Occurrence and distribution of toxic *Pseudo-nitzschia* events in Washington State: Analysis of scientific findings and policy responses

by

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ABSTRACT

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Jason Randall Lim

Algae are photosynthetic organisms that form the base of the food chain in aquatic ecosystems. Large accumulations of algal blooms and the presence of toxic algal species can have harmful impacts on humans and marine wildlife. The occurrences of these harmful algal bloom (HAB) events worldwide have become more prevalent, most likely due to human activities. For this thesis, an extensive literature review was done on twenty-five years of research on global and local occurrences of an algal genus known to have harmful impacts, *Pseudo-nitzschia*. The goal of the literature review was twofold: to identify environmental factors associated with *Pseudo-nitzschia* blooms and to predict the production of domoic acid (DA), a potent neurotoxin. Based on the current science, policy recommendations were developed to prevent and mitigate future toxic *Pseudo-nitzschia* events. Results of the literature review indicate that increased nutrient concentrations causing coastal eutrophication and shifts in nutrient composition are associated with the increased frequency of *Pseudo-nitzschia* blooms. A variation in the irradiance of sunlight has also been identified as influences on bloom activity. Climate changes, through variations in rainfall, can increase the input of nutrients into marine waters. While DA production is associated with twelve *Pseudo-nitzschia* species, DA production does not always coincide with the presence of *Pseudo-nitzschia* blooms. Within these twelve species, DA production varies across growth stages of a given population and nutrient concentration and composition. Changes in the physical environment such as increased salinity and pH result in increased DA production. DA may also serve an ecological role as an iron/copper chelator. Evidence has shown that *Pseudo-nitzschia* is versatile at adapting and thriving under various environmental conditions. Since it is still not possible to accurately predict these outbreaks, it has been difficult to develop policy responses that are species specific. Current policy and management strategies have been reactionary rather than precautionary. Policies are based on general ecological knowledge of the established connections between increased nutrients and algal biomass. Therefore, as the scientific knowledge furthers the understanding of biological and ecological dynamics of Ps*eudo-nitzschia,* policies can be refined to address species affecting specific regions. Future recommendations are as follows: 1) Develop collaborative efforts between Washington State and British Columbia. 2) Update policies addressing nutrient reductions. 3) Deploy ocean sensor technologies and develop models that forecast *Pseudo-nitzschia* bloom events.

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List of Abbreviations

ASP	Amnesic Shellfish Poisoning
B.C.	British Columbia
CFP	Ciguatera Fish Poisoning
CHRP	Coastal Hypoxia Research Program
CO_2	Carbon dioxide
COTF	Coastal Oceans Task Force
CSCOR	NOAA's Center for Sponsored Coastal Ocean Research hypoxia programs
DA	Domoic Acid
DAP	Domoic Acid Poisoning
dDA	Dissolved Domoic Acid
DSP	Diarrhetic Shellfish Poisoning
ECC	Environmental Collaboration Council
ECOHAB	Ecology and Oceanography of Harmful Algal Blooms Program
EPA	Environmental Protection Agency
EUROHAB	European Harmful Algal Bloom program
FDA	Food and Drug Administration
F&O	Canada Fisheries and Oceans
GEOHAB	Global Ecology and Oceanography of Harmful Algal Blooms
HAB	Harmful Algal Blooms
HABHRCA	Harmful Algal Bloom and Hypoxia Research and Control Act
HABSOS	Harmful Algal Blooms Observing System
MERHAB	Monitoring and Event Response for Harmful Algal Blooms Program
Mg	milligrams
µm	micrometers
Mm	millimeters
N	Nitrogen
NCCOS	Center for Sponsored Coastal Ocean Research (NOAA)
NGOMEX	Northern Gulf of Mexico Ecosystems and Hypoxia Assessment Research Program
NIEHS	National Institute for Environmental Health Sciences
NMFS	National Marine Fisheries Service
NOAA	National Oceans and Atmospheric Administration
NOS	National Ocean Service
NSF	National Science Foundation
NSP	Neurotoxic Shellfish Poisoning
NWFSC	Northwest Fisheries Science Center
ONRC	Olympic Natural Resource Center
ORHAB	Olympic Region Harmful Algal Blooms
OSU	Oregon State University
P	Phosphorus
PCM HAB	Prevention, Control, and Mitigation for Harmful Algal Blooms research
pDA	Particulate Domoic Acid
PNWH20	Pacific Northwest Center for Human Health and Oceans Studies
PPM	Parts per million
PSP	Paralytic Shellfish Poisoning
QIN	Quinault Indian Nation
Si	Silica
SiO2	Amorphous silica

TMDL	Total Maximum Daily Load
U of O	University of Oregon
UW	University of Washington
WCCOHH	West Coast Center for Oceans and Human Health (NOAA)
WDFW	Washington Dept. of Fish and Wildlife
WDOH	Washington Dept. of Health
WIP	Watershed Implementation Program
WSDA	Washington State Department of Agriculture

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Chapter 1

An Introduction to Algae, Harmful Algal Blooms, *Pseudo-nitzschia* and the Mechanisms of Domoic Acid toxicity

Introduction/ Significance of Harmful Algal Blooms

Algae consist of microscopic, single-celled organisms to large seaweeds; they are aquatic plants that form the base of the food-web. A small percentage of algal species produce toxins or accumulate in biomass depending on certain environmental conditions. These toxins have negative health impacts on fin-fish, mammals, and birds, and the movement through the food-web can lead to human illness. Other algae are nontoxic, but can clog the gills of fish and invertebrates. Some algae accumulate in large numbers forming blooms that cover the surface of the water blocking sunlight and thus affecting submerged aquatic vegetation and corals. As blooms decay, their decomposition by bacteria can deplete oxygen levels resulting in hypoxic (low oxygen) and anoxic (absence of oxygen) conditions. Blooms discolor the surface of the water and can appear in different colors including: blue-green, brown, and even reddish- orange depending upon the algal species, the aquatic ecosystem, and the concentration of the organisms. These outbreaks are commonly called “red tides.” However, because not all algal blooms are red, the term that has become more widely accepted is "harmful algal blooms" (or HABs). The term “red tides” erroneously includes many blooms that discolor the water but cause no harm, and also excludes blooms of highly toxic cells that cause problems at low cell (color-less) concentrations.

HABs are a global problem that impact both freshwater and marine environments. Most HABs are caused by blooms of microscopic algae or phytoplankton. HAB occurrence is associated with a complex set of physical, chemical, biological, hydrological, and meteorological conditions making it difficult to determine the specific causative environmental factors. The occurrences of HAB events worldwide have become more frequent, more widely distributed, and more severe worldwide likely due to human activities.

The impacts of HABs that directly harm humans include illness and mortality following consumption of impacted vectors such as fin-fish or shellfish or indirect exposure to HAB toxins. In addition to the physical effect on humans, HABs can lead to substantial economic losses to coastal communities and commercial fisheries with the closure of shellfish and fin-fish farms when these toxins accumulate in high concentrations.

The group of phytoplankton or microalgae of most concern to human health are dinoflagellates. Dinoflagellates are a group of unicellular flagellated protists that can also produce harmful toxins. The genus *Alexandrium* produces saxitoxins, which causes paralytic shellfish poisoning (PSP). *Prorocentrum* and the planktonic forms of *Dinophysis* produce okadaic acid, which cause diarrhetic shellfish poisoning (DAP). The species, *Karenia Brevis,* produce brevetoxins, which cause neurotoxic shellfish poisoning (NSP). The species *Gambierdiscus toxicus* produces ciguatoxins that cause ciguatera fish poisoning (CFP). Most dinoflagellates are marine plankton, but they are commonly found in fresh water habitats. Many dinoflagellates are known to be photosynthetic, but a large fraction of these are mixotrophic, meaning they have the ability to be photosynthetic and ingest their prey. Dinoflagellates are the largest group of marine eukaryotes aside from the diatoms (Stoecker, 1999). Their status as primary producers makes them an important part of the aquatic food chain. Some species, called *zooxanthellae,* are endosymbionts of marine animals and play an important part in the biology of coral reefs. However, this thesis will focus on another group of phytoplankton, diatoms and more specifically the diatom genus, *Pseudo-nitzschia*. *Pseudo-nitzschia* has been identified to produce a harmful toxin to humans called domoic acid (DA). *Pseudo-nitzschia* has also been identified as increasing in occurrence and distribution worldwide which has paralleled the degradation of aquatic ecosystems due to human activities.

Background on *Pseudo-nitzschia*

Between November and December of 1987, a poisoning event occurred in eastern Canada due to the consumption of contaminate shellfish. This event resulted in 200 hospital admissions in the provinces of Quebec and New Brunswick and left four elderly people dead. The patients were experiencing varying degrees of an acute illness, characterized by gastrointestinal illnesses along with unusual nervous system abnormalities (Perl et al. 1990). The source of these illnesses was traced back to a batch of blue mussels (*Mytilus edulis)* from Cardigan Bay, Prince Edward Island Canada. The toxin was later identified as domoic acid (DA), which had not been identified previously as harmful to humans. The producer of domoic acid came from a marine diatom genus, *Pseudo-nitzschia.* This event was the first documented case in which a diatom genus was shown to produce harmful toxins. Clinically the syndrome is called amnesic shellfish poisoning (ASP), for the memory loss associated with DA toxicity. In 1991, DA toxins were discovered as a potent toxin in brown pelicans off the coast of California in Monterey Bay (Work et al. 1993). In 1998, ASP was as the cause for the deaths of California sea lions (Scholin et al. 2000). During these two events, a new vector was found for DA in anchovies and sardines, which were prey to brown pelicans and California sea lions.

Since 1991 when DA was first measured, it has been detected every year in Pacific razor clams (*Siliqua patula*), Dungeness crab (*Cancer magister*), and other shellfish from coastal Washington State waters (Horner et al. 1993). High levels of DA toxicity on the outer Washington State coast has resulted in the emergency closures of harvest areas in 1991, 1998, 1999, and every year thereafter. DA levels have been detected in Puget Sound, prior to 2003 by the routine monitoring program implemented by the Washington State Department of Health (WDOH) (Fig.1. shows the global distribution of toxic and non-toxic species of *Pseudo-nitzschia*). 2003 was the first year that DA levels had exceeded the regulatory limit of 20 parts per million (ppm). Shellfish farms and harvesting in Puget Sound had experienced delays or have closed operations in September 2003, and again, in October to November 2005.

Table 1. List of Documented Closures of Shellfish Harvesting Caused by Domoic Acid

Closures of molluscan shellfish harvesting, caused by domoic acid. nr: not reported.

Location	Dates	Organisms affected	Dominant species of *Pseudo-nitzschia*	Reference
Eastern Prince Edward Island, Canada	December 1987–January 1988	Blue mussel (*Mytilus edulis*)	*P. multiseries*	Bates et al. (1989)
Northern Prince Edward Island, Canada	Autumn of 1991, 1992, 1994, 2000, 2001	Blue mussel (*Mytilus edulis*)	*P. multiseries*	Bates (2004)
Bay of Fundy, Canada	September 1988–December 1988	Soft-shell clam (*Mya arenaria*)	*P. pseudodelicatissima*[a]	Gilgan et al. (1990), and Haya et al. (1991)
Coastal Washington, USA	1991, 1993, 1995, 1998–1999, 2002, 2003, 2004, 2005, 2006	Razor clam (*Siliqua patula*)	*P.* cf. *pseudodelicatissima*, *P. australis*	Adams et al. (2000), Wekell et al. (2002), Trainer and Suddleson (2005), and Washington State Department of Health database.
Quatsino Sound, British Columbia, Canada	August 1992, October 1993	Dungeness crab (*Cancer magister*)	nr	Department of Fisheries and Oceans (1992)
Barkley Sound, British Columbia, Canada	November 1993	Razor clam (*Siliqua patula*); Dungeness crab (*Cancer magister*)	nr	Department of Fisheries and Oceans (1992)
New Zealand	Various dates between 1994 and 1999; review pending for 2000–2010	New Zealand scallop (*Pecten novaezealandia*); Greenshell™ mussel (*Perna canaliculus*)	*P. australis*	Hay et al. (2000), L. Rhodes (pers. comm.)
Galicia, Spain	1995, 1996	Scallop (*P. maximus*, *P. jacobeus*)	*P. australis*	Arévalo et al. (1998), and Fraga et al. (1998)
West coast of Scotland	June 1999–April 2000	King scallop (*Pecten maximus*)	*P. australis*	Gallacher et al. (2001)
Southeast coast of Ireland	December 1999–May 2000	King scallop (*Pecten maximus*)	*P. australis*	James et al. (2005), and Bogan et al. (2007a,b)
Coastal Portugal	Periodic closures lasting 1 week; mostly in May of various years	Common cockle (*Cerastoderma edule*); Peppery furrow shell (*Scrobicularia plana*); blue mussel (*Mytilus edulis*)	*P. australis*	Vale and Sampayo (2001, 2002)
California, USA	2000–2009	Blue mussel (*Mytilus edulis*), lobster viscera	*P. australis*, *P. multiseries*	Trainer et al. (2000), and Langlois (2001–2009)
Southern Gulf of St. Lawrence, Canada	April 2002–June 2002	Blue mussel (*Mytilus edulis*)	*P. seriata*	Bates et al. (2002)
Baie de Seine, France	November 2004–"several months"	King scallop (*Pecten maximus*)	*P. australis* or *P. multiseries*	Nézan et al. (2006)
West coast of Ireland	April 2005	Blue mussel (*Mytilus edulis*)	nr	Bogan et al. (2006)
Puget Sound, Washington, USA	September 2003; October 2005–November 2005	Blue mussel (*Mytilus edulis*)	*P. australis*, *P. pseudodelicatissima*	Bill et al. (2006), and Trainer et al. (2007)
Oregon, USA	1991, 1998, 2003, 2004, 2005	Razor clam (*Siliqua patula*), blue mussel (*Mytilus edulis*)	*P. australis*	Tweddle et al. (2010)
Denmark, various locations	March–April 2005	Blue mussel (*Mytilus edulis*)	*P. seriata*	Lundholm et al. (2005a)
Santa Catarina State, Brazil	January 2009–February 2010	Brown mussel (*Perna perna*)	nr	Reported in Fernandes and Brandini (2010)

Table 1. Documented closures of shellfish harvesting areas caused by elevated DA toxicity and identified dominant *Pseudo-nitzschia* species. Including geographic location and length of closure time, and specific shellfish species. Source: (Trainer, Vera L., Stephen S. Bates, Nina Lundholm, Anna E. Thessen, William P. Cochlan, Nicolaus G. Adams, and Charles G. Trick. "Pseudo-nitzschia Physiological Ecology, Phylogeny, Toxicity, Monitoring and Impacts on Ecosystem Health." *Harmful Algae* (2011): 1-30.)

Toxigenic species of *Pseudo-nitzschia* species have been found along most of the world's coastal waters and upwelling systems, including the Juan de Fuca eddy near the mouth of Puget Sound. While the impacts of DA toxicity have been most prevalent in temperate areas, *Pseudo-nitzschia* has also been found in tropic and sub-tropical regions. There is also a presence of both toxic and non-toxic species of *Pseudo-nitzschia* in both the Arctic and Antarctic waters. Recently, *Pseudo-nitzschia* and DA have been detected in the open ocean including high-nutrient, low chlorophyll (HNLC) regions, and additionally in bays, gulfs, and bights. Because *Pseudo-nitzschia* have shown their presence in diverse marine environments, it is important to understand the biology of the causative organisms to develop effective policies to control these events.

Figure 1. Global Distribution of Toxic and Non-Toxic Species of *Pseudo-nitzschia*

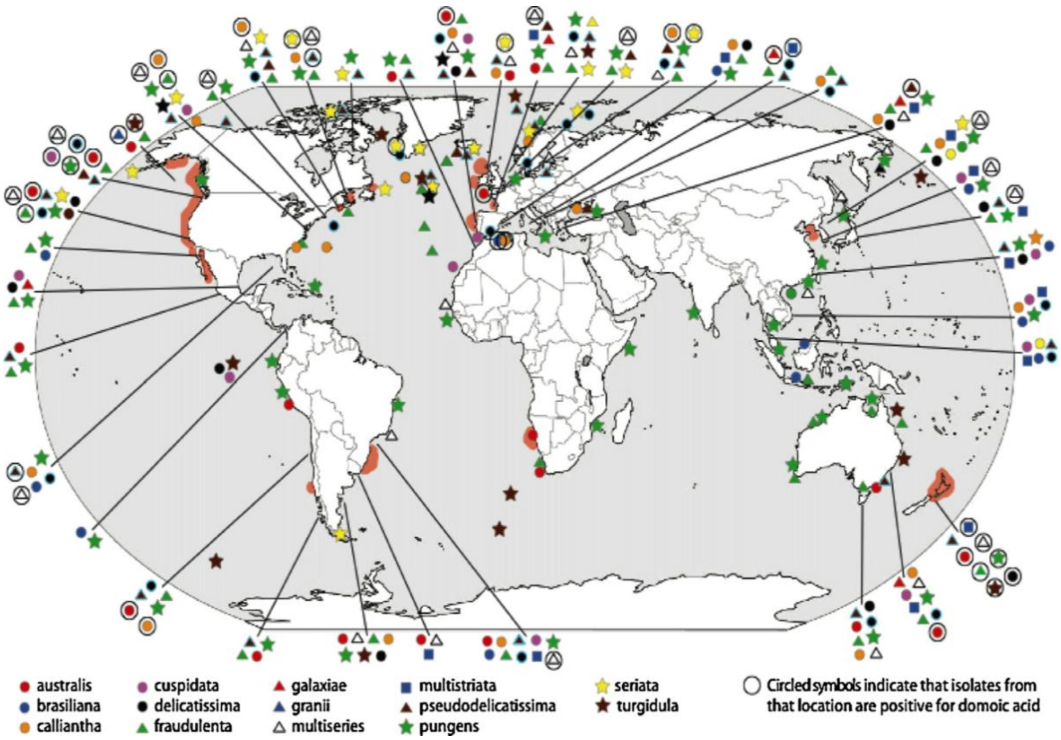

Fig. 1. Global distribution of identified toxic and non-toxic species of *Pseudo-nitzschia* blooms and DA events. Species that have demonstrated to produce DA in culture are circled. Coastal areas highlighted in red are locations of shellfish harvesting closures due to elevated levels of DA and locations of animal mortality events.
Source: (Trainer, Vera L., Stephen S. Bates, Nina Lundholm, Anna E. Thessen, William P. Cochlan, Nicolaus G. Adams, and Charles G. Trick. "Pseudo-nitzschia Physiological Ecology, Phylogeny, Toxicity, Monitoring and Impacts on Ecosystem Health." *Harmful Algae* (2011): 1-30.)

Biology of Diatoms

Based on fossil evidence, diatoms and other microalgae have been present for about 265 million years, and pennate diatoms, (in which *Pseudo-nitzschia* are a type) have been present for approximately 70 million years. Diatoms constitute a large source of the earth's oxygen supply, producing approximately the equivalent of all tropical rainforests (approximately 20% to 25% of the world's oxygen supply) (Field, 1998).

Diatoms (class *Bacillariophyceae*) are a type of mainly aquatic, photosynthetic algae. Similar to many other algae, diatoms can live as unicellular organisms, colonial, or filamentous. As with all plants even terrestrial and marine macroalgae (seaweeds), diatoms contain chlorophyll and other pigments that capture the energy from sunlight to convert carbon dioxide and water molecules into carbohydrates via photosynthesis. Diatoms contain chloroplasts that have been found to have numerous photosynthetic pigments. The chloroplasts of diatoms consist of lamellae with three thylakoids, girdle lamella, and four membranes that surround the chloroplast, giving the chloroplasts a typically golden brown color (Hasle et al. 1997). Photosynthetic pigments include chlorophylls a and c, beta-carotene, fucoxanthins, diatoxanthin, and diadinoxanthin (Hasle et al. 1997).

Diatoms also contribute approximately 40% to 45% of the total production of organic carbon in the marine ecosystem (Mann 1999). Diatoms, thus, play a significant role in the carbon cycle. Because of their large cell size and rapid sink rate, diatoms can remove carbon dioxide (CO_2) from the atmosphere and stores the CO_2 at deep depths of the oceans. Diatoms are significant source of food and therefore, play a vital role in the food chain.

Diatoms are found in marine and freshwater ecosystems as well as brackish waters. Diatoms have been found in snow, sea ice, and in the sand of beaches, found all around the world, from the tropics to the poles. In water, diatoms live in or on sediments (which can attribute to the long term impacts on benthic feeders), and can also be attached to rocks (epilithic), plants (epiphytic) and animals (epizootic), or be free floating but they are known for being part of the drifting planktonic mass (Trainer et al. 2008).

Planktonic diatoms that co-habit with dinoflagellates and cyanobacteria, among other microalgae make up the phytoplankton community (Greek, phyton meaning plant, and planktos meaning wanderer) are called microalgae, or microphytes.

There are some diatoms that are found as single cells, and others form long chains or colonies that can measure up to several centimeters in length. Diatoms form chains by linking to the adjacent cell, either by joining the protruding spines or setae, or by abutting end to end. This overlapping allows for some internal expansion room and is essential during the reproduction process. The cell size ranges from 2 micrometers (µm) to 2 millimeters (mm), and are uninucleate.

The name diatom comes from the Greek word diatomos, which dia=through, and temnein= to cut, meaning to cut in two. This is because diatom cells are composed of two overlapping halves (Bates et al. 1998).The cell wall of diatoms is called the frustule, and is composed of amorphous silica (SiO_2) or glass. The frustule is covered by an organic membrane, which allows nutrients to pass through for cell growth. All diatom skeletons are made of silica and consist of two parts or frustules that fit inside each other like a petri dish: the epitheca and the hypotheca. The upper half (epi) is called the epitheca, and the lower half (hypo) is called hypotheca. The theca is composed of an epivalve, and the hypovalve. The two valves include a flat or semi-flat valve face and a sloping mantle. The valves have lines called striae. The epitheca and hypotheca support and encircle the middle of the cell by bands of silica (girdle bands) that hold the two halves together. Each of the valves contain a longitudinal slit called the raphe. The raphe is associated with providing movement to the cell, and is attached to a substrate. The raphe secretes mucus that attaches to particles or the substrata, which leaves a trail of this mucus as diatoms move (Trainer et al. 2008). Pennate diatoms such as *Pseudo-nitzschia* have raphes present on both valves, termed biraphid.

However, motility in *Pseudo-nitzschia* is not known, for they are normally suspended in the water column. The raphe is also present in the interior of the edge of the valve, known as the keel. The keel is supported by structures called fibulae. The striae is composed of rows of small pores, called poroids, alternating with rib like strips called interstriae. The orientation of interstriae determines the order in which diatoms are classified. Diatoms are classified into two orders based on shape; centric diatoms have valves with a radial symmetry and pennate diatoms have valves with bilateral symmetry.

Figure 2. Image of Centric and Pennate Diatom

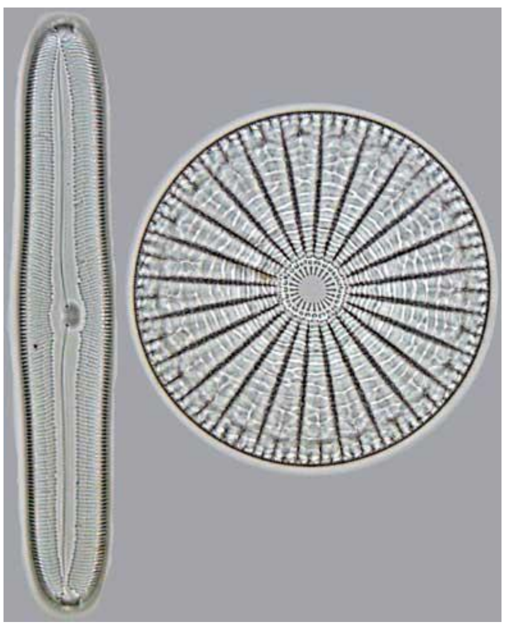

Fig. 3. Valve image of a pennate diatom and centric diatoms. Pennate diatoms are bilaterally symmetrical in valve-view. Centric diatoms are radially symmetrical in valve-view.
Source: (http://botit.botany.wisc.edu/botany_130/Diversity/heterokonts/diatoms/diatoms.html)

Sexual Reproduction

Reproduction of diatoms can be either sexual or asexual through cellular division. Though cellular division, reproduction occurs by binary fission, and two new individuals form within the parent cell frustule. In this method, during the processes of mitosis and cytokinesis, the two valves (hypotheca and epitheca) separate slightly and the division of the protoplast occurs parallel to the valves. The daughter cell receives a theca cell as the new epitheca. Division of the cell is complete when each of the individual cells the hypotheca for each of the daughter cells is formed. The progressive reduction in individuals' size is overcome because of the flexibility of the new cell walls or by sexual reproduction.

The predominant method of reproduction in pennate diatoms is sexual reproduction. Only individuals less than a certain size can reproduce sexually. Sexual reproduction in the centric diatoms is oogamous, meaning that this process has a motile sperm or nonmotile spermatium that reaches a nonmotile egg. Clones of the opposite mating type are needed (heterothallic), and produce non-flagellated gametes of the same size (isogamous). In both centric and pinnate diatoms, the gametes fuse together. The pennate diatom reproductive cycle differentiates from the centric diatom reproductive cycle. In centric diatoms, the same cell can produce both flagellated male gametes (which are usually small) and the nonmotile (larger) female gametes (homothallic and oogamous). The pennate order has an isogamous sexual reproduction meaning that the male and female gametes (egg and sperm) are indistinguishable. The "offspring" of diatoms are called auxospores. Within the auxospore, a large cell develops, and is eventually released, and the cell size of the diatom is restored. There is evidence from Davidovich and Bates, (1998) that sexual reproduction of *Pseudo-nitzschia* species may influence the dynamics of blooms (population growth) and the production of DA (discussed in Chapter 2). These new diatoms will increase in volume while forming vegetative cells and solid silica shells. (Hasle et al.1997).

Pseudo-nitzschia Biology

Figure 3. Image of *Pseudo-nitzschia* colony

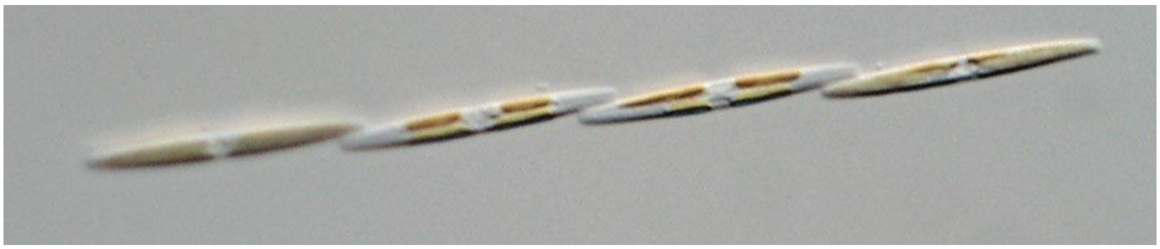

Fig. 4. Image of *Pseudo-nitzschia*. colony in girdle view (notice "step" or "chain" link)
Source: (Harmful Diatoms Introduction Module II." *University of Copenhagen*. IOC Science and Communication Centre on Harmful Algae, Web. 15 July 2012. <classroom.oceanteacher.org/...php/.../II-DiatomsIntroduction.pdf>..)

The genus *Pseudo-nitzschia* are pennate diatoms, with being the cells are lanceolate shaped, which are long and narrow, and gradually taper at each end (Figure 4 is an image showing the general shape of a *Pseudo-nitzschia* species). However, the features that distinguish *Pseudo-nitzschia* from other pennate diatoms are the tips. The tips overlap slightly so that the cells form chain-like bonds in a stepped formation. Each cell attaches to the next, near the tip at slight angles, resulting in chains with spiral forming staircase chains. However in some species, when the chains cease growing, they fall apart into single cells (Hasle et al. 1997). *Pseudo-nitzschia* comprises approximately 30 species. *Pseudo-nitzschia*, produces a potent neurotoxin called domoic acid (DA). Twelve species have been documented to produce DA. Ten of the toxic species have been documented on the west coast of the United States. Because all diatoms are made of silica, they are generally denser than seawater. Therefore, they require certain oceanic conditions, such as mixing, currents, and upwelling events, which are wind-driven movement of dense cool, and generally nutrient-replete water towards the surface to replace the warmer and usually nutrient-deplete surface waters. These oceanic conditions allow diatoms to grow within the photic zone (sunlit upper layer of the ocean). Ocean currents may transport diatom cells to areas that are favorable for growth. Cells may then proliferate rapidly and may also then lead to a bloom event.

Figure 4. Image of *Pseudo-nitzschia* cells

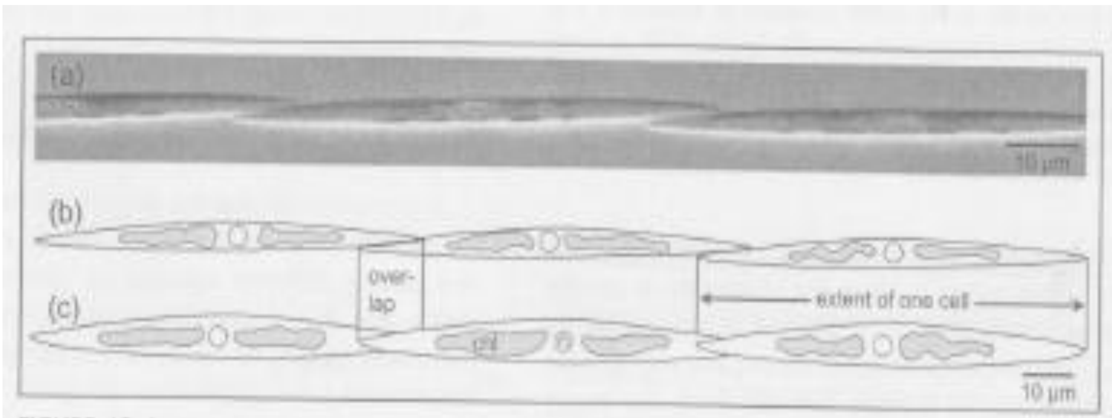

Fig. 5. Chain of *Pseudo-nitzschia* cells. (a) chain of *P. multiseries* under light microscopy showing the girdle view. (b) drawing of Pseudo-nitzschia showing girdle view. (c) drawing of valve view (top). (chl= chloroplast and n=nucleus). Source: (Trainer, Vera L., Barbara M. Hickey, and Stephen S. Bates. "Chapter 12 Toxic Diatoms." *Oceans and Human Health*. By Patrick J. Walsh. Amsterdam: Elsevier, 2008. 219-37.)

Domoic Acid (DA)

Phytoplankton cells are found suspended in the water column or in the sediment. Mollusks, such as clams, oysters, scallops, and mussels feed directly on the cells suspended in the water column by filtering the water. Mollusks can filter up to 500 liters of seawater per day (Trainer et al. 2008). This feeding strategy leads to the consumption of large amounts of the phytoplankton, most of which are beneficial to the mollusk. However, approximately 100 out of 5000 phytoplankton species (about 2%) that produce phycotoxins are deadly to molluscan shellfish (Trainer et al. 2008).These phycotoxins can accumulate in the digestive tract of the mollusks. The concentrated phycotoxins that remain in the digestive tract are then passed on to predators including California sea lions, Northern fur seals, anchovies, and sardines as well as cormorants, and humans, who consume the infected mollusks. Domoic acid in humans, marine mammals and seabirds can cause permanent memory loss, brain damage, and in severe cases, death. The clinical syndrome is called amnesic shellfish poisoning (ASP) or more currently domoic acid poisoning (DAP).

DA historically, has been used in Japan for treating victims who needed intestinal worms removed in children. It was shown that DA in low doses given to children (at 20 milligrams (mg)) and adults did not have harmful effects (Trainer et al. 2008). However, the victims with the most severe neurological symptoms had dosages of 290 mg of DA, which was an order of magnitude greater than the doses given to the children of Japan (Bates et al.1998).

Domoic acid is a water soluble and heat stable secondary amino acid. DA is also a part of the kanoid class of organic compounds. DA is structurally identical to glutamic acid (glutamate), which is a compound that is vital for the proper function of the nervous system.

> Glutamate is a major excitatory neurotransmitter in mammal's central nervous system. It is responsible for functions within the brain, including cell-to cell communication and hippocampal long-term potentiation, a process important in learning and memory. The function as a neurotransmitter plays a role in the transmission of nerve impulses from one neuron to another. Glutamate binds onto receptor sites on the membrane of the nerve fiber. This causes the receptor molecule to undergo a change in its shape. This opens up a microscopic channel in the membrane, which allows the influx of sodium or calcium into the axon. This results in the neuron being triggered, sending an impulse through the axon. The glutamate is released by the presynaptic neuron into the synaptic cleft and binds to the receptor sites on the dendrite of the postsynaptic neuron. This causes the next neuron to fire. The firing on neurons is controlled because the neuron bulb rapidly absorbs the glutamate or inactivated by specific enzymes; this removal closes the sodium and calcium channels (Figure 6. Shows a diagram of DA binding to a neuron). However, this could result in cell damage and death

when excessive amounts of glutamate are released from neuron cells and cannot be removed (Bates et al.1998).

Figure 5. Diagram of DA binding to nerve cell

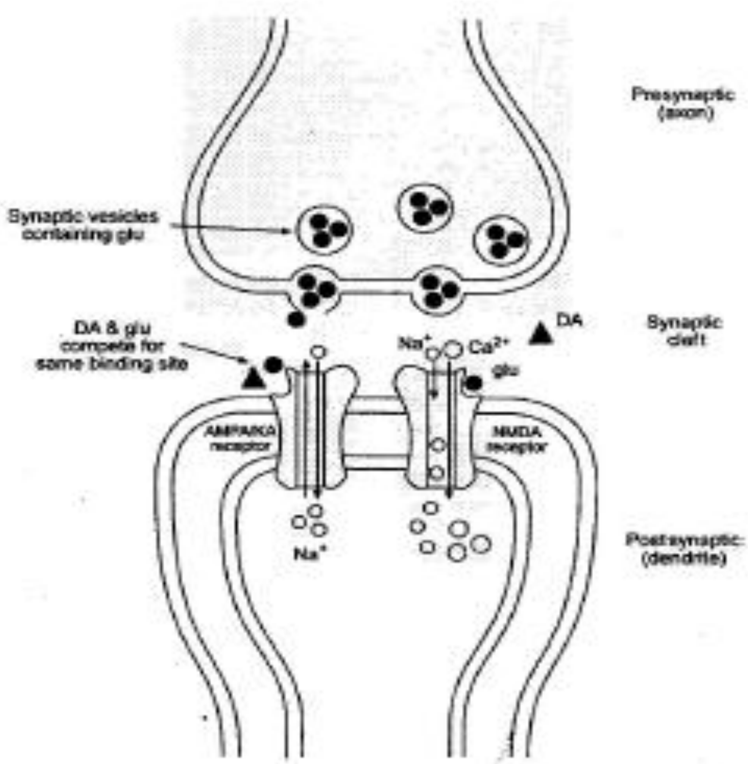

Fig. 6. Diagram of a nerve showing DA and glutamate fit into the AMPA/kainite (KA) receptor on the neuron surface. DA binds to the AMPA/KA site leads to the depolarization and activation of the NMDA receptors. Calcium then enters through the NMDA receptors. Prolonged binding of DA to the neuron results in swelling with water, causing the neuron to burst and eventually nerve cell death due to DA.
Source: (Trainer, Vera L., Barbara M. Hickey, and Stephen S. Bates. "Chapter 12 Toxic Diatoms." *Oceans and Human Health*. By Patrick J. Walsh. Amsterdam: Elsevier, 2008. 219-37.)

DA and glutamate molecules are structurally similar, they both fit into the same receptor and compete for the same binding site. The five-sided structure of the DA molecule causes it to bind to the receptor, resulting in a more potent effect per molecule than a glutamic acid molecule. When glutamate secedes to low concentrations, the glutamate is removed. However, the DA molecules are not, which results in neurotoxicity. The neuron then becomes inundated with calcium. The cell must use the adenosine triphosphate (ATP) reserves to get rid of the excess calcium, and the neuron begins to swell with fluid that causes it to burst. DA binds with these receptors in the hippocampus part of the brain. The hippocampus is associated with the processing of new memories, which explains the memory loss that occurs in patients with DA. The neural impacts are where the clinical name of amnesic shellfish poisoning is derived. The behavioral effects of ASP are also consistent among different mammal species (California Sea Lions and Northern Fur Seals). From the 1987 event in Canada older humans were particularly sensitive to the effects of DA, and in that case impacts were specific to males over the age of 70, many of whom died. Long-term exposure of humans to low concentrations of DA in shellfish is also unclear. Studies have suggested that there is increased risk of chronic, when there is long-term exposure. This is particularly concerns populations that subsist heavily on a shellfish diet and especially infants and the elderly.

This chapter reviewed the background and significance of harmful algal bloom events. It also discussed the background and biology of the genus targeted for study in this thesis: *Pseudo-nitzschia*. The structure and mechanism that explains the function of domoic acid toxicity in the nervous system was also discussed in this chapter. Blooms and toxic DA events arise under several different oceanographic conditions and the challenge is to tease out the factors that influence and control blooms and DA events are the most important.

Chapter 2
Review of Factors Controlling *Pseudo-nitzschia* Blooms and DA Events

Nutrient Concentrations and the blooms of *Pseudo-nitzschia*

Pseudo-nitzschia has been found in nutrient rich areas such as upwelling zones or near rivers. Ranges of nutrient concentrations associated with *Pseudo-nitzschia* blooms in various locations with different temperature and salinity include 8 – 22 μM $NO3^-$, 2.4 – 35 μM Si, 0.2 – 2 μM $PO4^-$ (Dortch et al. 1997, Trainer et al. 2000, Loureiro et al. 2005). It is important to note the large variation in these ranges, which could indicate that *Pseudo-nitzschia* blooms don't require very high nutrients concentrations to bloom since blooms are observed under a wide range of concentrations. Field studies suggest that increased nutrient loads are most likely due to anthropogenic nutrient loading, including sewage and agricultural run-off. Evidence for nutrient loading and the increase in abundance of *Pseudo-nitzschia* come from studies done by Dortch et al. (1997), Parsons et al. (2002), and Anderson et al. (2002). Dortch et al. (1997) found that *Pseudo-nitzschia* spp. has been the most abundant and often the most dominant diatom in the Louisiana and Texas shelf waters. Based on long-term historical data, evidence suggests that *Pseudo-nitzschia* spp. abundance may have increased in Louisiana and Texas shelf waters since the 1950s. The hypothesis for the increase in abundance of *Pseudo-nitzschia* is most likely due to the doubling of nutrient loading from the Mississippi and the Atchafalaya rivers and increased eutrophication on the shelf waters. However, seasonal variability also plays a role in *Pseudo-nitzschia* abundance, including regular shifts in wind, light, temperature and river flow. *Pseudo-nitzschia* peaks in abundance in the spring, corresponding to the average maximum river flow with another small peak in the fall during wind events that mix the stratified water column. There have been fall blooms of

Pseudo-nitzschia spp. that occurred during a period of low flow from the rivers, thus the nutrient inputs from the rivers alone cannot explain the seasonal cycle of *Pseudo-nitzschia* during the fall bloom. Despite the nutrient inputs from the river, high nutrient concentrations are observed near the mouth of the river and not on the shelf. Results also show that the highest cell numbers were often associated with lowest concentrations of nutrients. The historical data suggests that increased nutrient inputs discharged from rivers has increased the abundance of *Pseudo-nitzschia* spp. to where field observations suggest that a significant relationship between high concentrations of nutrients and *Pseudo-nitzschia* abundance cannot be established with certainty.

Evidence for nutrient concentrations and *Pseudo-nitzschia* abundance has also been reported in Parsons et al. (2002). The authors collected sediment core samples from the Louisiana Bight, located west of the Mississippi River. From the five core samples that were taken, all samples showed increases in *Pseudo-nitzschia* abundance coinciding with increasing nutrient concentrations in the Mississippi River. Because diatom frustules are composed of silica, the diatom frustules and valves were preserved in the sediment. The results from those cores indicated an increase in *Pseudo-nitzschia* abundance. The apparent increase in abundance of *Pseudo-nitzschia* appeared to reflect a response to increased nutrient loading and eutrophication. The hypothesis that could possibly explain the apparent increase in abundance of *Pseudo-nitzschia* in the northern Gulf of Mexico since the 1950s is increased fertilizer use in the Mississippi river watershed. The observed dominant species was *P. delicatissima,* a species capable of producing DA. Increased levels of nitrogen (N) and phosphorus (P) inputs from the Mississippi and Atchafalaya rivers coupled with nutrient limitations of silica (Si) resulted in increasing nitrate levels, and decreasing silica: nitrogen ratios. This result supports the conclusion that increases in *Pseudo-nitzschia* abundance are consistent with the increases in nutrient concentrations form increased use of nitrogen and phosphorus based fertilizers from terrestrial sources, and could potentially be a possible explanation for coastal eutrophication and the abundance of *Pseudo-nitzschia* and other HABs.

A review by Anderson et al. (2002) also shows that eutrophication may be one of several mechanisms that could explain the increase in abundance of *Pseudo-nitzschia.* Anderson et al. (2002) reviewed research from other authors that linked eutrophication and the abundance of harmful algal blooms including *Pseudo-nitzschia*. From this review, the authors concluded that eutrophication is a global problem and that many coastal areas throughout the world have been affected by the increased nutrient loading from anthropogenic sources since approximately the mid-20th century. They conclude that nutrient loading fuels the abundance of HABs, and increases in algal biomass as estimated by chlorophyll have also been shown to parallel the increases in nutrient concentrations. The evidence suggests stimulation of *Pseudo-nitzschia* blooms by nutrient enrichment. The authors also conclude that there is a link between eutrophication and the *Pseudo-nitzschia* blooms and other HAB events. However, they also conclude that there is a complex system of both direct and indirect pathways and it may not be a simple link between eutrophication and *Pseudo-nitzschia* events. Other factors may include species composition, the nutritional state of the organisms at the time of nutrient loading, the role of grazers, and the physical features of the environment including; storms, wind patterns, upwelling, and long-term seasonal patterns such as the El-Niño Southern Oscillation (ENSO).

In summary, this review shows that *Pseudo-nitzschia* blooms increase due to nutrient loading and eutrophication. The coastal studies from the northern Gulf of Mexico resulted in an apparent increase in *Pseudo-nitzschia* abundance since the mid-20th century. This could be a reflection of the increase in nitrogen and phosphorus based fertilizer use since the 1950's. However, the link between nutrient concentration and eutrophication may not fully explain the increase in abundance and apparent increased frequency of *Pseudo-nitzschia* bloom events. Results also showed that the highest cell numbers were often associated with the lowest concentrations of nutrients. As discussed later in this chapter *Pseudo-nitzschia* blooms occur in nutrient-replete waters (Juan de Fuca eddy, WA), as well as some of the most nutrient variable (Monterey Bay, CA and open-ocean) waters, which shows the versatility and adaptability of *Pseudo-nitzschia* in varying nutrient ecosystems. While there is evidence to suggest that increased nutrient loading and eutrophication has increased the abundance of *Pseudo-nitzschia,* a simple link may not explain it entirely.

Nutrient Composition and Blooms of *Pseudo-nitzschia*

There have been studies conducted to establish the possible link between nutrient enrichment /coastal eutrophication and the increase in abundance of *Pseudo-nitzschia.* In addition, there have also been studies that investigated the effect of nutrient composition. Turner & Rabalais (1991), Sommer (1994), Dortch et al. (1997), and Parsons et al. (2002) found from sedimentological samples taken from the Mississippi River an increase in *Pseudo-nitzschia* abundance since the 1950's. These core samples suggest that the increase in *Pseudo-nitzschia* abundance could be a response to eutrophication.

Turner and Rabalais (1991) examined water quality changes in the Mississippi River from the early 1900s to late 20th century and studied three identified indicators for water quality: phosphorus, silicate, and dissolved inorganic nitrogen (DIN), the total of all the nitrogen forms or nitrate+nitrite+ammonium. This data shows that water quality changes have occurred over time and coincide with an increase in fertilizer use. The results show an increase in nitrogen and phosphorus loading in Louisiana and Mississippi had made its way into the Gulf of Mexico via the Mississippi and Atchafalaya Rivers. The movement in nitrogen and phosphorus loading has coincided with an increase in nitrogen and phosphorus fertilizer use post- World War II. There was also a coinciding decline in silicate as phosphorus fertilizer applications increased, which supports the hypothesis that freshwater diatom growth in the streams and lakes that feed the Mississippi and Atchafalaya rivers was stimulated by the increased phosphorus loads. The increased freshwater diatom growth leads to a loss of silica from the water column, and therefore a decrease of silica in the river systems. The authors conclude that the changes in water quality coincided with the increased use in nitrogen and phosphorus based fertilizers, which altered the Si:N ratio. Therefore, their findings suggest that significant reduction of eutrophication is not likely to occur without a reduction in fertilizer use (Turner and Rabalais, 1991).

Because diatoms require nutrients, specifically silica, and species of flagellates and dinoflagellates don’t require silica, the mechanism of decreasing silica: nitrogen or silica: phosphorus ratios could also explain a shift from a diatom dominate assemblage to a flagellate assemblage. Evidence from field studies done by (Turner and Rabalais (1991), Dortch et al. (1997), and Parsons et al. (2001)) in the northern Gulf of Mexico and the Juan de Fuca eddy show nutrient replete areas as well as increases in nitrogen and phosphorus inputs into coastal area, and yet not an observed increase in silica. There is evidence from a study by Sommer (1994) showing that *Pseudo-nitzschia* increases in abundance in laboratory batch cultures of *Pseudo-nitzschia pungens* (*P. pungens*) grown under changing silica: nitrogen ratios. In batch cultures, *P. pungens* became the dominant species at high ratios of silica to non-siliceous nutrients, including nitrogen and phosphorus. However, the author also concludes that *P. pungens* have a particularly high silicate requirement and therefore high optimal Si: N ratios are required. Inclusion of diatoms with lower silica requirements would displace the transition from flagellate to diatom dominance under lower silica: nitrogen ratios (Sommer, 1994). Sommer (1994) also concludes there are limitations of controlled laboratory conditions compared to the variability of the natural environment (light, mixing, and wind patterns). The variability could alter the availability of silica, which would therefore change the silica: nitrogen ratio.

A field study by Parsons et al. (2002) provides evidence that supports the increase of Si:N. Their data showed an increase in abundance of *Pseudo-nitzschia* in all of the samples (from the frustules preserved in the cores), with significant correlation of increased nitrate levels and decreased silicate to nitrate ratios. Nitrogen inputs doubled and silica inputs decreased by 50% their data suggests that *Pseudo-nitzschia* has increased in abundance in the northern Gulf of Mexico since the 1950s. The hypothesis for the decreasing silicate to nitrogen ratios is that there has been increased nitrogen from increased fertilizer use in the watershed of the Mississippi River. These coastal studies show a response to riverine nutrients, changing nutrient ratios and eutrophication. This study from Parson et al. (2002) shows there is evidence to suggest a possible link that the increased abundance of *Pseudo-nitzschia* has been a response to anthropogenic riverine nutrients, changing nutrient ratios and eutrophication.

In summary, results from these studies indicate that nutrient composition plays a role in the *Pseudo-nitzschia* blooms. Evidence suggests that anthropogenic sources that have increased loading of nitrogen and phosphorus has altered and decreased the silica: nitrogen and silica: phosphorus ratios. Changes in the ratio were shown in cultures from the results from Sommer (1994) and in field studies from Turner and Rabalais (1991). Parsons et al. (2002) showed an increase in *Pseudo-nitzschia* abundance. *Pseudo-nitzschia* have nutrient requirements, are of which is which silica. Silica is vital for the development of the frustule. Because of increased nutrient loading of nitrogen and phosphorus, the silica: nitrogen and silica: phosphorus ratios are decreased, which means that, silica could become limiting for growth of *Pseudo-nitzschia*. Diatoms including *Pseudo-nitzschia* require silica whereas difference in silica requirements of flagellates and dinoflagellates don't require silica. This could have profound implications for coastal phytoplankton communities, as the difference gives flagellates a competitive advantage in the phytoplankton assemblage. In-terms of management and policy implications', limiting the amount of fertilizer use is fundamentally sound than controlling the distribution of fertilizer use.

Seasonal and Interannual Climate Patterns and *Pseudo-nitzschia* Blooms

A key distinction between upwelling zones and river plumes is that rivers can have anthropogenic nutrients while nutrients from upwelling are from natural decomposition. Seasonal patterns such as winds and heavy rainfall events can stimulate *Pseudo-nitzschia* blooms because of atmospheric sources of nutrients. Evidence for seasonal variability impacts on the formations of blooms come from studies by Trainer et al. (2000) and Trainer et al. (2002). Wind events can be especially important for transporting toxic blooms inland from upwelling sites offshore or providing necessary mixing which brings nutrients into the photic zone. Trainer et al. (2000) focused their analysis off of the coast of California, and found wind patterns from the north during the spring and summer months. Their finding was due to upwelling, which brought nutrient-rich waters from depth into the euphotic zone (uppermost layer of a body of water that is exposed to sunlight for photosynthesis to occur). However, there was contradictory evidence indicating that upwelling was reduced in 1998 because of the previous year's ENSO event. Additionally, rainfall reached record levels in late winter and early spring of the previous year (1997). Nutrient-rich water from upwelling was the hypothesized mechanism, because of several chemical and physical characteristics that suggested upwelling had occurred. Therefore, the authors concluded that increased nutrient supply from upwelling events could possibly be an environmental trigger of *Pseudo-nitzschia* blooms. In another study by Trainer et al. (2002), in 1998, a DA event occurred off the coast of Washington in a circulation cell known as the Juan de Fuca eddy. The Juan de Fuca eddy is the result of the interaction between the outflows of water from the Strait of Juan de Fuca, southward wind-driven currents along the continental slope and the underlying topography, a spur of the Juan de Fuca submarine canyon (Trainer et al. 2002). This event led to the hypothesis that phytoplankton biomass collected in the eddy with certain conditions is the initiation site for *Pseudo-nitzschia* blooms and DA events off of the Washington coast. There were favorable upwelling winds (southward) and southward currents cause movement of *Pseudo-nitzschia* cells and DA towards the Washington coast. Upwelling was also evidenced in a study by Loureiro et al. (2005). Loureiro et al. (2005) found that upwelling regions off of the coast of Portugal contain high concentrations of *Pseudo-nitzschia*. *Pseudo-nitzschia* are used as upwelling indicators during spring and summer, because riverine nutrient inputs stimulate toxic *Pseudo-nitzschia* blooms.

Bates et al. (1998) found that heavy rainfall after a drought could've caused a dramatic increase in *Pseudo-nitzschia* abundances in the river outflow in eastern Canada in 1987. This hypothesis was also supported by Dortch et al. (1997) in the Gulf of Mexico, Horner and Postel (1993) off of the Washington coast, Trainer et al. (1998) in Puget Sound and Trainer et al. (2000) off the coast of California. The mechanism that could explain bloom events after periods of heavy rainfall is from freshwater nutrient run-off from freshwater rivers nearby (Mississippi and Atchafalaya Rivers in the northern Gulf of Mexico, San Lorenzo River in northern Monterey Bay, and the Estrella near Morrow Bay). While rainfall may potentially be a trigger for some blooms of *Pseudo-nitzschia*, they may not be a contributing factor to all *Pseudo-nitzschia* bloom events.

Sunlight intensity has also been identified as a contributing factor for *Pseudo-nitzschia* blooms (Parsons et al. 1998). The authors noted that *Pseudo-nitzschia* blooms occur in the spring and fall, when irradiance is relatively low. In Sommer et al. (2004), in culture, *P. multiseries* became the dominant species over other phytoplankton species at low irradiance with a short photoperiod. However, according to a review by Bates et al. (1998), low light may contribute to the demise of autumn blooms. Fehling et al. (2005) simulated spring and summer photo period conditions on *P. seriata* and their results showed that the longer summer day length resulted in enhanced growth rates, cell yield, and toxin production. However, *P. delicatissima* achieved greater cell density under the shorter spring photo period. Their results show that day length and available light could influence which species of *Pseudo-nitzschia* becomes dominant.

Long-term climate patterns such as the El Niño Southern Oscillation (ENSO) can affect *Pseudo-nitzschia* abundances by controlling upwelling near the west coast of the United States. Fryxell et al. (1997) found that during weak ENSO years, upwelling was high and therefore so was the abundance of *Pseudo-nitzschia.* 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 were years with large toxic events on the west coast of the United States, and were strong ENSO years. Trainer et al. (2000) hypothesized that ENSO impacted *Pseudo-nitzschia* abundance through their work off the coast of California during a bloom formation in June of 1998. Nutrient concentrations were low, it was hypothesized that *Pseudo-nitzschia* abundance during a bloom event, was reduced because of the intensity of upwelling due to ENSO. ENSO was also evidenced in Trainer et al. (2002) when high DA levels were present in razor clams in 1998, but not in 1997. The summer of 1998 followed an ENSO event, where southward upwelling winds were weak in 1997. However winds were strong and persistent in the summer of 1998, which supported the ENSO hypothesis.

In summary, seasonal variability including wind patterns, upwelling, and rainfall have been identified as probable mechanisms of the formation of *Pseudo-nitzschia* blooms. Long-term climate patterns' including ENSO has also been identified as another possible factor for the formation of blooms. These weather and climate patterns are mechanisms because they influence the delivery of nutrients. *Pseudo-nitzschia* and all algae need light for growth and survival. Irradiance from sunlight and therefore day length and the intensity of sunlight also influences the formation of blooms. The exact mechanisms of *Pseudo-nitzschia* blooms are uncertain and could be caused by multiple factors.

Life-cycle of *Pseudo-nitzschia* and DA Production

According to Round (1990), *Pseudo-nitzschia* are 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. The sexual cycle differs between pennate and centric diatoms. Centrics are characterized by oogamous reproduction involving the formation of flagellated male gametes and non-motile female gametes. *Pseudo-nitzschia*, like many pennate diatoms, can reproduce sexually (Trainer et al. 2007). A *Pseudo-nitzschia* cell will become sexualized when cell length has decreased below a threshold size. Evidence for this sexual maturity comes from Davidovich and Bates (1998) and Bates and Davidovich (2002). From Bates and Davidovich (2002), this threshold size is known as the first cardinal point, which in *P. multiseries* is approximately 63% of the length of largest cells. Sexual reproduction must occur before the cells reach a minimum length, which in *P. multiseries*, is approximately 30 µm. For the species of *P. delicatissima*, this size range is from 19-80 µm and during this size window, cultures of *Pseudo-nitzschia* were mixed together to stimulate sexual reproduction. Mating in *Pseudo-nitzschia* was achieved by mixing clones of the same species, but of opposite sex, and were mixed during the exponential growth phase. These results suggest that parent cells must be healthy and photosynthesize to produce energy for sexual reproduction. 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 are non-flagellated, but the behavior of the gametes differs between sexes. One cell produces two active gametes (- male) and the other cell produces two passive gametes (+ female). The frustules of both cells open, permitting the active gametes to enter and fuse with the passive gametes. This fusion is not always successful in both pairs of gametes or in all pairing of parent cells. After gamete fusion, the resulting zygote expands to form larger auxospores inside where the initial cell is formed.

Davidovich and Bates (1998) have found that clonal cultures of *Pseudo-nitzschia* decrease in size over time, as described previously, and also lose their ability to produce DA. This process has been found in *P. multiseries*, *P. pseudodelicatissima*, and *P. calliantha.* Offspring of *P. multiseries* clones that have lost their ability to produce DA can be toxic, sometimes even more toxic than the initial toxicity of their parents. Bates and Davidovich (2002) observed the mating process in a laboratory setting, and found fewer auxospores compared to vegetative cells. Thus, paired cells and auxospores would be rare in natural populations. However, this process would be difficult to observe in the natural setting. Preliminary work has shown an interesting relationship between epibiont bacteria and *Pseudo-nitzschia* sexual reproduction. There is evidence to support that in laboratory settings clones can have significant variability in DA production. The variability in DA production can be attributed to genetics or other factors including nutrient composition and concentration, light, and irradiance.

DA production also varies depending on the growth stage of the *Pseudo-nitzschia* population. Evidence for the relationship between growth rates and DA production came from Bates et al. (1998), Pan et al. (1996), Pan et al. (2001), and Lundholm et al. (2004). Bates et al. (1998) found that cell division stopped *Pseudo-nitzschia* produced DA in cultures of *Pseudo-nitzschia*. In other batch cultures, DA production started at the onset of the stationary phase and DA content of the cells in cultures became more prevalent during the late exponential phase of growth. The variation in DA production is possibly due to a period of transition when some cells have stopped growing and are producing DA while other cells are still dividing. Pan et al. (1996) found that in continuous culture, toxin content increases when growth is slowed by decreased dilution rates. This growth effect means that many factors that slow growth would also indirectly increase toxin production. The slow growth rate may be due to limiting nutrient conditions of growth. While under nutrient limiting conditions for growth, *Pseudo-nitzschia* may then transition to increased production of DA as a defense mechanisms against grazers or serve other ecological purposes.

Studies of DA production in laboratory cultures have demonstrated that the relationship between growth rate and levels of DA production has generally been minimal or non-detectable during the exponential growth phase. During the stationary phase, DA production increases as cell division slows. As a result of silicate or phosphorus limitations, however, there has been conflicting evidence regarding the pattern of minimal or non-detectable DA production during exponential growth as shown by a study done by Pan et al. (2001). In their study, a culture of *P. pseudodelicatissima* from the Northern Gulf of Mexico produced the highest DA levels during early exponential growth. Their results showed high DA production and cellular DA concentrations and low density growth rate during the exponential growth stage and that there was no DA production during the stationary phase. Other studies have also shown DA production prior to the onset of the stationary phase in different strains of *Pseudo-nitzschia* as shown in *P. australis* from Garrison et al. (2002) and *P. fraudulenta* from Thessen et al. (2009). Study results suggest that nutrient limitations place stress on different strains of *P. nitzschia*, and the resulting slow division rate. The study also shows that the resulting slow division rate, rather than cessation of cell growth is the reason for the increased production of DA levels.

The increase in toxin production during slow growth periods 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. It was hypothesized that DA production was specifically linked to Si limitation in *P. multiseries*. However, *Pseudo-nitzschia* cultures began to produce toxin when growth was limited by Si or by other factors in the presence of adequate nitrogen and light (Bates et al. 1991). There is evidence to support that some species of *Pseudo-nitzschia* produce DA during the stationary phase of growth. However, evidence has also shown species of

Pseudo-nitzschia will halt their growth rate and produce high levels of DA (discussed later in the chapter under nutrient limits on DA production) under certain nutrient limiting conditions. The question remains if increased DA production results from a specific life stage such as a stationary phase, or if it is due to a specific growth rate or by limiting nutrients? A possible mechanism that could explain this could be that *Pseudo-nitzschia* switch from growth to DA production under certain limiting nutrient conditions.

In summary, there is evidence that sexual reproduction may be important for the production of DA. There is evidence that from *Pseudo-nitzschia* cultures decrease in size over time, and lose their ability to produce DA however, when mated the daughter cells become toxic (sometimes even more toxic than the parent cells). Because these cells were grown in culture and are difficult to observe in nature, it is difficult to establish daughter cell toxicity with certainty. Evidence shows increased DA production under a stationary phase of growth. The question that remains is whether slowed *Pseudo-nitzschia* growth rates and DA production occurs specifically during a life phase (hypothesized stationary phase of growth) or if it is due to the outcome of nutrient limiting nutrients.

Role of Nutrient Limitation in DA Production

While there have been many studies linking DA production with iron requirements of *Pseudo-nitzschia* (Rue and Bruland 2001), Maldanado et al. (2001), Wells et al. (2005)), there have been several studies indicating that there are other environmental factors that play a role in DA production. Under both phosphorus and silicate-limitations (particularly under silicate-limitations) DA levels per *Pseudo-nitzschia* cell increase during the senescence stage, compared to limited DA production during the exponential stage of growth. Evidence of nutrient limitations associated with increased levels of DA per *Pseudo-nitzschia* cell increase was first documented by Pan et al. (1996). Pan et al. (1996) found an association between high DA production, high levels of alkaline phosphate activity (APA), and high cellular nitrogen and phosphorus ratios strongly suggesting that phosphate limitations actually enhanced and therefore increased the DA production. Their results also showed that DA synthesis requires a substantial amount of biogenic energy. The evidence was that DA production was high when the uptake of carbon, nitrogen, phosphorus, silica and cell-division was low, however chlorophyll a was high (Pan et al. 1996). Chlorophyll a is an essential pigment in photosynthesis. In another study done by Pan et al. (1996b) the authors investigated DA production by *P. multiseries* under various silicate concentrations. Their results suggest that DA production was suspended during and shortly after silicate enrichment. However, when silicate levels was severely limiting, there was enhanced DA production. Fehling et al. (2004) isolated a species of *P. seriata* from waters off the west coast of Scotland and found that *P. seriata* produced DA when phosphorus or silicate nutrients were limiting. However, *P. seriata* produced higher levels of DA when stressed by a silicate limitation during a stationary phase of growth. DA production was low in the exponential phase under both phosphorus and silicate limiting conditions. In phosphate limiting conditions, the highest DA levels were produced during an immediate post-exponential growth phase. In contrast, although DA production increased during the slower exponential phase of the silicate-limited conditions, DA production was at its highest, coincident with a period of chlorophyll synthesis and increase in carbon biomass (Fehling et al. 2004). Under phosphorus and silicate limitations (particularly a silicate limitation) DA levels per *Pseudo-nitzschia* cell resulted in an increase at the early senescence stage with a low level of production during the exponential growth and early-senescence stage.

Supporting evidence to the studies done by Pan et al. (1996), Pan et al. (1996b), Fehling et al. (2004) and the review by Bates et al. (2008) are from a field study done by MacIntyre et al. (2011). MacIntyre et al. (2011) compared a microalgal community structure during a bloom year (2005) and a non-bloom year (2006) in the Gulf of Mexico. They found a bloom of *Pseudo-nitzschia* spp. that followed a high discharge of nutrients from the local aquifers that were heavily loaded with nitrates. These results provide supporting evidence linking nutrient status and DA production. Using environmental correlations from phytoplankton communities in the Gulf of Mexico, they found correlations between cellular DA levels and low silicate, and high dissolved inorganic carbon and high light. These conclusions are consistent with laboratory experiments showing that DA accumulated in *Pseudo-nitzschia* cells when conditions for macronutrient uptake and growth were out of balance with photosynthetically driven carbon uptake. The hypothesis regarding increased toxicity within the cells from an increase in photon pressure and availability of primary carbon can be explained by photosynthetic production of NADP and APT in the light reactions exceeding its utilization.

DA is derived from acetate through two possible pathways, the first being the synthesis of an isoprenoid structure. The other pathway is the synthesis of glutamate from products of the tricarboxylic (citric) acid cycle (TCA) and acetate as acetyl-CoA. Both can be derived directly from the Calvin cycle products via pyruvate in glycolysis, either in the stroma or in the cytosol. The pathway is up-regulated under conditions where there is an excess of Calvin cycle production. This synthesis of the isoprenoid structure from acetyl CoA requires three ATP and two NADPH. The synthesis of DA is therefore likely to be favored under conditions where sinks for primary carbon and NADPH and ATP are reduced by the limitation of silicate or phosphate. The results suggest that DA production is not related to the growth of *Pseudo-nitzschia* cells, but related to the accumulation of DA within the cells, when the *Pseudo-nitzschia* cells were put under nutrient limiting conditions, specifically silicate and phosphorus. The cells continued to produce DA just as the division rate decreased. This metabolic difference increases the level of DA per cell; the DA is not being diluted by partitioning the DA into new cells (MacIntyre et al. 2011). This conclusion suggests that DA levels do not respond to specific environmental conditions but rather to the growth of characteristics of DA-accumulating *Pseudo-nitzschia* cells.

There are numerous field studies that suggest nutrient limitation as there is most evidence pointing to silicate limitation as a potential trigger of DA production. Evidence for these results come from field studies done by Anderson et al. (2006), and Schnetzer et al. (2007). Anderson et al. (2006) investigated a bloom event of *P. australis* in the Santa Barbara Channel (SBC) during the spring of 2003. They found negative correlations between cell abundance, particulate DA, and nutrient concentrations of silicic acid, nitrogen, and phosphate. Schnetzer et al. (2007) presented a study on bloom events during two separate bloom events of *P. australis* in coastal waters of the Southern California bight. Schnetzer et al. (2007) found similar results to Anderson et al. (2006). Schnetzer et al. (2007) interpreted that significant high levels of DA and low (silicate:

phosphate) and (nitrogen: phosphate) ratios as possible enhancement of DA production by phosphate or silicate limitations. However, the authors reported no significant relationships for cellular DA and the concentrations or ratios of nutrients. Anderson et al. (2007) suggest that silicate limitations play a complex role in DA production in the natural environment. This relationship, however, *Pseudo-nitzschia* bloom events on the west coast of the United States has not produced the same relationship. From an initial study done by Marchetti et al. (2004), the authors compared the environmental conditions within the Juan de Fuca eddy to the conditions in the surrounding waters to assess the nutrient conditions on phytoplankton dynamics. The results from this initial study concluded that in the macro-nutrient replete waters of the Juan de Fuca eddy support a growing population of *Pseudo-nitzschia* cells that produce DA among the absence of a measurable level of silicate limitation. In a similar study by Trainer et al. (2009), the authors conducted a four year study of the Juan de Fuca eddy and the surrounding waters. The conclusions of their study were similar to the conclusions reached by Marchetti et al. (2004) that showed no correlation of *Pseudo-nitzschia* or DA concentrations to macronutrient concentrations (Trainer et al. 2009). These conclusions concluded that DA production is linked to nutrient physiology, and suggest that the production of the toxin is more complex than previously thought and therefore likely influenced by the host of environmental factors that may be unique to a particular region

Nitrogen

Nitrogen, unlike silica or phosphorus, has been identified as a requirement for DA production. The evidence for the nitrogen requirement came from studies done by Bates et al. (1991), Bates et al. (1993). In Bates et al. (1991), there was evidence that a nitrogen substrate that spawned growth may influence the exponential growth rate. There was also evidence that DA production began at the onset of the stationary phase of different *Pseudo-nitzschia* species induced by the limitations of silica. Bates et al. (1993) demonstrated differential growth and toxin responses to nitrogen substrates. More recent studies have also supported differential exponential growth rates as a function of nitrogen substrate for *P. australis* in field studies done by Cochlan et al. (2006) and Howard et al. (2007), off of Monterey Bay California. In these studies, the authors also show that toxicity during the exponential phase was inversely related to growth rate. The nitrogen substrate supporting the slowest growth will likely produce the most toxic cellular cells during the exponential growth phase.

In studies by Kudela et al. (2008) and Hagstrom et al. (2011) DA production at a measurable level have been observed in nitrogen limiting conditions in cultures of *P. australis*, and *P. multiseries*. These studies concluded that the interspecies differences and variability in the environment have resulted in the lack of generalization attributes to the nitrogen substrate fueling growth rate or cellular toxicity, and is due to the variability of the intrastrain of *Pseudo-nitzschia*. Evidence for intrastrain variability comes from a study done by Thessen et al. (2009). The authors used different strains of *P. multiseries*, *P. fraudulenta*, and *P. calliantha* that were isolated from the mid-Atlantic coastal region of the United States. They observed trends where higher growth rates achieved on ammonium and lower growth rates on urea. The toxicity (total DA, particulate DA, and dissolved DA) examined during the silicate induced stationary phase, showed no consistent pattern with respect to nitrogen sources or relationship to the prior exponential growth phase. There is evidence that the nitrogen substrate affects DA production in addition to a direct effect on growth. However, the toxin content in either the exponential or stationary phase of growth can be consistently predicted as a function of the nitrogen substrate due to species and strain variability of *Pseudo-nitzschia.* There is now evidence that shows that *Pseudo-nitzschia* can thrive under a variety of macronutrient conditions by effectively using nitrate, ammonium and urea as a nitrogen source. This evidence points to the likely result of anthropogenic nutrient loading including sewage and agricultural run-off.

In summary, this review highlighted the role of nutrient limitations on the production of DA. The results from the different studies show that there is evidence for increased nitrogen and phosphorus loads from terrestrial sources and therefore altering nutrient compositions and limiting silica: nitrogen ratio. The results show silica limitations as the potential environmental trigger in DA production. However other studies show varying results as in nutrient replete areas such as the Juan de Fuca eddy which is a "hotspot" for *Pseudo-nitzschia* that produce DA. While there is evidence supporting increased nutrient loads from anthropogenic sources on increase DA production, there are still varying conclusions. The possible relevance of these findings to policy is that increased nutrient loads from agriculture, sewage, and run-off could potentially lead to increasing amounts of toxic DA events.

Role of Salinity and pH

Salinity and pH have been found to be important factors associated with DA production. Seawater has a relative stable pH (pH of 8.2), as it is buffered by the carbonate system. However, uptake of dissolved inorganic carbon (DIC) during photosynthesis and primary production by high concentrations of phytoplankton cells can result in elevated pH values as high as 9 (Trimborn et al. 2005), (Doucette et al. (2008). Evidence on salinity and pH influences on DA production have been found in preliminary studies by Lundholm et al. (2004), Trimborn et al. (2008), and Doucette et al. (2008), all of whom tested varying levels of pH and or salinity in laboratory experiments. Lundholm et al. (2004) investigated the effects of pH on the production of DA of *Pseudo-nitzschia*. Specifically, DA production began during the late exponential growth phase, and it was only found in the cells during this phase of growth. Small amounts of DA were detected in the mid- to late exponential growth phase in other species including; *P. australis, P. Seriata, and P. pungens* along with *P. multiseries* (Lundholm et al. 2004). Results from Lundholm et al. (2004) did show increased DA production resulting from increasing levels of pH. The mechanics of pH effects on DA production has been hypothesized as two potential explanations. The first possible explanation is that the enzymatic processes involved in production of DA have a certain pH optimum, and therefore a shift in intracellular pH may affect DA production. Another possible explanation could be that pH-mediated changes in the speciation of metals impact DA production due to increased toxicity or reduced bioavailability of the metal, such as copper. This hypothesis comes from Maldanado et al. (2002) that copper toxicity (and iron limitation) induced the production of DA.

A study done by Trimborn et al. (2004) also supported the results from Lundholm et al. (2004) about increased DA production from increasing pH. The authors investigated the effects of pH-induced changes in the seawater carbonate chemistry on the inorganic carbon acquisition and the production of DA in *Pseudo-nitzschia multiseries (P.*

multiseries). The results from Trimborn et al. (2008) showed that DA content increased significantly in *P. multiseries* with increasing pH. Trimborn et al. (2008) hypothesized that it is likely external pH could affect internal pH, which could cause a shift in intracellular pH affecting the DA production.

Salinity has also been identified as an important factor in the production of DA. Evidence for the potential role of salinity in DA production came from studies done by Thessen et al. (2005) and Doucette et al. (2008). In Thessen et al. (2005) and Doucette et al. (2008) investigated the influence of salinity on DA concentrations per cell in P. *multiseries.* The two studies resulted in similar results, which suggested that DA production in *P. multiseries* was greatest at higher salinity levels. A hypothesized mechanism for this finding could be the competing energy requirements for the two processes (growth and production of DA). At high levels of salinity, photosynthetic energy levels are sufficient for both growth and DA production. However, the added stress imposed by lower levels of salinity may shift the energy balance toward osmoregulatory processes essential for maintaining a high growth rate and away from DA synthesis processes. The production of DA requires a supply of bioenergetic metabolites generated by photosynthesis. Additional energy is needed to grow rapidly while maintaining an osmotic balance at a likely sub-optimal salinity reduces that available for toxin synthesis, which could explain the observed toxin decline in DA production (Doucette et al., 2008)

In summary, results suggest that increased salinity increases the production of DA and that studies done on *Pseudo-nitzschia* in field studies in the northern Gulf of Mexico (Dortch et al. 1997), (Pan et al. 2001) indicate that particulate DA concentrations were highest in higher salinity shelf waters (as compared to estuarine waters).These findings could explain the lack of DA outbreaks in low salinity estuarine waters, which is important because estuarine areas are extensively used as shellfish growing and harvesting areas. Elevated pH levels might suggest that with the possibility of ocean acidification due to climate change, *Pseudo-nitzschia's* ability to adapt to various conditions of the ocean may give *Pseudo-nitzschia* species a competitive advantage. Also there is evidence elevated pH levels that inhibiting growth increases DA production. From these results it will also be important to monitor pH levels and salinity and be able to use these variables as factors for an early warning system in being able to predict either bloom events or production of high DA levels.

Possible role of DA as Fe or Cu Chelator

While most studies on DA production have been primarily focused on the harmful effects and concentrations of DA production, there have only been a few studies have addressed the role of DA in *Pseudo-nitzschia* physiology. The molecular structure of DA suggests the possibility that the toxin serves as an iron or copper chelator. Evidence of DA as an iron or copper chelator come from studies by Rue and Bruland (2001), Maldonado et al. (2002), Wells et al. (2005), Bates et al. (2002), Marchetti et al. (2004), and Trainer et al. (2009). The first study on this possible physiological role came from a study done by Rue and Bruland (2001), who found that the structure of DA was identical to known iron-complexing agents produced by terrestrial plants such as mugineic acid. The similarity in chemical structures of DA to other phytosiderophores (a class of chelate compounds that sequester iron) suggested a physiological role of DA as a trace metal chelator. The results showed that some species may produce DA to bind trace metals in order to either increase the availability of essential micronutrient, such as iron, or to decrease the availability a potentially toxic trace metal, such as copper. The ecological role of DA an iron-siderophore, keeps iron soluble and bioavailable for retrieval by *Pseudo-nitzschia,* or it could also act as a means of copper detoxification. This leads to the possibility that certain specific trace metals are critical to the ecological success of *Pseudo-nitzschia* species that produce DA.

This physiological role of DA is documented and further supported in literature done by Maldonado et al. (2002). Maldonado et al. (2002) investigated how iron and copper affected growth and DA production by *P. multiseries* and *P. australis*. The results showed that DA production was inversely related to cell growth rates when the cells were limited by low iron or copper availability. When the cellular levels of DA increased, there was also an additional increase in the levels of dissolved DA under the iron or copper stressed conditions: ninety-five percent of DA was actively released into the metal stressed condition. The presences of DA in the environment enhanced iron transport into low-iron grown cells.

Additionally, another study was conducted by Wells et al. (2005) also confirmed that DA is a functional component of the unusual high affinity iron acquisition system of these organisms. Wells et al. (2005) presented a conceptual model (Fig. 4) of DA production which showed release similar to high affinity iron uptake system found in yeast *S. cerevisiae*. They use this model as a possible explanation that DA could serve an ecological purpose. Wells et al. (2005) investigated that *Pseudo-nitzschia* blooms were generated by the alleviation of iron limitations in high nitrate-low chlorophyll (HNLC) regions. Results showed that DA is a functional component of the high-affinity copper regulated, iron acquisition system in *Pseudo-nitzschia spp*. (Wells et al., 2005). In the absence of an adequate copper supply, iron-limited natural populations of *Pseudo-nitzschia* will become increasingly toxic, and thus the high DA production levels will provide *Pseudo-nitzschia* cells with a competitive ecological advantage.

Figure 6. Conceptual model of high affinity iron uptake system

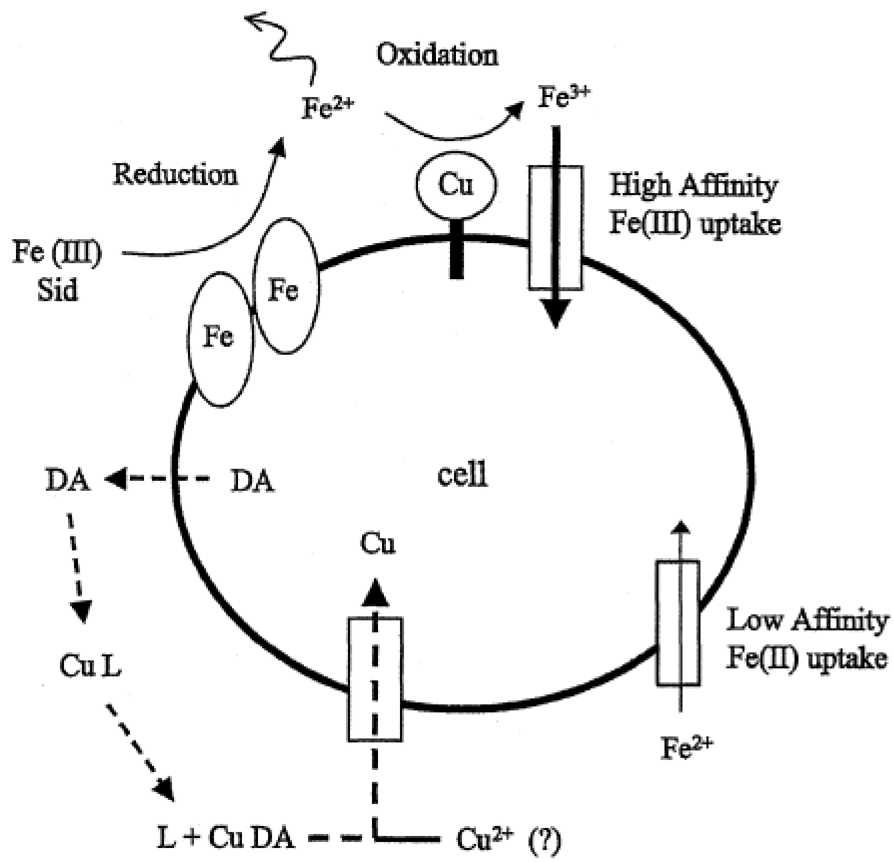

Fig. 7. Conceptual model of the high affinity iron uptake system found in yeast S. cerevisiae, comprising a membrane bound, iron-containing iron reductase, a multi-copper iron oxidase and high affinity Fe (III) transporter. In this sequence, organically bound Fe(III) is reduced, releasing Fe (II) from the cell. Fe (II) has a various lifetime depending on temperature, before diffusion transports Fe(II) away for the cell it is oxidized enzymatically with the resulting Fe (III) being bound and transported into the cell. Copper uptake in this case is shown by the release of DA (Wells et al. 2005).
Source: (Wells, Mark L., Charles G. Trick, William P. Cochlan, Margaret P. Hughes, and Vera L. Trainer. "Domoic Acid: The Synergy of Iron, Copper, and the Toxicity of Diatoms."*Limnology and Oceanography* 50.6 (2005): 1908-917).

However, there is inconsistency in the specific concentrations of iron or degree of iron stress (iron as a limiting trace metal) that result in DA production by *Pseudo-nitzschia*.

This discrepancy was found from a study done by Bates et al. (2002). In this study, results showed that DA levels did not increase, but decreased as a result of depriving *Pseudo-nitzschia* of iron. There were also differences in the results of experiments conducted done by three studies that examined the relationship of Fe and DA. In Maldonado et al. (2002) and Wells et al. (2005), the cells of *Pseudo-nitzschia* were in cultures and growing exponentially, in contrast to the Bates et al. (2002) study where the cells were in a stationary phase. The difference in life phases could attribute the differences in levels of DA production in the three studies. From both Maldonado et al. (2002) and Wells et al. (2005), *Pseudo-nitzschia* cells were in iron depleted conditions and therefore less stressed by the iron compositions. In comparison, in the Bates et al. (2002) study, the cells of *Pseudo-nitzschia* were in a dormant and stationary phase and therefore highly stressed by the iron limited concentrations. Iron and its availability play a vital role in the process of chlorophyll synthesis. With the iron limited cell conditions, there is less iron available for the synthesis of chlorophyll and nitrogen metabolism. These are two processes that are essential for maintaining production levels of DA which would not be able to occur in iron-depleted *Pseudo-nitzschia* cells, which then possibly have the ability to produce DA. (Bates et al. 2002)

These studies were performed in laboratory settings and therefore the ability to control specific macro and micro nutrient conditions and therefore create specific environments could ultimately not be able to reproduce the complexity of the coastal marine ecosystem. The three previous studies presented were primarily focused in laboratory settings, however, there have been studies done in the field that considered the relationship between DA production and the coastal ecosystem. In Marchetti et al. (2004)

and Trainer et al. (2009) a four year study was conducted off the Washington coast in the Strait of Juan de Fuca, taking water samples from mooring stations. Study results showed no predictable relationship between environmental conditions and the presence of *Pseudo-nitzschia* and levels of DA production (Marchetti et al., 2004). These stations recorded cell concentrations of particulate DA (pDA), dissolved DA (dDA) and cellular DA concentrations did not correlate with the macronutrient levels. However, these stations showed that when dissolved iron concentrations were limiting, *Pseudo-nitzschia* abundance and particulate domoic acid (pDA) and cellular DA levels were highest (or maximal). These results show that there is evidence that within this ecosystem (Strait of Juan de Fuca) exists a natural iron-regulating cycle. Iron becoming depleted by biological (phytoplankton blooms) or chemical (distance from shore) processes, which limits the iron input from sediments. In a later study done by Ribalet et al. (2010), the authors concluded that a complex iron relationship exists, suggesting when ideal conditions occur. These conditions called a "hotspot" occur when iron-rich coastal waters and low-iron oceanic waters meet and thus create a natural iron enriched area. However, DA levels has not been observed or documented in the region.

In summary, the differences between mesocosm-laboratory experiments and the field studies highlight contrasting results and their conclusions address the uncertainties that iron plays in the natural ecosystem. Laboratory experiments show differing results regarding the production of DA. Some studies show that iron could play a physiological role as an iron-copper chelator and produce DA. Other studies show that iron limiting and iron depleted conditions are important, and dependent on the life stage of *Pseudo-nitzschia*; either in a competitive growing stage or the stationary phase thus producing different results. These results are in contrast to the field experiments where there is a possible physiological iron regulating system in coastal environments. However, this raises concerns of the iron fertilization experiments. There has been evidence to suggest that oceanic *Pseudo-nitzschia* only produce low DA levels or none at all (Marchetti et al. 2004, 2008). Mesocosm experiments of iron fertilization experiments conducted by Trick et al. (2010) concluded that many of the iron-fertilization experiments, where low levels of iron are added to macronutrient-rich regions of the ocean (high-nitrate, low chlorophyll; HNLC) to assess the carbon-sequestering capacity of the ocean. These experiments resulted in a stimulation of specific species of *Pseudo-nitzschia*. After fertilization of open-ocean HNLC waters with iron, its nutrient composition approaches that of coastal waters and results in a *Pseudo-nitzschia* bloom dynamics similar to that observed in coastal or upwelling regions (Trick et al 2010, Silver et al. 2010). Also, in the study conducted by Trick et al. (2010), they presented results showing that *Pseudo-nitzschia* (which was in low numbers) at a HNLC station could retain a level of DA in conjunction with iron and copper. Trick et al. (2010) concluded that toxin production occurs with iron fertilization in HNLC regions, that the addition of iron is a key component and the levels of iron is also critical in the production of DA, and that increasing the copper availability further enhances DA. These studies also show that there is a strong link between the physiological need for iron by *Pseudo-nitzschia* and DA production and illustrates the possible dangers of geo-engineering. Unintended consequences of Fe-fertilization include the production of DA by *Pseudo-nitzschia* in the fertilized waters.

Conclusion

Summary of *Pseudo-nitzschia* bloom dynamics findings

This chapter discussed the various factors that contribute to the growth of *Pseudo-nitzschia* blooms and the production of DA. This review found that an increase in abundance and frequency of *Pseudo-nitzschia* blooms has been associated with increases in nutrient loading and eutrophication from increased use of nitrogen and phosphorus based fertilizers. However, there is also evidence that the highest cell numbers were often associated with lowest concentrations of nutrients. *Pseudo-nitzschia* blooms have also occurred in nutrient-replete waters (Juan de Fuca eddy, WA), as well as some of the most nutrient variable (Monterey Bay, CA and open-ocean) waters, which shows the versatility and adaptability of *Pseudo-nitzschia* in varying nutrient regimes. Another important finding is regarding the role of nutrient composition and the blooms of *Pseudo-nitzschia*. The decreasing silica: nitrogen and silica: phosphorus ratios, silica could become limiting for growth of *Pseudo-nitzschia*. While competitors such as flagellates and dinoflagellates don't require silica, decreased amounts of silica in relation to nitrogen and phosphorus could have profound implications for coastal phytoplankton communities, by giving flagellates a competitive advantage in the phytoplankton assemblage. Seasonal variability including wind patterns, upwelling, and rainfall have been identified as probable mechanisms of the formation of *Pseudo-nitzschia* blooms. Long-term climate patterns' including ENSO have also been identified as another possible factor for the formation of blooms. These weather and climate patterns influence the delivery of nutrients. Sunlight irradiance and therefore day length and the intensity of sunlight have also been identified as influences on the formation of blooms.

Summary of Findings in DA production

Some of the key findings in the literature on the reasons for DA production are also not fully understood. There is evidence that sexual reproduction may be important for the production of DA. There is uncertainty specifically during a life phase (hypothesized stationary phase of growth) or the outcome of nutrient limiting nutrients that *Pseud-nitzschia* slow the growth rates and produce DA. There is also uncertainty regarding the role of nutrient limitations on the production of DA. There is contradictory evidence about the role of nutrient composition and the production of DA. Lastly, there is evidence that shows silica limitations as the potential environmental trigger in DA production. However, studies also show varying results in nutrient replete areas such as the Juan de Fuca eddy, which is a "hotspot" for *Pseudo-nitzschia* that produce DA. Although evidence shows an increased production of DA from increased nutrient loading from anthropogenic sources, there are still varying conclusions.

Salinity and pH have also shown to have possible roles in the production of DA. Results suggest that increased salinity increases the production of DA in field studies conducted in the northern Gulf of Mexico (indicated that particulate DA concentrations were highest in higher salinity shelf waters as compared to estuarine waters). This result could explain the lack of DA outbreaks in this and other low salinity estuarine waters, which is important because estuarine areas are extensively used as shellfish growing and harvesting areas. From elevated pH levels with the possibility of ocean acidification due to climate change, *Pseudo-nitzschia's* ability to adapt to various conditions of the ocean may give *Pseudo-nitzschia* species a competitive advantage. While elevated levels of pH inhibit growth, there is also evidence that elevated pH increases DA production. Some studies suggest that DA could play a physiological role as an iron-copper chelator. While other studies have shown that iron limiting and iron depleted conditions were important, and dependent upon the life stage *Pseudo-nitzschia* (a growing stage or the stationary phase). These results contrasted to the field experiments where there is a possible physiological iron regulating system in coastal environments.

Chapter 2 has shown that there is evidence to support that *Pseudo-nitzschia* are versatile at adapting to varying environmental conditions, such as the effective use of macronutrients, and survival in varying nutrient regimes. It is also possible that DA serves an ecological purpose in increasing the availability of iron or decreasing the availability of copper, which is toxic to *Pseudo-nitzschia.* These results illustrate the possible dangers of geo-engineering. Unintended consequences of Fe-fertilization would be the production of DA by *Pseudo-nitzschia* present in the fertilized waters. While *Pseudo-nitzschia* and other HABs are a natural part of the phytoplankton and marine ecosystem, the apparent increase in abundance and global distribution of *Pseudo-nitzschia* has been likely due to human activities. Increased nutrient loading and eutrophication has played a possible role in the increase of both blooms of *Pseudo-nitzschia* and the toxic DA events. The current state of knowledge can be used to better inform policy makers and managers in the development of management and policy strategies to safeguard human health and reduce nutrient loads. Other policy recommendations include enhanced collaboration amongst different entities, with the goals of monitoring, restoring, and conserving the health of Washington Coastal waters. Also, continuing to develop technologies including mooring stations and monitoring platforms that can be deployed in a networked array as a better means of prediction of the conditions that lead to *Pseudo-nitzschia* blooms and toxic DA events in the future. The findings in the scientific literature provide a whole suite of valuable information for informing the development of policy and management strategies in the future.

Chapter 3

Policy Responses to HABs and *Pseudo-nitzschia* in the United States and Washington State

National Plan

Harmful algal bloom and hypoxic events caught attention at the federal level of government in the late 1990's both the U.S. Congress and the Clinton administration recognized HAB and hypoxic events (severe oxygen depletion) as some of the most complex phenomena challenging management of aquatic and marine ecosystems. Advancements in the scientific understanding of HABs and hypoxia have progressed since the early 1990's, but major impediments still remain for prediction, control, and mitigation of these complex phenomena. Practical and innovative approaches in addressing eutrophication, hypoxia, and HABs in US waters are essential for managing of aquatic and marine ecosystems. These practical and innovative approaches fulfill a stronger investment in the health of the coasts and oceans called for by the U.S. Ocean Action Plan and recent reports on ocean policy. Recognizing this need, in 1998, the United States Congress passed/authorized the Harmful Algal Bloom and Hypoxia Research and Control Act of 1998 (HABHRCA, Public Law 108-456,). Congress reauthorized and expanded HABHRCA by passing the Harmful Algal Bloom and Hypoxia Amendments Act of 2004 (HABHRCA 2004, Public Law 105-383). As of June 2012, HABHRCA 2011 was still in the Committee on Natural Resources.

Harmful Algal Bloom Hypoxia Research and Control Act

In 1998, the United States Congress passed the Harmful Algal Bloom and Hypoxia Research and Control Act (HABHRCA) (Public Law 108-456). This act established a national harmful algal bloom and hypoxia program, to develop and coordinate a comprehensive and integrated strategies and regional action plan to address and reduce HAB and hypoxia events. The act was reauthorized by Congress and signed into law by President George W. Bush in December of 2004 as the Harmful Algal Bloom and Hypoxia Amendments Act of 2004 (Public Law 105-383) which reaffirmed and expanded the mandate for NOAA to advance scientific understanding and the detection, monitoring, assessment, and prediction of harmful algal blooms and hypoxia. HABHRCA mandated NOAA to develop programs to research methods of prevention, control, and mitigation of HABs. The Bush administration further recognized the importance of HABs as a high priority national issue by specifically calling for the implementation of HABHRCA in the President's U.S. Ocean Action Plan (Ocean Blueprint for the 21st Century). HABHRCA also mandated the reestablishment of the Federal Interagency Task Force on HABs and hypoxia which implemented the production of the following assessments, reports, and programs: Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) research program, the Monitoring and Event Response for Harmful Algal Blooms (MERHAB) research program, and the Prevention, Control, and Mitigation for Harmful Algal Blooms (PCM HAB) research program. NOAA's Center for Sponsored Coastal Ocean Research (CSCOR) hypoxia programs included the Coastal Hypoxia Research Program (CHRP) and the Northern Gulf of Mexico Ecosystems and Hypoxia Assessment (NGOMEX) Research Programs. All of these programs, federal and state governments along with academic and research institutions were involved in addressing the issues of HABs and hypoxia.

HABHRCA also authorized funding to be appropriated to the Secretary of Commerce for research, education, and monitoring activities related to the prevention, reduction, and control of harmful algal blooms and hypoxia. Specifically, funding was authorized for both ongoing and new programs and to NOAA to carry out research and assessment activities, including the procurement of necessary research equipment, at research laboratories of the National Ocean Service (NOS) and the National Marine Fisheries Service (NMFS) and the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) project under the Coastal Ocean Program established under section 201c of Public Law 102-567. NOAA's National Ocean Service (NOS) was also charged with carrying out peer-reviewed research projects on management measures that could be taken to prevent, reduce, control, and mitigate HAB events. The NOS administered federal and state annual monitoring and analysis programs for harmful algal blooms. NOAA also established the National Centers for Coastal Ocean Science (NCCOS) to carry out local and regional assessment teams. The Interagency Task Force on Harmful Algal Blooms and Hypoxia oversees regional HAB research and management programs for NOAA in the listed regions below:

- Regional HABHRCA Efforts
    - Gulf of Mexico
    - Great Lakes
    - Northeastern United States
    - Pacific Coast Region
    - Washington State

NCCOS also partners with state agencies, academic and research institutions focused on understanding the causes of HABs and use this information to help state agencies develop more effective methods of monitoring and prediction. The short term goal was to prevent (or minimize) the impacts of HABs. The long term goal is to develop an understanding of the causes of blooms that will lead to forecasting capabilities and possible prevention strategies. This research was conducted through three NCCOS programs established in response to the HABHRCA: the multi-agency ECOHAB focuses on understanding the causes of HABs; MERHAB focuses on improving the abilities of coastal managers to protect human health and minimizing impacts of HABs on coastal economies. In addition, NCCOS maintains internal capabilities for responding to HAB events and conducting biotoxins research to complement these extramural competitive research programs.

Overview of programs addressing HAB events in Washington State

There are many environmental factors that play a role in the formation of *Pseudo-nitzschia* blooms and DA production that can play a central role in shaping a coherent, long-term environmental policy based on scientific assessment. Due to the variable toxicity of each *Pseudo-nitzschia* species and cosmopolitan distribution, *Pseudo-nitzschia* poses a unique management challenge. Here in Washington State, there are several state agencies, research and academic institutions that have formed a network of partnerships that collaborate on addressing HAB events (Figure 8 shows the network of organizations, tribes, and other institutions working together in Washington State addressing *Pseudo-nitzschia* and HAB events). The WDOH implements the Marine Biotoxins monitoring program. The Marine Biotoxins program, involved in *Pseudo-nitzschia* identification and enumeration, DA quantification and testing of potential sentinel shellfish, specifically mussels and clams. The Washington State Department of Fish and Wildlife (WDFW) also collect commercial shellfish and implement closures when DA levels are above the regulatory limit of 20ppm. There are also several partnerships between the WDOH, WDFW, and NOAA that implement research programs such as ECOHAB and MERHAB as well as a volunteer "citizen scientist" monitoring program called the SoundToxins program. Additionally there is a multi-stakeholder partnership program, called the Olympic Regional Harmful Algal Bloom (ORHAB) program, where state agencies, research and academic organizations collaborate in shellfish and coastal monitoring efforts around the state of Washington. However, abundance data alone is rarely a sufficient basis for management decisions. Oceanographic characteristics of a region should be taken into account when developing a sampling program so that any heterogeneous distributions of *Pseudo-nitzschia* in the water column can be defined. This management strategy provides an early warning system of possible *Pseudo-nitzschia* and other HAB related events that allow managers and officials at Department of Health to either close or limit shellfish harvesting and thereby reduce the risk to human health. This strategy is reactionary and while this strategy has been effective at safeguarding human health, it has often led to massive coastwide closures of shellfish harvesting areas, which leads to negative impacts on the shellfish industry and sectors of the economy related to the shellfish industry (Trainer et al. 2011). Reactionary policy is designed to respond to a harmful level as opposed to precautionary policies that seek to prevent the harmful events.

Figure 7 Organizations in Washington State addressing *Pseudo-nitzschia* and DA events

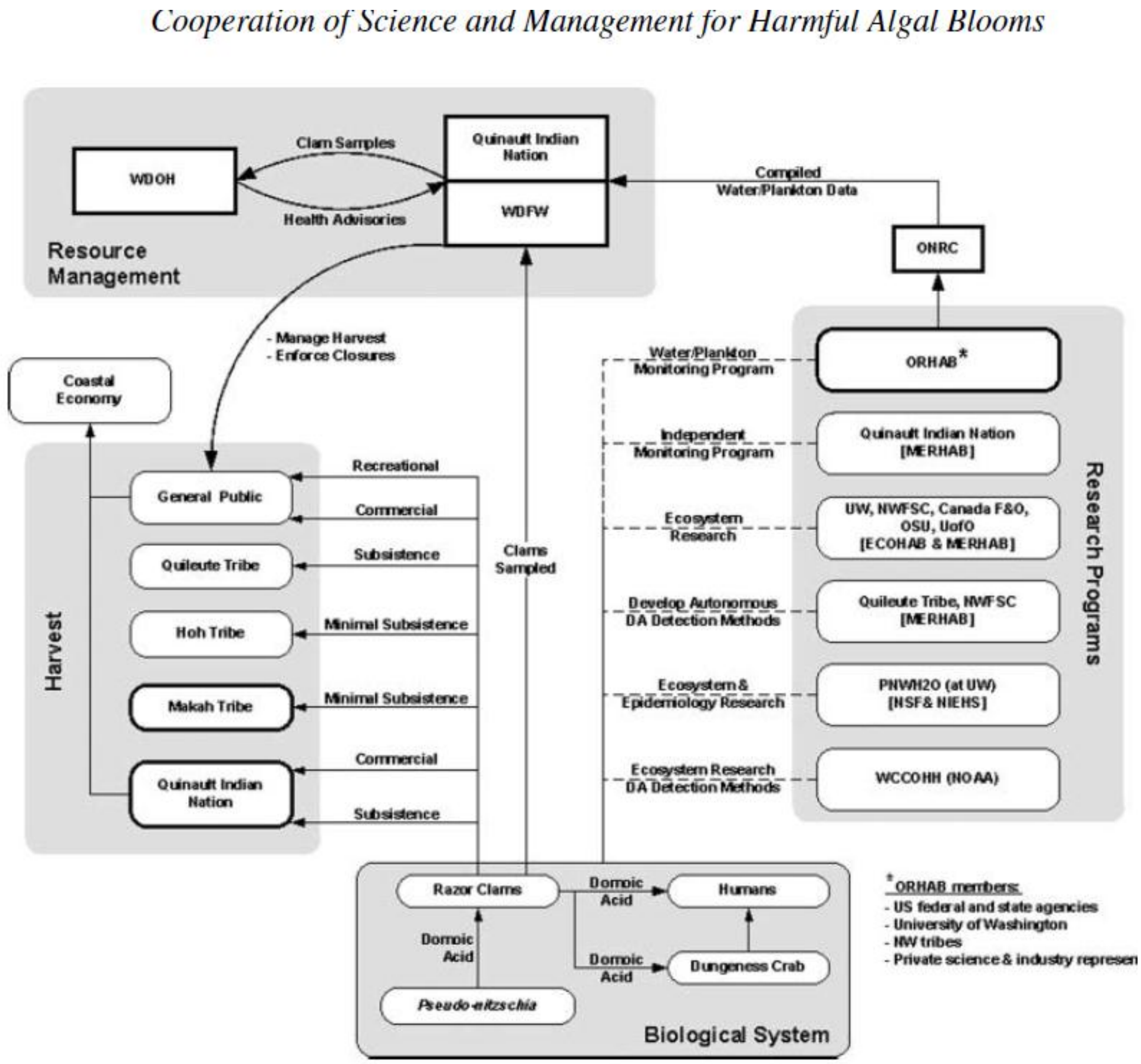

Fig. 8. The organization and institutions (action arena) surrounding the Washington razor clam fishery and the problem of DA contamination.
Source: (Chadsey, Meg, Vera L. Trainer & Thomas M. Leschine (2012): Cooperation of Science and Management for Harmful Algal Blooms: Domoic Acid and the Washington Coast RazorClam Fishery, Coastal Management, 40:1, 33-54)

Washington State Department of Health

The Washington Department of Health (WDOH) Marine Biotoxins Program, established in 1992 to protect humans from illness and death caused by shellfish contaminated by biotoxins. The program encompasses both commercially and recreationally harvested mollusks (those with a hinged shell) including clams, mussels, oysters, geoduck, and scallops. In this program mussels are also routinely used by WDOH to test for DA, but clams and oysters from commercial sites are also used. The regulatory limit of DA in shellfish tissue was established by the United States Food and Drug Administration (FDA) at 20 parts per million (ppm). The Marine Biotoxins Program monitors biotoxin levels in molluscan shellfish year-round. Shellfish in both recreational and commercial harvest areas are routinely tested for biotoxins known to be present in Washington marine waters, such as PSP and DA. When DA reaches the limit of 20 ppm or regulatory limits of other toxins the Department of Health closes the harvest area, and continues to test the closed area, and when lab results confirm that biotoxin concentrations drop to safe levels the department reopens the area to harvest.

When an area is closed on or near a public beach, a notification to the local health department is issued, and a news release is broadcasted about the closure. WDOH also posts the closure bulletin on their web site (http://ww4.doh.wa.gov/gis/mogifs/biotoxin.htm) and includes the up-to date recorded hotline (Shellfish Safety Hotline 1-800-562-5632) to let recreational harvesters know that shellfish in that area are not safe to eat. Warning signs are also placed on the beach. When there is a closure is in an area that is commercially harvested, the WDOH contacts all licensed companies harvesting in that area and notifies them to halt the harvesting. They also recall any commercial product on the market that came from the closed area. The shellfish testing is performed at WDOH's Public Health Laboratories in Seattle. Currently there is no certified reliable biotoxin test that can be performed outside of a laboratory environment.

Additionally, the Washington Department of Fish and Wildlife (WDFW), formerly the Washington Department of Fisheries and coastal Tribes (including the Quinault, Quileute, and Hoh tribes) collect razor clams in a number of management areas along the open coast. These are analyzed by WDOH and the U.S. National Marine Fisheries Service, Northwest Fisheries Science Center as part of the Olympic Region Harmful Algal Bloom (ORHAB) project. WDFW sets the shellfish harvesting regulations as well as the status of harvesting areas.

Olympic Regional Harmful Algal Bloom Program

In Washington State, shellfish are important in the commercial, recreational, and tribal subsistence fishery industries. The institutions charged with managing shellfish farms include the Washington Departments of Health (WDOH) and Fish and Wildlife (WDFW), and native tribes. These institutions were confronted with an unexpected environmental challenge in 1991: razor clams on Washington's outer coast became contaminated with DA. The detection of DA required rapid response from the managing agencies, including a coast-wide closure of shellfish harvesting (Horner and Postel 1993). Unlike mussels, which can rid themselves of the toxin following a toxic event, razor clams have the ability to retain DA for longer periods of time. The initial Washington closure remained in effect for nearly a year, which impacted a commercial fishery, operated by the Quinault Indian Nation (QIN) and deprived coastal communities of a major source of food and tourism. With the initial assistance of NOAA's Northwest Fisheries Science Center (NWFSC) and later support from NOAA's National Center for

Coastal Ocean Science (NCCOS), the Quileute Tribe began to monitor phytoplankton for the presence of *Pseudo-nitzschia* and shellfish tissues for the presence of DA in 1997. WDOH, WDFW, and the QIN expanded razor clam sampling and tissue analysis during this same period, also with assistance from NWFSC and NCCOS. Another DA outbreak in 1998 hastened the necessity for information useful and available to managers who were seeking to avoid emergency beach closures and risks to human health. In 1991 a unique partnership emerged to deal with DA contamination of Washington's razor clam fishery, consisting of academic, federal, tribal, and state researchers and managers. This group included the NWFSC, the University of Washington, the QIN, the Makah Tribe, the Washington State Departments of Fish and Wildlife, Health, and Ecology, Battelle Environmental Lab and the Pacific Shellfish Institute, which was consolidated in 1998 into the Olympic Region Harmful Algal Bloom (ORHAB) Partnership (Table 2, ORHAB partners).

Table 2 List of ORHAB partners and expertise

ORHAB partners and expertise

Partner	Expertise
Battelle Marine Sciences Laboratory	Remote Sensing
Makah Tribe	Beach and Nearshore Sampling
National Centers for Coastal Ocean Science	Program Management; Funding
Northwest Fisheries Science Center	Project Management; Toxin, Chlorophyll and Nutrient Analysis; Field Studies
Northwest Indian College	Training
Olympic Coast National Marine Sanctuary	Moorings and Cruises
Pacific Shellfish Institute	Nearshore Sampling
Quinault Indian Nation	Beach and Nearshore Sampling
Saigene Corporation	Marking Techniques
University of Washington	Oceanography; Beach and Nearshore Sampling; Phytoplankton Identification; Outreach
Washington Dept. of Ecology	Estuarine Studies
Washington Dept. of Fish and Wildlife	Beach and Nearshore Sampling
Washington Dept. of Health	Shellfish Tissue Analysis

Table 2 shows the ORHAB partnership group and the expertise of each partner.
Source: (Chadsey, Meg, Vera L. Trainer & Thomas M. Leschine (2012): Cooperation of Science and Management for Harmful Algal Blooms: Domoic Acid and the Washington Coast Razor Clam Fishery, Coastal Management, 40:1, 33-54)

The ORHAB partnership established a monitoring program for HABs. The objectives of ORHAB include investigating the origins of toxic algae blooms, monitoring where and when the blooms occur, assessing the environmental conditions conducive to blooms and toxicity of shellfish located near shellfish farms, and exploring methods that can be used to reduce HAB impacts on humans and the environment. Initially, the ORHAB partnership received funding from NOAA's NCCOS and MERHAB program for five years. A the end of the five year funding period there began a move towards a primary reliance on state dollars generated by a surcharge on recreational shellfish licenses. The focus of the partnership is primarily on HAB event prediction and monitoring. These state funds provide for two HAB specialists, one working for WDFW and the other for the University of Washington. NOAA initially funded the Quinault Shellfish HAB Sampling and Monitoring program from 2004-2007. The program supported the Quinault Indian Nation's (QIN) efforts to expand shellfish sampling within the Washington State coastal area managed or co-managed by QIN and incorporate new HAB sampling technologies to build an independent testing ability. Shellfish sampling continued after 2007 with funding from the Quinault Indian Nation (QIN) to provide a third HAB specialist. NOAA and the Quileute Tribe are developing and testing new methods of detecting DA in shellfish farms. Although employed by separate agencies, these local experts work closely together to monitor for HAB events along the entire Washington coast. The ORHAB specialists regularly present and discuss their findings with staff biologists and public health experts from WDFW, QIN and the Washington Department of Health (WDOH). In addition, scientists from NOAA and the UW provide oversight and advice on a regular basis. Insight gained from the ORHAB partnership and the recently completed ECOHAB-PNW project has led to a better understanding of where HAB events originate and what environmental factors promote their growth.

Olympic Regional Harmful Algal Bloom Program Approach

Trends have emerged regarding the seasonality, duration, and magnitude of *Pseudo-nitzschia* blooms that impact coastal shellfish. Several *Pseudo-nitzschia* species can occur simultaneously and can be difficult to identify. Precise identification often requires extensive electron microscopy. However, given financial and resource limitations it is not viable to identify every sample collected in the monitoring program using such an approach. Electron microscopy allows for the identification of selected samples of *Pseudo-nitzschia* to be identified to the species level. To overcome the financial restraints, ORHAB currently deploys a combination of analytical techniques, including bi-weekly determinations of total *Pseudo-nitzschia* cells using light microscopy and combined assessment of DA levels in seawater to give an effective early warning of shellfish toxicity. This approach has assisted shellfish resource managers on the outer coast of Washington State by providing them with an early warning of DA accumulation by shellfish. The success of the ORHAB program is a result of intensive monitoring over its life history. ORHAB technicians sample at several locations on the Washington coast and a majority of the areas include areas of razor clam harvest and shellfish farms. These technicians identify phytoplankton and quantify total numbers of *Pseudo-nitzschia*. Because many species of *Pseudo-nitzschia* are observed in Washington State's outer coastal waters, these species are grouped into categories according to size and morphological similarities including: *P. pungens, P. multiseries (*long and narrow), *P. heimii, P. fraudulenta, P. australis* (short and broad), and *P. delicatissima, and P. pseudodelicatissima* (small and narrow). These size groups include both toxic and nontoxic species. Because *P. pungens* is categorized as low toxicity and *P. multiseries* produces substantial amounts of toxin, it is important to know the total cellular DA in a sample in order to determine the potential for toxin transfer to shellfish (Trainer and Suddleson 2005). Razor clams are sampled about twice a month and tested for DA.

Ecology of Harmful Algal Blooms Program in the Pacific Northwest

The ECOHAB Program was initiated nearly a decade ago as a scientific program designed to increase our understanding of the fundamental processes underlying the impacts and population dynamics of HABs (Anderson 1995). The ECOHAB Program has identified three major research themes that encompass the priority issues of national importance. These three major research themes include 1. Organisms—with a goal of determining the physiological, biochemical, and behavioral features that influence bloom dynamics; 2. Environmental regulation—with a goal of determining and parameterizing the factors that govern the initiation, growth, and maintenance of these blooms; and 3. Food web and community interactions—with a goal of determining the extent to which food webs and trophic structure affect and are affected by the dynamics of HABs. Information in these areas, in turn, supports a critical goal of the ECOHAB program and the development of reliable models to forecast bloom development, persistence, and toxicity.

The federal partners in ECOHAB are NOAA, the National Science Foundation (NSF), the Environmental Protection Agency (EPA), the National Aeronautics and Space Administration (NASA), and the Office of Naval Research (ONR). Each agency brought its own unique interests and missions into this coordinated research program. ECOHAB in the Pacific Northwest was funded from 2002-2007 by a team of NOAA, NSF and NASA researchers, led by the University of Washington. This team studied the physiology, toxicology, ecology and oceanography of toxic *Pseudo-nitzschia* species off the Pacific Northwest coast. Toxins produced by *Pseudo-nitzschia* cause closures of razor clam and dungeness crab fisheries. Their research will lead to improved capabilities to predict the onset and path of these toxic bloom events.

SoundToxins Program

SoundToxins is a volunteer based monitoring program designed to provide early warning of harmful algal blooms and *Vibrio parahaemolyticus* events in order to minimize both human health risks and economic losses to Puget Sound fisheries.

SoundToxins engages “citizen scientists” with a diverse partnership of Washington State shellfish and finfish growers, staff from environmental learning centers, and native tribes. The SoundToxins program is funded by the NOAA fisheries science center’s West Coast Center for Oceans and Human Health as part of the NOAA Oceans and Human Health Initiative. The goal of this partnership is to establish a cost-effective monitoring program that will be led by state managers, tribal harvesters, and commercial fish and shellfish farmers. There are two main objectives of the program: 1. determine which environmental conditions promote the onset and flourishing of HABs and increased concentrations of *V. parahaemolyticus*; and 2. to determine which combination of environmental factors can be used for early warning of these events (http://www.soundtoxins.org/index.php). To accomplish these goals, seawater samples are collected weekly by the volunteer participants from different sites throughout Puget Sound and are analyzed for salinity, temperature, nutrients, chlorophyll, (paralytic shellfish toxins and DA) and the specific phytoplankton species. Phytoplankton species diversity is described and the four target species specifically identified and enumerated are *Pseudo-nitzschia* species, *Alexandrium catenella*, *Dinophysis* species, and *Heterosigma akashiwo*. The preceding review of programs demonstrates the diversity of state, national, and international programs that have interests in HAB research, monitoring, and management activities. This review serves to demonstrate the current difficulty in coordinating such a diverse and large group of agencies and programs.

Policy Addressing Nutrient Composition

One of the main/important scientific findings is that increased nutrient loading fosters blooms of *Pseudo-nitzschia* and that production of DA is based on nutrient composition. The increased nitrogen and phosphorus loads from the anthropogenic sources have decreased the Si: N and Si: P ratios. Examples of policy responses by the state of Washington have been the RCW 70.95L.020 and the passage of ESHB1489. These two laws could be part of the first steps reducing other nutrients including nitrogen. The Revised Code of Washington (RCW) is the compilation of all permanent laws now in force. RCW's are composed of a collection of session laws. These laws are enacted by the Washington State legislature, and signed by the governor, or enacted via the initiative process, arranged by topic, with amendments added and repealed laws removed. These laws do not include temporary laws such as appropriations acts. The passage of these two laws represents an important step towards reducing nutrient concentrations and composition.

RCW 70.95.L.020

RCW 70.95L.020 regulates the phosphorus content in laundry detergent. According to the language in the law after July 1, 1994, a person may not sell or distribute for sale a laundry detergent that contains 0.5 percent or more phosphorus by weight and a person may not sell or distribute for sale a dishwashing detergent that contains 8.7 percent or more phosphorus by weight (RCW 70.95L.020). In 2008 the law expanded to counties located east of the crest of the Cascade Mountains with populations greater than 400,000 as determined by the office of financial management population estimates, a person may not sell or distribute for sale a dishwashing detergent that contains 0.5 percent or more phosphorus by weight. From July 1, 2008 to June 30, 2010, the law expanded into counties located west of the crest of the Cascade mountains with populations greater 180,000 and less than 220,000 as determined by office of financial management population estimates, a person may not sell or distribute for sale a dishwashing detergent that contains 0.5 percent or more phosphorus by weight except in a single-use package containing no more than 2.0 grams of phosphorus. By July of 2010, a person may not sell or distribute for sale a dishwashing detergent that contains 0.5 percent or more phosphorus by weight in the state. However, RCW 70.95L.020 does not apply to the sale or distribution of detergents for commercial and industrial uses.

ESHB 1489

In April of 2011, Governor Gregoire signed ESHB 1489 the "Clean Fertilizers, Healthier Lakes and Rivers" into law. This law restricts the use of all turf fertilizers containing phosphorus with applications to frozen and impervious surfaces. The definition of turf defined by 1489 is "land, including residential property, commercial property, and publicly owned land, which is planted in closely mowed, managed grass. The definition of turf does not include pasture land, land used to grow grass for sod, or any other land used for agricultural production or residential vegetable or flower gardening (Bill 1489, 2011). The regulation of turf fertilizers is for any commercial fertilizer that is labeled for use on turf. The use, sale and promotion of fertilizers that do contain phosphorus is allowed when certain instances are presented including: application for establishing grass or repairing damage grass during the growing season; application to an area where the soil is phosphorus deficient, proven with a soil test preformed not more than thirty six months of the application; application to pasture, interior house plants, flower and vegetable gardens located on either public or private property, land used to grow grass for sod, or any agricultural or silvcultural production (Bill 1489, 2011). Only commercial fertilizer that has been registered with the Washington State Department of Agriculture (WSDA) can be distributed throughout the state. The sale and application to lawn fertilizer that is labeled as containing phosphorus is prohibited. Retailing exemptions and accountabilities, any retailers with phosphorus containing turf fertilizers in store may sell the product only if there is proof that it was in stock before January 1, 2012 and may sell it until it is sold out. In the violation of the restrictions posed by this bill, any person shall be found guilty of a misdemeanor and fined or presented with a written violation depending on the degree of the violation. All violations will be treated as misdemeanors and the appropriate law enforcement will give the proper sanctions. This legislation also prohibited local governments from adopting less restrictive ordinances on the use of fertilizers that contained phosphorus. This bill is enforced by the Department of Agriculture. The Department of Agriculture also decides what the proper measures for violators will be (Bill 1489, 2011).

Policy Analysis

With the passage of the Harmful Algal Bloom Hypoxia Research and Control Act in the mid-1990s, there have been many federal and state level programs connecting scientific findings on HAB and *Pseudo-nitzschia* events to management (WDOH MarineBiotoxins Program and SoundToxins) and policy strategies (HABHRCA, RCW 70.95L.020, and ESHB 1489). The importance of mitigating and predicting HAB and hypoxia events with the enactment of the HABHRCA increased precedent thusly increased the funding for research and development of programs that manage coastal areas with regional programs such as ORHAB in Washington State (State of Washington implemented a shellfish monitoring program in 1992). The enactment of the HABHRCA created new programs such as ORHAB and the SoundToxins program, and new funding for research programs such as ECOHAB and MERHAB. The HABHRCA originally passed in 1998 and again in 2004. However, the re-authorization of the HABHRCA (2011) was last in the subcommittee on energy and environment committee on science, space and technology in the U.S. House of Representatives (Committee Reports 112th

Congress (2011-2012)). There are potential setbacks to this legislation, which include the potential political hurdles the United States' Congress. One of the challenges that the composition of the United States Congress changes with the elections, and the balance of power could be shifted. Also even if the reauthorization of the HABHRCA may pass the U.S. Congress a shift in power of the majority party could result in the HABHRCA not being reauthorized, major changes in the language of the legislation, or a cut in funding which would make the HABHRCA less effective in addressing HAB research and events.

In Washington State, there is cooperation between the science and management of HAB and *Pseudo-nitzschia* events (as shown previously in Figure 8). However, the management strategy of closing shellfish harvesting areas based on monitoring for DA is a reactionary management strategy (most coastal regions also implement a similar strategy). Commercial shellfish are routinely monitored and tested for toxins, and the harvest areas are then closed when the regulatory (20ppm) toxin levels are exceeded. This management strategy has been proven to be beneficial in protecting human health. However, the strategy has also led to coastwide closures of shellfish harvest areas, which have negative impacts on the shellfish industry and the Washington State economy (recreational shellfish and tourism/related industries) (Trainer et al. 2011). Another potential drawback of this strategy is that sentinel shellfish (mussels) in cages, may not provide the most complete warning of DA events (Trainer et al. 2011 and Chadsey et al. 2012). This drawback demonstrates that coastwide "blanket" closures that prohibit the harvesting of all shellfish species may not be necessary, because oysters accumulate little or no DA at the same location where other shellfish species are over the regulatory limit (Trainer et al. 2011).

The scientific findings also indicate that it is still not possible to predict with accuracy of outbreaks of bloom events or the production of DA making policy responses that are species specific difficult. Both management and policy strategies do not address specific species of *Pseudo-nitzschia*. Legislation addresses nutrient reduction, which targets the growth of *Pseudo-nitzschia* the genus and not specific species. Managers and officials at WDOH, WDFW, and NOAA try to manage the genus, but try to target the management of the known species of *Pseudo-nitzschia* (*P. australis, P. multiseries, and P. pseudodelicatissima*). However, there is still a lack of knowledge in possible specific characteristics that make certain species toxic and to isolate those characteristics in which policies and management strategies can prevent, control, and mitigate the toxic species.

Another strategy addressing HAB events is the implementation of legislation. Washington State has enacted two policies that are tailored from scientific literature findings. In the literature an increase in *Pseudo-nitzschia* blooms has been associated with the increase in nutrient concentrations, specifically nitrogen and phosphorus, due to increased usage of synthetic fertilizers, increase in agriculture and food production, and energy use and production. Increased amounts of nitrogen and phosphorus also change the nutrient composition (decreasing the silica: nitrogen and silica: phosphorus ratios). These changes in nutrient composition have also been associated with the increase in *Pseudo-nitzschia* blooms. The two related pieces of policy legislation enacted in Washington State are RCW 70.95L.020 and ESHB 1489, both of which address the reduction in phosphorus loading. RCW 70.95L.020 aims for the reduction of phosphorus in dishwater detergents, and restricts the sale of dishwater detergents that contain 0.5 percent or more phosphorus by weight in the state. ESHB 1489, enacted into law in April of 2011, restricts the use of phosphorus in turf fertilizers. While both RCW 70.95L.020 and ESHB 1489 address phosphorus loading and restrict the use of phosphorus in dish detergents and fertilizers both pieces of legislation are limited in their scope. ESHB 1489 does not apply to pasture land, land used to grow grass for sod, or any other land used for agricultural production or residential vegetable or flower gardening. ESHB 1489, also does not apply in certain application scenarios including application for establishing grass or repairing damage grass during the growing season, application to an area where the soil is phosphorus deficient, proven with a soil test preformed not more than thirty six months of the application; application to pasture, interior house plants, flower and vegetable gardens located on either public or private property, land used to grow grass for sod, or any agricultural or silvicultural production. Although RCW 70.95L.020 was originally passed in 1994, it was not in full effect, statewide until 2010. Both RCW 70.95L.020 and ESHB 1489 do not apply to the sale or distribution of detergents and fertilizers for commercial and industrial uses.

Most importantly, however, RCW 70.95L.020, ESHB 1489, or any piece of legislation do not address the restrictions of the use of nitrogen in products such as fertilizers. Nitrogen inputs doubled as a result of the increased global use of nitrogen based fertilizers since post- World War II (Turner and Rabalais 1991). The widespread use of synthetic fertilizer took off after World War II when innovations allowed nitrogen fertilizer to be produced inexpensively and on a grand scale. There has been an increase in the global application of synthetic nitrogen based fertilizers on farm fields, as well as an increase in manure generated from agricultural livestock such as chickens and cows.

A possible alternative around excess use of nitrogen would be more efficient use of fertilizer. The challenge then is to find a way to provide plants with enough nutrients to maintain high yields while also minimizing nitrogen leakages. However, farmers do not use nitrogen efficiently and safely. There aren't incentives to do so, because fertilizer is inexpensive, and polluters don't pay. The situation might change if nitrous oxide became regulated under climate legislation. Even if agriculture-related nitrous oxide emissions do get capped, policies would have to address efficiency directly.

Farmers should be rewarded at least as much for conserving nitrogen and building organic matter in soil. The Chesapeake Bay program (further discussed later in the chapter) formally agreed to cooperate with the United States Environmental Protection Agency, in order to fully address the extent, complexity, and sources of pollutants entering the Chesapeake Bay. However, Chesapeake Bay and other nitrogen-threatened ecosystems need more than cooperation to restore the health of the ecosystem.

Policymakers need "political will" to develop policies that address the reduction in the application and global use of nitrogen based fertilizers. The one common aspect that both management and policy strategies from federal and state levels of government is that both strategies employ a science-based approach. These actions are based on general ecological understanding of the established connections between increased nutrients and algal biomass. Substantial efforts have been made at both the federal and state levels of government in addressing *Pseudo-nitzschia* bloom and DA events. Therefore as the scientific knowledge continues the understanding of biological and ecological dynamics of Ps*eudo-nitzschia*, policies can be refined to address specific species affecting specific regions. With the understanding of the advances and shortfalls of current organizations and policy, there are programs in place that provide platforms for enhanced collaboration between different states. The Environmental Cooperation Council (ECC) and the Coastal Ocean Taskforce (COTF) serve as mechanisms already in place in which Washington State and B.C. participate in collaborative efforts in addressing coastal ecosystem health. However, the ECC and COTF need a program to serve as a model for enhanced collaboration with support from the EPA or other federal programs and also a platform where short-term and long-term goals for preservation, conservation, and restoration of Washington- B.C. coastal health.

Collaboration between the B.C. and Washington State

A known hotspot for *Pseudo-nitzschia* is the Juan de Fuca eddy. Because *Pseudo-nitzschia* are present in the Strait of Juan de Fuca, *Pseudo-nitzschia* can be found on both sides of the Canadian and U.S. border. A HAB problem in one country may have been initiated through nutrient delivery or another source from another country. Furthermore, the transport of species and water via currents and shipping poses additional mechanisms by which these problems spread from one country to another. A coordinated effort between the United States and Canada would not be the first effort towards collaboration. The European Union has established the European Harmful Algal Bloom (EUROHAB) program. There is also an international collaboration called, the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB). There is also a lack of policies that target the reduction of overall nutrient concentration from terrestrial sources including nitrogen and phosphorus in Washington State and British Columbia (B.C.). There is a necessity for international and state to state collaboration in sharing knowledge and research to reduce nutrient concentration. A reduction of overall nutrient reduction would restore the health of B.C. and Washington coastal waters, as well as, the environmental factors that trigger the bloom of *Pseudo-nitzschia* and the production of DA. There are many partnerships and collaborative agreements between Washington and B.C. which allow the development of policies international policy and integrate communication, including the Environmental Cooperation Council (ECC). There are also other examples where collaboration may spawn. On the east coast of the United States, the Chesapeake Bay Program consists of a partnership of various state, federal, academic and local watershed organizations to build and adopt policies that support Chesapeake Bay restoration. The Climate Action Partnership, Chesapeake Bay Program, and the many agreements and collaborative efforts between B.C. and Washington including the ECC and the COTF, are examples of collaborative efforts reduce nutrient concentrations, and works towards restoring the health of Puget Sound and coastal Washington waters.

Environmental Cooperation Council

In February of 2011, the state of Washington and the province of British Columbia (B.C.) entered into climate agreements so that efforts are in place to reduce carbon pollution and advance the low-carbon economy. These two agreements focus on limiting carbon emissions from government operations and raises awareness on the impacts of sea level rise on coastal areas. The pair of agreements strengthens the collaboration between B.C and Washington State. Other examples of collaboration between B.C. and Washington include the Pacific Coast Collaborative**,** Washington-British Columbia Memorandum of Understanding on Coastal Climate Change

Adaptation, the Salish Sea Ecosystem/Puget Sound-Georgia Basin Ecosystem Research Conference, and the Environmental Cooperation Council (ECC).

The Pacific Coast Collaborative includes B.C., Washington, Oregon, California, and Alaska working collaboratively on energy, transportation, climate change, and ocean issues. For example, the participants have addressed the following issues in the past; ocean debris, transportation fuels, clean-energy, and energy-efficient building standards and best practices. The Washington-British Columbia Memorandum of Understanding on Coastal Climate Change Adaptation includes joint science workshops, the exchange of information on sea-level rise projections and mapping, information on Green Shores programs, and Washington and B.C. “king tide” photo initiatives. The Salish Sea Ecosystem/Puget Sound-Georgia Basin Ecosystem Research Conference is one of the largest, most comprehensive scientific research and policy conferences that focus on issues impacting the region known as the Salish Sea. British Columbia and Washington take turns hosting the biennial conference.

The Environmental Cooperation Council (ECC) was established by the Environmental Cooperation Agreement, by the Governor of Washington State and Premier of British Columbia on May 7, 1992. The main objective of the ECC was to ensure a coordinated action plan and information sharing on environmental matters of mutual concern. The principal members of the ECC include the B.C. Ministry of the Environment, the Department of Ecology, EPA Region 10, the Federal Environment of Canada, and the Fisheries and Oceans Canada Pacific Region. There are several critical cross-border environmental issues that require joint attention by Washington State and BC. In an effort to address these issues, the ECC established and directed the work of Task Forces, who facilitate information sharing, coordination and cooperation on issues of mutual interest, including the Washington-British Columbia Coastal and Ocean Task Force (COTF).

Coastal and Ocean Task Force

The COTF was established to provide a mechanism to enhance collaboration between Washington and B.C. on joint coastal and ocean issues. The geographic area of interest includes Puget Sound, the Georgia Basin, and the outer coasts of Washington and British Columbia. The Task Force provides an arena where B.C. and Washington can share information and collaborate on activities that protect and restore coastal and marine habitats; encourage the development of ecosystem management approaches for ocean and coastal resources; and foster sustainable coastal communities and development.

These partnerships and agreements represent collaborative values already shared by B.C. and Washington State. The agreements also provide the necessary mechanisms to which management strategies and future policies can develop when tackling the problem of HABs. Specifically, the ECC can provide the format in developing a coordinated effort between B.C. and Washington management and policy strategy focused on *Pseudo-nitzschia* bloom and toxic DA events.

There are collaborative mechanisms already in place for Washington State and B.C. to work together. However, there is no integrated long-term program in place to prevent and mitigate HAB events along the U.S. and Canadian coastal borders. The Chesapeake Bay program could serve as the model for which to build a long-term collaborative partnership in addressing issues of coastal health.

Chesapeake Bay Program

Pseudo-nitzschia transcends boundaries if they begin in the Strait of Juan de Fuca because *Pseudo-nitzschia* crosses borders between B.C. and the state of Washington. An example of such a program of collaboration between different states that share a common estuary is the Chesapeake Bay Program. The state of Washington and the province of British Columbia could design an agreement similar to the Chesapeake Bay Program. The Chesapeake Bay program is a collaborative five state effort with the primary goal of restoration and conservation of the Chesapeake Bay estuary. The program partners include federal and state agencies, local governments, non-profit organizations and academic institutions. The program has set a long-term reduction of nutrient loading from identified watersheds of 40% by the year 2025. Washington and B.C. could establish a similar program to the Chesapeake Bay Program in order to enhance collaboration between the two entities with enhanced management and policy strategies to mitigate the occurrence of *Pseudo-nitzschia* and other HAB events along B.C. and Washington coastal and estuary waters.

The Chesapeake Bay Program was first formed in 1983 as the first estuary program in the United States targeted by the United States Congress for restoration and protection. In the late 1970s, U.S. Senator Charles Mathias (R-Md.) sponsored a Congressional study which, funded $27 million for five-years to analyze the Chesapeake Bay’s rapid loss of wildlife and aquatic life. The study, which was published in the early 1980s, identified excess nutrient pollution as the main source of the Bay's degradation. These initial research findings led to the formation of the Chesapeake Bay Program as the means to restore the Bay.

In 1983, a document was signed by the governors of Maryland, Pennsylvania, and Virginia, the mayor of Washington D.C., the administrator of the Environmental Protection Agency (EPA) and the Chair of the Chesapeake Bay Commission. The signatories of this agreement became the Chesapeake Executive Council. This document became known as the Chesapeake Bay Agreement. The agreement recognized that a cooperative approach was necessary in addressing the Chesapeake Bay's pollution issues. The main goal was to formulate a coordinated long-term adaptive management strategy in restoring the health of Chesapeake Bay.

In an unprecedented move by the Chesapeake Executive Council, the first numeric goals were to reduce pollution and restore the Chesapeake Bay ecosystem. Chiefly among the goals set was the reduction of nitrogen and phosphorus entering the bay by 40% by the year 2000. Numeric goals with specific deadlines, while unprecedented in other agreements, it has become a hallmark of the program. In 1992, the council agreed to target reductions of nitrogen and phosphorus from the source: from terrestrial sources and upstream from the many rivers that feed into Chesapeake Bay.

Another milestone occurred in 2000 when the Chesapeake 2000 program was signed by the governors of New York and Delaware (West Virginia signed on in 2002). This comprehensive initiative set a strategy to guide the restoration efforts for the next decade. The agreement established 102 goals to accomplish five main objectives including: reducing pollution, restoring habitats, protecting the biological resources, promoting sound land use practices, and engaging the public. The agreement laid the groundwork and put into motion a plan for restoration efforts in early 2000 throughout the decade. However, the agreement did not set a transparent network in achieving those goals. The program and its partners achieved significant restoration gains in certain areas, such as land conservation, forest buffer restoration and reopening some fish passages. However, limited progress was made toward many other health and restoration measures, including oyster abundance, and most importantly, the reduction of nutrient pollution from anthropogenic sources including agriculture and urban areas. Nearing the end of the Chesapeake 2000 agreement, the Executive Council decided to implement short-term restoration goals called "milestones". These milestones were short-term goals that were set by the each state and would need to be met every two years. A long-term objective was for all restoration measures deemed necessary for Chesapeake Bay restoration was to be in place no later than 2025. The implementation of these short-term two year goals would help the states achieve the long-term goal.

At the end of the ten-year agreement, the EPA established the Chesapeake Bay Total Maximum Daily Load (TMDL). The TMDL is a federally mandated pollution reduction plan that sets limits on the amount of nutrient and sediment load that can enter the Bay's joining rivers and still meet the long-term water quality goals. Each of the seven Bay states was tasked in creating their own Watershed Implementation Plan (WIP). Federal, state and local governments coordinated with one another through the Chesapeake Bay partnership in developing each state's WIP. Each WIP would detail specific steps necessary for each state and all the geographic jurisdictions to take and meet the nutrient and pollution reduction goals by 2025. The WIPs provide transparency, accountability, and guide measures for each jurisdiction and state to meet the restoration goals by 2025 and beyond.

Conclusions and Recommendations for Future Work

The negative impacts of the genus *Pseudo-nitzschia* are growing worldwide, and society's need for research on these phenomena is more pressing than ever. There have been increased public health, tourism, fishery, and ecosystem impacts from HABs that have led to heightened scientific and regulatory attention and an increased awareness of the value of ocean ecosystem to human society (Anderson et al. 2011).

> In *Making Environmental Policy* by Daniel J. Fiorino the author states "Because of many budget shortfalls and cuts to state agencies and programs the reality of there is more to do out there than there are resources, knowledge, and political capital with which to do it…the capacities of EPA and its state and local counterparts were stretched beyond their limits (Fiorino p. 164)."

There has been significant progress made in both the policy and management realms in addressing *Pseudo-nitzschia* and the broader HAB impacts. However, there still drawbacks to both policy and management. Current policies do not address the nitrogen loading and overall nutrient loading. The current management strategies are reactive and have a tendency to lead to coast wide closures of shellfish harvest areas. A long-term collaborative effort between Washington State and British Columbia with the goal of protection, conservation, and restoration of coastal waters goals that have been identified by both as an important issue to address in the future. There are already collaborative mechanisms setup in the Environmental Cooperation Council (ECC) and the Coastal Ocean Task Force (COTF) and therefore Washington and British Colombia can pattern a long-term plan after the Chesapeake Bay Program. The Chesapeake Bay program is a collaborative partnership amongst the five states that share the Chesapeake Bay, supported by the federal government and other research and academic institutions. They all collaborate and set long-term plans with the aim of protecting, conserving, and restoring the health of Chesapeake Bay. Another vision for the future is that collaboration along with advancements in technologies will lead to the deployment of instruments and monitoring stations along the coast that will serve as an early warning system.

HABs represent a biological component of coastal waters that challenge present technologies, in part because of the need for species- or toxin specific detection capabilities (Anderson et al. 2011). The development of HAB forecasting capabilities for the state of Washington, province of British Columbia, and the entire West Coast of the transport and impact of toxic *Pseudo-nitzschia* blooms will require continued research on their biology, genetics, regular long-term monitoring, and the development of forecast models. The primary goal of *Pseudo-nitzschia* and the HAB forecasting system is to provide an early warning network for the detection and transport of *Pseudo-nitzschia* blooms and DA toxicity events using an integrated suite of monitoring sensors. Sensors include satellites, mooring stations, and stationary platforms that together measure ocean water properties including salinity, pH, and temperature, and the movement of currents. These monitoring stations would also measure *Pseudo-nitzschia* cell numbers, and DA, all of these factors would be in real-time, to allow for communications with shore-based lab testing.

This real-time data collected from mooring stations could be used to create a database of information that could be used in computer generated and simulated oceanographic models that would predict the environmental conditions impacting future *Pseudo-nitzschia* blooms and toxic events. These models will allow WDOH, WDFW, and the British Columbia Ministry of the Environment to take preventive actions that would increase monitoring efforts, close specific shellfish beds, and most importantly warn at-risk communities to safeguard their public health, local economies, and shellfish farms. Additionally, an integrated forecasting system will allow the proactive management of resources such as an early warning to commercial crab and clam fishers who are impacted by DA-related closures. This rapid real-time transmission of information to managers is critical in communicating the exposure of DA and public health.

Currently, the National Coastal Data Development Center's HABs observing system HABSOS is in collaboration with ORHAB in establishing a program that provides real-time data that will deliver weekly summaries of data and alert partners when dangerous levels of cells and toxins have been observed. HABSOS provides an online, integrated information system for managing HAB data, events, and effects. Managers will have a tool for rapid access to current information on Washington State outer coast HAB events and similar events across the nation. Currently, there is an online bulletin at the Pacific Northwest HAB website for the Washington State coast, which is currently in its pilot stage (see http:// pnwhabs.org/pnwhabbulletin/index.html).

In most coastal regions of the world, closures of shellfish harvesting based on monitoring for DA are reactionary, shellfish are routinely tested for toxins and harvest closures are instated when the regulatory threshold toxin level is exceeded. This management approach has been successful in protecting human health. However, it has also often led to coast-wide closures of shellfish harvesting areas, which negatively impact the shellfish industry, the local economies that depend on shellfish, and the state's economy (Trainer et al. 2011). Shellfish, typically mussels in cages, does not always provide the best warning of DA events.

The success of a combination of more proactive approaches to monitoring that allow for targeted closures has been demonstrated. The ORHAB monitoring partnership is an example of proactive monitoring approach. ORHAB uses a simple combination of analytical techniques, which provide an effective early warning of shellfish toxicity events. However, in order to sustain monitoring the integration of new technologies and methods should be considered. Sensors including mooring stations, monitoring platforms, and satellites that collect a suite of biological, physical, chemical factors that can be integrated into a computer database, which could then generate models to help forecast *Pseudo-nitzschia* blooms and toxic DA events. This would rapidly assist managers during toxic bloom events. ORHAB partners have already demonstrated to state legislators on how integral the monitoring program is for effective and timely management of shellfish resources. ORHAB is only the first step in being able to predict future toxic events and protect public health. Current programs including ORHAB coupled together with new technologies will provide the most up to date and accurate forecasting capabilities for the early warning of *Pseudo-nitzschia* and HAB events.

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