	**0.2432***	**0.0306**
2005	**-0.2640***	**0.0019**	**0.1586**	**0.0625**	**0.3215***	**0.0002**
2006	**-0.2951***	**0.0002**	**0.3077***	**0.0001**	**0.0644**	**0.5048**
2007	**-0.1855**	**0.1081**	**0.1875**	**0.1045**	**-0.2470***	**0.0348**

* Indicates significance at 0.05 level.

Figure 2.1 Map of the Chesapeake Bay region showing the 31 sampling stations.

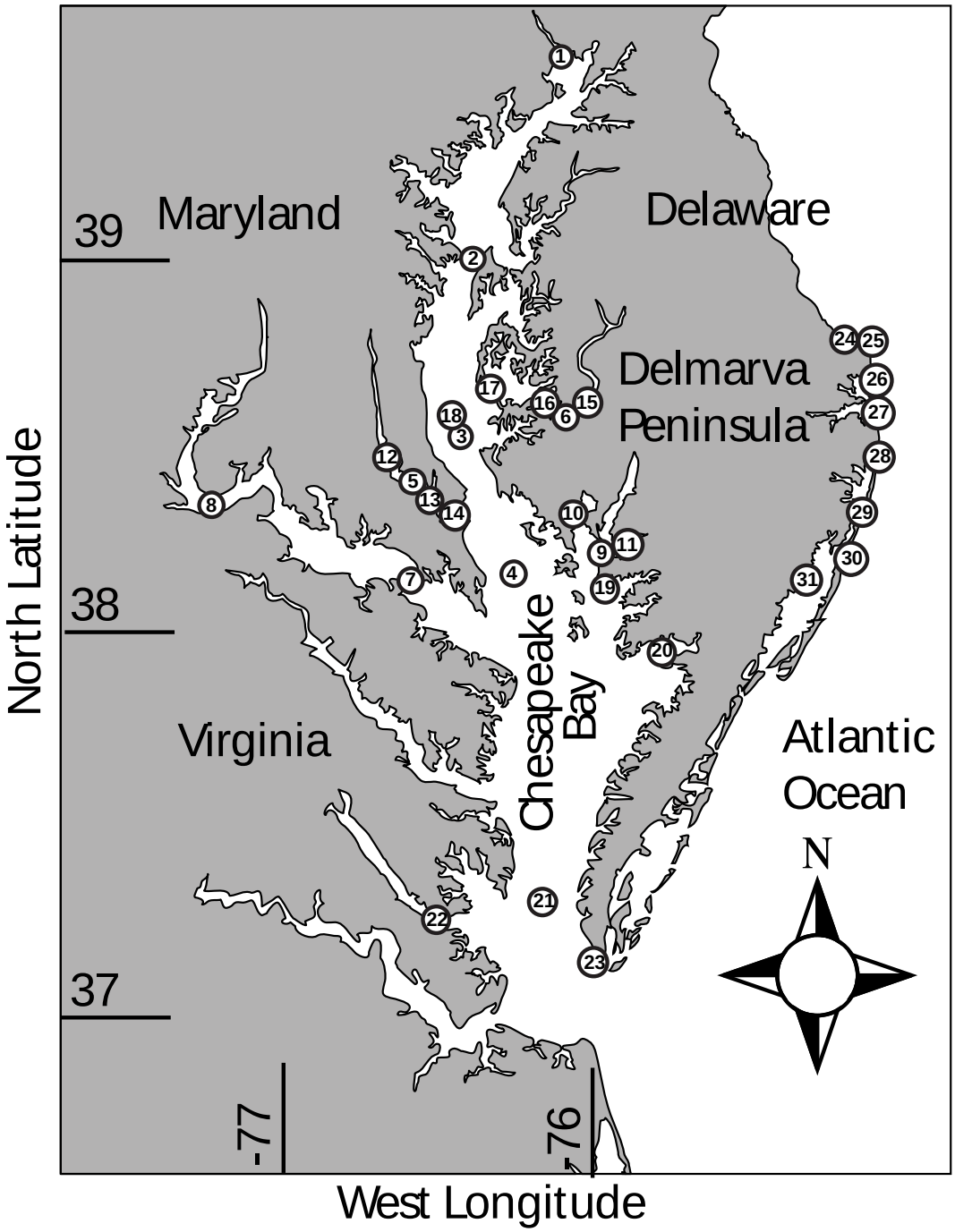


Figure 2.2 Maximum abundances (cells mL^{-1}) of *Pseudo-nitzschia* species from 2002-2007 in the Chesapeake Bay region.

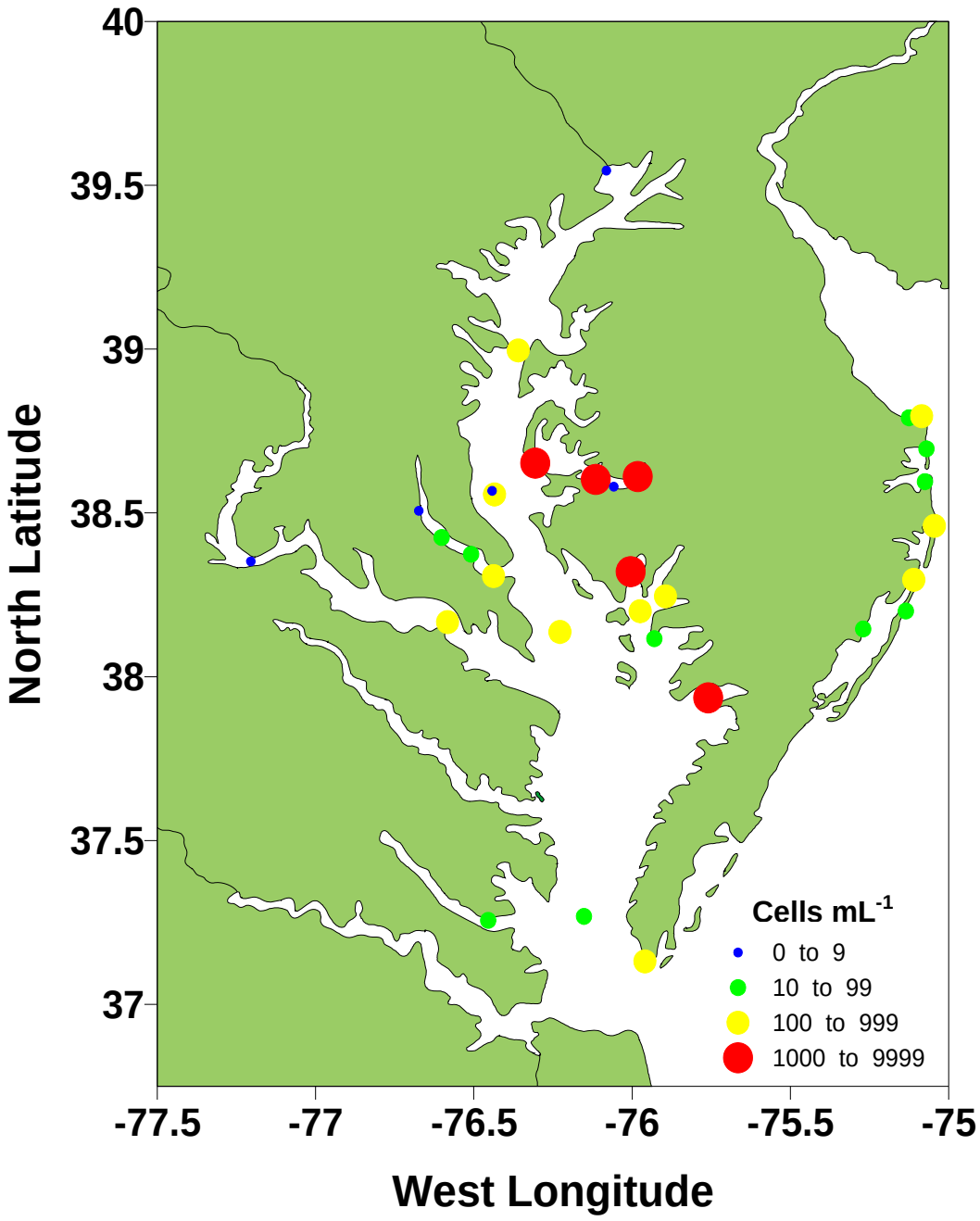


Figure 2.3 Maximum total concentrations of domoic acid in the particulate and dissolved fraction (pg DA mL^{-1}) from 2003-2007 in the Chesapeake Bay region.

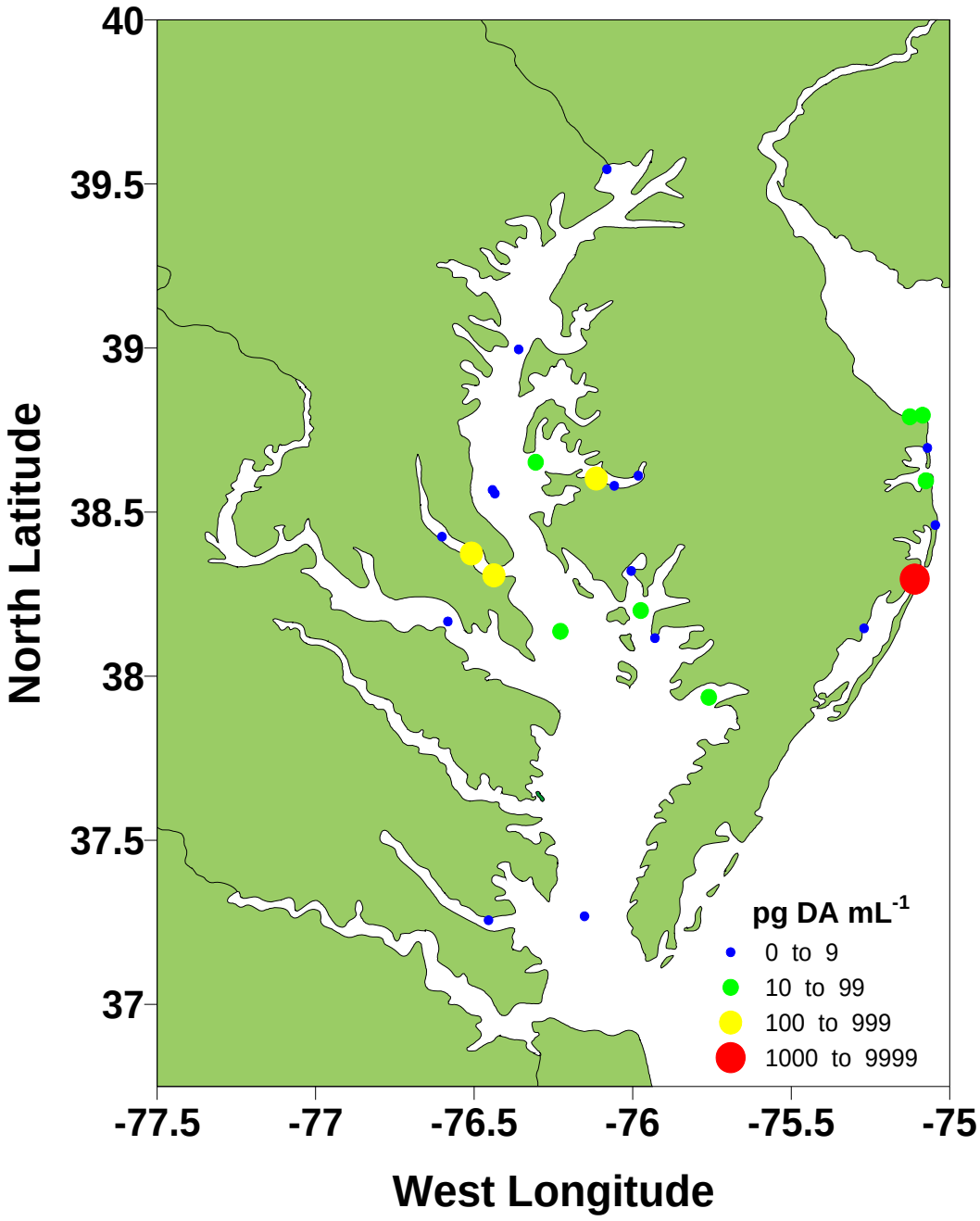


Figure 2.4 Monthly surface temperature (open circles, °C) and salinity (closed squares) data for stations in the northern and mainstem Chesapeake Bay. Shaded areas indicate months when water samples were specifically collected for *Pseudo-nitzschia* enumeration and identification. Arrows indicate when samples contained >100 cells mL^{-1} *Pseudo-nitzschia*. Station numbers are given in upper right corner and correspond to Figure 2.1.

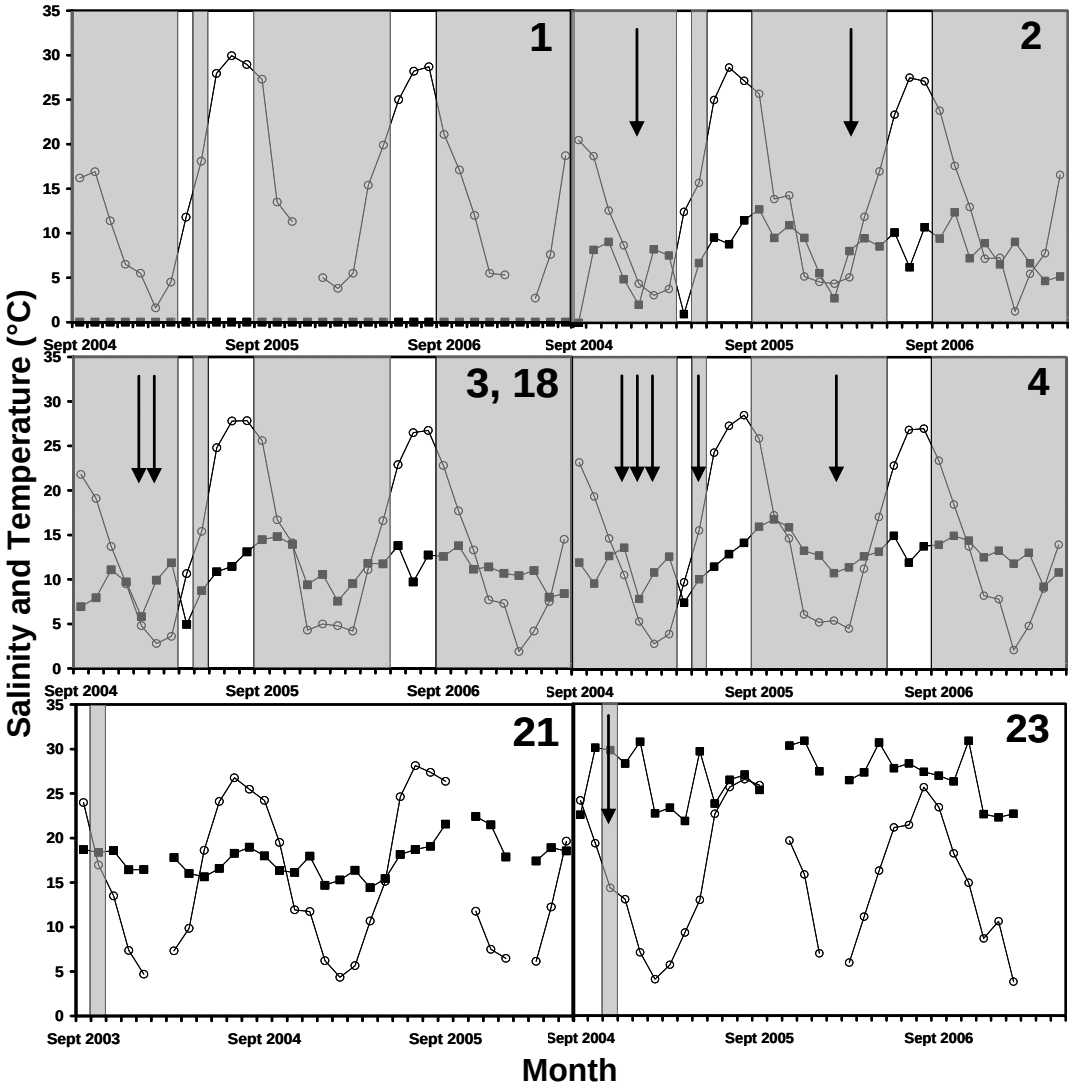


Figure 2.5 Monthly surface temperature (open circles, °C) and salinity (closed squares) data for stations in tributaries of the Chesapeake Bay other than the Choptank and Patuxent Rivers. Shaded areas indicate months when water samples were collected for *Pseudo-nitzschia* enumeration and identification. Arrows indicate when samples contained >100 cells mL^{-1} *Pseudo-nitzschia*. Station numbers are given in upper right corner and correspond to Figure 2.1.

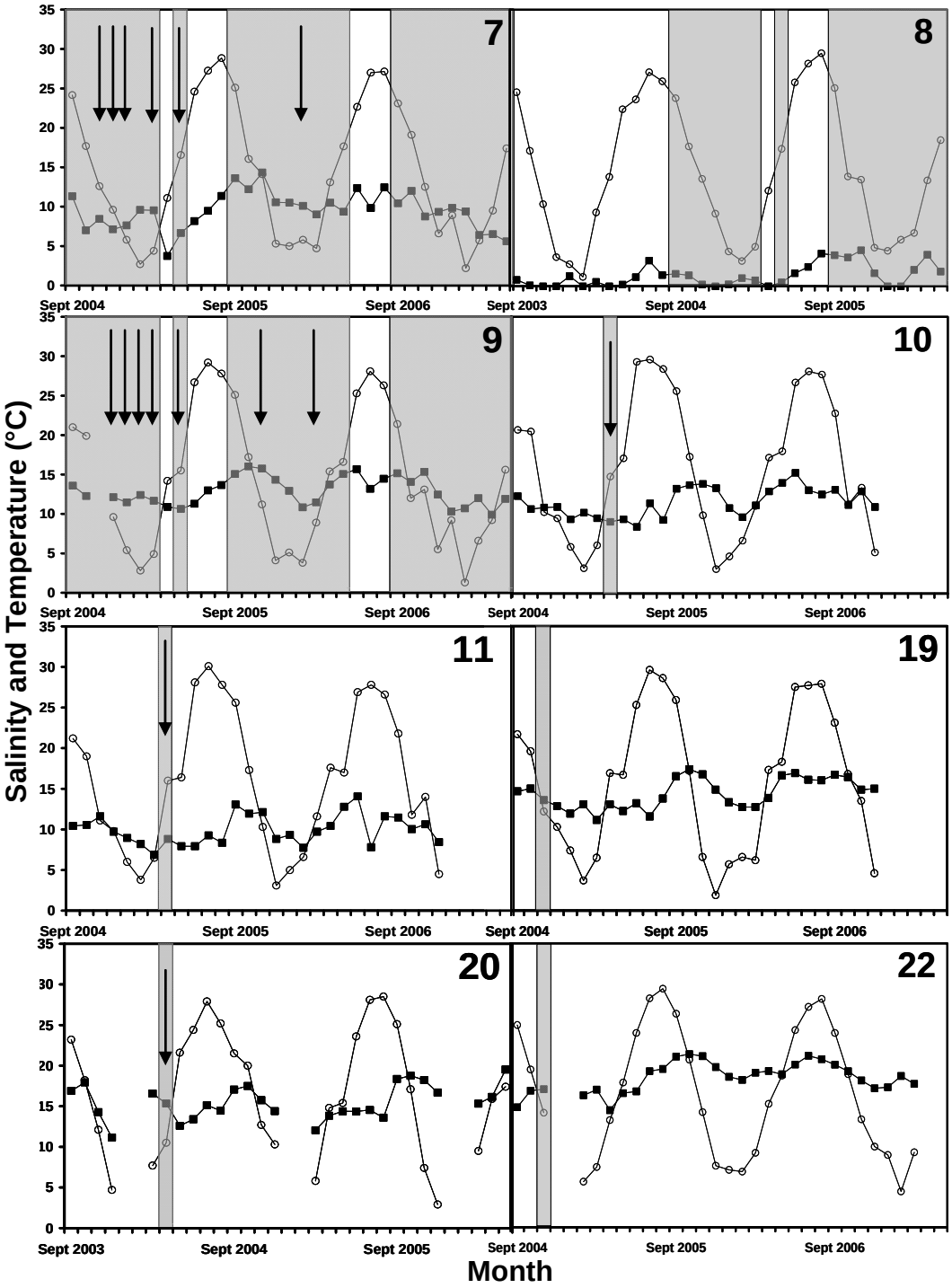


Figure 2.6 Monthly surface temperature (open circles, °C) and salinity (closed squares) data for stations in the Choptank River. Shaded areas indicate months when water samples were collected for *Pseudo-nitzschia* enumeration and identification. Arrows indicate when samples contained >100 cells mL^{-1} *Pseudo-nitzschia*. Station numbers are given in upper right corner and correspond to Figure 2.1.

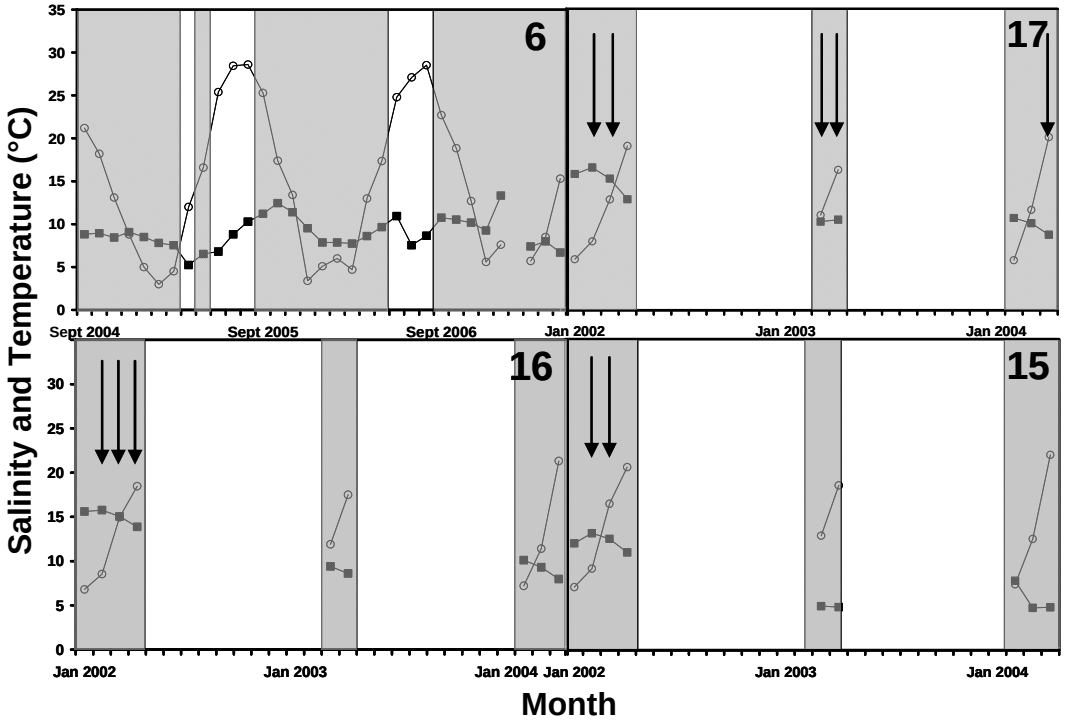


Figure 2.7 Monthly surface temperature (open circles, °C) and salinity (closed squares) data for stations in the Patuxent River. Shaded areas indicate months when water samples were collected for *Pseudo-nitzschia* enumeration and identification. Arrows indicate when samples contained >100 cells mL^{-1} *Pseudo-nitzschia*. Station numbers are given in upper right corner and correspond to Figure 2.1.

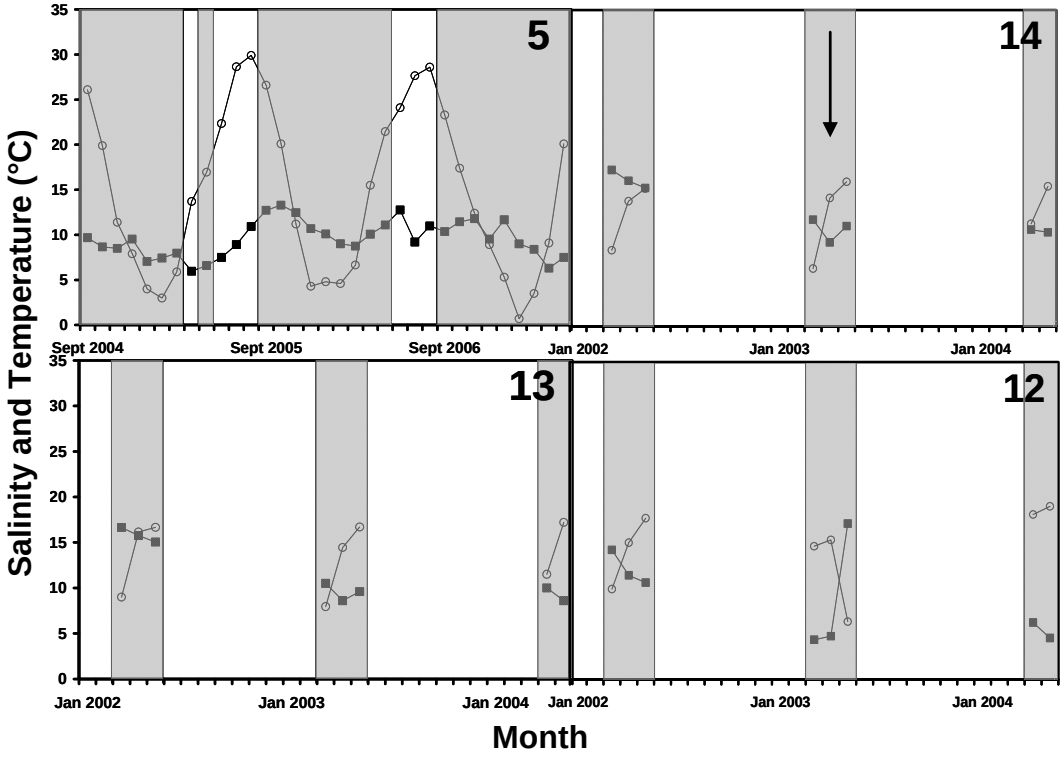


Figure 2.8 Monthly surface temperature (open circles, °C) and salinity (closed squares) data for stations in the Atlantic coast and coastal bays. Shaded areas indicate months when water samples were collected for *Pseudo-nitzschia* enumeration and identification. Arrows indicate when samples contained >100 cells mL^{-1} *Pseudo-nitzschia*. Station numbers are given in upper right corner and correspond to Figure 2.1.

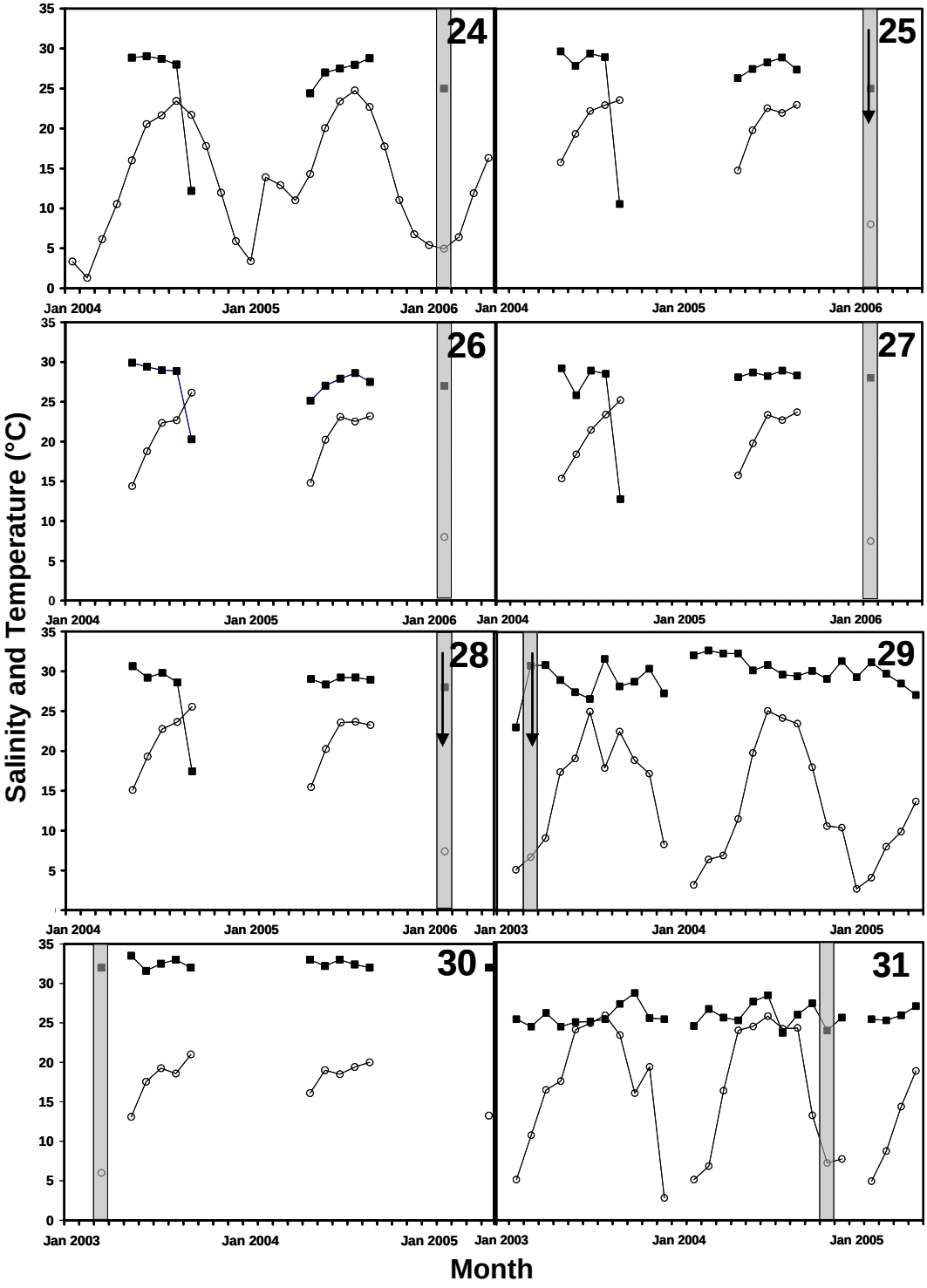


Figure 2.9 (A) Abundances (cells mL^{-1}) of *Pseudo-nitzschia* spp. at all stations year-round. Data from September to May 2002-2007 are from our collections. Data in June, July and August were taken from MD DNR (www.chesapeakebay.net). (B) DA concentrations (pg mL^{-1}) at all stations year-round. In both panels, each filled circle represents one sample.

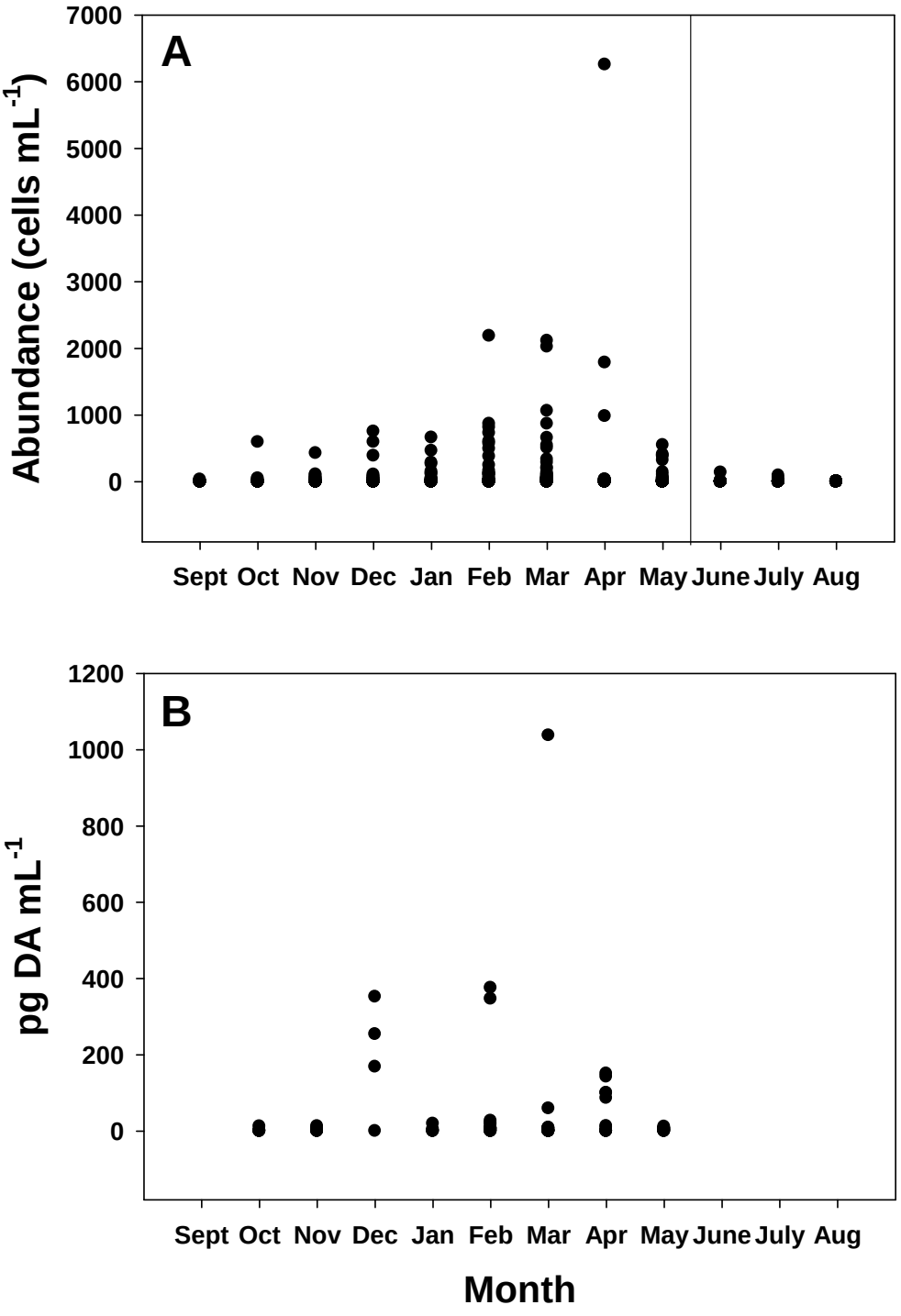


Figure 2.10 Plot of temperature (°C) and salinity data collected from 2002 to 2007 during fall, winter and spring at stations throughout the Chesapeake Bay area. Size of filled diamond indicates abundance of *Pseudo-nitzschia* spp. in orders of magnitude. Lines indicate minimum salinity limit and maximum temperature limit beyond which *Pseudo-nitzschia* do not occur in this study.

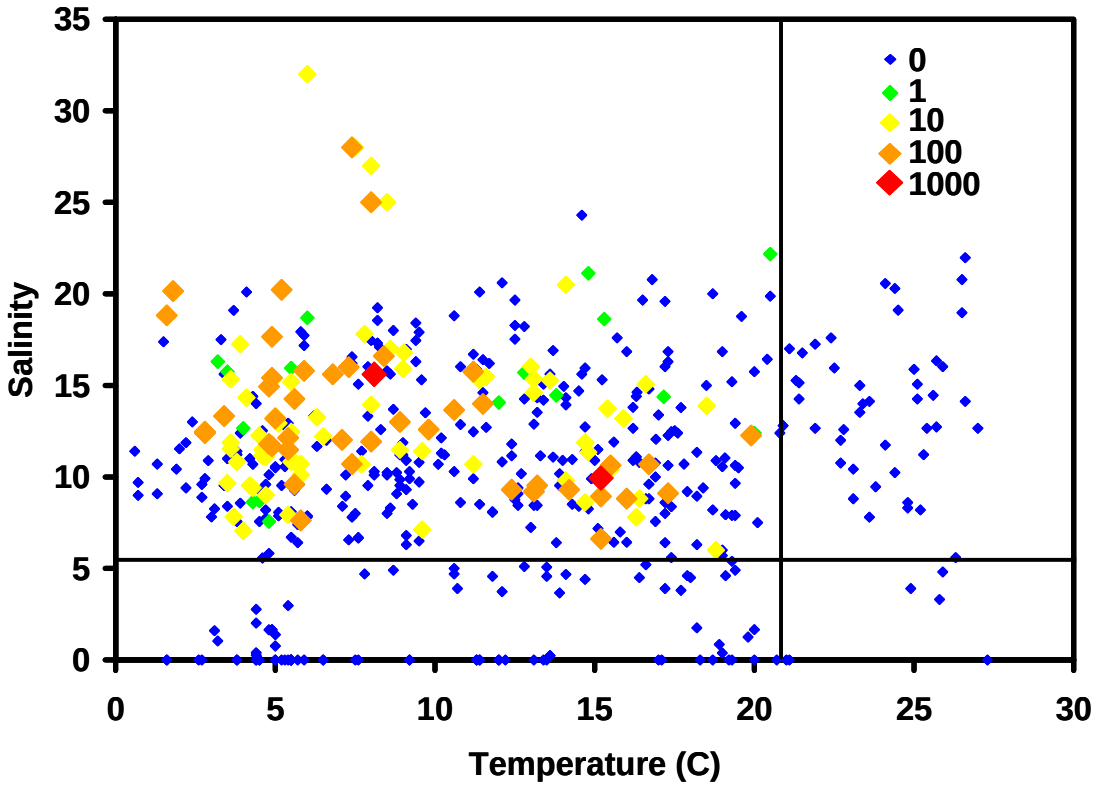
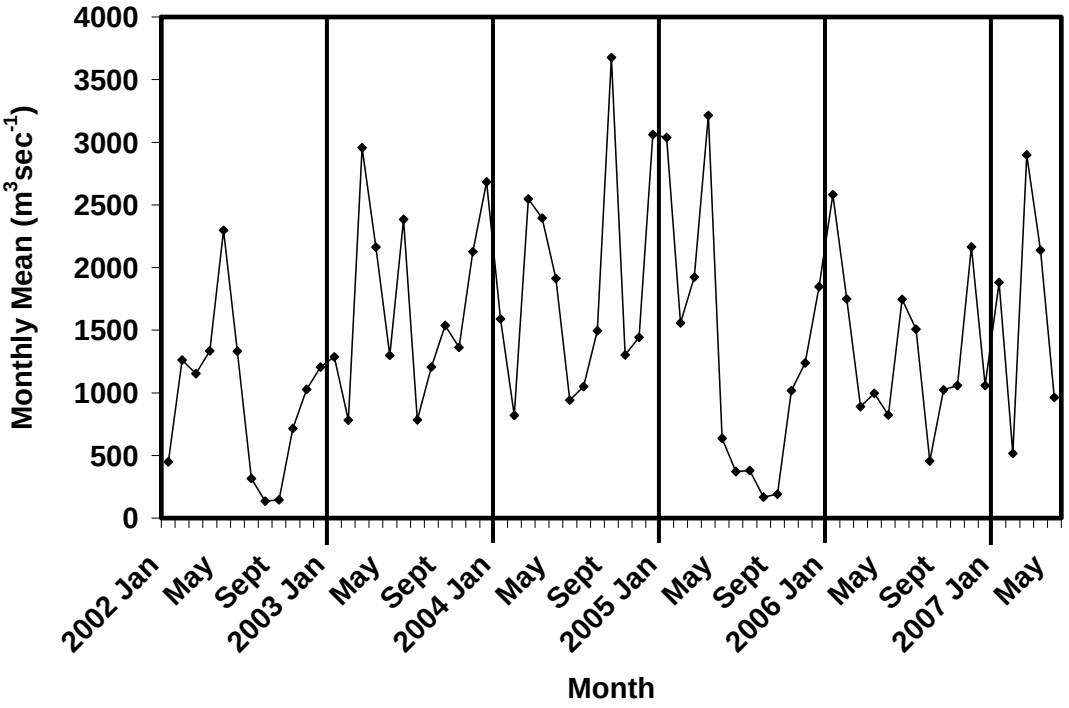


Figure 2.11 Plot of average monthly flow from the Susquehanna River through the Connewingo Dam (m^3sec^{-1}) during the sampling period (January 2002 to May 2007). Vertical lines separate years.



Chapter 3: Intra- and interspecies differences in *Pseudo-nitzschia* growth and toxicity while utilizing different nitrogen sources

Abstract

Clonal cultures of plankton are widely used in laboratory experiments and have contributed greatly to knowledge of microbial systems. However, many physiological characteristics vary drastically between strains of the same species, calling into question our ability to make ecologically relevant inferences about populations based on studying one or a few strains. This study included nineteen strains of three species of the diatom *Pseudo-nitzschia* isolated primarily from the mid-Atlantic coastal region of the United States. Toxin (domoic acid) production and growth rates were measured in cultures using different nitrogen sources (NH_4^+, NO_3^- and urea) and growth irradiances. The strains exhibited broad differences in growth rate and toxin content even between strains isolated from the same water sample. Both *P. multiseries* clones produced toxin, yet preferentially utilize different nitrogen sources. Only some of the *P. calliantha* and *P. fraudulenta* isolates were toxic and domoic acid content varied by orders of magnitude. All three species had variable intraspecies growth rates on each nitrogen source, but *P. fraudulenta* strains had the broadest range. Light-limited growth rate and maximum growth rate in *P. fraudulenta* and *P. multiseries* varied with species. These findings show the importance of defining intra- and interspecies variability in ecophysiology and toxicity. Ecologically relevant functional diversity in the form of ecotypes or cryptic species appears to be present in the genus *Pseudo-nitzschia*.

Introduction

Much of what we know about plankton physiology comes from experiments using laboratory cultures. Most *Pseudo-nitzschia* culture studies focus on *P. multiseries* with the results extrapolated to other species and strains (Bates 1998). However, broad species and strain differences have been well documented (Gallagher 1980, 1982, Elrifi & Turpin 1985, Goldman & Dennett 1985, Holmes et al. 1991, Wood & Leatham 1992, Larsen & Bryant 1998) and draw into question the validity of making conclusions about microalgal species physiology based on one or a few strains.

Such high variability between strains casts doubt on the species concept in microalgae in general. While the creation of a species is the result of a biological reality, secondary characteristics are used (frustule morphology, ribosomal genes) to detect and define them. Manhart and McCourt (1992) stated that:

> "Practicing phycologists often seem to strive to delineate biological species while basing descriptions solely upon morphological data. The assumption is that morphological species closely approximate biological species of algae, but only rarely is this hypothesis tested. If species assignment is a hypothesis of relationship, then many (perhaps most) implicitly described biological species of algae represent untested hypotheses."

This implies a basic fault in microalgal species definitions which could explain high strain variability. However, Wood and Leatham (1992) argue that difficulties arise when culture studies involving only a few clones attempt to

define interspecies differences without assessing within species variation, not necessarily as the result of a fault in the species definition. The seemingly enormous number of isolates necessary to accurately define within species variation restricts the number of studies able to include such an analysis.

The alternative, making conclusions about a species based on one strain, would likely lead to erroneous results. Toxin production in 17 strains of *Alexandrium tamarense* showed broad differences in toxin production, the lowest approximating zero mouse units (MU) per 10^4 cells and the highest at 1.1 MU per 10^4 cells (Ogata et al. 1987). No one strain was representative of the species. Taking the analysis one step further, 15 sub-strains taken from one strain had a 0.6 MU per 10^4 cells range in toxin production. An analysis of PSP toxin composition in two strains of *A. tamarense* showed one strain, SB31, produced mostly a sulfocarbamoyl derivative C2 while the other strain, SB32, produced mostly GTX3 and GTX4 (Cembella et al. 2002). Studies such as these show broad differences among strains and argue that finding one "representative" strain is highly unlikely if not impossible. Yet, an analysis of recent publications shows only 40% of studies that use culture experiments consider the possibility of significant strain differences when making conclusions (Burkholder & Glibert 2006).

Genetic variability in field populations and strains of *Pseudo-nitzschia* has been widely documented; however, physiological variability has not been as thoroughly investigated (Evans et al. 2004, Orsini et al. 2004). Many *Pseudo-nitzschia* culture studies present results from one strain (Bates et al. 1991,

Douglas & Bates 1992, Hillebrand & Sommer 1996, Pan et al. 1996a,b, Fehling et al. 2004, Armstrong Howard et al. 2007). Others compare one strain of multiple species (Jackson et al. 1992, Hargraves et al. 1993, Wang et al. 1993, Maldonado et al. 2002, Fehling et al. 2005). Most studies comparing multiple cultures of one species have focused on toxin production in *P. australis, P. seriata* or *P. multiseries* (Bates et al. 1989, Garrison et al. 1992, Douglas et al. 1993, Villac et al. 1993a, Lundholm et al. 1994, Bates et al. 1999) with one study investigating toxicity in *P. pseudodelicatissima* (Pan et al. 2001). Only four studies used multiple strains of the same species to investigate other physiological processes in addition to toxicity. With four strains of *P. multiseries* and two strains of *P. pungens*, Bates et al. (1993) analyzed the effect of NO_3^- and NH_4^+ on growth and toxin production. Lundholm et al. (2004) employed two strains of *P. multiseries* to study the effect of pH on growth and toxin production. Bates et al. (1995) used three strains of *P. multiseries* to examine the role of bacteria in domoic acid (DA) production. Thessen et al. (2005) studied two strains of *P. delicatissima*, two strains of *P. multiseries* and three strains of *P. pseudodelicatissima* to assess the effect of salinity on growth rate.

The meaning of high intraspecific genetic diversity in natural populations is controversial (Fenchel 2005, Foissner 2006). High genetic diversity in some protist taxa has been considered an indicator of cryptic species and functional diversity (Dolan 2005, Foissner 2006, Scheckenbach et al. 2006). Others argue that variation in rRNA is an accumulation of neutral mutations that does not correlate with physiology or show biogeographic patterns (Fenchel 2005).

However, there have been populations of microalgae comprised of distinct physiological or genetic groupings which showed dynamic seasonal abundances. The presence of multiple ecotypes has been demonstrated in populations of *Skeletonema costatum* in Narragansett Bay (Gallagher 1980, 1982). These ecotypes have different physiological characters, making them better adapted to different environmental conditions resulting in a succession of ecotypes throughout the year. Similar results have been found with *Ditylum brightwellii* in Puget Sound (Rynearson et al. 2006). Reproductively isolated cryptic variants within *P. delicatissima* and *P. pseudodelicatissima* have been identified using morphology, genetic sequences and mating experiments (Amato et al. 2007). The ecological significance of this diversity is not well understood.

This paper is a presentation of strain differences between three species of *Pseudo-nitzschia*: *P. multiseries*, *P. fraudulenta* and *P. calliantha* for growth rate, toxin production, nitrogen use and saturating growth irradiance. It is also the first presentation of genetic and toxin data from Chesapeake Bay area *Pseudo-nitzschia* strains.

Materials and Methods

Culture isolation, identification and maintenance

Pseudo-nitzschia were isolated from field samples via micropipetting (Andersen & Kawachi 2005) and incubated as separate cultures in an inorganic nutrient enriched seawater medium for diatoms, f/2* (Andersen et al. 1997), at a temperature and salinity close to ambient conditions at the time and place of

collection and a 14:10 L:D cycle (Table 3.1). Morphological identification was performed using a derivation of methods in Lundholm et al. (2002a). A 10 mL aliquot of culture was preserved in 1% gluteraldehyde and digested using 2 mL of 30% H_2SO_4 and 10 mL of a saturated $KMnO_4$ solution in a 100 mL Erlenmeyer flask. The flask was covered with parafilm and swirled periodically throughout the digestion. After 24 h, a saturated oxalic acid solution was added until the sample became transparent. Samples were rinsed 3-4 times with distilled, deionized water and pelleted via centrifugation. The pellet was resuspended in approximately 1 mL of water. One drop was placed on a 200 μm mesh copper grid with a formvar film. Transmission Electron Microscopy (TEM) was used to identify species of *Pseudo-nitzschia* based on frustule morphology from measurements of cell properties in photographic negatives (Skov et al. 1999, Lundholm et al. 2002a,b, Lundholm & Moestrup 2002, Hasle & Lundholm 2005).

Genetic and phylogenetic analysis

DNA extractions from cultures were performed using the Puregene® DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Samples were centrifuged at 4000 g and the supernatants decanted. Pellets were resuspended in 300 μl of cell lysis buffer supplied with the kit and the manufacturer's protocol was followed for the remainder of the extraction procedure. Eluted DNA was stored at -20^{o} C until analyzed by polymerase chain reaction (PCR).

The nuclear encoded large subunit (D1-D3 region of the LSU; 28S) locus was amplified from *Pseudo-nitzschia* cultures using two primer sets. Primers

D1R forward (Scholin et al. 1994; 5'-ACCCGCTGAATTTAAGCATA-3') and LSUSeqRev (5'-AGTGCTAGCAACAGACATCAACT-3') resulted in a 517 base pair amplicon, and D3Ca reverse (Scholin et al. 1994; 5'-ACGAACGATTTGCACGTCAG -3') and LSUSeqFor (5'-CTGAAACGGAAGCGAAGGAAA-3') resulted in a 434 base pair amplicon. The two amplicons overlapped by 144 base pairs.

The nuclear encoded internal transcribed region (ITS1-5.8S-ITS2) was amplified using two primer sets. Primers 1815F (Bowers et al. 2006; 5'-GGAAGTTGGGGGCAATAACAGG-3') and ITS Rev (Bowers et al. 2006; 5'-CATCGTTGTGGGAACCWAGACAT-3') were used to generate an amplicon from the first half of the ITS region, while ITS For (reverse complement of ITS Rev) and D1R Rev (reverse complement of D1R For mentioned above) were used to generate the second half. Amplicon sizes varied based on the species amplified. These two amplicons did not overlap, so species-specific primers were designed upstream of the ITSFor/Rev primer region to be used in conjunction with D1R Rev in order to generate overlapping sequences: *P. fraudulenta* For (5' – CGTTTGCCTCAAAAGTCAACTTG – 3'); *P. calliantha* For (5' – TTTGGCTCGTGACTTTTGTTGC – 3'); and *P. multiseries* For (5' – TTGCCCGCCACTCTTTACGA – 3').

Each 50 µl PCR reaction contained 1.5 U of MegaFrag™ Taq polymerase (Denville Scientific, Metuchen, NJ); 10X PCR buffer and 4 mM $MgCl_2$ supplied with Taq polymerase; 2 mM each dNTP (Invitrogen, Alameda, CA),

0.25 mg mL^{-1} bovine serum albumin (Idaho Technology, Idaho Falls, ID), 0.8 µM each primer (Qiagen/Operon, Alameda, CA), 1 µl DNA template and molecular biology grade water to a final volume of 50 µl. Cycling was performed on the DNA Engine Dyad Peltier Thermocycler (Bio-Rad Laboratories, Inc., Waltham, MA) as follows: initial denaturation at 94°C for 2 min, followed by 45 cycles of 94°C for 10 s, annealing temperature ranging from 56°C to 60°C (based on primer pair used) for 30 s, extension step of 68°C for 30 s to 40 s (depending on amplicon size), and a final extension at 68°C for 6 min 20 s. PCR products were examined on a 1% ethidium bromide-stained agarose gel, and bands were extracted from the gel following the procedure supplied with the MinElute kit (Qiagen, Alameda, CA).

All primers outlined above were used for sequencing amplicons. Gel-extracted bands were sequenced using the DYEnamic™ ET Terminator Cycle Sequencing kit (Amersham Biosciences, Piscataway, NJ). The sequencing reactions contained the following: 2 µl dye (diluted 1:5), 1 µl of desired primer (0.4 µM final concentration), 0.5 - 1 µl of gel purified product, and sterile H_2O to 5 µl. Cycling parameters were as follows: 25 cycles of 95°C for 20 s, 55°C for 15 s and 60°C for 1 min. After cycling, sequencing reactions were centrifuged through Sephadex G50 to remove unincorporated dye (Amersham Biosciences, Uppsala, Sweden). Sequencing was performed on the 3100 capillary sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned and inspected for nucleotide ambiguities using Sequencher (version 4.1.2, Gene Codes Corporation, Ann Arbor, MI) and then aligned to other *Pseudo-nitzschia* species

available from GenBank utilizing the software MacClade (version 4.04; Maddison & Maddison; Sinauer Associates, Inc., Sunderland, MA). The BLAST search program available through the National Center for Biotechnology Information website (www.ncbi.nih.gov/) was used to determine the closest sequence matches available in GenBank (Altschul et al. 1997). Parsimony analysis was performed using PAUP* 4.0b10 (Swofford 2002).

Toxin Content of Cultures

Potential toxicity of each culture was tested in f/2* medium with 53 μM Si (to induce Si limitation during stationary phase of growth; Bates et al. 1991, Pan et al. 1991) with bubbling at an irradiance approximating 150 μmol photons m^{-2} sec^{-1} and a 14:10 L:D cycle in 15°C (Bates 1998). *In vivo* fluorescence of a subsample was measured at the same time daily to monitor growth phase and timing of toxin sample collection. An emphasis was placed on documenting DA concentrations in exponential and stationary phases at least one week after peak fluorescence to capture maximum intracellular toxin (Bates 1998). For the toxin sample, 50 mL of culture was removed and filtered through a Whatman GF/F filter. Both the filter and filtrate were stored at -80°C in the dark for determination of particulate (DA in the cell) and dissolved (DA in the filtrate) toxin by high performance liquid chromatography (HPLC; Quilliam 2003) or the ELISA method (Garthwaite et al. 1998) using the ASP direct cELISA test kit (Biosense, Bergen, Norway). HPLC analysis was used to quantify particulate DA initially, but the limit of detection (375 pg DA mL^{-1}) was too high for many

samples. The ELISA method has lower limits of detection (9.5-388 pg mL^{-1}) and provided results for more samples. A preserved sample (1 % gluteraldehyde final concentration) for cell counts was taken with the toxin sample. An aliquot of each preserved sample was stained with 0.03% proflavin hemisulfate, filtered onto a 2 μm polycarbonate filter, and mounted on a glass slide with immersion oil for enumeration via epifluorescent microscopy (excitation 450-490 nm, dichromatic beam splitter 500 nm, barrier filter 515 nm; Nikon filter set EF-4 B-2A) at 400X magnification.

Varying Nitrogen Source

To test the effect of nitrogen on growth rate and toxin production, each strain was adapted to grow in f/2* with NO_3^-, NH_4^+, urea and Si at f/20 (88 μmol N, 11 μmol Si) concentrations at 15°C with 150 – 200 μmol photons m^{-2} sec^{-1} (14:10 L:D cycle) through two batch culture generations. When the cultures were adapted, 5 replicate 125 mL flasks were inoculated. Chlorophyll *a* was measured at the same time daily by *in vivo* fluorescence of a subsample of the flasks and used to calculate specific growth rates (Wood et al. 2005). Toxin and preserved samples were taken one week after the beginning of stationary phase to compare toxin production and cell abundance. Dissolved DA $cell^{-1}$ was calculated by dividing dissolved DA by cell abundance.

Differences in growth rates on each nitrogen source were determined for each strain by one-way ANOVA using Tukey's studentized range test (SAS®, version 9.1, SAS Institute, Inc., Cary, NC, USA) at the 5% level. Overall

differences among all treatments were analyzed with a model I one-way nested ANOVA with a type III sums of squares where replication was uneven (SPLUS®, version 6, Insightful Corporation, Seattle, WA, USA). Variation was determined within nitrogen source, species, strain and error by parsing the sum of squares. Identical statistical analysis was performed on the toxin data. Correlation between growth rate and toxin content was analyzed using Spearman's correlation coefficient (SPLUS®, version 6).

Growth versus Irradiance

Three strains of two species were adapted through two batch culture generations to the experimental temperature and irradiance. The experiment was performed in f/2* media at a salinity of 32 in five replicate 10 mL glass tubes at each discrete irradiance. Treatments started at 20 μmol photons m^{-2} sec^{-1} with a 14:10 L:D cycle and increased at approximately 50 μmol photons m^{-2} sec^{-1} intervals until growth rate saturation. Cultures were monitored daily using *in vivo* fluorescence. Growth rates were calculated as described above. Replicates were averaged and a standard deviation was calculated. The data were fitted to a non-linear least squares regression using an equation initially derived for photosynthesis (Platt et al. 1980) and modified to describe growth:

$$\mu = \mu_o + \mu_{max}(1 - e^{((-E\alpha)/\mu max)})e^{((-E\beta)/\mu max)}$$

where μ_o is the y intercept, μ_{max} is the maximum potential growth rate, α is the initial light-limited slope, E is the irradiance and β is the slope of the photoinhibited part of the curve. E_k, the irradiance saturating for growth, was

calculated for each temperature treatment by dividing μ_{max} by α. Significant difference between treatments for each regression parameter was calculated using a Gabriel Approximation (Sokal & Rohlf 1995).

Results

Identification of Cultures

All fifteen cultures were identified as one of three species: *P. multiseries*, *P. calliantha* or *P. fraudulenta* (Fig. 3.1; Table 3.1). Morphometric measurements of the frustules fall within previously reported values for each species (Table 3.2). ITS and LSU rRNA sequences of the cultures show identical or close relationships to sequences deposited on GenBank from strains of the same morphological species isolated globally. The *P. multiseries* culture Pn-1 had 100% LSU sequence similarity to three strains (NWFSC005 and NWFSC011 from Washington and OFPm984 from Japan) and had two base pairs difference from a California isolate CV19 (Fig. 3.2A). Pn-1 had 100% ITS sequence similarity to California strain mu3 and was two base pairs different from the Japanese strain OFPm984 (Fig. 3.2B). Table 3.1 provides GenBank accession numbers for LSU and ITS sequences derived from Chesapeake Bay isolate Pn-1.

Sequences for *P. fraudulenta* cultures Pn-9, Pn-10, Pn-11, Pn-12 and Pn-15 were identical for both the ITS and LSU loci. LSU sequences were identical to a strain from Spain, Limens1, and less than ten base pairs different from three strains (SZN-B21, SZN-B40 AND SZN-B22) from Italy (Fig. 3.2A). The ITS sequence

data revealed two base pairs difference from the Spain Limens1 isolate (Fig. 3.2B). Table 3.1 provides GenBank accession numbers for LSU and ITS sequences derived from Chesapeake Bay isolates Pn-9, Pn-10, Pn-11, Pn-12 and Pn-15.

LSU sequences for *P. calliantha* cultures Pn-2, Pn-3, Pn-4, Pn-6, Pn-7, Pn-8 and Pn-13 were identical to each other, while the ITS sequences exhibited polymorphisms at two positions. Although there were no *P. calliantha* sequence data available for the LSU locus on GenBank, BLAST results showed that the LSU sequence was closest to thirteen *P. pseudodelicatissima* isolates (P-11 from Portugal, NWFSC047, NWFSC040 and NWFSC006 from Washington and the remaining isolates from Italy). There was high genetic variability between all of these isolates, and our sequence showed approximately 0.7 - 5% divergence from those sequences (Fig. 3.2A). Our ITS sequences shared 99% sequence similarity to three *P. calliantha* isolates from Vietnam (Fig. 3.2B). Table 3.1 provides GenBank accession numbers for LSU and ITS sequences derived from Chesapeake Bay isolates Pn-2, Pn-3, Pn-4, Pn-6, Pn-7, Pn-8 and Pn-13.

Toxicity of Cultures

All batch cultures increased their abundance by at least one order of magnitude during this experiment. Lag phase, growth rate and time to onset of stationary phase varied. Out of 16 strains tested, seven produced domoic acid (Table 3.3). All three species cultured had at least two toxic isolates. The *P. multiseries* isolates had the two highest DA concentrations, per mL and per cell

(Figs. 3.3, 3.4). *P. calliantha* and *P. fraudulenta* isolates produced between 10^3 and 10^2 pg DA mL^{-1} (between 1 and 10^{-4} pg DA $cell^{-1}$) with *P. calliantha* producing the least (Pn-3) and the most (Pn-8) toxin per mL (Fig. 3.3). Per cell, *P. fraudulenta* (Pn-12) produced the most (0.16 pg DA $cell^{-1}$) and *P. calliantha* (Pn-3) produced the least ($2.62X10^{-4}$ pg DA $cell^{-1}$) amount of toxin (Fig. 3.4). Growth rates and maximum abundances of strains that did not produce DA are not different from isolates that did produce DA (Table 3.3). Two of the three species cultured, *P. calliantha* and *P. fraudulenta*, had non-toxic isolates (Table 3.3). Three strains of *P. calliantha* (Pn-2, Pn-5 and Pn-18) died within months of isolation, before toxin content was investigated.

The highest total DA levels occurred during stationary phase in all strains. However, in *P. multiseries* and one *P. fraudulenta* strain (Pn-12), some DA was also present before stationary phase while the culture was still growing (Figs. 3.3, 3.4). The two *P. multiseries* strains (Pn-1, CLN47) show similar DA production patterns, with DA present throughout the entire growth cycle, gradually increasing as cell division slowed and reaching a maximum in stationary phase. The two *P. calliantha* strains (Pn-3, Pn-8) also show similar DA production patterns with DA produced only once the cultures had stopped growing. The two *P. fraudulenta* strains (Pn-12, Pn-9) show different DA production patterns. One strain (Pn-12) produces DA during late exponential phase while the other (Pn-9) does not produce toxin until stationary phase. High DA content of the first sample in some strains (Pn-1, CLN47 and Pn-12) is probably from the inoculum used to start the

culture and does not signify any real production of DA in those cultures at the beginning of growth.

Varying Nitrogen Source and Growth Rate

Nitrogen source (NH_4^+, NO_3^- and urea) affected growth rates in *Pseudo-nitzschia* cultures, but not in a way that could be predicted based on nitrogen source and species (Fig. 3.5; Table 3.3). Statistical analysis revealed significant growth rate differences between nitrogen source, species and strain with strain being the highest source and replication the lowest source of variability (Table 3.4). The two *P. multiseries* strains had different nitrogen responses, with Pn-1 growing fastest on NO_3^- and NH_4^+ (0.75 d^{-1}) while CLN47 grew equally well (0.6 d^{-1}) on all sources. The five *P. fraudulenta* strains showed three different growth responses with Pn-10 and Pn-12 growing fastest on NO_3^- and NH_4^+ (0.74-0.88 d^{-1}), while Pn-11 and Pn-15 grew fastest on NH_4^+ (1.21 d^{-1} and 1.16 d^{-1} respectively) and Pn-9 grew fastest on urea (1.02 d^{-1}). The two *P. calliantha* strains had different growth responses, but similar growth rates. Pn-13 grew fastest on NO_3^- and NH_4^+ (0.7 d^{-1}). Pn-8 grew fastest on NO_3^- (0.86 d^{-1}). Nitrogen was an important source of variation in growth rate, but growth was also significantly affected by strain.

Strains of *P. multiseries* (Pn-1), *P. fraudulenta* (Pn-10) and *P. calliantha* (Pn-13), despite being different species, showed similar relative growth responses and growth rates. *P. fraudulenta* strains isolated from the same water sample (Pn-9, Pn-10, Pn-11 and Pn-12) have the largest differences in relative growth

response and growth rate. The only trend in all of these data is higher growth rates on NH_4^+ and lower growth rates on urea, with the exception of one *P. fraudulenta* culture (Pn-9).

Varying Nitrogen Source and Toxicity

DA concentrations were affected by nitrogen source just as unpredictably as growth rate (Figs. 3.6, 3.7; Table 3.3). Statistical analysis revealed significance in toxin content between nitrogen source, species and strain with strain being the highest source of variability in DA content $cell^{-1}$ and mL^{-1}, while for particulate DA mL^{-1} the highest source of variability is species (Table 3.4). The only insignificant effect was that of nitrogen on total DA $cell^{-1}$. Two strains of *P. multiseries* produced the most DA while growing on NH_4^+ (Pn-1, 3984 fg $cell^{-1}$) and NO_3^- (CLN47, 369 fg $cell^{-1}$; Tables 3.3, 3.5; Figs. 3.6, 3.7) and contained the highest total toxin of all strains tested. DA concentration in one strain of *P. calliantha* (Pn-8) and one strain of *P. fraudulenta* (Pn-9) did not vary significantly with nitrogen source. One strain of *P. fraudulenta* (Pn-12) contained more DA while growing on NH_4^+ (23.7 fg $cell^{-1}$) and urea (43.3 fg $cell^{-1}$) than NO_3^- (3.1 fg $cell^{-1}$). The other *P. fraudulenta* strain (Pn-9) contained more particulate toxin when growing on NO_3^- (0.29 fg $cell^{-1}$). Only the two *P. multiseries* (Pn-1 and CLN47) strains always produced detectable DA. This is probably due to the *P. calliantha* and *P. fraudulenta* strains having low toxin content, near the detection limit of the assay, leading to a high error term in the results. Growth rates and toxin production did not track each other except in *P. multiseries* (Pn-1), where

the highest growth rate (0.75 d^{-1}) accompanied the highest toxin content (3984 fg $cell^{-1}$). Statistical analysis reveals no significant correlation between growth rate and toxin content for CLN47, Pn-8 and Pn-12 (Table 3.6). Growth rate is correlated with dissolved DA normalized to cell number and volume and total DA normalized to cell number in strain Pn-9. Growth rate in Pn-1 is strongly correlated with particulate, dissolved and total DA normalized to both cell number and volume.

Toxin content normalized to cell number and volume were nearly identical. CLN47, Pn-1 and Pn-8 show the same pattern in DA content per cell and per mL. One *P. fraudulenta* strain (Pn-12) contained more particulate DA per mL while growing in NH_4^+ and urea with no significant difference in dissolved DA (0.06-1.18 ng mL^{-1}). Per cell, there was no significant difference in particulate DA (0-0.78 fg $cell^{-1}$).

Growth versus Irradiance

Growth versus irradiance experiments were performed on three strains of two species at three temperatures (Fig. 3.8). *P. fraudulenta* (Pn-15) would not grow at all light levels at all temperatures; therefore the curve at 20°C is incomplete. Curve parameters α (d^{-1}[μmol photons m^{-2} sec^{-1}]$^{-1}$)[†] and μ_{max} (d^{-1}) showed significant differences between species at the same temperature (Table 3.7). At 10°C, α (0.0263 d^{-1}[μmol photons m^{-2} sec^{-1}]$^{-1}$) in *P. fraudulenta* was different from α in the *P. multiseries* strains (Pn-1 and CLN47) which were not

[†] Unit for α reduces to μmol photons m^{-2}*86400. While conceptually correct, this method uses unusual units because α is typically used to describe P vs. E curves instead of μ vs. E curves.

different from each other (0.0067, 0.0127 d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$ respectively). At 15°C, α in *P. fraudulenta* (0.0239 d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$) was different from only one of the *P. multiseries* strains (0.0047 d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$, Pn-1) which were not different from each other (0.0109 d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$, CLN47). At 20°, α was not significantly different between strains of *P. multiseries* (0.0094 - 0.0098 d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$; Table 3.7). Alpha was not significantly different between temperatures within the same strain. At 10°C, μ_{max} (d^{-1}) did not show any difference between strains. At 15°C, however there was a difference in μ_{max} between *P. fraudulenta* (0.68) and one of the *P. multiseries* strains (0.33, CLN47). There was no statistically significant difference between μ_{max} at 20°C (0.78-2.0), probably due to the high variability in *P. multiseries* (Pn-1). None of the strains showed significant changes with temperature. Comparisons of β were not possible because not all strains were photoinhibited. Saturating irradiance (E_k) was calculated for each treatment where possible, but had such high variability that none of the data were significant (Table 3.7).

Discussion

Sequences for the LSU (large subunit of the ribosome) and ITS (internal transcribed spacer) regions were successfully combined with morphology to identify *Pseudo-nitzschia* strains to the species level. Both morphological and genetic data were conclusive and consistent for identification of *P. multiseries, P. fraudulenta* and *P. calliantha*, indicating an absence of cryptic species within

those groups. However, these isolates are probably only a small representation of a more genetically diverse *Pseudo-nitzschia* population in the Chesapeake Bay. In fact, real-time molecular assays designed against the three species isolated from the Bay only identified these species in a small percentage of environmental water samples where *Pseudo-nitzschia* had been identified using light microscopy (data not shown). Field studies using rRNA sequences and microsatellite markers have found high genetic diversity in *Pseudo-nitzschia* populations (Orsini et al. 2004, Evans et al. 2005). The lack of such variability in *Pseudo-nitzschia* cultures world-wide could be an artifact of the culturing process favoring strains with similar genetics. Alternate locus sequencing or microsatellite analysis could reveal more diversity in cultures. Physiological parameters tested in this study were not consistently related to species, suggesting the presence of ecotypes and high functional diversity in the species tested. Genetic and functional diversity among and within *Pseudo-nitzschia* species needs to be explored further in order to gain a better understanding of the strains present and how they are related to natural populations.

In batch culture, *Pseudo-nitzschia* typically produces DA when in stationary phase, once cell division has stopped and the culture is limited by P or Si, for example, with replete N and light (Bates 1998). This is seen in Pn-1, Pn-3, Pn-8 and Pn-9 as well as in other studies with *P. multiseries* (Subba Rao et al. 1990, Bates et al. 1991, 1993a, 1995, Douglas & Bates 1992; Wohlgeschaffen et al. 1992, Douglas et al. 1993, Whyte et al. 1995, Kotaki et al. 1999, Lundholm et al. 2004), *P. seriata* (Lundholm et al. 1994, Fehling et al. 2004) and *P. australis*

(Cusack et al. 2002). In CLN47 and Pn-12, there is evidence of toxin production during late exponential phase, similar to results for *P. pseudodelicatissima* (Pan et al. 2001), *P. australis* (Garrison et al. 1992) and for *P. multiseries* (Pan et al. 1996a). A possible explanation for DA production in late exponential phase, before the culture has stopped growing, is that this phase may be a period of transition, when some cells have stopped dividing and are producing DA while others are still growing (Pan et al. 1996a, Bates 1998). High DA content during lag phase is probably from the inoculum used to start the culture; however, it is possible that the shock of being transferred led to DA production in lag phase cultures (Bates 1998). It is also generally assumed that particulate DA peaks about one week after the beginning of stationary phase, then decreases as dissolved DA increases (Bates 1998). Total DA content did not peak in any of our cultures within the 26 d duration of the experiment, with the exception of Pn-12.

Initially, samples were analyzed via HPLC. Toxin content in one strain of *P. multiseries* (Pn-1) and four strains of *P. calliantha* (Pn-4, Pn-6, Pn-7 and Pn-8) was determined with this method. After testing these cultures, it was determined that the limit of detection of the HPLC was too high for these studies and the ELISA method was subsequently employed. ELISA has a lower, but variable, limit of detection. Individual kits have their own quantitation limit (LOQ) and detection limit (LOD = 1/3 of LOQ) which, in the kits used in this study, varied from 28.4 to 1165 pg mL^{-1} and 9.5 to 388 pg mL^{-1}, respectively. Concentrations that are below the LOQ, but above the LOD are considered semi-quantitative.

The test is positive for DA, but the accuracy is less than if the concentration had been above the LOQ. Since each test kit can process a maximum of 36 samples, not all samples from the same culture could always be processed on the same plate and therefore have the same LOD and LOQ. Working with very low amounts of DA on multiple plates could explain the unusual zero values in *P. fraudulenta* (Pn-12) and *P. calliantha* (Pn-8). Some *P. calliantha* strains (Pn-3, Pn-13 and Pn-14), all *P. fraudulenta* strains (Pn-9, Pn-10, Pn-11, Pn-12, Pn-15) and one *P. multiseries* strain (CLN47) were tested using ELISA. The possibility exists that some *P. calliantha* strains (Pn-4, Pn-6 and Pn-7) produced DA, but not enough to detect with HPLC.

It is generally thought that NH_4^+ is more readily taken up by phytoplankton than NO_3^- due to its inhibition of nitrate reductase and lower energy requirement for use (McCarthy et al. 1977, Losada & Guerrero 1979, Syrett 1981). However, studies have shown that inhibition of NO_3^- uptake is rarely as complete or as common as sometimes believed (reviewed in Dortch 1990). Uptake and reduction of NO_3^- can be uncoupled and diatoms in particular take up large amounts of NO_3^- especially under high light, low temperature conditions (DeManche et al. 1979, Dortch et al. 1979, Collos 1982, Lomas & Glibert 1999, 2000). Diatoms are known to grow equally well on NH_4^+ and NO_3^- (Eppley & Renger 1974) and studies on phytoplankton assemblages show utilization of whatever nitrogen source is readily available (McCarthy et al. 1977, Dortch 1990). Uptake rates of NO_3^-, NH_4^+ and urea by diatoms vary considerably and depend on the physiological state of the cell, but uptake of all three nitrogen

species in diatoms has been documented (Eppley et al. 1969, McCarthy 1972). All of these findings are reflected in the current data by the ability of all strains to grow on all nitrogen sources given.

These results demonstrate the ability of *Pseudo-nitzschia* to grow and become toxic on multiple nitrogen sources. *P. multiseries* can grow on NH_4^+, NO_3^-, urea, glutamine and NO_2^- with NH_4^+ supporting the slowest growth (Hillebrand & Sommer 1996). NH_4^+ concentrations above 200 μM do not support growth due to NH_4^+ toxicity (Hillebrand & Sommer 1996), and only at NH_4^+ concentrations of 55 - 110 μM were growth rates comparable to those in NO_3^- (Bates et al. 1993a). The present study used 88 μM nitrogen. Growth rates (d^{-1}) for *P. multiseries* were slightly less (0.36 - 0.33 for NH_4^+, 0.42 – 0.55 for NO_3^- and 0.38 – 0.60 for urea) than those in our experiments (0.61 – 0.76 for NH_4^+, 0.45 – 0.8 for NO_3^- and 0.3 – 0.68 for urea; Hillebrand & Sommer 1996). A study of cultured *P. australis* showed equal growth on NO_3^- and NH_4^+, but reduced growth on urea, with the latter culture containing the highest DA (Armstrong Howard et al. 2007). This is unlike any of the present cultures, except Pn-12 (*P. fraudulenta*). Another study showed higher DA content in *P. multiseries* treated with NH_4^+ than NO_3^- (4.2 pg $cell^{-1}$ in NH_4^+ and ~1.6 pg $cell^{-1}$ in NO_3^- ; Bates et al. 1993a) which is comparable to the DA content in *P. multiseries* (Pn-1) in our study (4.0 pg $cell^{-1}$ when grown in NH_4^+ and 1.9 pg $cell^{-1}$ when grown in NO_3^-). These data show that specific growth rate and toxin content of *Pseudo-nitzschia* can be affected by nitrogen source, but cannot be predicted based on nitrogen and species. There is clearly no strain in this study

that is representative of the growth response or toxin content of the genus or any of the three species.

There are two important concerns with the toxin samples for the nitrogen experiments. First, even though all cultures were sampled at the same time relative to their growth phase, one week after the fluorescence peak is not enough time for these strains to reach maximum DA content (Fig. 3.3). Some of the variation between treatment, species and/or strain could be due instead to time of sample collection relative to DA production cycle in batch culture. Second, the dissolved DA fraction could have been artificially increased by the filtering process. While the vacuum pressure used to filter samples was low (<130 mm Hg), weakened cells in stationary phase could have been lysed, artificially increasing the dissolved fraction. Future experiments might investigate gentler separation techniques such as use of dialysis tubing or gravity filtration.

Statistical analysis (ANOVA) revealed that most variation in toxin content and growth rate is due to strain (except particulate DA mL^{-1}) arguing that strain is more important than species in defining the physiology of *Pseudo-nitzschia*. However, species was the second most important source of variation. If more species were included in this study, especially more toxic species, this relative importance could shift so that species is more important than strain. The actual treatment, nitrogen source, resulted in the lowest source of variation in toxin content and growth rate, sometimes lower than the replicate cultures (particulate DA and total DA $cell^{-1}$), but it was responsible for a higher proportion of variation in growth rate than in toxin content. Nitrogen source had more of an effect on

particulate and total DA mL^{-1} than DA $cell^{-1}$, suggesting that the affect of nitrogen source on toxin content is an indirect effect of growth rate on cell abundance. Most of the variation in toxin content between the set of strains used in this study can be attributed to strain differences, indicating that intraspecies variation is larger than interspecies variation especially among low toxin-producing species.

Toxin content of the present strains was just as variable as growth rate, but the fastest growing cultures were not necessarily the most toxic or *vice versa* (Figs. 3.9, 3.10). Total DA, particulate DA and dissolved DA varied with nitrogen source in both of the *P. multiseries* strains (CLN47, Pn-1) and one of the *P. fraudulenta* strains (Pn-12), with no real pattern or relationship to growth rate. For example, the highest DA content in CLN47 (371 fg $cell^{-1}$) occurred in the NO_3^- treatment, but growth rate was neither the highest nor the lowest on this nitrogen source. This suggests that nitrogen source has an affect on DA production besides the direct effect on growth rate. Previous studies showed a relationship between growth rate and DA content using one strain of *P. multiseries* in continuous and batch culture (Pan et al. 1996a,b). As growth rate increased in continuous cultures, DA content decreased. This study found an inverse relationship between growth rate and toxin content only in Pn-9 (Table 3.6). Growth rate and toxin content in Pn-1 was found to be positively correlated and no relationship was found in the other strains. However, these experiments were performed on stationary phase batch cultures, which are different from slowly growing continuous cultures.

The growth versus irradiance curves seem to show differences in α and μ_{max} among temperature treatments (Fig. 3.8). However, high variability associated with the 20°C treatments render these differences statistically insignificant (Table 3.7). Using a turbidostat to calculate a growth vs. irradiance curve (Falkowski et al. 1985) instead of using replicate batch cultures at discrete irradiances could result in less error. Replication could be achieved by collecting data for multiple curves instead of replicates of points on a single curve. Only one other study contains μ vs. E data for *Pseudo-nitzschia* (Pan et al. 1996b), where an α of 0.0027 d^{-1} [μmol photons m^{-2} sec^{-1}]$^{-1}$ was observed at 10°C in the *P. multiseries* culture NPBIO, but the curve is not very well described below 200 μmol photons m^{-2} sec^{-1}, the light-limiting part of the curve in that study (Fig. 3.8). Nevertheless, the α is within the range observed for *P. multiseries* in the present experiments (Table 3.7). The incomplete curve at 20°C for Pn-15 (Fig. 3.8) can be explained by intolerance of this strain to higher temperatures; this strain would not adapt to 20°C at low irradiance. Pn-15 was isolated from cold waters (Table 3.1) and could represent an ecotype that is adapted to survive at low temperatures. Pn-15 had its highest growth rates at 10°C (Fig. 3.8). A cold-water strain of *P. granii* also did not grow below 100 μmol photons $m^{-2}sec^{-1}$ at 20°C. Chlorophyll in these *P. granii* cells decreased above 14°C suggesting that the decline in growth rate at lower irradiance may have been due to an inability to capture adequate light to support increased enzyme activity associated with higher temperatures (El-Sabaawi & Harrison 2006).

Both bacteria and culture age can influence toxicity of cultures. We did not address variation in epibiotic bacteria, a potential source of variation in toxin levels. For example, two cultures of the same strain (CLN-1 and CLN-1 NRC) of *P. multiseries* that had been maintained in separate laboratories for 2 years differed in their epibiotic bacteria and toxin production (Kaczmarska et al. 2005). The culture with more diverse bacterial flora produced the most DA (CLN-1). Therefore, it is possible that bacteria play an important role in DA content variation between strains. There was also concern that results might be affected by differences over time in culture as many physiological properties have been known to change with culture age, especially *Pseudo-nitzschia* toxin production (Bates et al. 1999). However, most of the cultures were approximately the same age (one year) during this study (Table 3.9).

Results do support the following: Defining diatom species based on morphology alone can be inadequate. A more thorough approach is required which includes morphological investigations, genetic sequencing, mating experiments and physiological experiments (Mann 1999). Numerous *Pseudo-nitzschia* spp. have been described by combinations of morphology and gene sequences (Lundholm et al. 2002a,b, 2003, 2006, Lundholm & Moestrup 2002). Only one *Pseudo-nitzschia* study combines morphology, genetic sequencing and mating experiments (Amato et al. 2007). As the impact of strain differences is recognized, diatom studies should include an investigation of the biological and phylogenetic species concepts in addition to the more traditional morphological species concept (e.g., Behnke et al. 2004, Mann et al. 2004).

The ability of *Pseudo-nitzschia* to grow under multiple nitrogen, light and temperature conditions indicates a broad ecological niche. Not all strains were able to grow optimally under all treatments, but at least one strain did grow optimally under all conditions tested. This, in combination with high genetic variability in natural populations (Evans et al. 2005), suggests that *Pseudo-nitzschia* is able to take advantage of multiple regimes in a changing environment. These data suggest the existence of multiple ecotypes in natural populations of *Pseudo-nitzschia*, similar to *Skeletonema costatum* and *Ditylum brightwellii* (Gallagher 1982, Rynearson et al. 2006). These ecotypes could undergo population succession throughout the year. Since all of the strains tested in this study came from the same region, nothing can be concluded about biogeography; however, it would be reasonable to investigate strains from multiple regions as *Pseudo-nitzschia* is a cosmopolitan diatom genus (Hasle 2002).

Table 3.1 Cultures of *Pseudo-nitzschia* spp. used in the present study.

Clone	Species	GenBank Accession #		Collection Site Information[a]			
		ITS	LSU	Station	Date	Temp (°C)	Salinity
Pn-1	*Pseudo-nitzschia multiseries*	DQ445651	DQ445638	Choptank River	11/18/2002	11	17
Pn-2	*Pseudo-nitzschia calliantha*	DQ445652	DQ445639	Choptank River	4/28/2003	15	9
Pn-3	*Pseudo-nitzschia calliantha*	DQ445653	DQ445640	Choptank River	4/14/2003	10	11
Pn-4	*Pseudo-nitzschia calliantha*	DQ445654	DQ445641	Chesapeake Bay	10/3/2003	21	17
Pn-5	?			Chesapeake Bay	10/3/2003	21	17
Pn-6	*Pseudo-nitzschia calliantha*	DQ445655	DQ445642	Chesapeake Bay	10/4/2003	20	12
Pn-7	*Pseudo-nitzschia calliantha*	DQ445656	DQ445643	Patuxent River	10/13/2003	20	11
Pn-8	*Pseudo-nitzschia calliantha*	DQ445657	DQ445644	Choptank River	10/20/2003	17	10
Pn-9	*Pseudo-nitzschia fraudulenta*	DQ445659	DQ445645	Assateague Island	3/28/2004	6	32
Pn-10	*Pseudo-nitzschia fraudulenta*	DQ445660	DQ445646	Assateague Island	3/28/2004	6	32
Pn-11	*Pseudo-nitzschia fraudulenta*	DQ445661	DQ445647	Assateague Island	3/28/2004	6	32
Pn-12	*Pseudo-nitzschia fraudulenta*	DQ445662	DQ445648	Assateague Island	3/28/2004	6	32
Pn-13	*Pseudo-nitzschia calliantha*	DQ445658	DQ445649	Choptank River	5/17/2004	23	9
Pn-14	*Pseudo-nitzschia calliantha*			Kiptopeke Virginia	11/11/2004	14	30
Pn-15	*Pseudo-nitzschia fraudulenta*	DQ445663	DQ445650	Asilomar California	10/7/2005	11	32
CLN47	*Pseudo-nitzschia multiseries*			CL147 X CL191[b]	6/16/2005		
Pn-16	*Pseudo-nitzschia calliantha*	EF621757	EF621754	Tangier Sound	11/14/2006	13	15
Pn-17	*Pseudo-nitzschia calliantha*	EF621758	EF621755	Tangier Sound	11/14/2006	13	15
Pn-18	*Pseudo-nitzschia calliantha*	EF621759	EF621756	Tangier Sound	11/14/2006	13	15

[a]Collection site information column contains data on water samples from which cultures were isolated.
[b]Strain CLN47 was isolated after sexual reproduction between strains CL147 and CL191.

Table 3.2 Morphometric summary of *Pseudo-nitzschia* cultures used in the present study.

Strain	Species	Length (µm)	Width (µm)	Central interspace	Poroids (in 1 µm)	Striae (in 10 µm)	Fibulae (in 10 µm)	Band striae (in 10 µm)	Rows of poroids
*	*P. multiseries*	55-169	2.8-5.3	Absent	4-7	9-16	9-16	19-30	3-4 (5)
Pn-1	*P. multiseries*	55-82 (66.3) {9.0} [6]	2.4-4 (3.2) {0.5} [8]	Absent	4-8 (5.7) {1.7} [4]	10-16 (11.9) {1.7} [10]	10-16 (12.5) {2.0} [8]	18-26 (21) {3.6} [4]	3-4
CLN47	*P. multiseries*	52-76 (64.0) {10.0} [4]	3.3-3.8 (3.6) {0.2} [4]	Absent					
*	*P. calliantha*	41-98	1.3-2.6	Present	4-6	34-39	15-22	42-48	1.0
Pn-2	*P. calliantha*	55-61 (57.8) {4.8} [5]	1.5-3.0 (2.4) {0.6} [6]	Present	6-7 (6.75) {0.5} [4]	32-40 (36) {5.6} [2]	10-11 (10.5) {0.7} [2]	40 (40) {0} [1]	1
Pn-3	*P. calliantha*	62-66 (64.5) {2.3} [4]	1.7-2.3 (2.0) {0.2} [5]	Present	4-6 (5.3) {1.2} [3]	34-42 (38) {5.7} [2]	15-22 (18.5) {5} [2]		1
Pn-4	*P. calliantha*	29-37 (31.7) {4.6} [3]	2-2.5 (2.2) {0.2} [4]	Present	5-6 (5.2) {0.7} [3]	35-38 (36.3) {1.2} [7]	16-19.2 (18) {1.1} [6]	34-49.6 (43.2) {8.2} [3]	1
Pn-6	*P. calliantha*	43-71 (56.1) {14.7} [4]	1.9-2.8 (2.3) {0.4} [5]	Present	5-6 (5.5) {0.7} [2]	39-40 (39.5) {0.7} [2]	20-22 (21) {1.4} [2]		1
Pn-7	*P. calliantha*	22-33 (26.7) {4.2} [5]	1.7-3.5 (2.5) {0.6} [6]	Present	4-6 (5) {1.4} [2]	32-34 (33) {1.4} [2]	12-13 (12.5) {0.7} [2]		1
Pn-8	*P. calliantha*	46-55 (48.7) {3.6} [6]	1.8-2.5 (2.0) {0.3} [4]	Present	5-6 (5) {0.4} [5]	36.6-40 (38.8) {1.4} [5]	17.1-21.7 (19.2) {2.0} [5]	37.7-40 (39.2) {1.3} [3]	1
Pn-13	*P. calliantha*	84-87 (85.3) {1.5} [3]	1.9-2.7 (2.3) {0.3} [7]	Present	4-5 (4.3) {0.6} [3]	24-27 (25.6) {1.1} [8]	10-14 (12.3) {1.3} [8]	30-35 (31.8) {2.4} [4]	1
Pn-14	*P. calliantha*	42-60 (50.7) {6.3} [5]	2-2.3 (2.1) {0.1} [3]	Present	5-6 (5.7) {0.6} [3]	34-42 (37.3) {4.2} [3]	20-24 (22) {2} [3]	44 (44) {0} [1]	1
Pn-16	*P. calliantha*	73-81 (77.7) {3.1} [6]	2.1-2.8 (2.4) {0.3} [5]	Present	4 (4) {0} [1]	32 (32) {0} [1]	17 (17) {0} [1]	38 (38) {0} [1]	1
Pn-17	*P. calliantha*	53-70 (58.4) {6.8} [9]	2.1-3.0 (2.5) {0.3} [9]	Present	4 (4) {0} [1]	36 (36) {0} [1]	19 (19) {0} [1]		1
Pn-18	*P. calliantha*	86.9 (86.9) {0} [1]	2.6 (2.6) {0} [1]	Present	4 (4) {0} [1]	35 (35) {0} [1]	17 (17) {0} [1]		1
*	*P. fraudulenta*	50-164	4.0-10	Present	4-7	17-26	12-26	35-40	2-3
Pn-9	*P. fraudulenta*	71-78 (74.4) {3.2} [5]	3-4 (3.8) {0.5} [4]	Present	5.6-7 (6.1) {0.8} [3]	21-24 (22.4) {1.0} [9]	18-20.6 (19.5) {0.8} [8]	33-40 (37.3) {2.6} [7]	2-3
Pn-10	*P. fraudulenta*	52-70 (60.0) {6.0} [6]	3-4 (3.4) {0.6} [3]	Present	6 (6) {0.6} [2]	20-24 (21.6) {2.0} [4]	16 (16) {0} [1]	36-40 (38) {2.0} [3]	2-3
Pn-11	*P. fraudulenta*	62-66 (64.3) {2.1} [3]	3-4 (3.7) {0.6} [3]	Present		20-23 (21.7) {1.2} [4]	20.8-22 (21.5) {0.6} [4]	36 (36) {0} [1]	2-3
Pn-12	*P. fraudulenta*	61-71 (64.5) {3.9} [5]	3.8-5.2 (4.6) {0.6} [4]	Present	6-6.4 (6.2) {0.3} [2]	20-24 (22.1) {2.0} [3]	22-24 (22.9) {1.0} [3]	28.8 (28.8) {0} [1]	2-3
Pn-15	*P. fraudulenta*	69-89 (80.2) {10.0} [4]	4.9-6 (5.6) {0.6} [3]	Present	5-6 (5.3) {0.6} [3]	20-23 (21.4) {1.1} [5]	11-20 (17) {3.5} [5]	32 (32) {0} [1]	2-3

The data is given as range of measurement (mean) {standard deviation} [replicates]. * Indicates measurements are referenced in other studies (Hasle 1965; Rivera 1985; Hasle et al. 1996; Skov et al. 1999; Stonik et al. 2001; Lundholm et al. 2003; Caroppo et al. 2005).

Table 3.3 Growth and toxicity of *Pseudo-nitzschia* cultures used in the present study.

Strain	Species	Salinity	Culture Volume (L)	Nitrogen Source	dissolved DA		particulate DA		growth μ (d^{-1})	Maximum Abundance
					(ng mL^{-1})	(fg $cell^{-1}$)	(ng mL^{-1})	(fg $cell^{-1}$)		
Pn-2	*P. calliantha*	15	1.5	NO_3^-	ND[a]	ND	ND	ND		
Pn-3	*P. calliantha*	15	1.5	NO_3^-	ND	ND	0.27	0.26	0.58	1.78 X 10^6
Pn-4	*P. calliantha*	15	1.5	NO_3^-	ND	ND	ND	ND	0.45	7.47 X 10^5
Pn-6	*P. calliantha*	15	1.5	NO_3^-	ND	ND	ND	ND	0.61	1.04 X 10^6
Pn-7	*P. calliantha*	15	1.5	NO_3^-	ND	ND	ND	ND	0.82	2.72 X 10^5
Pn-8	*P. calliantha*	15	1.5	NO_3^-	1.7	0.86	6.3	5.7	0.42	1.97 X 10^6
Pn-8	*P. calliantha*	15	0.065	NO_3^-	0.4 ± 0.49	2.0 ± 2.82	0.101 ± 0.0239	1.8 ± 2.51	0.55 ± 0.047	
Pn-8	*P. calliantha*	15	0.065	NH_4^+	0.3 ± 0.22	3.5 ± 5.41	0.14 ± 0.050	4.7 ± 7.7	0.87 ± 0.154	
Pn-8	*P. calliantha*	15	0.065	urea	1.2 ± 2.67	19.8 ± 44.26	0.17 ± 0.057	3.5 ±2.66	0.42 ± 0.056	
Pn-13	*P. calliantha*	15	1.5	NO_3^-	+[b]	+	+	+	0.68	3.64 X 10^5
Pn-13	*P. calliantha*	15	0.065	NO_3^-					0.72 ± 0.071	
Pn-13	*P. calliantha*	15	0.065	NH_4^+					0.71 ± 0.064	
Pn-13	*P. calliantha*	15	0.065	urea					0.44 ± 0.028	
Pn-16	*P. calliantha*	15	1.5	NO_3^-	ND	ND	ND	ND	0.9	1.41 X 10^5
Pn-17	*P. calliantha*	15	1.5	NO_3^-	ND	ND	ND	ND	0.9	4.61 X 10^5
Pn-9	*P. fraudulenta*	32	1.5	NO_3^-	ND	ND		0.667	0.56	7.33 X 10^5
Pn-9	*P. fraudulenta*	32	0.065	NO_3^-	0.6 ± 0.71	32 ± 43.3	0.009 ± 0.011	0.29 ± 0.389	0.75 ± 0.051	
Pn-9	*P. fraudulenta*	32	0.065	NH_4^+	0.9 ± 0.55	20 ± 11.0	ND	ND	0.62 ± 0.038	
Pn-9	*P. fraudulenta*	32	0.065	urea	0.2 ± 0.21	4.7 ± 5.95	ND	ND	1.02 ± 0.137	
Pn-10	*P. fraudulenta*	32	1.5	NO_3^-	ND	ND	ND	ND	0.6	2.49 X 10^5
Pn-10	*P. fraudulenta*	32	0.065	NO_3^-					0.84 ± 0.035	
Pn-10	*P. fraudulenta*	32	0.065	NH_4^+					0.85 ± 0.058	
Pn-10	*P. fraudulenta*	32	0.065	urea					0.58 ± 0.067	

Table 3.3 cont.

Strain	Species	Salinity	Culture Volume (L)	Nitrogen Source	dissolved DA		particulate DA		growth μ (d^{-1})	Maximum Abundance
					(ng mL^{-1})	(fg $cell^{-1}$)	(ng mL^{-1})	(fg $cell^{-1}$)		
Pn-11	*P. fraudulenta*	32	1.5	NO_3^-	ND	ND	ND	ND	0.46	2.18×10^5
Pn-11	*P. fraudulenta*	32	0.065	NO_3^-					0.73 ± 0.036	
Pn-11	*P. fraudulenta*	32	0.065	NH_4^+					1.22 ± 0.177	
Pn-11	*P. fraudulenta*	32	0.065	urea					0.55 ± 0.103	
Pn-12	*P. fraudulenta*	32	1.5	NO_3^-	0.525	4.6	0.057	1.6	0.37	1.59×10^5
Pn-12	*P. fraudulenta*	32	0.065	NO_3^-	0.06 ± 0.139	2.7 ± 5.92	0.015 ± 0.0092	0.42 ± 0.257	0.75 ± 0.039	
Pn-12	*P. fraudulenta*	32	0.065	NH_4^+	0.8 ± 0.38	30 ± 17.0	ND	ND	0.88 ± 0.048	
Pn-12	*P. fraudulenta*	32	0.065	urea	1.2 ± 0.24	53 ± 19.7	0.019 ± 0.0269	0.8 ± 1.03	0.58 ± 0.181	
Pn-15	*P. fraudulenta*	32	1.5	NO_3^-	ND	ND	ND	ND	1.2	2.27×10^5
Pn-15	*P. fraudulenta*	32	0.065	NO_3^-					1.05 ± 0.033	
Pn-15	*P. fraudulenta*	32	0.065	NH_4^+					1.16 ± 0.020	
Pn-15	*P. fraudulenta*	32	0.065	urea					1.05 ± 0.030	
CLN47	*P. multiseries*	32	1.5	NO_3^-	3.76	57.2	69.6	1059	0.8	1.14×10^5
CLN47	*P. multiseries*	32	0.065	NO_3^-	1.2 ± 0.33	8.9 ± 2.98	48 ± 29.1	362 ± 254.3	0.65 ± 0.018	
CLN47	*P. multiseries*	32	0.065	NH_4^+	0.19 ± 0.039	1.8 ± 0.35	5.0 ± 2.77	48 ± 23.6	0.61 ± 0.054	
CLN47	*P. multiseries*	32	0.065	urea	0.16 ± 0.094	1.0 ± 0.54	3.9 ± 5.53	24 ± 32.6	0.68 ± 0.070	
Pn-1	*P. multiseries*	15	1.5	NO_3^-	17.3	20	398	458	0.45	1.14×10^6
Pn-1	*P. multiseries*	15	0.065	NO_3^-	30 ± 3.7	584 ± 169.4	65 ± 11.2	1250 ± 351.8	0.76 ± 0.104	
Pn-1	*P. multiseries*	15	0.065	NH_4^+	103 ± 11.5	2134 ± 154.2	87 ± 25.1	406 ± 53.7	0.76 ± 0.175	
Pn-1	*P. multiseries*	15	0.065	urea	2.9 ± 1.10	61 ± 16.8	19 ± 3.5	1807 ± 514.3	0.30 ± 0.100	

[a]ND indicates that DA was not found.
[b]+ indicates that toxin was found, but not quantified.

Table 3.4 Nested ANOVA tables for growth and toxicity experiments using different nitrogen sources.

Growth (Fig. 3.5)	df	SS	MS	F	P
nitrogen	2	1.22	0.611	77.1	<0.001
species nested in nitrogen	6	1.79	0.298	37.6	<0.001
strain nested in species nested in nitrogen	18	3.39	0.188	23.7	<0.001
replicate cultures	106	0.84	0.008		

Dissolved DA cell^{-1} (Fig. 3.7)	df	SS	MS	F	P
nitrogen	2	1751435	875717	216.7	<0.001
species nested in nitrogen	6	6875495	1145916	283.5	<0.001
strain nested in species nested in nitrogen	6	12139455	2023243	500.6	<0.001
replicate cultures	56	226342	4042		

Dissolved DA mL^{-1} (Fig. 3.6)	df	SS	MS	F	P
nitrogen	2	4038	2019	180.5	<0.001
species nested in nitrogen	6	16393	2732	244.3	<0.001
strain nested in species nested in nitrogen	6	28338	4723	422.3	<0.001
replicate cultures	56	626	11		

Particulate DA cell^{-1} (Fig. 3.7)	df	SS	MS	F	P
nitrogen	2	935125	467562	13.8	<0.001
species nested in nitrogen	6	8974923	1495821	44.3	<0.001
strain nested in species nested in nitrogen	6	10097263	1682877	49.8	<0.001
replicate cultures	58	1958029	33759		

Particulate DA mL^{-1} (Fig. 3.6)	df	SS	MS	F	P
nitrogen	2	3522	1761	15.5	<0.001
species nested in nitrogen	6	31698	5283	46.5	<0.001
strain nested in species nested in nitrogen	6	18149	3025	26.6	<0.001
replicate cultures	58	6595	114		

Total DA cell^{-1} (Fig. 3.7)	df	SS	MS	F	P
nitrogen	2	136266	68133	1.7	0.200
species nested in nitrogen	6	21394034	3565672	87.8	<0.001
strain nested in species nested in nitrogen	6	28286001	4714333	116.1	<0.001
replicate cultures	60	2436325	40605		

Total DA mL^{-1} (Fig. 3.6)	df	SS	MS	F	P
nitrogen	2	11648	5824	67.3	<0.001
species nested in nitrogen	6	85654	14276	165.0	<0.001
strain nested in species nested in nitrogen	6	90050	15008	173.5	<0.001
replicate cultures	56	4845	87		

Table 3.5 Results of ANOVA and Tukey's studentized range multiple comparisons test ($\alpha = 0.05$) for domoic acid content (normalized to culture volume and cell number) in *Pseudo-nitzschia* strains grown on NO_3^-, NH_4^+ and urea. Pn-1 and CLN47 are *P. multiseries*. Pn-8 is *P. calliantha*. Pn-9 and Pn-12 are *P. fraudulenta*.

Strain	per mL									per cell								
	particulate			dissolved			total			particulate			dissolved			total		
	NH_4^+	NO_3^-	urea	NH_4^+	NO_3^-	urea	NH_4^+	NO_3^-	urea	NH_4^+	NO_3^-	urea	NH_4^+	NO_3^-	urea	NH_4^+	NO_3^-	urea
Pn-1	A*	A*	B	A*	B	C	A*	B	C	A*	A*	B	A*	B	C	A*	B	C
Pn-8	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Pn-9	A	A	A	A	A	A	A	A	A	A	B*	A	A	A	A	A	A	A
Pn-12	A*	B	A*	A	A	A	A*	B	A*	A	A	A	AB	B	A*	A	A	A
CLN47	AB	A*	B	AB	A*	B	AB	A*	B	B	A*	B	B	A*	B	B	A*	B

Data (in Figures 3.6 and 3.7) for the particulate fraction, dissolved fraction and total DA (dissolved + particulate) content was tested separately. Treatments with different letters indicate significantly different means. *Indicate treatments with higher toxin content.

Table 3.6 Spearman's rank coefficient (ρ) for growth rate (μ) and DA content. Data were grouped and analyzed by strain.

Strain	Particulate DA (ng mL^{-1})		Dissolved DA (ng mL^{-1})		Total DA (ng mL^{-1})		Particulate DA (fg $cell^{-1}$)		Dissolved DA (fg $cell^{-1}$)		Total DA (fg $cell^{-1}$)	
	ρ	p value	ρ	p value	ρ	p value	ρ	p value	ρ	p value	ρ	p value
CLN47	**-0.1210**	**0.6569**	**-0.1430**	**0.6006**	**-0.1210**	**0.6569**	**-0.1210**	**0.6569**	**-0.2332**	**0.396**	**-0.1210**	**0.6569**
Pn-1	**0.7377***	**0.0059**	**0.6571***	**0.0142**	**0.6893***	**0.0101**	**0.6893***	**0.0101**	**0.6231***	**0.0201**	**0.6643***	**0.0132**
Pn-8	**-0.4554**	**0.0989**	**0.0916**	**0.7471**	**0.1826**	**0.5154**	**-0.2860**	**0.2987**	**0.2138**	**0.4455**	**-0.1293**	**0.6474**
Pn-9	**-0.0974**	**0.7196**	**-0.5479***	**0.0473**	**-0.5385**	**0.0513**	**-0.1046**	**0.7002**	**-0.5868***	**0.0337**	**-0.5868***	**0.0337**
Pn-12	**-0.2426**	**0.3953**	**-0.0530**	**0.8469**	**-0.0578**	**0.8339**	**-0.1944**	**0.4947**	**-0.1757**	**0.5364**	**-0.1788**	**0.5294**

*Indicates significance at 0.05 level

Table 3.7 Growth vs. irradiance curve parameters for three strains of two species of *Pseudo-nitzschia* grown in three temperatures. Pn-1 and CLN47 are *P. multiseries*. Pn-15 is *P. fraudulenta*.

Strain	temp (°C)	α (d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$)	β (d^{-1}[µmol photons m^{-2} sec^{-1}]$^{-1}$)	μ_{max} (d^{-1})	E_k (µmol photons m^{-2} sec^{-1})
Pn-1	**10**	**0.0067 (0.0016)^{A}**	**2.4X10^{-4} (0.0002)**	**0.24 (0.045)**	**36 (15)**
	15	**0.0047 (0.0016)^{C}**	**0**	**0.32 (0.148)**	**70 (55)**
	20	**0.0098 (0.0026)**	**0.003 (0.0107)**	**2.0 (3.4)**	**200 (410)**
CLN47	**10**	**0.0127 (0.0030)^{B}**	**2X10^{-4} (0.00047)**	**0.33 (0.071)**	**26 (12)**
	15	**0.0109 (0.0025)**	**0**	**0.33 (0.057)^{A}**	**30 (12.0)**
	20	**0.0094 (0.0052)**	**0**	**?**	**?**
Pn-15	**10**	**0.0263 (0.0001)^{AB}**	**0**	**?**	**?**
	15	**0.0239 (0.0047)^{C}**	**0.0004 (0.0003)**	**0.68 (0.072)^{A}**	**28 (9)**
	20	**?**	**?**	**0.78 (0.04)**	**?**

Values for α, β, μ_{max} and E_k are given with standard error in parentheses. Identical letter superscripts indicate significant difference between parameters at the 0.05 level. Photoinhibition (β) was not tested statistically due to missing values. Data are in Figure 3.8.

Table 3.8 Replicates (n) for experiments testing growth and toxin production in cultures grown on different nitrogen sources. Data is in Figures 3.5, 3.6 and 3.7.

Strain	Species	toxin			growth		
		NO_3^-	NH_4^+	urea	NO_3^-	NH_4^+	urea
Pn-1	***P. multiseries***	**5[a]/5[b]**	**5/5**	**5/5**	**5**	**5**	**5**
Pn-3	***P. calliantha***				**4**	**5**	**5**
Pn-8	***P. calliantha***	**5/5**	**5/5**	**5/5**			
Pn-9	***P. fraudulenta***	**5/5**	**3/5**	**4/4**	**5**	**5**	**5**
Pn-10	***P. fraudulenta***				**5**	**5**	**4**
Pn-11	***P. fraudulenta***				**5**	**5**	**5**
Pn-12	***P. fraudulenta***	**5/5**	**5/4**	**5/5**	**5**	**5**	**5**
Pn-13	***P. calliantha***				**5**	**5**	**5**
Pn-15	***P. fraudulenta***				**5**	**5**	**5**
CLN47	***P. multiseries***	**5/5**	**5/5**	**5/5**	**5**	**5**	**5**

[a]replicates for the particulate fraction
[b]replicates for the dissolved fraction

Table 3.9 Age of cultures in years at the time of experimentation.

Strain	Species	Culture Age (year)		
		Toxin[a]	Nitrogen[b]	Irradiance[c]
Pn-1	***P. multiseries***	**1.5**	**2.5**	**2.2**
Pn-3	***P. calliantha***	**1**		
Pn-4	***P. calliantha***	**0.3**		
Pn-6	***P. calliantha***	**0.3**		
Pn-7	***P. calliantha***	**0.5**		
Pn-8	***P. calliantha***	**0.5**	**1.5**	
Pn-9	***P. fraudulenta***	**0.25**	**1.2**	
Pn-10	***P. fraudulenta***	**0.75**	**1.2**	
Pn-11	***P. fraudulenta***	**0.75**	**1.2**	
Pn-12	***P. fraudulenta***	**0.75**	**1.3**	
Pn-13	***P. calliantha***	**0.6**	**1**	
Pn-15	***P. fraudulenta***	**0.25**	**0.2**	**0.5**
CLN47	***P. multiseries***	**1.2**	**1.3**	**1.5**
Pn-16	***P. calliantha***	**0.2**		
Pn-17	***P.calliantha***	**0.2**		

[a]Results in Figures 3.3 and 3.4
[b]Results in Figures 3.5, 3.6 and 3.7
[c]Results in Figure 3.8.

Table 3.10 Limit of quantitation and limit of detection for ELISA method used to analyze data presented in Figs. 3.3 and 3.4. Particulate DA for Panel A and Panel C were obtained via HPLC, thus no LOD is presented.

Panel	Number	particulate		dissolved	
		LOQ	LOD	LOQ	LOD
A	1	375		345	115
B	1	163.2	54.4	1020	340
	2	28.8	9.6	1020	340
	3	792	264	1020	340
	4	792	264	396	132
C	1	375		420	140
	2	375		1020	340
	3	375		1165	388.3
D	1	111.2	37	1165	388.3
	2	28.4	9.47	420	140
E	1	55.2	18.4	345	115
	2	44.8	14.9	345	115
	3	55.2	18.4	1020	340
	4	28.8	9.6	1020	340
F	1	111.2	37	420	140
	2	34	11.3	420	140

Fig. 3.1 TEM micrographs of digested frustules of *Pseudo-nitzschia* cultures. A, B and C (scale bar = 1 μm) show arrangement of poroids. D, E and F (scale bar = 5μm) show presence or absence of central interspace and transapical axis. A and F are *P. fraudulenta* (Pn-12), B and E are *P. calliantha* (Pn-13) and C and D are *P. multiseries* (Pn-1).

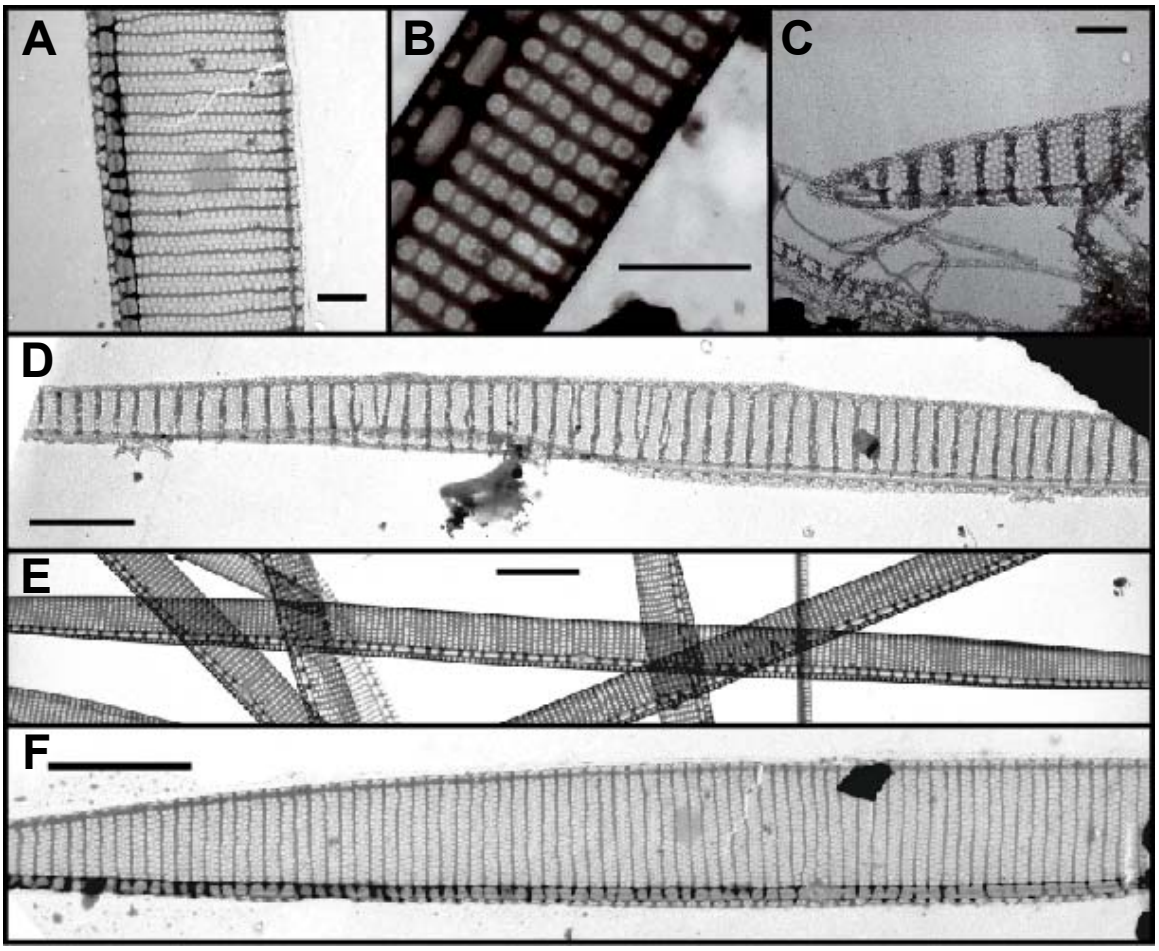


Fig. 3.2 Parsimony tree inferred for the D1-D3 variable region of LSU (A; 720 positions included) and for the ITS1-5.8S-ITS2 region (B; approx. 990 positions included) from Chesapeake Bay isolates of *Pseudo-nitzschia* (in bold) and sequence data available on GenBank. Analysis was performed using heuristic searches (25X random addition of sequences) with TBR (tree bisection and reconnection) branch swapping. Gaps were treated as a fifth character state in figure 3.2A, and the approximate number of character changes are shown on branches. Each species includes culture identification, location and GenBank accession number.

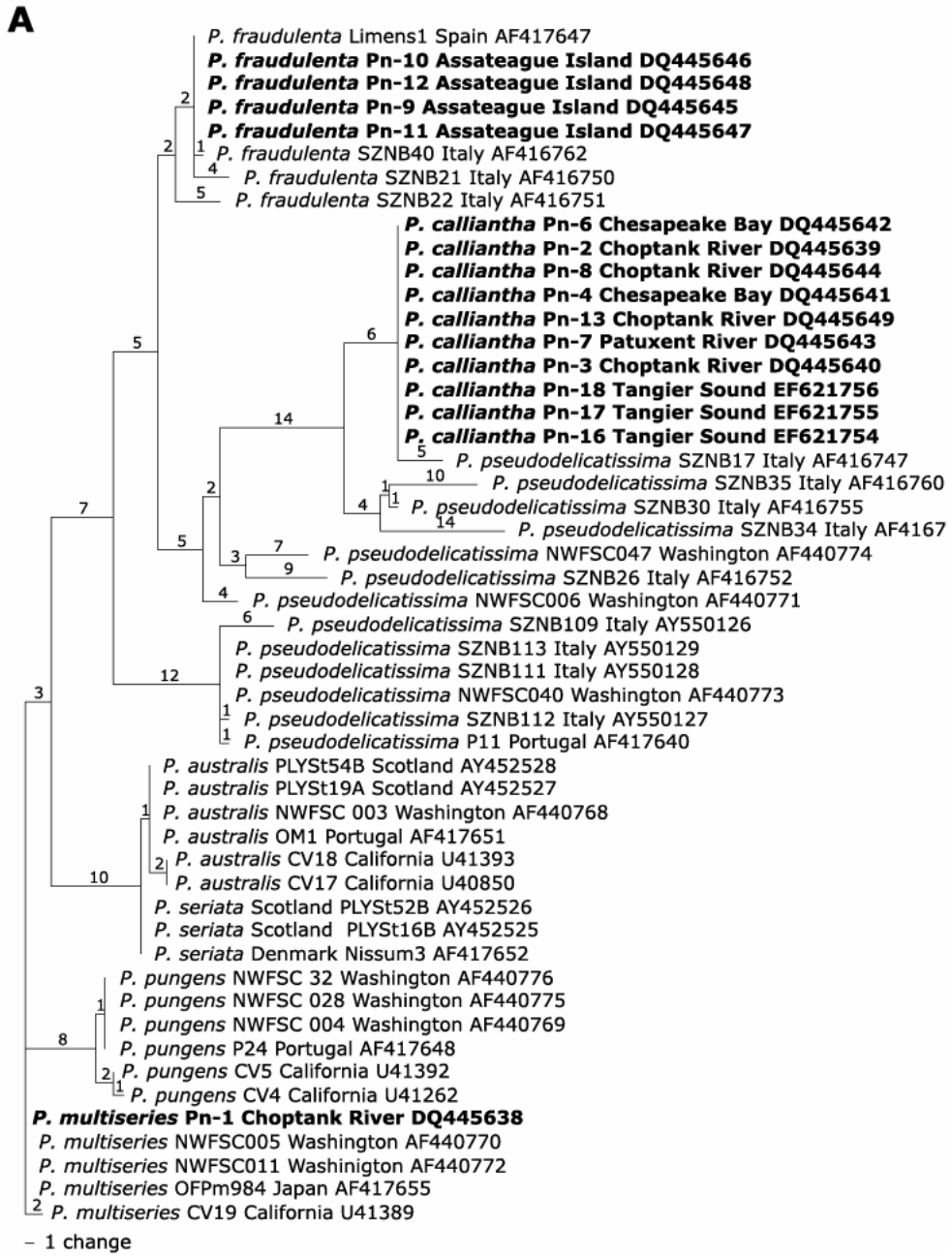


Fig. 3.2 cont.

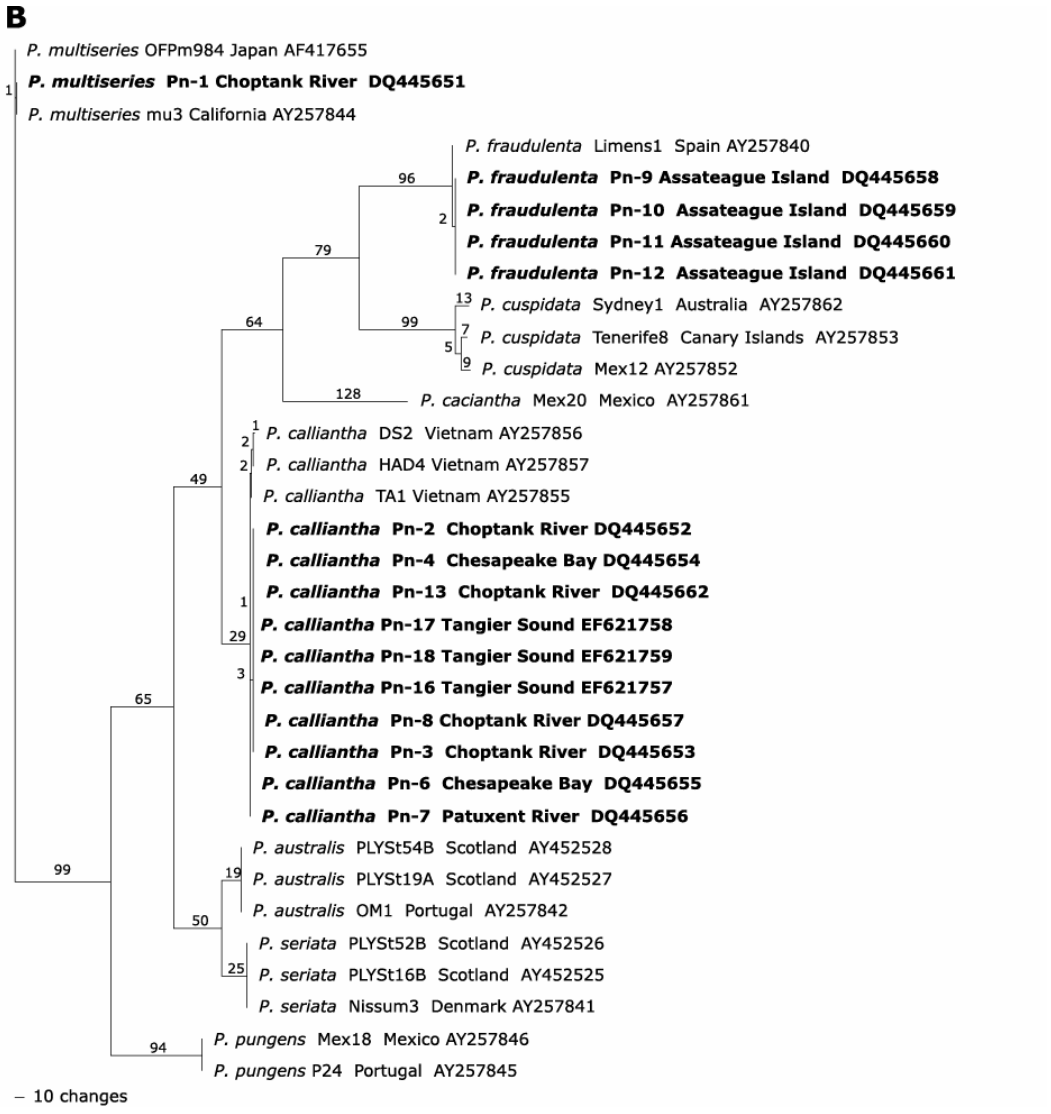

B
P. multiseries OFPm984 Japan AF417655
P. multiseries Pn-1 Choptank River DQ445651
P. multiseries mu3 California AY257844
P. fraudulenta Limens1 Spain AY257840
P. fraudulenta Pn-9 Assateague Island DQ445658
P. fraudulenta Pn-10 Assateague Island DQ445659
P. fraudulenta Pn-11 Assateague Island DQ445660
P. fraudulenta Pn-12 Assateague Island DQ445661
P. cuspidata Sydney1 Australia AY257862
P. cuspidata Tenerife8 Canary Islands AY257853
P. cuspidata Mex12 AY257852
P. caciantha Mex20 Mexico AY257861
P. calliantha DS2 Vietnam AY257856
P. calliantha HAD4 Vietnam AY257857
P. calliantha TA1 Vietnam AY257855
P. calliantha Pn-2 Choptank River DQ445652
P. calliantha Pn-4 Chesapeake Bay DQ445654
P. calliantha Pn-13 Choptank River DQ445662
P. calliantha Pn-17 Tangier Sound EF621758
P. calliantha Pn-18 Tangier Sound EF621759
P. calliantha Pn-16 Tangier Sound EF621757
P. calliantha Pn-8 Choptank River DQ445657
P. calliantha Pn-3 Choptank River DQ445653
P. calliantha Pn-6 Chesapeake Bay DQ445655
P. calliantha Pn-7 Patuxent River DQ445656
P. australis PLYSt54B Scotland AY452528
P. australis PLYSt19A Scotland AY452527
P. australis OM1 Portugal AY257842
P. seriata PLYSt52B Scotland AY452526
P. seriata PLYSt16B Scotland AY452525
P. seriata Nissum3 Denmark AY257841
P. pungens Mex18 Mexico AY257846
P. pungens P24 Portugal AY257845
10 changes

Fig. 3.3 Abundance (open squares) and DA concentration normalized to culture volume (diamonds) over time in six *Pseudo-nitzschia* strains of three species grown in silicate-limited medium with NO_3^- as a nitrogen source. DA concentrations represent the sum of particulate and dissolved fractions. Open diamonds represent semi-quantitative data (<LOQ, but >LOD). Closed diamonds represent quantitative values (>LOQ). Numbers shown within diamonds indicate LOD and LOQ for each sample in pg mL^{-1} which can be found in Table 3.10. Zero values were plotted as values of one. (A) Pn-1, *P. multiseries*. (B) CLN47, *P. multiseries*. (C) Pn-8, *P. calliantha*. (D) Pn-3, *P. calliantha*. (E) Pn-12, *P. fraudulenta*. (F) Pn-9, *P. fraudulenta*.

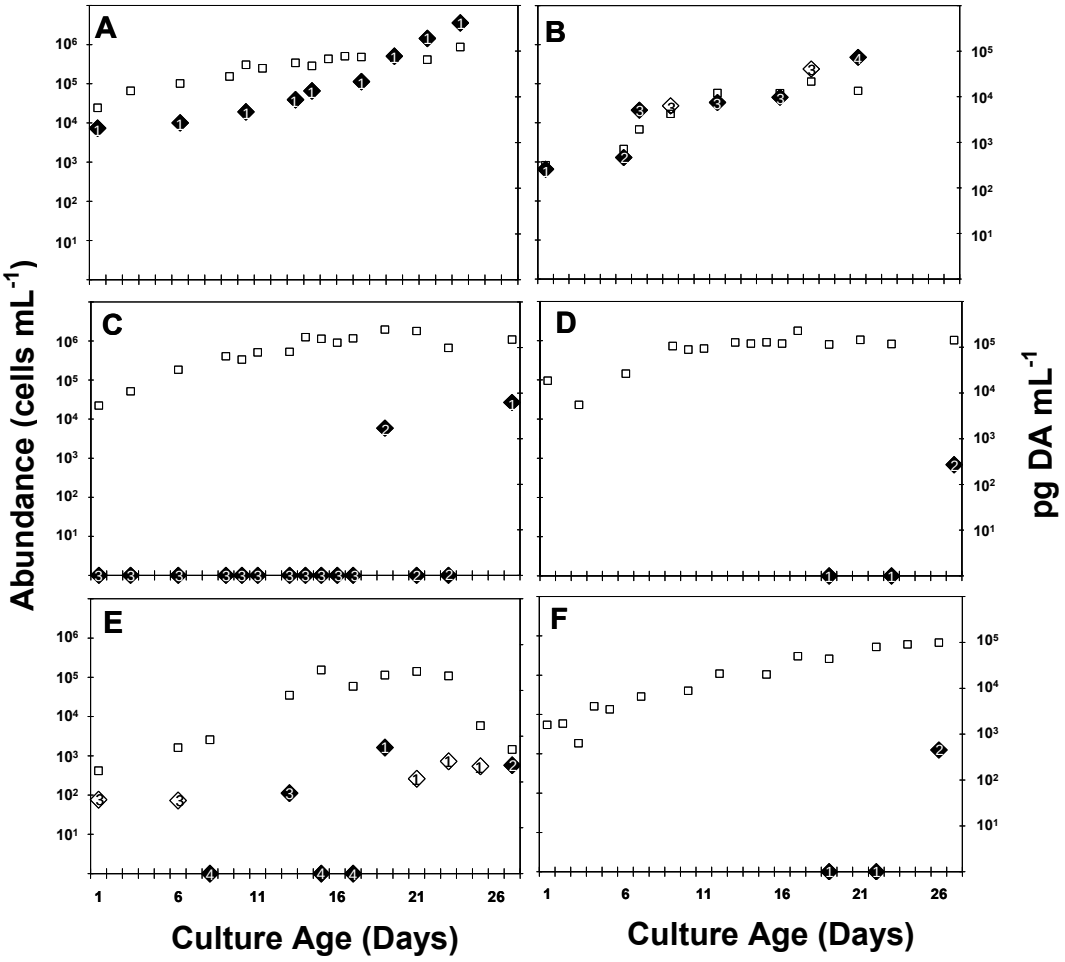


Fig. 3.4 Abundance (open squares) and DA concentration normalized to cell number (diamonds) over time in six *Pseudo-nitzschia* strains of three species. DA concentrations represent the sum of particulate and dissolved fractions. Closed diamonds represent quantitative values (definitions as in Fig. 3.3). LOD and LOQ for each sample are given in Table 3.10. Zero values were plotted as values of 1. (A) Pn-1, *P. multiseries*. (B) CLN47, *P. multiseries*. Open diamonds indicate samples where dissolved fraction was below LOQ. (C) Pn-8, *P. calliantha*. (D) Pn-3, *P. calliantha*. (E) Pn-12, *P. fraudulenta*. (F) Pn-9, *P. fraudulenta*.

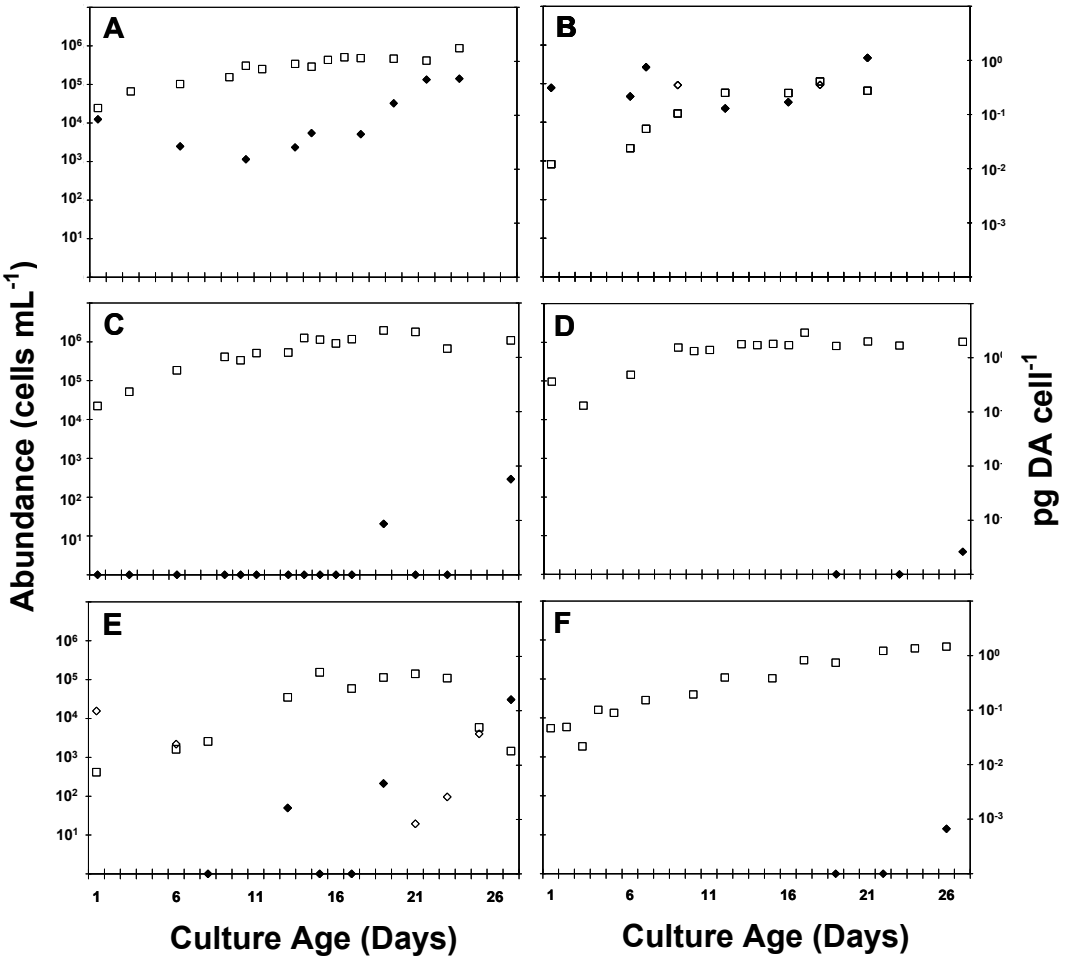


Fig. 3.5 Specific growth rates (μ) for strains of *Pseudo-nitzschia* grown on NO_3^-, NH_4^+ and urea. Error bars represent one SD. Means with identical letters above bars are not significantly different at 0.05 using Tukey's studentized range test for each strain. Strain designations are given along the x axis and species names are given at the top of the figure. Replication is shown in Table 3.8.

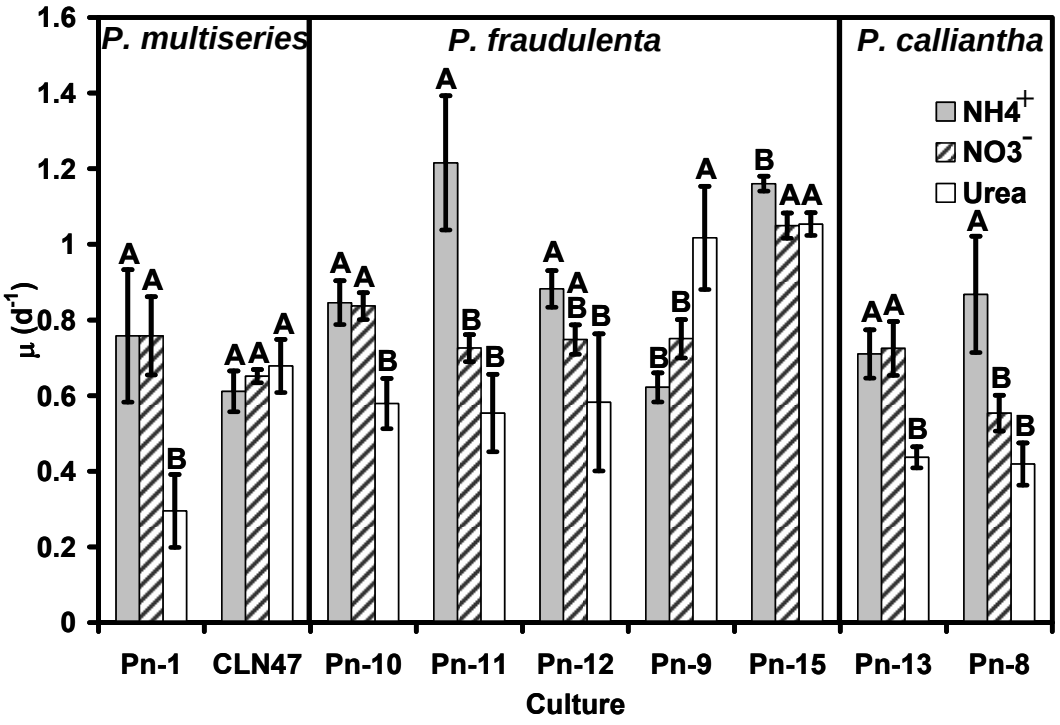


Fig. 3.6 DA content (ng mL^{-1}) one week after fluorescence peak in strains of *Pseudo-nitzschia* grown on NO_3^-, NH_4^+ and urea in silicate-limited media. Filled bars show particulate DA and open bars show dissolved DA. Error bars represent one SD. Letters showing significant difference are in Table 3.5. Replication is shown in Table 3.8. Note the differences in scale. (A) Pn-1, *P. multiseries* (B) CLN47, *P. multiseries*. Mean dissolved DA in NH_4^+ and urea treatments contain semi-quantitative data (<LOQ, but >LOD). (C) Pn-8, *P. calliantha*. Mean dissolved DA in the NH_4^+ treatment contains semi-quantitative data. (D) Pn-12, *P. fraudulenta*. Mean dissolved DA in the NH_4^+ and NO_3^- treatments and particulate DA in the NO_3^- and urea treatments contain semi-quantitative data. (E) Pn-9, *P. fraudulenta*. Mean dissolved DA in all treatments and particulate DA in the NO_3^- treatment contain semi-quantitative data.

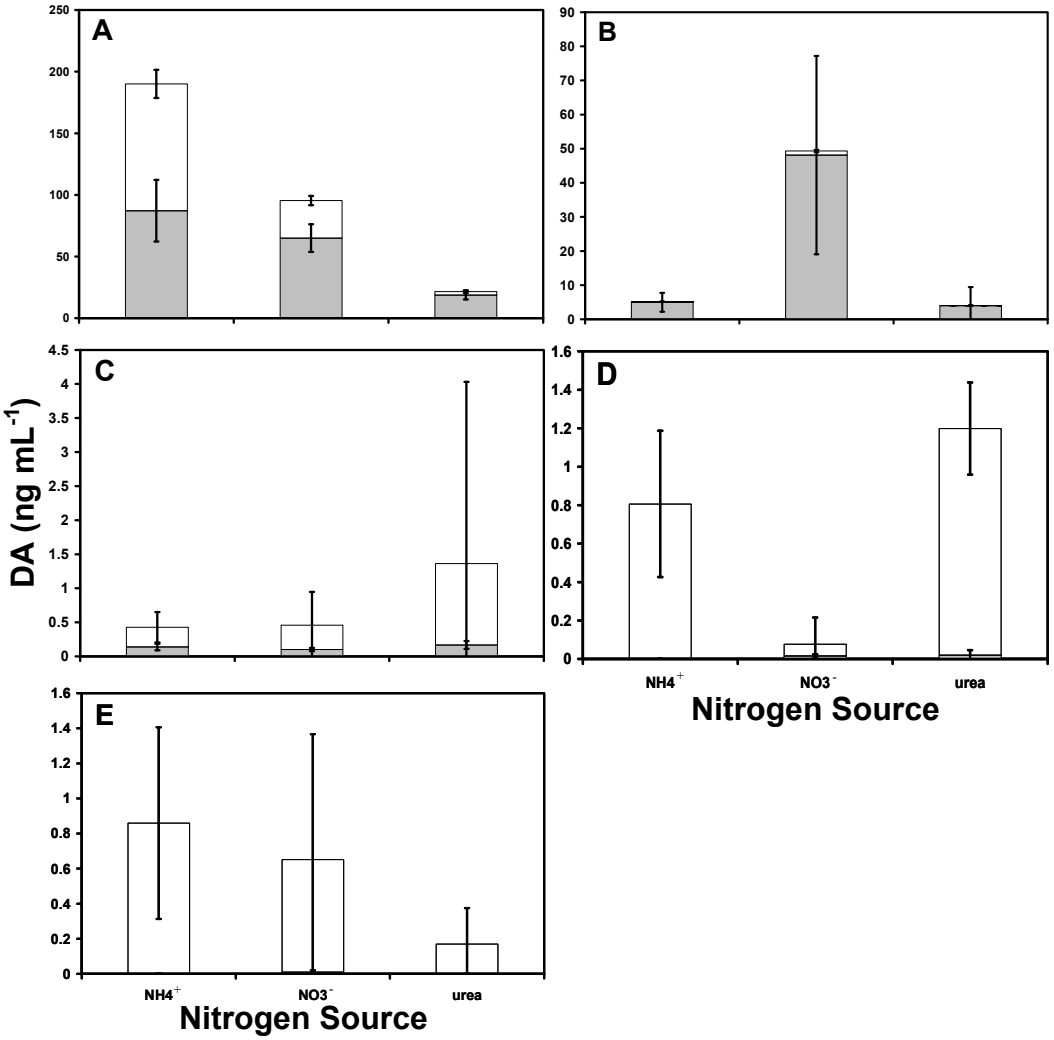


Fig. 3.7 DA content (fg cell^{-1}) one week after fluorescence peak for strains of *Pseudo-nitzschia* grown on NO_3^-, NH_4^+ and urea in silicate limited media. Filled bars show particulate DA and open bars show dissolved DA. Error bars represent one SD. Letters showing significant difference are in Table 3.5. Replication is shown in Table 3.8. Note the difference in scale. (A) Pn-1, *P. multiseries* (B) CLN47, *P. multiseries.* Mean dissolved DA in NH_4^+ and urea treatments contain semi-quantitative data (<LOQ, but >LOD). (C) Pn-8, *P. calliantha.* Mean dissolved DA in the NH_4^+ treatment contains semi-quantitative data. (D) Pn-12, *P. fraudulenta.* Mean dissolved DA in the NH_4^+ and NO_3^- treatments and particulate DA in the NO_3^- and urea treatments contain semi-quantitative data. (E) Pn-9, *P. fraudulenta*. Mean dissolved DA in all treatments and particulate DA in the NO_3^- treatment contain semi-quantitative data.

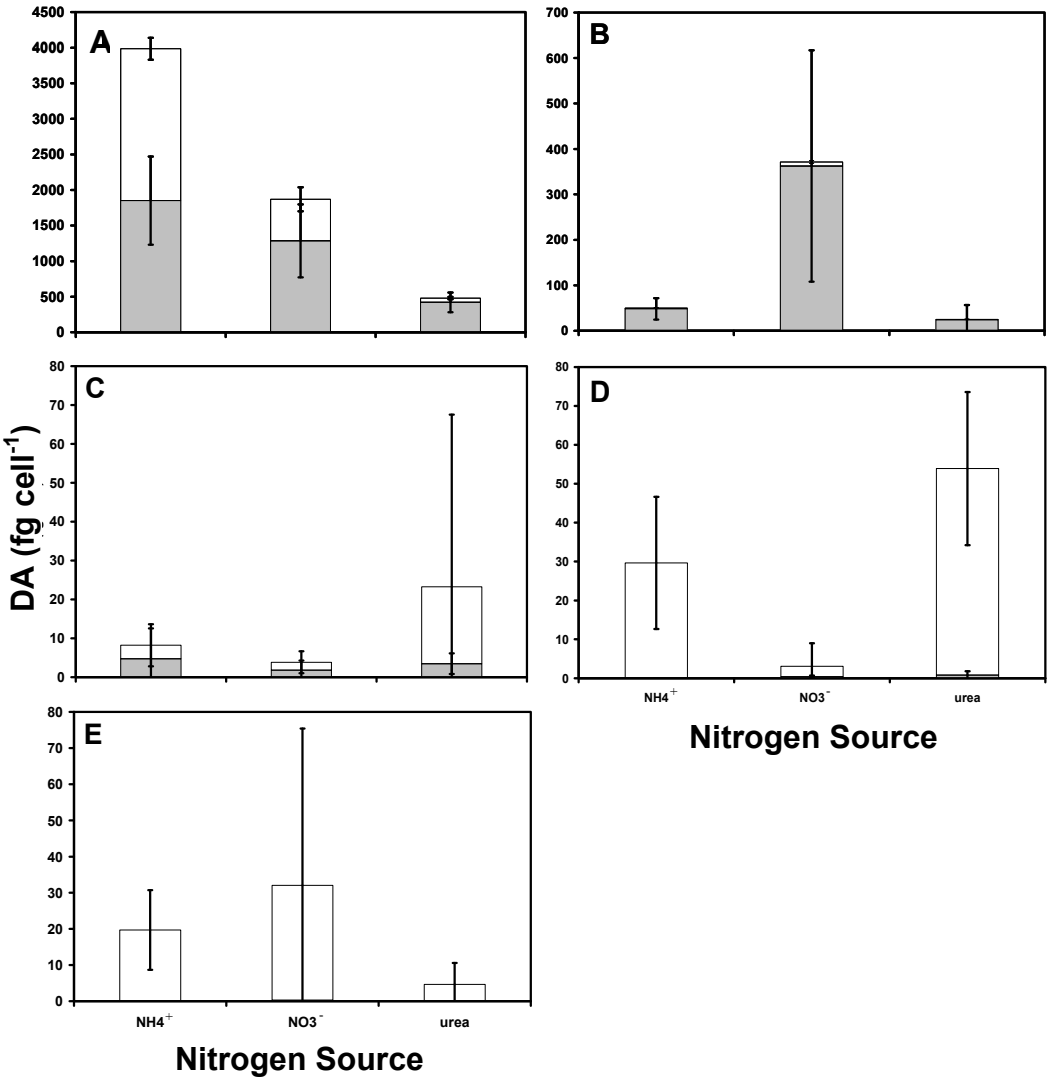


Fig. 3.8 Growth vs. irradiance curves for three strains of two species of *Pseudo-nitzschia* at three temperatures (10°, 15°, 20°C) in nutrient replete medium. (A) Pn-1, *P. multiseries* (B) CLN47, *P. multiseries* (C) Pn-15, *P. fraudulenta*. Photosynthetic parameters derived from curves are summarized in Table 3.7.

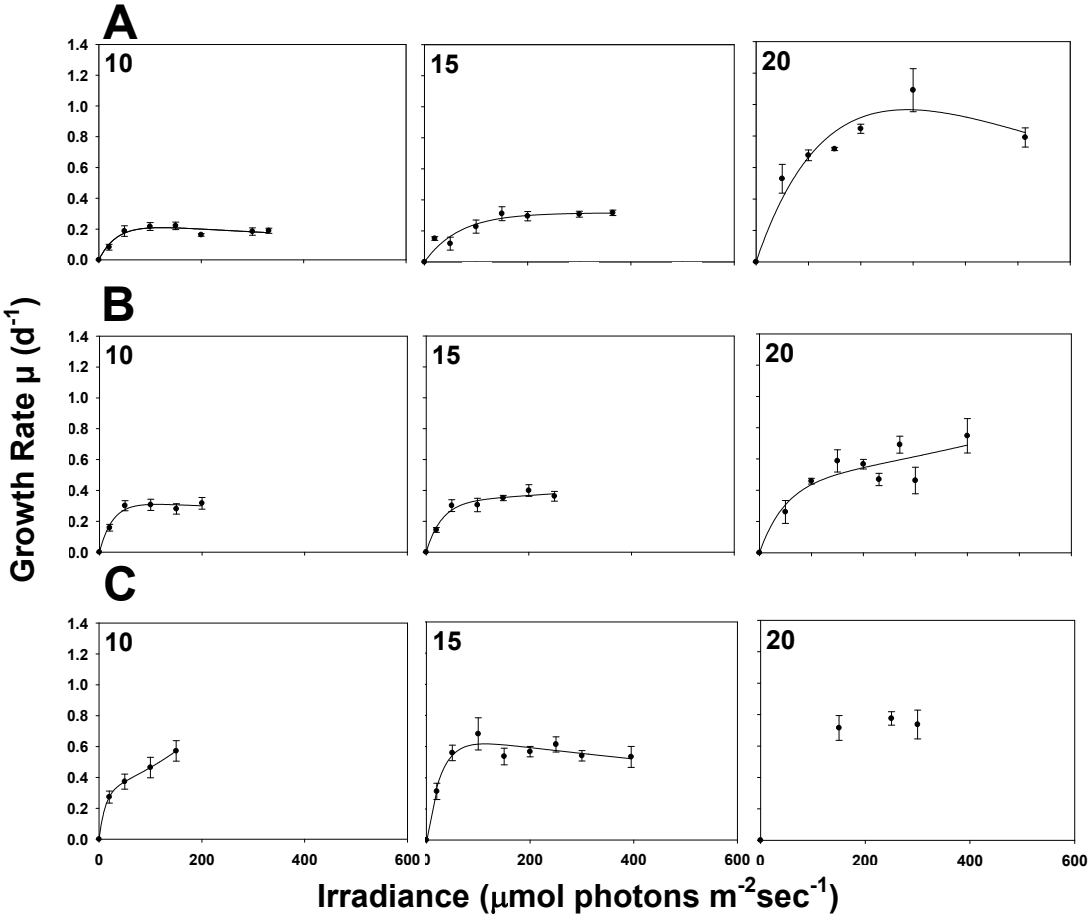


Figure 3.9 Growth rates in exponential phase (shaded bars) and total DA content (ng mL^{-1}; open bars) one week after fluorescence peak in strains of *Pseudo-nitzschia* grown in batch culture on NO_3^-, NH_4^+ and urea in silicate-limited media. Error bars represent one SD. Spearman's correlation coefficients are in Table 3.6. Replication is shown in Table 3.8. Note the difference in scale. (A) Pn-1, *P. multiseries* (B) CLN47, *P. multiseries.* (C) Pn-8, *P. calliantha.* (D) Pn-12, *P. fraudulenta.* (E) Pn-9, *P. fraudulenta*.

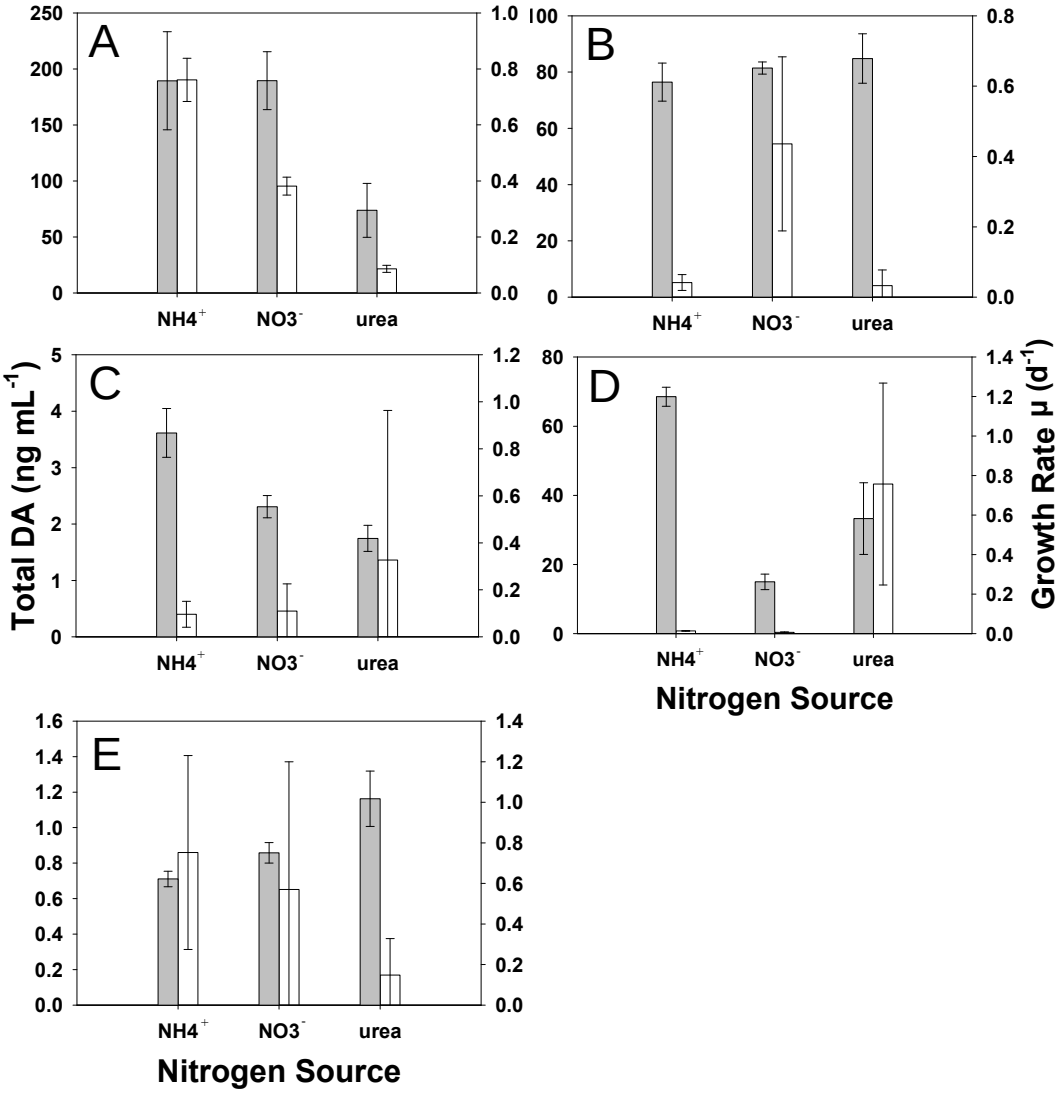
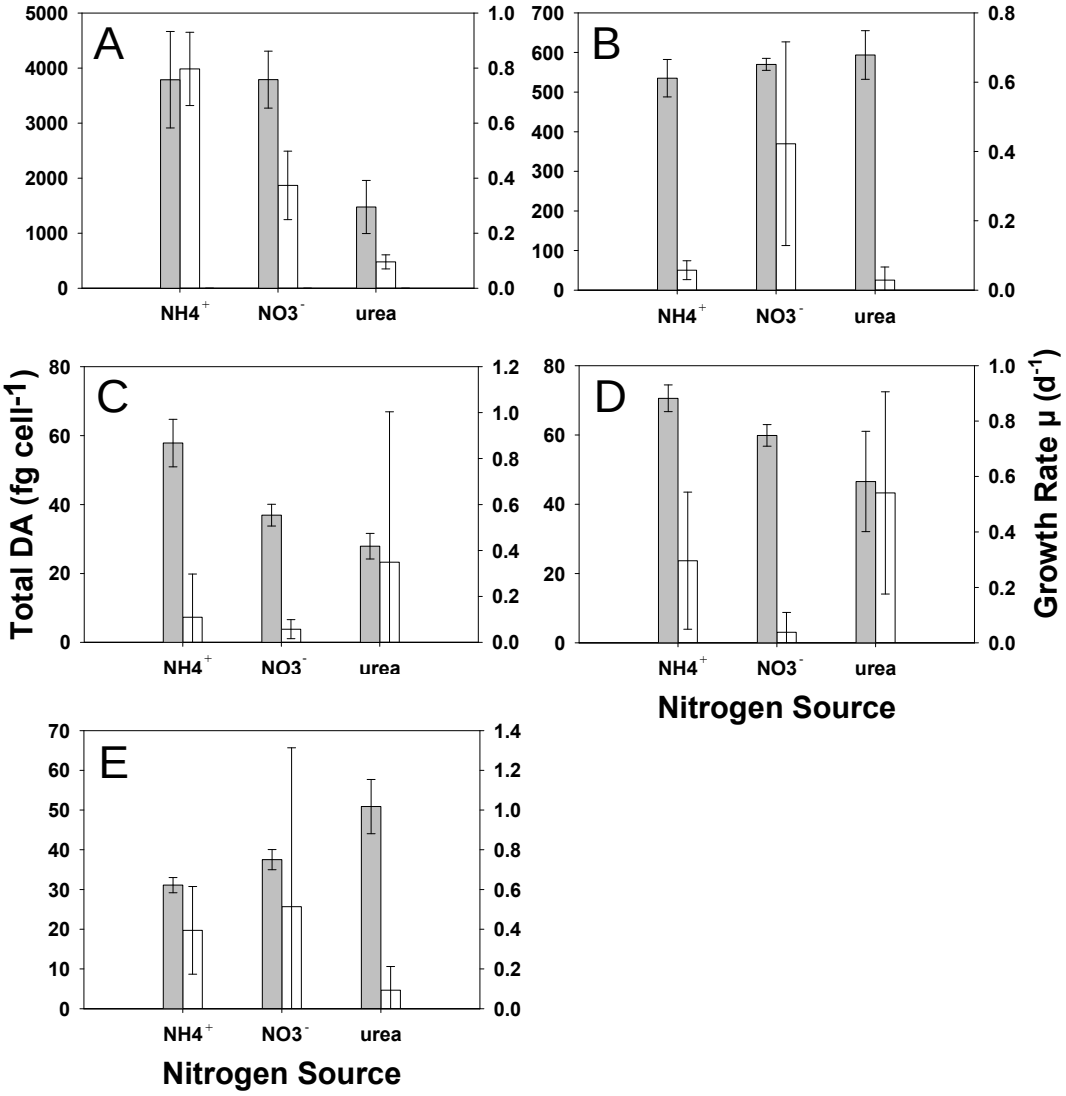


Figure 3.10 Growth rates in exponential phase (shaded bars) and total DA content (fg cell^{-1}; open bars) one week after fluorescence peak in strains of *Pseudo-nitzschia* grown in batch culture on NO_3^-, NH_4^+ and urea in silicate-limited media. Error bars represent one SD. Spearman's correlation coefficients are in Table 3.6. Replication is shown in Table 3.8. Note the difference in scale. (A) Pn-1, *P. multiseries* (B) CLN47, *P. multiseries.* (C) Pn-8, *P. calliantha.* (D) Pn-12, *P. fraudulenta.* (E) Pn-9, *P. fraudulenta*.



Chapter 4: The effect of a rapid increase in irradiance on domoic acid production and nitrate uptake by *Pseudo-nitzschia multiseries*

Abstract

Pseudo-nitzschia are diatoms that can be found worldwide, but are common and often toxic during periods of low temperature and rapid fluctuations in irradiance such as spring and fall and in upwelling zones. It has previously been shown that diatoms differ from flagellates and green algae in their carbon and nutrient assimilation and their ability to cope with rapid increases in irradiance. These differences could explain the dominance of diatoms in cool, high nutrient, well-mixed environments. A batch culture of *Pseudo-nitzschia multiseries* was exposed to a 10-fold increase in light (from 20 to 200 μmol photons $m^{-2}sec^{-1}$) at 15°C during exponential and stationary growth phase to investigate the relationship between high light stress, NO_3^- reduction and DA production. Of the nitrogen taken up as NO_3^- from the surrounding media, 3.3% was released as NO_2^- and 4.8% was released as NH_4^+. There was no change in cellular carbon or nitrogen. DA was not produced in the high light treatment, but was produced at a rate of 1.18 X 10^{-4} pg DA $cell^{-1}hr^{-1}$ in the low light controls. Stationary phase cultures did not take up NO_3^- or release NO_2^- and NH_4^+. *Pseudo-nitzschia* does increase NO_3^- uptake during periods of low temperature and high irradiance, but subsequent NH_4^+ release and electron consumption is very low. These results may be due to xanthophyll cycling preventing a rapid increase in light harvesting.

Introduction

Marine diatoms often form large blooms during periods of low temperature, turbulent mixing and high, pulsed NO_3^- delivery, such as spring, fall turnover and in upwelling zones. Often under these conditions, the mixed layer depth exceeds the depth of the euphotic zone, exposing these diatom-dominated populations to rapidly and widely changing irradiance levels from darkness to full sunlight. The dominance of diatoms under these conditions has been explained ecologically as the result of high growth rates or ability to compete for pulsed nutrients (Margalef 1978). Physiological adaptations allowing for diatom dominance under these conditions have been investigated but are not well understood. For example, field studies show diatoms have a higher photosynthetic efficiency than green algae under fluctuating light conditions (Wagner et al. 2006). Laboratory studies of the xanthophyll cycle in diatoms show that a rapid increase in light intensity can be dissipated quickly by non-photochemical quenching (Lavaud et al. 2004). Some studies propose a physiological model linking NO_3^- uptake in excess of growth, light fluctuation and low temperature (Lomas & Glibert 1999, Lomas et al. 2000).

Cells exposed to rapid and wide fluctuations in light can experience transient energy stress due to imbalances between light harvesting and energy utilization. This stress can be increased by temperature limitation of metabolic enzymes such as RUBISCO (Lomas & Glibert 1999). In order to be successful in these cool, well mixed environments, cells would need a strategy to cope with irradiance widely fluctuating on short time scales. One possible means of coping

with such stress is luxury uptake and subsequent reduction of NO_3^-. Previous studies found a negative relationship between NO_3^- uptake and temperature in natural assemblages dominated by diatoms during short-term periods of rapid light increase (Lomas & Glibert 1999). Laboratory experiments also showed NO_3^- uptake in diatom cultures exposed to a rapid increase in irradiance (from 40-120 μmol photons $m^{-2}sec^{-1}$ to 300-750 μmol photons $m^{-2}sec^{-1}$) at low temperature (Lomas et al. 2000). If diatoms are reducing NO_3^- to modulate electron flow during periods of rapid irradiance shifts that could explain the dominance of diatoms in regions of cool temperatures, high NO_3^- and turbulent mixing. In fact, experimentally derived temperature optima for nitrate reductase in diatoms ranged from 10-22.2°C (Kristiansen 1983, Lomas & Glibert 2000) and diatom cultures that took up NO_3 under low temperature high light conditions also released NO_2^- and NH_4^+ (Lomas et al. 2000).

Flagellates exhibit a different nitrogen uptake and release pattern under similar low temperature high light conditions. While cultured diatoms take up large amounts of NO_3^- and subsequently release NO_2^- and NH_4^+, cultured flagellates took up NO_3^- and NH_4^+ without release of NO_2^- or NH_4^+ (Lomas & Glibert 2000, Lomas et al. 2000). The experimentally derived temperature optimum for nitrate reductase in flagellates is 19.9-30.1°C (Lomas & Glibert 2000), higher than nitrate reductase in diatoms. These results show a difference in nitrogen uptake between flagellates and diatoms that could explain diatom dominance of cool, high NO_3^- and turbulent waters such as during seasonal turnover and upwelling events.

Pseudo-nitzschia is one of the diatom genera that often blooms during the spring and fall and in upwelling zones, suggesting that this genus also has a cold-water adaptation. Since *Pseudo-nitzschia* is known to produce the amino acid-based neurotoxin, domoic acid (DA), changes in nitrogen metabolism such as those described above could play an important role in DA production. If *Pseudo-nitzschia* reduces NO_3^- as a cold water adaptation, could the reduced form of nitrogen later released be DA instead of NH_4^+? DA concentrations in natural samples can be higher under low temperature, turbulent conditions, suggesting such an effect on toxin production (Trainer et al. 2000). In addition, DA production is a very energy intensive process that could be used to consume electrons.

Some of the direct effects of light and temperature on DA production have already been investigated. Cells need at least 100 μmol photons m^{-2} sec^{-1} to produce maximal amounts of DA (Bates 1998). Laboratory studies have shown that DA production slows at lower temperatures, but *P. multiseries* can still produce DA at 0°C (Bates et al. 1991, Smith et al. 1993). Previous studies on the effects of light and temperature on DA production allow time for adaptation before sampling, thereby missing any potential stress response. The effect of rapid increases in irradiance at low temperatures on DA production has not been investigated.

Materials and Methods

Growth vs. Irradiance Parameters

Before experimental irradiances could be chosen, growth versus irradiance parameters had to be determined. The experimental strain (*P. multiseries*, CLN47) was adapted through two batch culture generations to the experimental temperature and irradiance. The experiment was performed in f/2* (Andersen et al. 1997) media at a salinity of 32 in five replicate 10 mL glass tubes at each discreet irradiance. Treatments started at 20 μmol photons m^{-2} sec^{-1} and increased at approximately 50 μmol photons m^{-2} sec^{-1} intervals until growth rate saturation. Chlorophyll *a* was monitored at the same time daily by *in vivo* fluorescence and used to calculate specific growth rates (Wood et al. 2005). Replicates were averaged and a standard deviation was calculated. The data were fitted to a non-linear least squares regression using an equation initially derived for photosynthesis (Platt et al. 1980) and modified to describe growth:

$$\mu = \mu_o + \mu_{max}(1 - e^{((-E\alpha)/\mu max)})e^{((-E\beta)/\mu max)}$$

where μ_o is the y intercept, μ_{max} is the maximum potential growth rate (d^{-1}), α is the initial light-limited slope (d^{-1} [μmol photons m^{-2} sec^{-1}]$^{-1}$), E is the irradiance (μmol photons m^{-2} sec^{-1}) and β (d^{-1} [μmol photons m^{-2} sec^{-1}]$^{-1}$) is the slope of the photoinhibited part of the curve. E_k, the irradiance saturating for growth, was calculated for each temperature treatment by dividing μ_{max} by α (Fig. 4.1; Table 4.1).

Culture Maintenance

One strain of *P. multiseries*, CLN47, was maintained in natural seawater media amended with f/2* at 15°C at the irradiance which was half saturation for growth, 20 μmol photons m^{-2} sec^{-1} (Fig. 4.1; Table 4.1) set to a 14:10 L:D cycle for several months. Cells were grown in batch culture and growth phase was monitored at the same time daily via *in vivo* fluorescence of a subsample. Culture volumes were increased for experimentation by adding a volume of stationary phase inoculum that was 7-8% of the volume of the fresh media at each transfer (30 mL to 50 mL to 700 mL to 10 L). Media for the 10 L experimental culture was supplemented with f/2* levels of all nutrients except NO_3^-, which was added to the medium to obtain 88 μM total NO_3^- concentration. The flask was air bubbled and growth was monitored at the same time daily via *in vivo* fluorescence of a subsample. All glassware used in culturing was acid washed in 10% HCl overnight and rinsed with distilled deionized water before use.

Experimental Procedure

Two experimental 10 L cultures were used sequentially, one during exponential phase and another during stationary phase of growth. All glassware, graduated cylinders, sample collection vials and the sample collection apparatus were acid washed in 10% HCl and rinsed with distilled deionized water prior to the experiment and then rinsed with culture just before use. For the exponential phase experiment, when there was approximately 20 μM NO_3^- left in the growth media, 600 mL was removed using a graduated cylinder, poured into a 1 L glass

Erlenmeyer flask and kept in 20 μmol photons m^{-2} sec^{-1}. This was repeated for 6 replicate flasks. Preserved samples (15 mL, 1% gluteraldehyde) were collected from each flask by pipette before the start of the experiment. At t = 0, all flasks were spiked with NO_3^- to bring concentrations to 75 μM (exponential) or 35 μM (stationary). Three control flasks were left in low irradiance while three treatment flasks were immediately placed in 200 μmol photons m^{-2} sec^{-1} irradiance, which is higher than E_k and a ten-fold increase above the adapted, half-saturation irradiance (Fig. 4.1; Table 4.1). All flasks were sampled for NO_3^-, NO_2^-, NH_4^+, CHN (to quantify particulate carbon and nitrogen) and DA analysis at t = 0. Dissolved and particulate nitrogen samples were filtered using a light vacuum (<100 mm Hg) and precombusted (450°C for 1 h) Whatman GF/F glass fiber filters. DA samples were gravity filtered onto Whatman GF/F filters. Both the filter and the filtrate were frozen. Samples for NO_2^-, NH_4^+ and DA were collected at t = 30 min and t = 60 min and at time t = 180 min, the NO_3^- and CHN samples were again collected. Another preserved sample was removed from each flask after the t = 180 min sampling. An additional DA sample was taken at t = 390 min. The procedure was repeated in its entirety for the culture in stationary phase of growth.

Sample Analysis

Samples were analyzed for inorganic nitrogen (NO_3^-, NO_2^- and NH_4^+) using a Technicon AutoAnalyzer II (USEPA 1979, Lane et al. 2000). Particulate carbon and nitrogen were quantified using a CE-440 Elemental Analyzer (Exeter

Analytical Inc. Chelmsford, Massachusetts). Analytical resolution and limit of detection for each analysis can be found in Table 3.2. DA samples were analyzed using an ELISA ASP test kit (Biosense, Norway) for particulate (DA within the cell and therefore on the filter) and dissolved (DA released from the cell and therefore in the media) fractions. Particulate and dissolved DA were summed to get total DA. Preserved samples were filtered onto a 2 μm polycarbonate filter and placed onto a glass microscope slide with immersion oil and cover slip for enumeration of *Pseudo-nitzschia* using an epifluorescence microscope (excitation 450-490 nm, dichromatic beam splitter 500 nm, barrier filter 515nm; Nikon filter set EF-4 B-2A).

Statistical Analysis

Differences between treatments and controls and changes over time were determined by one way ANOVA using Fisher's LSD test for multiple comparisons at the 5% level (SigmaStat ver. 3.1, Systat Inc., San Diego, CA, USA). Separate one way ANOVAs were performed on the DA data, testing differences in the treatment and control flasks at t = 0, treatment flasks at t = 0 and t = 390 min and control flasks at t = 0 and t = 390 min. When data were not normally distributed a Kruskal-Wallis ANOVA on ranks was performed.

Results

Exponential Phase

After the 180 min exposure to higher irradiance, NO_3^- concentrations in treatment flasks were reduced by 14 ± 8 μM and increased by 10 ± 4 μM in the control (Fig. 4.2). NO_2^- concentrations increased by 0.6 ± 0.3 μM in the experimental media while NO_2^- in the control flasks remained unchanged over 180 min (Fig. 4.3). NH_4^+ concentrations in treatment flasks increased by 0.7 ± 0.3 μM after 30 minutes and decreased by 0.5 ± 0.3 μM in control flasks after 180 min (Fig. 4.4). Carbon and nitrogen within the cells were the same in treatments and controls and remained unchanged throughout the course of the experiment (Figs. 4.5, 4.6)

DA was present in the culture before initiation of the experiment. The cells were in late exponential phase, a time when this culture can produce DA (Chapter 3, this thesis). Particulate, dissolved and total DA were not different in treatment and control flasks at $t = 0$ ($p > 0.05$). Particulate and total DA increased over the course of the experiment in control flasks only; dissolved DA did not ($p = 0.013$, 0.014 and 0.450, respectively). At the end of the experiment, cells in the control flasks increased particulate DA by 0.05 ± 0.003 pg $cell^{-1}$ with a production rate of 1.18 X 10^{-4} pg DA $cell^{-1}hr^{-1}$ (Fig. 4.7).

Stationary Phase

During stationary phase, treatment flasks did not have different NO_3^- concentrations after 180 min of exposure to high light (Fig. 4.2). $NO_2^-$and NH_4^+

concentrations did not increase in treatment flasks (Figs. 4.3 and 4.4). Carbon and nitrogen within the cells also did not change over time (Figs. 4.5 and 4.6). Since this experiment was conducted during stationary phase of growth, DA levels were higher than in the exponential phase experiment ($p = 0.002$) and increased in the dissolved fraction. At the end of the experiment, particulate and total DA had not changed in the treatments nor the controls ($p > 0.05$) and dissolved DA had increased in the treatment flasks ($p = 0.008$; Fig. 4.7).

Discussion

Dissolved Inorganic Nitrogen Release

Exponentially growing cells in the treatment flasks reduced the NO_3^- in the media by 20%. Release of NO_2^- as a percentage of NO_3^- uptake was 3.3% and release of NH_4^+ as a percentage of NO_3^- uptake was 4.8%. More than 90% of the nitrogen taken up as NO_3^- in the treatment flasks was unaccounted for by the NO_2^-, NH_4^+, CHN and DA samples collected. Lack of a statistically significant increase in nitrogen within the cell suggests that the nitrogen was released, but not as NO_3^-, NO_2^-, NH_4^+ or DA. However, if the statistical result is ignored and an increase in particulate nitrogen is calculated using the average nitrogen content of the cells at t = 0 and t = 180 min (Fig. 4.6), the result is an increase in cellular nitrogen by ~3 pg N cell^{-1}. This could account for the 90% of the nitrogen taken up as NO_3^-, but not released as NO_2^-, NH_4^+ or DA, if the increase in cellular nitrogen is real.

This experiment was designed to be comparable to the irradiance shift experiments in Lomas et al. (2000). Both studies exposed diatoms adapted to an irradiance that is half-saturation for growth of the specific strain to an immediate 10 fold increase in irradiance at 15°C. The diatoms used in Lomas et al. (2000) were *Thalassiosira weissflogii* (CCMP1047), *Skeletonema costatum* (CCMP1332) and a *Chaetoceros* sp. with half-saturating irradiances from 40-120 μmol photons m^{-2} sec^{-1}, which is higher than the half-saturating irradiance for the *P. multiseries* strain used in this experiment, CLN47. The Lomas et al. (2000) study used 50-70 μmol photons m^{-2} sec^{-1} in the control and 500-700 μmol photons m^{-2} sec^{-1} in the treatment, which is much higher than the treatment irradiances used in this study (200 μmol photons m^{-2} sec^{-1}). However, PAR (photosynthetically available radiation) in the Chesapeake Bay in winter and spring can be > 1000 μmol photons m^{-2} sec^{-1} at the surface (www.chesapeakebay.net) which is much higher than the light levels used in either experiment.

All diatom strains used in this study and the Lomas et al. (2000) study took up NO_3^- after the irradiance shift up. The important difference between the Lomas et al. (2000) study and these results is the NH_4^+ release. Release of NO_2^- as a percentage of NO_3^- uptake is slightly higher in this study (3.3%) than for *Chaetoceros* sp. (2.2%), the same as release in *T. weissflogii* and slightly less than for *S. costatum* (3.9%). Release of NH_4^+ as a percentage of NO_3^- uptake is much lower in this study (4.8%) than for *T. weissflogii* (76.8%) and *S. costatum* (49.6%) but higher than for *Chaetoceros* sp. which did not release NH_4^+. These differences have important consequences energetically. Lomas et al. (2000)

calculated that 1-62% of the electrons harvested in the experimental irradiance were consumed to support the observed increase in NO_2^- and NH_4^+. The strains that released the most NH_4^+ consumed the most electrons. A similar calculation was performed for *P. multiseries* in this study using the equation:

$$\text{Electrons produced} = ETa*0.5$$

Where E is the irradiance (mol photons m^{-2} sec^{-1}), T is time (s), a* is the chlorophyll-specific absorption (m^2 mg^{-1} Chl *a*) and 0.5 is a constant (Falkowski & Raven 1997). Chlorophyll-specific absorption has not been measured for *Pseudo-nitzschia*, so a high (0.028) and a low (0.004) a* value for other diatoms in the literature were used (Falkowski et al. 1985, Sakshaug et al. 1991). Electrons consumed were calculated by multiplying the cell specific NO_2^- and NH_4^+ release by 4 and 10, respectively (Lomas et al. 2000), representing the relative number of electrons needed for transport and reduction. Electrons consumed to reduce NO_3^- to NO_2^- and NO_2^- to NH_4^+, to produce the concentrations found in the media at t = 180 min, were a negligible fraction (<<1%) of the electrons harvested in 200 µmol photons $m^{-2}sec^{-1}$ irradiance over 3 h. While *P. multseries* took up NO_3^- and released NO_2^- and NH_4^+, the amount of NH_4^+ released and the amount of electrons consumed were much less than the diatoms in Lomas et al. (2000). This argues against the reduction of NO_3^- into NO_2^- and NO_2^- into NH_4^+ as an adaptive energy dissipation pathway in *P. multiseries* strain CLN47 under these experimental conditions.

When grown in a specific irradiance for long periods of time, photosynthetic cells have the capability to photoacclimate to the given light

regime. This means changing either the size or number of photosynthetic units (Falkowski & Raven 1997). In microalgae, chlorophyll per cell can increase 5 – 10 fold in low irradiance (Falkowski 1980, Richardson et al. 1983). An increase in pigments could have enabled the cells in this study to dissipate excess light energy via the xanthophyll cycle instead of via nitrate reduction (Hagar & Stransky 1970). This process, whereby accessory carotenoid pigments absorb energy (specifically diadinoxanthin), effectively reduces the size of the photosynthetic unit at high light, i.e., E_k increases (Falkowski & Raven 1997). Specifically, photons are absorbed by the pigment diadinoxanthin and used to convert to diatoxanthin instead of participating in photosystem II. This type of photoprotective mechanism has been documented in laboratory cultures and natural populations of diatoms. For example, field studies in Baffin Bay show that at low temperatures, phytoplankton in the high light surface waters had increased pools of diadinoxanthin cycle pigments and higher nonphotochemical quenching than phytoplankton at depth (Kashino et al. 2002).

The amount of xanthophyll cycle pigments available for use can depend on the light regime to which a cell is acclimated. Diatoms grown in the laboratory in a high irradiance (100 μmol photons m^{-2} sec^{-1}) have larger pools of diadinoxanthin cycle pigments than diatoms grown in low irradiance (7 μmol photons m^{-2} sec^{-1}; Schumann et al. 2007) and diatoms acclimated to multiple light cycles in a 24 h period have larger pools of diadinoxanthin cycle pigments than diatoms acclimated to a single diel light cycle (40 μmol photons m^{-2} sec^{-1}; Lavaud et al. 2002). This suggests that a cell acclimated to constant low light would

contain lower quantities of diadinoxanthin cycle pigments and would be less able to dissipate electrons via this mechanism. However, a study of five species of diatoms acclimated to 40 μmol photons m^{-2} sec^{-1} showed de-epoxidation of diadinoxanthin within minutes of being exposed to 2000 μmol photons m^{-2} sec^{-1} (Lavaud et al. 2004). This suggests that diatoms acclimated to constant low light can dissipate excess energy via the xanthophyll cycle. It is unclear if the low light acclimated cells in this study contained enough diadinoxanthin to dissipate a rapid 10 fold increase in irradiance. Xanthophyll cycling could have been occurring in the treatments, which would confound measurement of nitrate reduction as an energy dissipation pathway. Pigments in the low light adapted *Pseudo-nitzschia* in this experiment were not analyzed.

Increased dissolved inorganic nitrogen release by diatoms during periods of cool temperatures, high NO_3^- availability and high water column turbulence can have important ecological implications. Diatoms have been observed to release nitrogen both as NO_2^- (Collos 1998, Lomas et al. 2000, Singler & Villareal 2005) and NH_4^+ (Lomas et al. 2000, Singler & Villareal 2005) in laboratory experiments and in natural populations. NO_2^- excretion by phytoplankton in the field is important for the creation of the primary NO_2^- maximum (Al-Qutob et al. 2002, Lomas & Lipschultz 2006) and supporting new production in some areas (Singler & Villareal 2005). In the field, NO_2^- excretion can vary from 4% to 63% of NO_3^- uptake (Miyazaki et al. 1975, Olson et al. 1980) and depends on NO_3^- concentration (Wada & Hattori 1971, Collos & Slawyk 1983) and light (Wada & Hattori 1971, Miyazaki et al. 1973, Miyazaki et

al. 1975, Olson et al. 1980, Losada et al. 1981). NO_2^- release can also be stimulated by Fe limitation due to the need for ferrodoxin in nitrite reductase (Milligan & Harrison 2000, Singler & Villareal 2005). In laboratory cultures, NO_2^- release varies from 0 to 50% of NO_3^- uptake in the light and 0 to 96% in the dark (Collos 1998). Greater NO_2^- excretion in the dark can be explained by the light requirement for ferrodoxin synthesis (Losada et al. 1981). NO_2^- release in this study was a small fraction of NO_3^- uptake (3.3%) which is consistent with increased light promoting nitrite reductase activity.

Domoic Acid Production

In these experiments, the production of DA was lower in the treatments than in the controls. This suggests that a high light pulse in combination with low temperature can reduce DA production by affecting at least one of the following necessary processes: Citric Acid Cycle reactions in the mitochondria, Calvin Cycle reactions in the chloroplast and/or NO_3^- reduction. The 15 carbon DA molecule is synthesized from a 5 carbon glutamate and a 10 carbon geranyl phosphate with the help of several enzymes (Laycock et al. 1989, Douglas et al. 1992, Pan et al. 1998, Ramsey et al. 1998). One of those enzymes, nitrate reductase, in diatoms operates optimally at 10-22°C, which is lower than the temperature optimum for another enzyme, RUBISCO (Packard et al. 1971, Kristiansen 1983, Dohler 1991, Lomas et al. 2000). At cool temperatures this would provide plenty of NO_2^- for subsequent reduction by nitrite reductase to form NH_4^+ which could then be used in glutamate synthesis. However,

RUBISCO drives the Calvin Cycle and, in diatoms, operates optimally at >30°C (Li et al. 1984, Smith & Platt 1985, Descolas-Gros & de Billy 1987, Lomas et al. 2000). This would limit DA biosynthesis in cool temperatures by reducing available geranyl phosphate, which is synthesized via the GAP/pyruvate pathway and requires glyceraldehyde 3-phosphate, a product of the Calvin cycle. In addition, a release of NH_4^+ into the media (as seen in the Lomas et al. 2000 experiments) could reduce the glutamate available within the cell for DA synthesis.

In the exponential phase experiments, the DA production rate was lower than those reported for other strains of *P. multiseries* in the laboratory under low Fe stress (1.25×10^{-2} pg DA $cell^{-1}h^{-1}$), toxic Cu stress (3.27×10^{-2} pg DA $cell^{-1}$ h^{-1}) and Si stress in continuous culture (0.13 pg DA $cell^{-1}h^{-1}$; Pan et al. 1996b, Maldonado et al. 2002). Reported production rates of *P. multiseries* strain NPBIO in batch culture under Si limitation ($1.26 \times 10^{-3} – 4.0 \times 10^{-5}$ pg DA $cell^{-1}h^{-1}$) approximate the production rate in this study (Pan et al. 1996a). In stationary phase, DA concentrations were higher and increased in the dissolved fraction over time, which is similar to previous batch culture studies showing higher DA concentrations in stationary phase and release of DA into the media as cell membranes begin to fail (Bates 1998).

These experiments are a preliminary investigation into an adaptive mechanism for coping with high light pulses and low temperature in *Pseudo-nitzschia* and the relationship to DA production. The results show that uncoupling growth and photosynthesis via high light pulses suppresses DA

production unlike uncoupling growth and photosynthesis through nutrient limitation in the laboratory. This could have important ecological implications for natural populations of *Pseudo-nitzschia*. Further work is necessary to confirm these results.

Table 4.1 Growth vs. Irradiance curve (Fig. 4.1) parameters for *P. multiseries* strain CLN47 grown at 15°C. Parameter values are given with standard error in parentheses.

Parameter	Value	Unit
α	**0.0109 (0.0025)**	$d^{-1}[\mu mol\ photons\ m^{-2}\ sec^{-1}]^{-1}$
β	**0**	$d^{-1}[\mu mol\ photons\ m^{-2}\ sec^{-1}]^{-1}$
μ_{max}	**0.33 (0.057)**	d^{-1}
E_k	**30 (12.0)**	$\mu mol\ photons\ m^{-2}\ sec^{-1}$

Table 4.2 Analytical resolution and limit of detection for dissolved inorganic nitrogen.

Analysis	Analytical resolution[a]	Limit of detection[b]	Unit
Dissolved ammonium	**0.06**	**0.21**	**µM**
Dissolved nitrite and nitrate	**<0.01**	**0.01**	**µM**
Dissolved nitrite	**0.01**	**0.03**	**µM**
Particulate carbon	**0.02**	**0.06**	**pg cell^{-1}**
Particulate nitrogen	**<0.01**	**0.003**	**pg cell^{-1}**

[a]Analytical resolution calculated as the standard deviation of seven replicates collected from one container
[b]Limit of detection calculated as the standard deviation*3

Figure 4.1 Growth (μ) versus Irradiance (μmol photons m^{-2} sec^{-1}) curve for *P. multiseries* strain (CLN47) used in this experiment at 15°C.

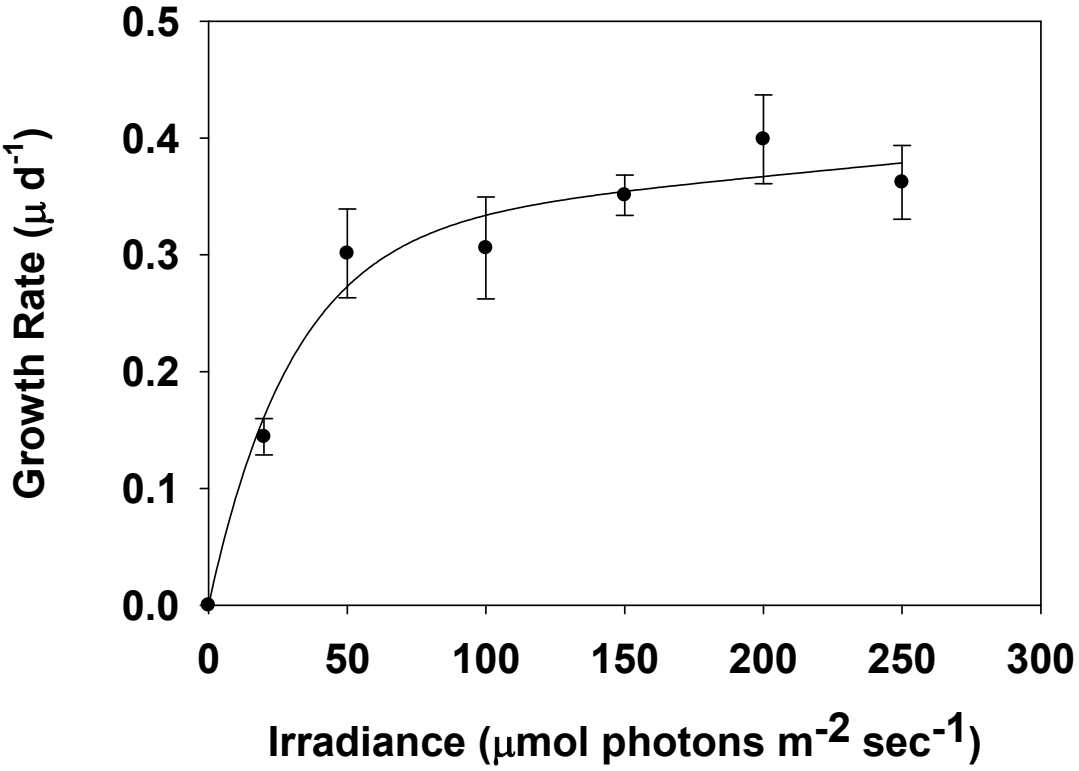


Figure 4.2 Concentrations (μM) of NO_3^- in (A) exponential and (B) stationary phase experiments in treatment (open bar) and control (closed bar) flasks after 180 minutes. * Indicates significant difference between treatment and controls ($p < 0.05$). Error bars represent one standard deviation.

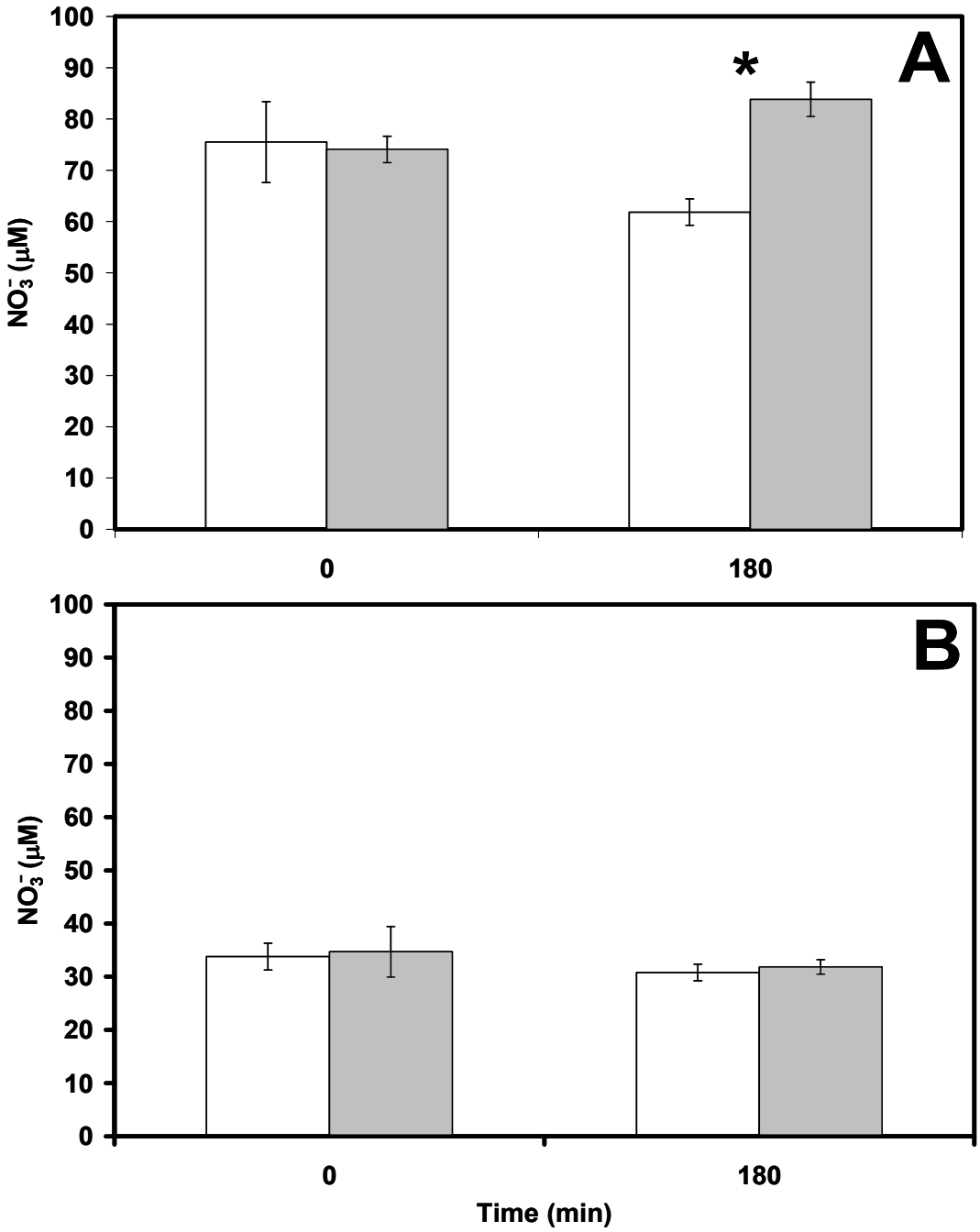


Figure 4.3 Concentrations (μM) of NO_2^- in (A) exponential and (B) stationary phase experiments in treatment (open bar) and control (closed bar) flasks after 180 minutes. * Indicates significant difference between treatment and controls ($p < 0.05$). Error bars represent one standard deviation.

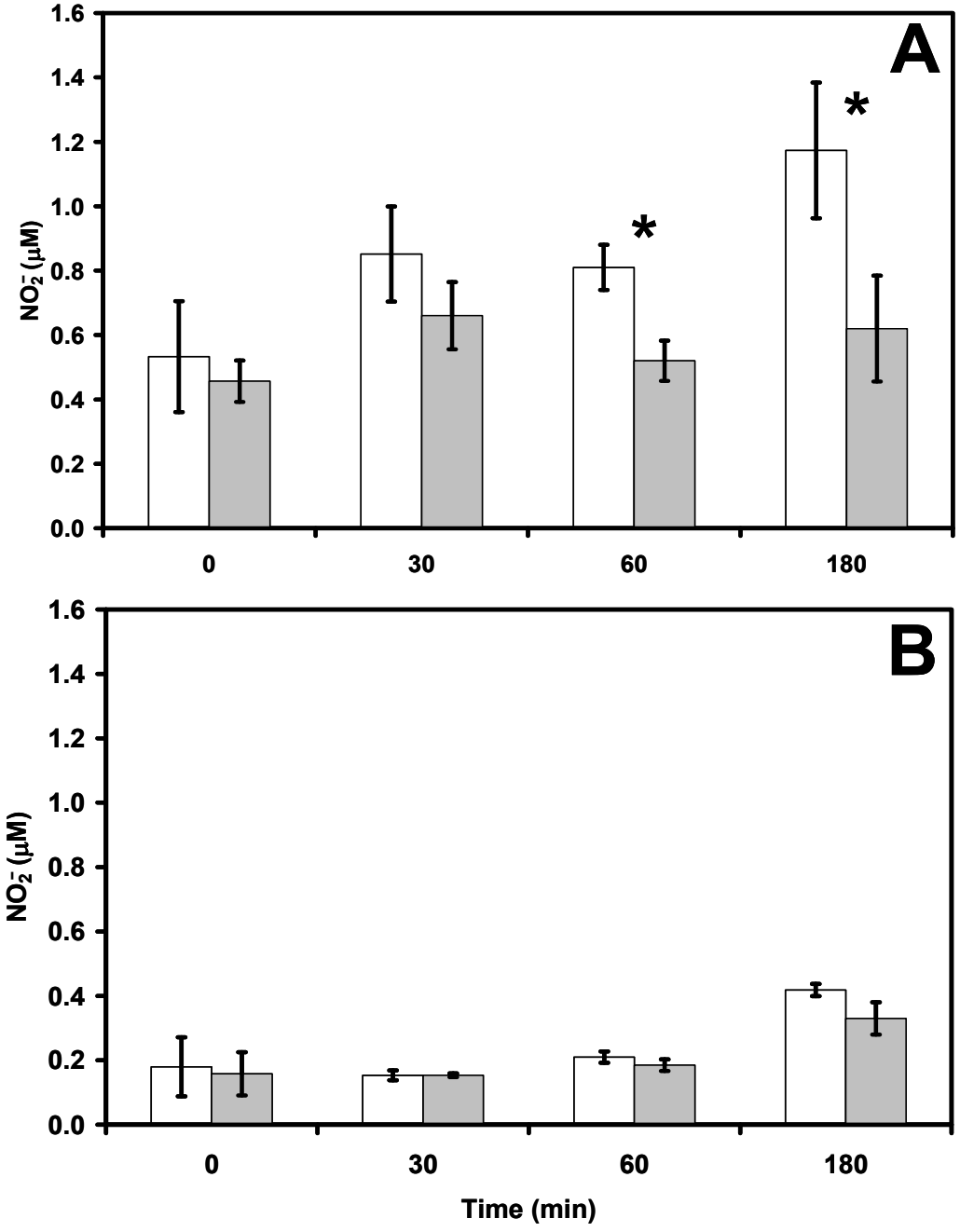


Figure 4.4 Concentrations (μM) of NH_4^+ in (A) exponential and (B) stationary phase experiments in treatment (open bar) and control (closed bar) flasks after 180 minutes. * Indicates significant difference between treatment and controls ($p < 0.05$). Error bars represent one standard deviation.

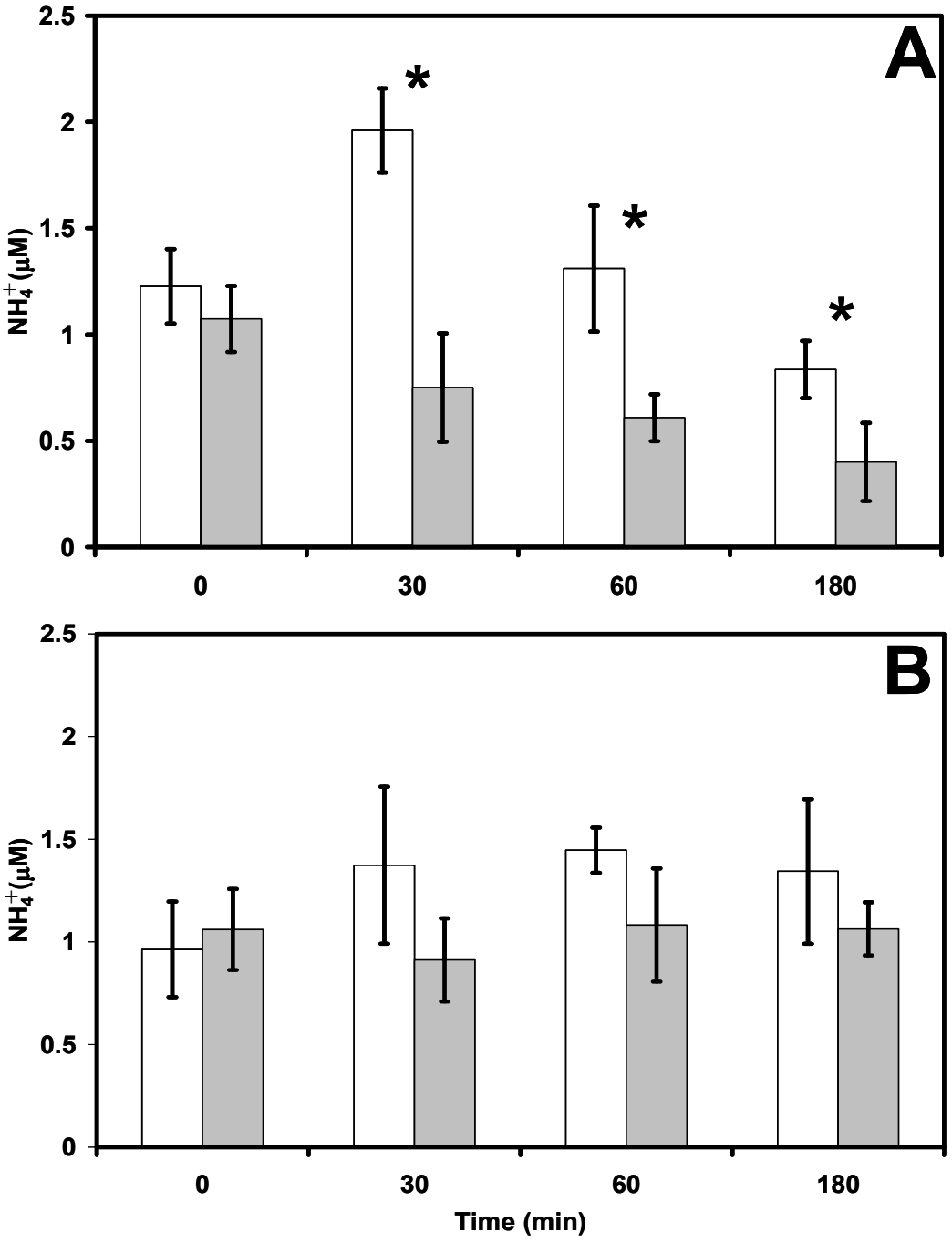


Figure 4.5 Carbon (pg cell^{-1}) within the cell in (A) exponential and (B) stationary phase experiments in treatment (open bar) and control (closed bar) flasks after 180 minutes. * Indicates significant difference between treatment and controls ($p < 0.05$). Error bars represent one standard deviation. The ANOVA p value for A is 0.125 and B is 0.734.

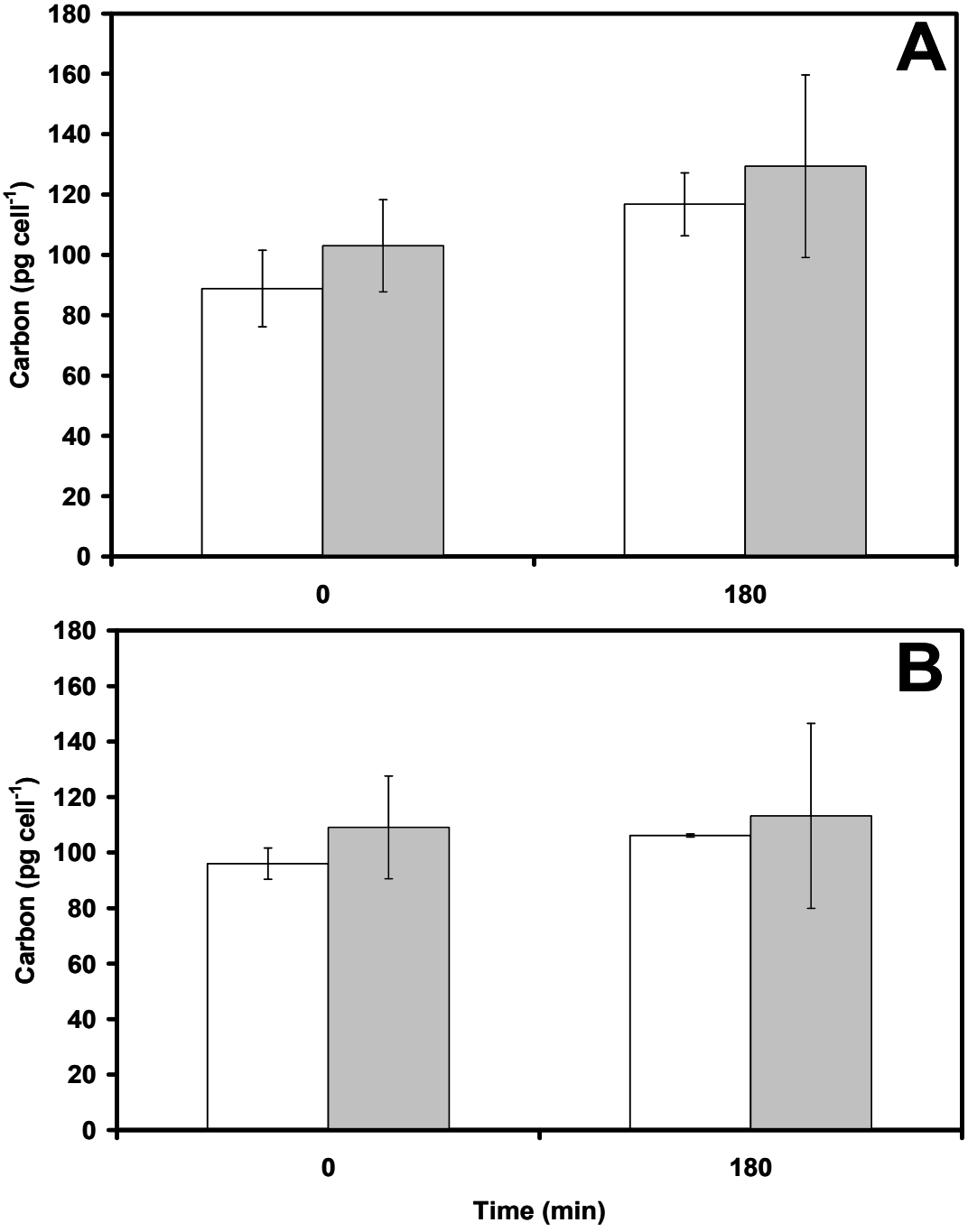


Figure 4.6 Nitrogen (pg cell^{-1}) within the cell in (A) exponential and (B) stationary phase experiments in treatment (open bar) and control (closed bar) flasks after 180 minutes. * Indicates significant difference between treatment and controls ($p < 0.05$). Error bars represent one standard deviation. The ANOVA p value for A is 0.125 and B is 0.583.

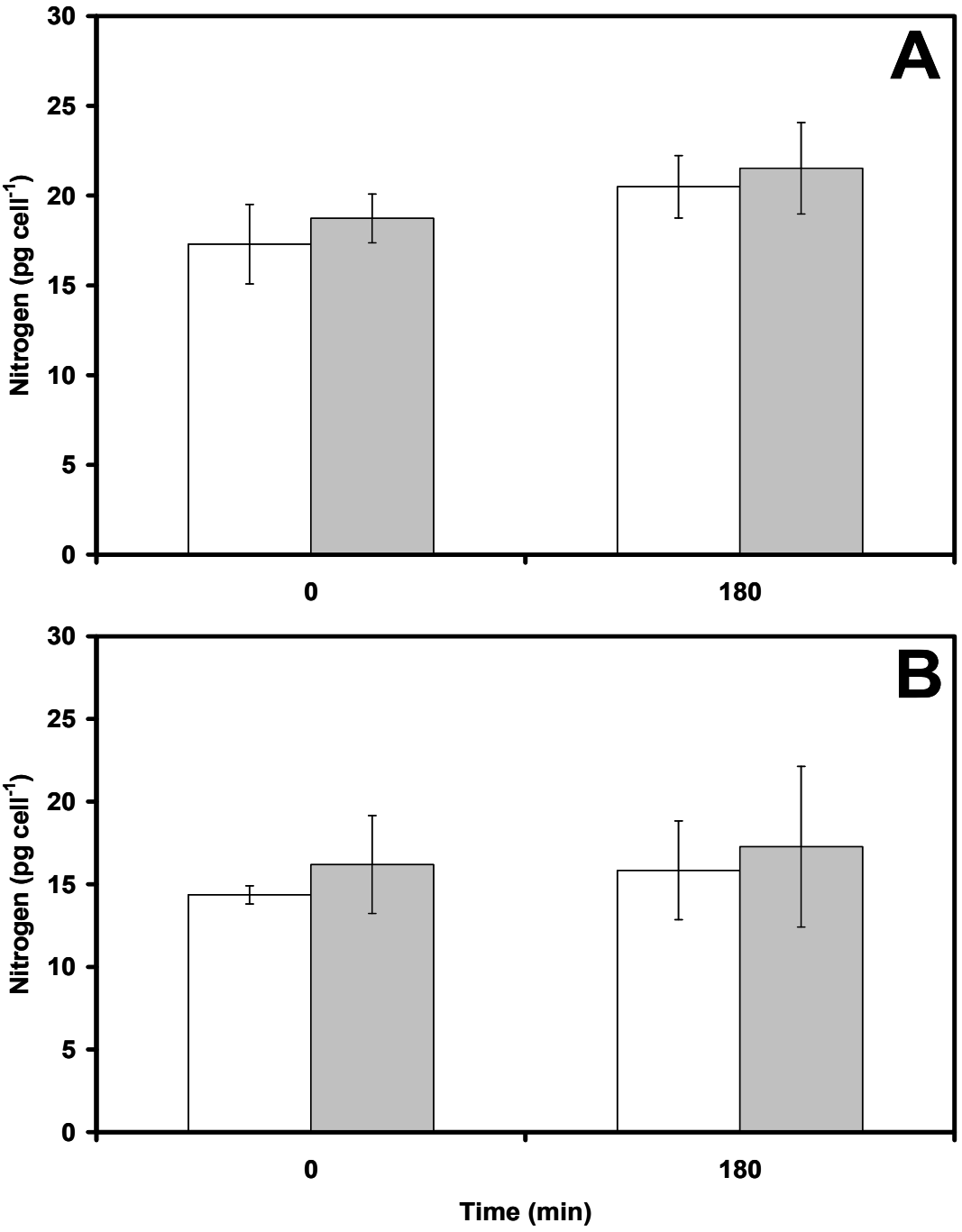
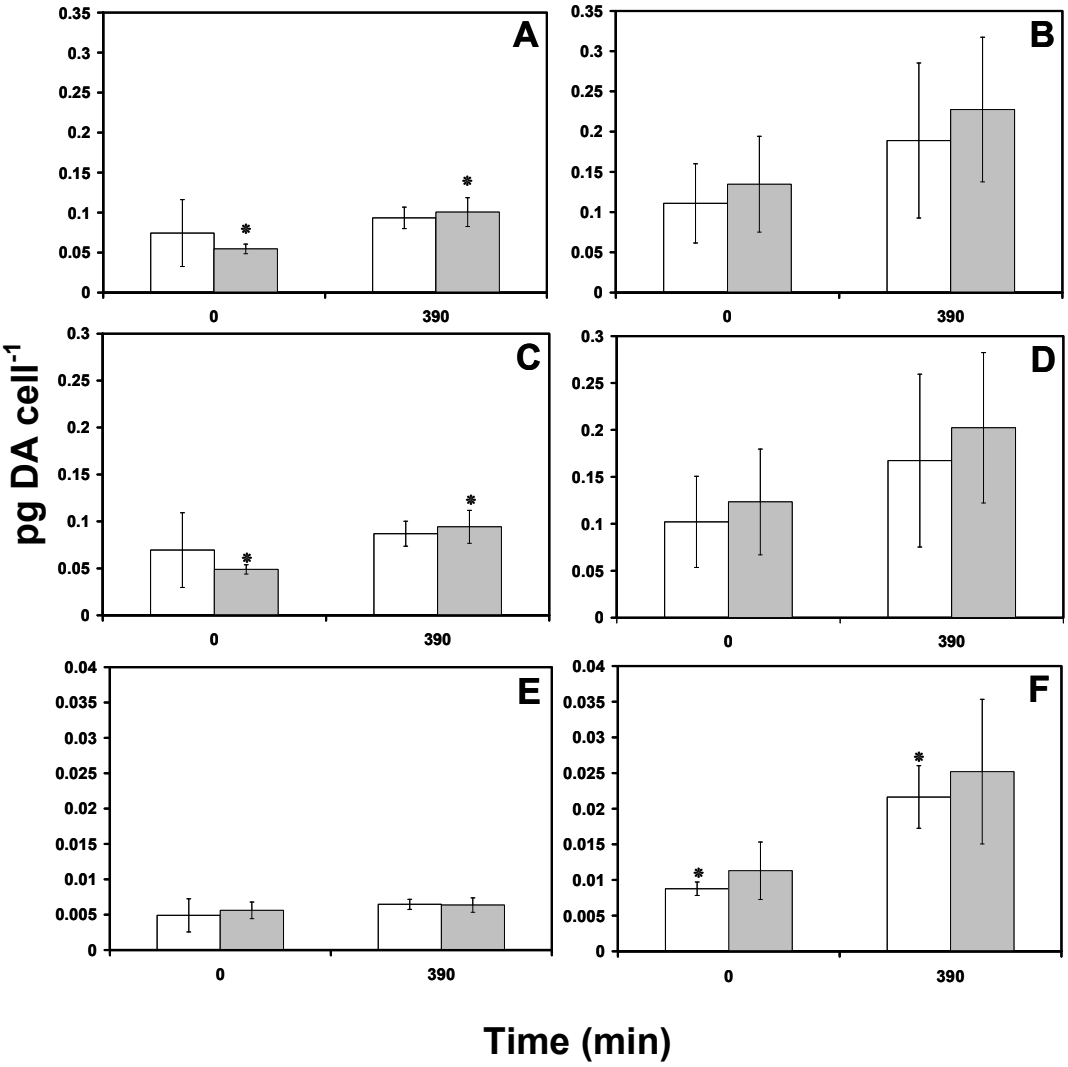


Figure 4.7 Domoic acid (pg cell^{-1}) in exponential (A, C, E) and stationary (B, D, F) phase experiments in treatment (open bar) and control (closed bar) flasks after 390 min. Panels A and B give total DA. Panels C and D give particulate DA. Panels E and F give dissolved DA. *Indicates significant difference between the marked bars at t = 0 and t = 390 ($p < 0.05$). Error bars represent one standard deviation.



Summary: Chapter 5

General Findings and Expansion of Previous Knowledge

The research conducted for this dissertation was designed to permit explorations of *Pseudo-nitzschia* ecology in a dynamic estuary, the Chesapeake Bay. Preliminary work identifying species and toxicity of strains isolated from the Chesapeake (A. Thessen, unpubl.) was contrary to what state environmental agencies commonly believed about the genus. This led to field studies conducted to determine spatial and temporal dynamics of species and toxin production, which were highly variable and generally low (Chapter 2). This led to the following questions: What is the intra- and interspecies variability of this genus? Could the light regime in the Chesapeake affect DA production?

***Pseudo-nitzschia* in the Chesapeake Bay can be toxic; however, domoic acid in field samples was relatively low and uncommon.** *Pseudo-nitzschia* is a cosmopolitan genus (Hasle 2002, Chapter 1, this thesis) often reported to produce DA in many environments. To date, the highest toxin concentrations in nature have been reported from high salinity, upwelling zones and coastal embayments (Bates et al. 1989, Scholin et al. 2000, Trainer et al. 2000, Vale et al. 2007). Toxin concentrations in the Chesapeake Bay, a low salinity, estuarine environment, have been generally low to date and prior to this study were thought non-existent. However, many of the species present in the Chesapeake are toxic elsewhere (Bates et al. 1989, Martin et al. 1990, Rhodes et al. 1998b). Nutrients in the Chesapeake are rarely limiting at the times *Pseudo*-nitzschia is most abundant (winter-spring; Fig. 5.1), which could partially explain the low DA.

Cells in the field are rarely exposed to the nutrient-limiting conditions in the field that stimulate DA production in the laboratory. The presence of low levels of DA in the Chesapeake has raised the following questions: What are the effects of repeated, subacute doses of toxin on humans and animals? How widespread is DA in Chesapeake food webs? Why are Chesapeake *Pseudo-nitzschia* low toxin producers?

The most common species is *P. calliantha*. *Pseudo-nitzschia calliantha* is a relatively new species, described in 2003 using a combination of morphological and molecular evidence (Lundholm et al. 2003). It is difficult to be sure about the historic range and toxicity of this species due to the absence of micrographs in many historical reports. Current reports are of *P. calliantha* in Europe, North America and Australia with little to no toxicity (Martin et al. 1990, Lundholm et al. 1994, 1997, Lundholm et al. 2003, Chapter 1). Since the most common species of *Pseudo*-nitzschia in the Chesapeake produces little to no DA, the lack of toxin in the field is not unusual. Pseudo-*nitzschia calliantha* has not been previously reported from the Chesapeake Bay (Marshall et al. 2005); however, it could have been present prior to 2003 as Marshall (1994) reported *P. pseudodelicatissima* (the species which was split to form *P. calliantha*) in samples collected from 1963-1993. The phytoplankton monitoring database by the Maryland Department of Natural Resources lists all *Pseudo-nitzschia* as either *P. pungens* or *P. seriata* without confirmation via electron microscopy. It is not

clear if this represents a species shift or limited taxonomic resolution for the Chesapeake populations.

***Pseudo-nitzschia* abundances in the Chesapeake are related to low temperatures and high salinities.** Abundances of *Pseudo-nitzschia* are highest in winter and early spring in saltier water, but broad tolerances in this genus prevent complete exclusion from warmer, fresher areas. Similar patterns have been documented in the Adriatic (Caroppo et al. 2005) and the Gulf of Mexico (Dortch et al. 1997). Fluctuations in temperature and precipitation in the Chesapeake Bay area can influence *Pseudo-nitzschia* abundances annually, through the thermal limits identified here and freshwater discharge which governs salinity, clarity and nutrient distributions (e.g., Malone et al. 1988, Harding 1994, Fisher et al. 1999, Roman et al. 2005). Overall phytoplankton dynamics in the Chesapeake are related to the effect of winter climate variability on river discharge and nutrient loads (Miller et al. 2006b, Miller & Harding 2007). These temperature and salinity effects likely influence where and when *Pseudo-nitzschia* occur in the Chesapeake (Chapter 2) and results in seasonal and annual fluctuation in abundances.

Intra- and interspecies variation in physiological responses in culture experiments was wide; there was no characteristic response from the genus or from any species. Species could be easily and consistently resolved using morphological and molecular data; however, species, defined in this way, was not

a good predictor of physiology (Chapter 3). Within species variation in growth rate and DA production shows the inadequacy of traditional morphological species definitions in describing physiology and emphasizes the importance of working with multiple strains. Furthermore, many physiological characters that are commonly applied to the genus *Pseudo-nitzschia* from mostly single strain experiments may not be as widely applicable as first thought. High functional diversity within species of Chesapeake *Pseudo-nitzschia* (Chapter 3) suggests the presence of multiple ecotypes as in Narragansett Bay populations of *Skeletonema costatum* (Gallagher 1982, Gallagher et al. 1984) and Puget Sound populations of *Ditylum brightwellii* (Rynearson et al. 2006) or cryptic species as proposed in European populations of *P. delicatissima* and *P. pseudodelicatissima* (Amato et al. 2007).

***Pseudo-nitzschia* grew and produced DA on all nitrogen sources tested.**

Previous research on diatoms shows many species have the ability to utilize multiple nitrogen sources (Eppley et al. 1969, McCarthy 1972), typically, the nitrogen source that is most readily available (McCarthy et al. 1977, Dortch 1990). *Pseudo-nitzschia* is not unique in this regard (Chapter 3). Previous studies have shown growth and toxicity on multiple nitrogen sources (Bates et al. 1993a, Hillebrand & Sommer 1996, Armstrong Howard et al. 2007), but these studies do not compare strains. These results (Chapter 3) show that intraspecies variation in growth rates and toxin production on a given nitrogen source can be high. At least one strain grew optimally and produced toxin on all nitrogen sources tested

(Chapter 3). The high intra- and interspecies variability noted for *Pseudo-nitzschia* suggests that no detectable difference in abundance or toxin should result from change in relative abundance of nitrogen species in the Chesapeake, such as through increased use of urea fertilizer (Glibert et al. 2006).

***Pseudo-nitzschia multiseries* took up 20% of available dissolved nitrate and released only 10% of this as nitrite or ammonia during a high light pulse at low temperature.** As a temperate estuary, cool springs and seasonal turnover typify the Chesapeake. A model has been proposed to explain the dominance of diatoms in cool environments with high nutrients and high water column turbulence in reference to nitrate reduction (Lomas & Glibert 1999). Hence, the effective use of nitrate by *Pseudo-nitzschia* (Chapter 4) might prove beneficial in cool well-mixed environments where this genus is often found. Slight differences between *Pseudo-nitzschia* and other diatoms exposed to a high light pulse in low temperatures exist (Lomas et al. 2000), e.g., that *Pseudo-nitzschia* takes up large quantities of nitrate, but does not release very much nitrite or ammonia, as suggested by the aforementioned model. Either *Pseudo-nitzschia* does not possess the same cold water adaptation or is using a different mechanism to protect itself from high irradiance pulses in low temperatures.

A rapid increase in irradiance in combination with other factors, likely including temperature limitation of enzymes, reduces DA production in the laboratory. Previous studies on the effects of temperature and light on DA

production do not focus on parameter shifts or periods of adaptation. Typically higher irradiances will result in higher DA production (Bates et al. 1991); however, in this experiment, a rapid increase in light actually decreased DA production. The exact mechanisms for this decrease are unknown and require further experimentation.

Domoic Acid in Chesapeake Bay Food Webs and Potential Impacts

Why are there no documented incidents of ASP or DAP in the Chesapeake Bay region despite the large human and waterfowl populations and multiple fisheries? From previous work (Perl et al. 1990, Work et al. 1993a,b, Scholin et al. 2000), three simultaneous requisite conditions for a toxic event must be in place: abundant toxic *Pseudo-nitzschia*, a planktivorous vector feeding on the *Pseudo-nitzschia*, and a higher trophic level feeding on the vector. Due to the water soluble nature of DA, unless all three circumstances occur at the same time, a toxic event will not occur. In addition, appropriate conditions must be met for maximum DA production by the cell. These situations rarely occur globally, but have become common in areas such as the west coast of North America.

Abundances of *Pseudo-nitzschia* can fluctuate widely seasonally and annually depending on factors such as nutrients, salinity and temperature. Potentially toxic species are present in the Chesapeake Bay, but field samples from the area do not contain high levels of domoic acid. *Pseudo-nitzschia* can grow at the low salinities found in the Chesapeake and can produce toxin at salinities as low as 10 (Thessen et al. 2005, Doucette et al. in press). However,

maximum toxin production occurs at salinities above 20 due to the cell's inability to provide energy for DA production while maintaining a high growth rate under osmotic stress, even when adapted to low salinities (Doucette et al. in press).

In addition to salinity effects, available light in the turbid waters of the Chesapeake Bay is often below the 100 μmol photons m^{-2} sec^{-1} required to saturate DA production (Bates 1998; Table 5.1). Calculation of mean PAR in the mixed layer by month for the Chesapeake Bay using published values of modal surface PAR on sunny days, mixed layer depth (Fisher et al. 2003) and diffuse attenuation coefficients (Harding et al. 1985) show that late spring and summer (April through August) is the only time when irradiances exceed 100 μmol photons m^{-2} sec^{-1} in the water column. Most of that time, during the summer, *Pseudo-nitzschia* is less abundant or absent. The month with the highest percentage of samples positive for DA in our study is April (Table 5.1), which is the month where temperatures are likely to be cool and irradiances are likely to be above 100 μmol photons m^{-2} sec^{-1}. It is important to note, that the modal PAR represents the irradiance that cells are most often exposed to, not necessarily the highest irradiance. Instantaneous surface measurements of PAR in the Chesapeake can exceed 1000 μmol photons m^{-2} sec^{-1} (www.chesapeakebay.net). In the Chesapeake, when temperature is optimal for *Pseudo-nitzschia* abundance, irradiance would be limiting to DA production. Light and salinity are two highly variable factors in the Chesapeake that can have an impact on DA production and provide a partial explanation for low DA in field samples.

One of the factors necessary for an intoxication event was not investigated in the current study: there was no assessment of DA content in potential vectors such as bivalves or planktivorous fish and this remains a potential focus for future research. There are several types of bivalves in the Chesapeake either harvested for human consumption or prey for system predators. The eastern oyster *Crassostrea virginica* has been the primary suspension feeding bivalve since colonial times (Kennedy 1996) and is harvested for human consumption. This species is known to feed on a wide range of plankton, including diatoms. Due to disease and over harvesting most *C. virginica* are located in the mesohaline (salinities of 10-20) portions of the Chesapeake and are therefore less likely to be exposed to maximal concentrations of DA. Laboratory feeding experiments show *C. virginica* grazing does not differ between toxic and nontoxic strains of *Pseudo-nitzschia*, but grazing is less and pseudofeces production is higher when given *Pseudo-nitzschia* than other diatoms. Smaller oysters show a reduced capability for grazing on *Pseudo-nitzschia* by producing large amounts of pseudofeces and no feces (Thessen et al. in prep). Possibly cell size and shape, rather than the DA, make *Pseudo-nitzschia* less palatable than many other diatoms (Tenore & Dunstan 1973).

Hard clams (*Mercenaria mercenaria*) are produced in aquaculture operations in upper mesohaline and polyhaline areas of the Chesapeake for human consumption (Roegner & Mann 1991). Since higher salinity favors maximum toxin production (Doucette et al. in press), DA contamination in these clams is possible because of their location, but not documented. To date, no experiments

have been published investigating hard clam grazing on *Pseudo-nitzschia*. However, hard clams decrease their grazing when given other types of toxic algae (Pate et al. 2006, Shumway et al. 2006).

In addition to bivalves, planktivorous fish are potential vectors of DA and plentiful in the Chesapeake. In other areas, many small, schooling fish like sardines and anchovies have caused DAP events or had DA in their tissues, but this has not happened in the Chesapeake despite the presence of many similar types of fish. Atlantic menhaden (*Brevoortia tyrannus*) are an abundant fish species that feed primarily on phytoplankton as an adult (Durbin & Durbin 1983) and in turn are an important food source for predatory fish and birds (Hartman & Brandt 1995). Most of the menhaden in the Chesapeake are juveniles that feed on zooplankton; adults remain in saltier waters near the Atlantic coast. They are harvested commercially for their oil, a major ingredient in many pet and human food products. Since DA is a water soluble molecule, the likelihood of the toxin being transferred to these products in the oil is low. However, eating menhaden whole (including the digestive tract) would pose a DAP risk to animals on the Atlantic coast. Other small fish such as the bay anchovy (*Anchoa mitchilli*) and the Atlantic silverside (*Menidia menidia*) are an important food source in the Chesapeake, but are secondary consumers themselves. This does not mean they cannot contain DA passed to them by their zooplankton prey, but it does decrease the likelihood of subsequent passage of DA to the next trophic level. Anchovies in other areas (*Engraulis mordax*) that have caused DAP events directly feed on large diatoms in addition to zooplankton, unlike the bay anchovy or the atlantic

silverside which feed on zooplankton exclusively (www.fishbase.org; Robins & Ray 1986, Whitehead et al. 1988). Other herbivorous fish such as the eastern silvery minnow (*Hybognathus regius*), the gizzard shad (*Dorosoma cepedianum*) and the spot tail shiner (*Notropis hudsonius*) are present in the Chesapeake, but only in low salinity waters, which decreases the likelihood of exposure to DA-producing *Pseudo-nitzschia* (www.fishbase.org).

Hence, the overall DAP and ASP risk in the Chesapeake is low. The "ideal vector" species would live in a high salinity part of the Chesapeake during the winter and early spring, eat primarily phytoplankton and serve as a primary food source for another consumer. Of the species discussed above, *Mercenaria mercenaria* is a possible candidate for causing ASP in humans, but data are sparse on *M. mercenaria* grazing on *Pseudo-nitzschia* and DA content. The hooked mussel (*Ischadium recurvum*) is a possible vector of DA to the seaducks that feed on them while over wintering in the Chesapeake (Perry et al. 2004). Hooked mussels can survive over a wide range of salinities and are typically found living on oyster reefs in the region; however, their grazing and toxin accumulation is not known. The only fish species that could act as a vector is atlantic menhaden, but only the adults, and primarily to animals that would eat the entire fish. The restricted number of potential vectors combined with environmental conditions that restrict DA production make the risk of DAP or ASP in the Chesapeake Bay low.

Ecological Meaning of Intra- and Interspecies Differences

In the rapidly and widely changing environment of the coastal ocean, maintaining a functionally diverse population (whether phytoplankton or other) would be advantageous, allowing at least some individuals to do well under nearly any given set of conditions. Instead of multiple narrow niches and diatom evolution proceeding toward more species, the niche is wide and diatom evolution would proceed toward fewer species with high functional diversity, or intraspecies variation. Laboratory studies conducted as a part of this thesis and field studies support this idea (Gallagher 1982, Evans et al. 2005, Chapter 3). Variation of this sort will thwart attempts to characterize a diatom species by a strict set of physiological responses, especially when only one strain is considered. Variation previously attributed to differences in species or genera could actually represent differences between strains. However, some of this variation could represent differences between reproductively isolated cryptic species resulting from sympatric speciation (Beszteri et al. 2005). Comprehensive studies comparing morphology, phylogeny, physiology and mating compatibility are necessary to characterize diatom species.

Until relatively recently, diatom taxonomy has remained linked to the study of the silica frustule. Many frustule characteristics are used in taxonomy without any insight into their adaptive significance or how they may have evolved. Evolutionary pressures that change physiology may or may not also affect frustule morphology and *vice versa*. However, since diatoms are sexually reproducing organisms, there should be a reliable species-level taxonomy based

on variation in phenotype. Now, genetic sequencing is being used in conjunction with frustule morphology to evaluate species (Lundholm et al. 2003, Lundholm et al. 2006). Molecular data can give new insights into morphologically based taxonomic characters, but are particularly useful when related to biological processes.

Recent studies examining molecular and morphological characters to better describe diatom taxonomy have made significant headway; however, a truly exhaustive study should include mating and physiological experiments to look for cryptic species and functional diversity. ITS (internal transcribed spacer) and LSU (large subunit) rRNA sequences did not provide insight into functional diversity in this study, but sequencing more physiologically relevant genes may prove useful. The whole genome sequencing of *Pseudo-nitzschia multiseries* currently underway (DOE Joint Genome Institute http://www.jgi.doe.gov/sequencing/DOEmicrobes2006.html) will be an asset to investigations of functional diversity by enabling researchers to measure regulation of genes for specific cellular processes and to develop an EST (expressed sequence tag) library. Microsatellites have been used to describe variation in natural populations of *Pseudo-nitzschia* (Evans et al. 2005) and could be useful in describing functional diversity and biogeography on a more detailed level than ribosomal RNA.

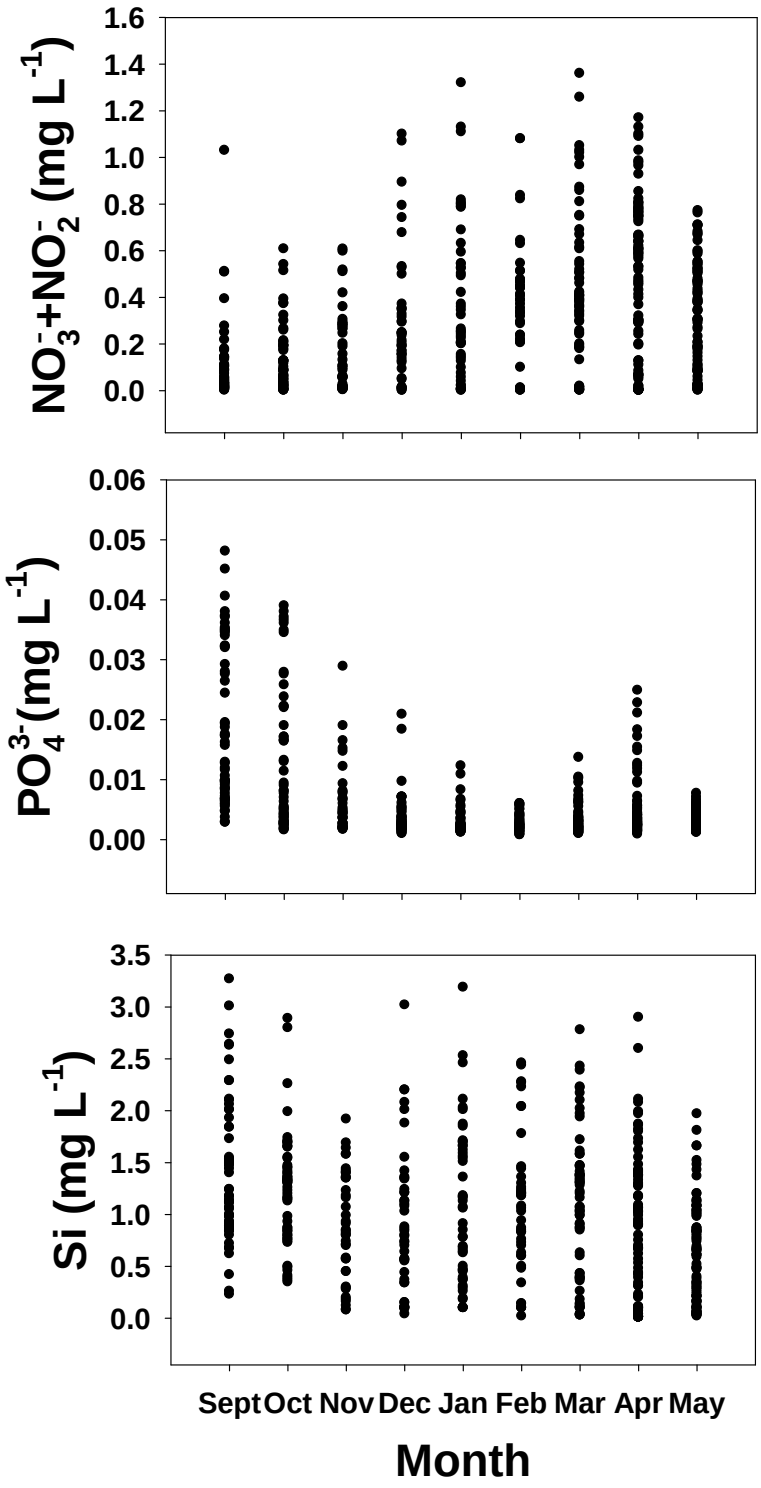
This thesis provides information on Chesapeake Bay *Pseudo-nitzschia* populations, strain variability and toxin production physiology. Many questions about taxonomy, toxin production and ecophysiology were answered, yet many

more questions concerning the effect of high light pulses on DA production, the prevalence of DA in Chesapeake food webs and the ecological meaning of intraspecies differences remain unanswered. Future research on Chesapeake *Pseudo-nitzschia* should focus on assessing the extent of DA in primary consumers, comparing mid-Atlantic strains of *Pseudo-nitzschia* using mating experiments and further probing the effects of decoupling photosynthesis and growth on DA production.

Table 5.1 Average PAR in the mixed layer, modal surface PAR and the percentage of samples positive for DA by month. Average PAR was calculated using modal PAR values on a sunny day for each month found in Fisher et al. (2003), a value of 5 m for mixed layer depth, 1 m^{-1} for diffuse attenuation coefficient and an equation that can be used to express the mean water column PAR for any aquatic system (Phlips et al. 1995).

Month	average water column PAR	modal surface PAR	% samples positive for DA	n
January	42.4	224	33	6
February	59.1	314	68	19
March	84.1	446	40	20
April	111	589	78	9
May	126	671	46	13
June	126	670	ND	
July	124	654	ND	
August	113	596	ND	
September	94.1	499	0	0
October	68.2	361	17	6
November	48.4	257	57	7
December	43.1	228	75	4

Figure 5.1 Dissolved nutrient concentrations in the Chesapeake Bay. Data were taken from the Maryland DNR database at www.chesapeakebay.net from 2002-2007, September through May.



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Appendix B: Glossary of Terms

Aconitase iron-containing enzyme involved in the Krebs Cycle; transforms citrate into isocitrate
See Chapter 1 Figure 3

Adductor muscle muscle present in bivalves that controls opening and closing of the shell

Agarose gel made from the polysaccharide agarose and used as a separation medium during electrophoresis

Amygdala part of the brain involved with fear and aggression

Amplicon small replicating DNA fragment

Annealing as used in PCR means the recombination of the two halves of DNA after temperature dissociation

Antihelminthic used to kill worms or removed internal parasites in animals or humans

Apical Plane Cross-sectional view of a cell showing the slice through the middle of the top and bottom valve faces

Appendicularan filter feeder with a primitive notochord; often found in the surface layers of the open ocean

Ataxia loss of muscle coordination

Auxospore Enlarged diatom cell that forms after sexual reproduction

Axenic without bacteria or other microorganisms besides the species being studied

Biogeography geographical distribution of living things

Blood-brain barrier a layer of tightly-packed cells in the walls of brain capillaries which prevent molecules from freely diffusing into the brain

cDNA microarray collection of single stranded DNA molecules synthesized in the laboratory using messenger RNA as a template and the enzyme reverse transcriptase which are then attached to a solid surface that will react with a known sequence; a tool to probe for up to thousands of genetic markers at a time

Central interspace interruption in the pattern of fibulae along the raphe canal near the midpoint of the length of a pennate diatom; also called a central nodule
See Chapter 1 Figure 2

Cephalopod division of phylum Mollusca that contains octopuses and squids

Chronic of long duration; continuing

Chytrid algae-like fungus that infects microbes

Cingulum girdle bands associated with a single valve

Clade A group of organisms that share a common ancestor

Clonal Culture created by a single cell isolation in which all cells are a descendant or clone of one cell and no sexual reproduction has occurred

Coastal bay body of water located between a coastline and a barrier island; characterized by shallow depth, high salinity and little exchange

Conopea Fine silica structure spanning from the keel to the valve walls along both sides of the entire length of the raphe canal
See Chapter 1 Figure 2

Cotyledon leaf of an embryonic plant

Cryptic species species that are reproductively isolated yet identical in appearance

Culture growth resulting from cultivation of microorganisms

Denaturation as used in PCR means the "unzipping" of the DNA strand into two halves comprised of the sugar/phosphate backbone and attached nucleotides

Deposit feeder organism that feeds on organic matter on the surface of the sediment

Digestive gland a gland that secrets digestive enzymes
See Hepatopancreas

Ecotype subdivision beneath species in which cells are morphologically similar, but functionally different

ENSO acronym for El Nino Southern Oscillation; warming of the Pacific Ocean near the equator that occurs every 5-7 years and is associated with poor fisheries and unusual weather patterns

Enumerate to count

Epibiont cell that lives on the surface of another cell; a bacteria living on the surface of a diatom

EST acronym for expressed sequence tag; short sequence of DNA used to determine gene expression

Exponential Growth period of growth in a batch culture when cell numbers are increasing exponentially; characterized by balanced growth, replete nutrients in growth media and sufficient light
Also referred to as exponential phase

Fibula (pl. fibulae) silica structure spanning the raphe canal inside the valve
See Chapter 1 Figure 2

Formvar resin used to coat grids and provide support for samples to be viewed by electron microscopy

Frustule whole diatom box composed of two valves

Genotoxic chemical or other agent that damages DNA, resulting in mutations

Gluconic acid acid formed by the oxidation of glucose

Gluconolactone cyclic form of gluconic acid; forms equilibrium mixture with gluconic acid in water

Glucuronate a derivative of glucuronic acid

Glucuronic acid acid of glucose found in the liver; used to detoxify compounds

Glutamate agonist a chemical that binds to a glutamate receptor to illicit a physiological response

Glutamate receptor a receptor located on neuron membranes that binds with glutamate and can cause a neuron to depolarize

Glutamic acid nonessential amino acid commonly occurring in plant and animal cells; important for nitrogen metabolism in plants; neurotransmitter

Glutamine synthetase enzyme used to add ammonium to α-ketoglutarate and make glutamate
See Chapter 1 Figure 3

Glutaraldehyde colorless chemical used as a biological fixative

Glycoprotein a protein with a carbohydrate component

Hepatopancreas gland that functions as a liver and a pancreas in crustaceans and mollusks

See Digestive gland

High voltage paper electrophoresis analytical method used to identify and quantify amino acids using an electrified paper strip

HNLC acronym for High Nutrient Low Chlorophyll;describes regions in the ocean characterized by high nitrogen and low chlorophyll where autotrophic growth is limited by iron

Hypocotyl part of an embryonic plant that pushes the cotyledons above ground and eventually becomes part of the stem

Intraperitoneal within the body cavity that contains the organs

Ion exchange chromatography analytical method allowing separation of molecules based on their charge

IR spectroscopy analytical method based on detection of a chemical by its absorption of infrared light

ITS acronym for internal transcribed spacer; sequence of ribosomal RNA that is present in the initial transcript from DNA, but is removed when the strand is processed into an actual ribosome

Kainic acid glutamate derived amino acid that can be extracted from macroalgae and has excitotoxic potential; similar to domoic acid

Lag phase period of time in batch culture, typically immediately after the start of a new culture and before exponential growth, in which little to no growth occurs

Lectin proteins on a cell surface that are not antibodies, but bind specifically

Limit of detection lowest amount of test substance that can be detected in an analytical sample below which it is considered absent

Limit of quantitation lowest amount of test substance that can be detected in an analytical sample where the accuracy is within acceptable levels; below this point data is semiquantitative

LSU acronym for large subunit; sequence that makes up the large subunit of the ribosomal RNA

Lugols iodine solution used as a preservative and stain for protists in water samples

Mantle part of the valve at the margin of the valve face which is set off at an angle

Mass spectroscopy analytical method using magnetic and/or electrical fields to identify molecules based on their charge

Mesohaline water of medium salinity; approximately 5 to 18 salinity

Metazoan animal comprised of many cells organized into tissues and organs

Micrograph photograph taken using a microscope

Microsatellite markers short segments of non-coding DNA that have a repeated sequence

Monospecific consisting of one species

Nitrate reductase enzyme used to reduce nitrate to nitrite
See Chapter 1 Figure 3

Nucleotide any of a group of organic molecules used as building blocks of DNA and RNA, they are made of a sugar, phosphate group and nitrogenous base

Oomycete fungus-like protist that can infect microbes

Parsimony tree phylogenetic tree diagram structured so that species with similar sequences are closer together

PCR anacronym for polymerase chain reaction; process for replicating DNA fragments for analysis

Phylogeny evolutionary history of a taxonomic group of organisms

Poroid regularly repeated perforation in the valve wall; patterns formed by poroids are using to aid identification of species
See Chapter 1 Figure 2

Primer a sequence of DNA that is complementary to DNA sequence to be analyzed and is needed to start a PCR reaction

Principle Components Analysis technique used to reduce multidimensional data sets into lower dimensions for analysis; data is reduced to the principle components

Proton nuclear magnetic resonance spectra analytical method based on the magnetic qualities of a nucleus that can identify individual atoms in a molecule

PSP acronym for Paralytic Shellfish Poisoning; food poisoning resulting from eating shellfish contaminated with algal toxins, characterized by paralysis which can lead to respiratory failure

Raphe canal feature of a diatom frustule that runs the length of a pennate cell
See Chapter 1 Figure 2

rRNA ribosomal ribonucleic acid; a type of RNA functioning in protein synthesis as a ribosome

RUBISCO acronym for ribulose bisphosphate carboxylase/oxygenase; plant enzyme that fixes carbon during photosynthesis

Secondary metabolite product of cell machinery that is not directly related to cell growth or nutrient uptake

SSU acronym for small subunit; sequence that makes up the small subunit structure of the ribosomal RNA

Stationary growth period of growth in batch culture in which nutrients and/or light are not enough to sustain growth; characterized by high biomass and no cell division
Also referred to as stationary phase

Strain all descendants of one cell

Stria (pl. striae) one or many rows of poroids
See Chapter 1 Figure 2

Sublethal less than lethal

Upregulate process by which a pathway or product is increased in use or abundance; an increase in expression of a gene resulting in more of a product; increase in a physiological process either to preserve homeostatis or take advantage of beneficial conditions

Upwelling a coastal and oceanographic phenomenon where cold, nutrient-rich water from depth is brought to the surface often by wind moving surface water away from a coast line

Utermohl method used for counting cells that have been allowed to settle in a chamber

UV spectroscopy analytical method that detects chemicals based on absorption of UV light

Valve one half of the two-part silica frustule surrounding a diatom cell

Vector organism that transmits a pathogen or chemical

Voltammetric technique electroanalytical method where an analyte is observed by measuring changes in current while the electrical potential is varied

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