Domoic Acid Production in Pseudo-Nitzschia (Bacillariophyceae) as a General Response to Unbalanced Growth: The Role of Photo-Oxidative Stress

April Woods
California State University, Monterey Bay

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DOMOIC ACID PRODUCTION IN *PSEUDO-NITZSCHIA (BACILLARIOPHYCEAE)* AS A GENERAL RESPONSE TO UNBALANCED GROWTH: THE ROLE OF PHOTOOXIDATIVE STRESS.

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Presented to the

Faculty of the

Moss Landing Marine Laboratories

California State University Monterey Bay

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Marine Science

__

by

April Woods

Spring 2016
DOMOIC ACID PRODUCTION IN *PSEUDO-NITZSCHIA (BACILLARIOPHYCEAE)* AS A GENERAL RESPONSE TO UNBALANCED GROWTH: THE ROLE OF PHOTO-OXIDATIVE STRESS.

Nicholas Welschmeyer, Chair
Moss Landing Marine Labs

G. Jason Smith
Moss Landing Marine Labs

Kenneth Coale
Moss Landing Marine Labs

Digitally signed by Kris Roney, Ph.D.
Kris Roney, Dean
Associate VP for Academic Programs and Dean of Undergraduate and Graduate Studies

Approval Date
ABSTRACT

DOMOIC ACID PRODUCTION IN _PSEUDO-NITZSCHIA_ (BACILLARIOPHYCEAE) AS A GENERAL RESPONSE TO UNBALANCED GROWTH: THE ROLE OF PHOTO-OXIDATIVE STRESS.

by April L. Woods

Toxic _Pseudo-nitzschia_ blooms, resulting from species-specific production of the neurotoxin domoic acid (DA), have caused human deaths and illnesses, numerous mass mortality events of marine birds and mammals, and economic loss brought on by fisheries and aquaculture closures. A spectrum of potentially growth limiting biotic and abiotic factors are implicated in production and release of DA in this diatom genus. Because the consequences of toxic _Pseudo-nitzschia_ blooms may be environmentally and economically severe, scientists are investigating the how and why of DA metabolism and biosynthesis to gain understanding of; what ecological purpose DA serves, and whether biomarkers exist in _Pseudo-nitzschia_ that would help monitor shifts toward toxin production and thereby refine identification of environmental parameters used to inform modeling of toxigenic DA event prediction indicators. In an effort to define a common mechanism of DA induction, the hypothesis of this thesis is that DA production is a general response to cellular stress and thus DA could be induced or attenuated through a direct stress signal. Up regulation of DA production was induced when toxin positive cultures were transitioned to growth under high irradiance. Biomarkers of oxidative stress accompanied the increase in DA, supporting the hypothesis that excess photon pressure generates a cellular stress response. A pharmacological chemical model of photosynthetic stress induction was used to mimic the physiological response of excess irradiance in order to provide a physiologically robust test of the hypothesis that DA biosynthesis is enhanced as a general response to photo-
oxidative stress. The data shown here do not support that outcome. Additional data collected, suggests a correlation in enhanced cellular resilience to stress among toxic species.
Table of Contents

ABSTRACT .............................................................................................................................................. iv
LIST OF TABLES ..................................................................................................................................... vii
LIST OF FIGURES ............................................................................................................................... viii
ACKNOWLEDGEMENTS ....................................................................................................................... xii
  Pseudo-nitzschia species and domoic acid ecology ........................................................................ 1
  Hypotheses regarding the physiological roles of DA ...................................................................... 4
  Algal Stress Perceptions and Responses ......................................................................................... 7
Hypothesis ................................................................................................................................................ 13
Questions ................................................................................................................................................ 13
  Isolate Identification ......................................................................................................................... 14
  Algal Culture Growth and Maintenance ......................................................................................... 14
  High Light Stress Experiments ....................................................................................................... 14
  Electron Transport Rate (ETR) ......................................................................................................... 15
  Protein Extraction and Quantification .............................................................................................. 15
  SuperOxide Dismutase (SOD) ......................................................................................................... 16
  Domoic Acid ELISA ........................................................................................................................ 17
  Hydrogen peroxide quantification ................................................................................................... 17
  Methyl Viologen as an Agent for Photochemical Stress Induction .............................................. 18
  Methyl Viologen Stress Experiments ............................................................................................... 19
  Taxonomic survey of stress management ......................................................................................... 20
  ABTS- An Assay for General Antioxidant Capacity ...................................................................... 20
  Domoic Acid Certified Reference Material ..................................................................................... 21
  Excess photon energy increases DA production and induces an Oxidative Stress Response ....... 22
  Chemically Induced oxidative stress: Methyl Viologen Effects on Domoic Acid production and Pseudo-nitzschia physiology ......................................................................................... 23
  10uM MV Acute (24 hour) experiments .......................................................................................... 23
  Acclimated response to chronic oxidative stress ............................................................................. 25
  Species Specificity in Stress Capacity .............................................................................................. 26
Discussion .............................................................................................................................................. 27
Conclusion .............................................................................................................................................. 33
LIST OF TABLES

Table I. Enzymatic activities and gene products associated with mitigation of oxidative stress. .......................................................................................................................................................................................... 42

Table II. Isolates Used in Experiments.......................................................................................................................................................................................... 43

Table III. Examples of Methyl Viologen used in algal experiments to induce oxidative stress. .......................................................................................................................................................................................... 47

Table IV. Change in cell concentration (cells/mL) in Pseudo-nitzschia australis over 48 hours of growth as a function of growth under either standard or increased irradiance conditions.......................................................................................................................................................................................... 48

Table V. One-way ANOVA results for the effects of 10uM MV acute experiments on each species’ change in protein content, cell abundance, DA content and SOD activity................. 49
LIST OF FIGURES

Figure 1. Structure of Domoic Acid................................................................. 51

Figure 2. Mechanism of Xanthine-Xanthine Oxidase mediated SOD assay. ................. 52

Figure 3. Example of a % color inhibition curve generated using a bovine SOD standard. . 53

Figure 4. Chemical structure of Methyl Viologen, MV (N,N'-dimethyl-4,4'-bipyridinium dichloride, C12H14Cl2N2). ....................................................................................... 54

Figure 5. Example of a typical Trolox standard curve used in antioxidant capacity assay. .. 55

Figure 6. Change in Domoic Acid concentration (fgDA/cell) in Pseudo-nitzschia australis as a function of growth under either standard or increased irradiance conditions....................... 56

Figure 7. Change in SOD activity (Units SOD/ mg protein) in Pseudo-nitzschia australis as a function of growth under either standard or increased irradiance conditions....................... 57

Figure 8. Rapid Light Curves were generated following 24 and 48hrs growth under standard or High Light conditions............................................................................................................. 58

Figure 9. Maximal Electron Transport Rate (µmol electrons m^{-2} s^{-1}) in Pseudo-nitzschia australis as a response to growth under standard irradiance conditions at 24 and 48hrs. .................. 59

Figure 10. Reduction in maximal Electron Transport Rate (µmol electrons m^{-2} s^{-1}) in Pseudo-nitzschia australis as a response to growth under increased irradiance conditions at 24 and 48hrs. ..................................................................................................................... 60

Figure 11. Change in maximal Electron Transport Rate (µmol electrons m^{-2} s^{-1}) in Pseudo-nitzschia australis as a response to growth under either standard or increased irradiance conditions .............................................................................................................................................. 61

Figure 12. Saturating Light levels (E_{s}) (µmol electrons m^{-2} s^{-1}) in Pseudo-nitzschia australis as a response to growth under standard irradiance conditions at 24 and 48hrs. ......................... 62
Figure 13. Saturating Light levels (Ek) (µmol electrons m-2 s-1) in Pseudo-nitzschia australis as a response to growth under increased irradiance conditions at 24 and 48hrs. .......................... 63

Figure 14. Change in saturating light levels (µquanta electrons m² s⁻¹) in Pseudo-nitzschia australis as a response to growth under either standard or increased irradiance conditions... 64

Figure 15. Pilot study to assess dose response of MV and effects potential effects on Domoic Acid production in a toxin positive Pseudo-nitzschia culture. ......................................................... 65

Figure 16. Raw data and summary of species growth (cells/mL and ng protein/cell) and cDA content evaluation following acute MV treatment by species at 24hrs .............................. 66

Figure 17. Change in percent yield cell abundance (cells/mL) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours ........................................ 67

Figure 18. Change in percent yield protein content (ng protein/cell) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours. ......................... 68

Figure 19. Raw data and summary of species SOD activity evaluation following acute MV treatment by species at 24hrs ...................................................................................... 69

Figure 20. Relative change in SOD activity (units SOD/ mg protein) for six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours......................... 70

Figure 21. Change in percent yield cellular DA (fg DA/cell) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours........................................ 71

Figure 22. Change in hydrogen peroxide generation (nMoles H₂O₂/L  nM H₂O₂) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours .......... 72

Figure 23. Change in photosynthetic efficiency (fv/fm) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours ............................................. 73
Figure 24. Cell abundance of control or 1uM MV treated cultures over a 7-day growth cycle of Pseudo-nitzschia australis .................................................................................................................. 74
Figure 25. Cellular DA concentrations of control or 1uM MV treated cultures over a 7-day growth cycle of Pseudo-nitzschia australis ........................................................................................................ 75
Figure 26. SOD activity (Units SOD/ mg protein) of control or 1uM MV treated cultures over a 7-day growth cycle of Pseudo-nitzschia australis ........................................................................................................ 76
Figure 27. Change in maximal electron transport rate (µmol electrons m⁻² s⁻¹) of control or 1uM MV treated cultures over a 7-day growth cycle of Pseudo-nitzschia australis ........................................... 77
Figure 28. Change in saturating light intensity (µquanta electrons m⁻² s⁻¹) of control or 1uM MV treated cultures over a 7-day growth cycle of Pseudo-nitzschia australis .......................................................... 78
Figure 29. Cellular Domoic Acid concentration of field isolates collected from Monterey Bay, CA, 2015, n = 42 .............................................................................................................................................. 79
Figure 30. Assay for naïve antioxidant capacity (nmol Trolox Equivalents/ cell) of field isolates collected from Monterey Bay, CA, 2015, n = 42 ............................................................................................. 80
Figure 31. Linear fit analysis of cellular DA content vs Trolox equivalents, a proxy for cellular antioxidant capacity, in field isolates collected from Monterey Bay, CA, 2015, n = 42 ....... 81
Figure 32. Linear fit analysis of cellular DA content vs Trolox equivalents, a proxy for cellular antioxidant capacity, in only the toxin positive species field isolates collected from Monterey Bay, CA, 2015, Pseudo-nitzschia australis n = 19, Pseudo-nitzschia multiseries n = 7, Pseudo-nitzschia seriata n = 5 ................................................................................................................................. 82
Figure 33. Assay for naïve SOD activity (Units SOD/ mg protein) of field isolates collected from Monterey Bay, CA, 2015, n = 42 ........................................................................................................... 83
Figure 34. Linear fit analysis of cellular DA content vs SOD activity, in field isolates collected from Monterey Bay, CA, 2015, n = 42. .......................................................... 84

Figure 35. Linear fit analysis of cellular DA content vs SOD activity in only the toxin positive species field isolates collected from Monterey Bay, CA, 2015, Pseudo-nitzschia australis n = 19, Pseudo-nitzschia multiseries n = 7, Pseudo-nitzschia seriata n = 5................................. 85

Figure 36. Domoic Acid has no associated anti-oxidant properties when assayed by ability to (A) quench H$_2$O$_2$ radicals or (B) synergistically or additively enhance SOD inhibition of superoxide radicals........................................................... 86
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Introduction

_Pseudo-nitzschia species and domoic acid ecology_

Members of the globally distributed diatom genus _Pseudo-nitzschia_ comprise a prominent bio-toxin producing and ecologically important phytoplankton consortium. Identified as a taxonomic group as early as 1882 (Cleve), interest in _Pseudo-nitzschia_ expanded greatly in 1987 when an unobserved phytoplankton bloom off the coast of Prince Edward Island, Canada resulted in contamination of local filter feeders with the novel amino acid, domoic acid soon leading to a mass human poisoning event. Consumption of tainted mussels (_Mytilus edulis_) killed three people and caused over 100 non-lethal cases of Amnesiac Shellfish Poisoning (ASP) (reviewed in Bates, 1989). A rigorous investigation quickly identified _Pseudo-nitzschia multiseries_ as the primary food source of mussels contaminated with high concentrations of the bio-toxin Domoic Acid (DA) and ultimately named _Pseudo-nitzschia multiseries_ as the _de facto_ DA producer. This was noteworthy especially because up to this point, no diatoms had been known to produce neurotoxins.

Increased focus on _Pseudo-nitzschia_, combined with the advancement of sophisticated taxonomic tools, has revealed numerous species within the genus, with both diverse and cryptic phenotypes (Lundholm, 2002). To date, there are 46 confirmed species of _Pseudo-nitzschia_, and of these, almost half are confirmed as toxigenic by way of their ability to make the bio-compound, DA (Guiry, 2016). Domoic acid (Fig. 1) is a water-soluble, tricarboxylic, neuroexcitatory amino acid that acts by activating receptors for glutamate and inhibits standard glutamate neurotransmission (Todd, 1993). In mammals, DA intoxication can cause temporary physical illness, permanent brain damage or death (Pulido, 2008). The toxin accumulates in the
tissues of fish and shellfish and moves through the trophic web to poison birds, marine mammals and humans through consumption of the tainted fish or shellfish (Fire, 2010, Bates, 1989, add more). Additional mass poisoning events, similar to the episode of 1987, have been avoided due to diligent global monitoring programs that now include routine DA testing of any market bound fisheries products. A regulatory limit of 20ug DA/g tissue (20 ppm) has been set for products intended for human consumption (USFDA, 2011). Recent research has suggested that chronic exposure to low amounts of DA; less than 20ug DA/g tissue; can also have adverse consequences to animal health. Funk et al., found in a murine model, when administered sub-clinical doses of DA, did not elicit a neurologic response, but did present with biomarkers of nephrological toxicity (Funk, 2014). This suggests that current management best practices are sufficient to avoid large scale events, but still leave vulnerabilities in long term consumption of otherwise presumed “safe” seafood products. Because of the active monitoring of market and recreational fisheries, current Pseudo-nitzschia HAB events have mostly affected sea birds and sea mammals (Scholin, 2000, LeFebvre 1999 and 2010) and through consumption of finfish as well as shellfish, so that the more general term Domoic Acid Poisoning (DAP) is often used in place of ASP.

Toxic blooms of Pseudo-nitzschia have serious impacts on ecosystem health, and cause economic losses in fisheries and aquaculture, yet as a cosmopolitan genus, Pseudo-nitzschia is frequently an important component of regional food webs. Consequently, it is important to understand what factors promote Pseudo-nitzschia to develop into blooms and persist for long periods at high concentration and what conditions select for the toxic phenotype. Most in situ monitoring efforts focus on a few of the previously observed DA producers, like Pseudo-nitzschia multiseries and Pseudo-nitzschia australis, but ignore lesser DA producers like Pseudo-
nitzschia pungens, Pseudo-nitzschia fraudulenta and others, in an increasing list of newly described species. These efforts and our understanding of environmental signals promoting toxigenic blooms is further confounded not only by inter-specific but critically by pronounced intra-specific variation in DA production phenotypes observed in isolates (Smith et al., 2001; Hubbard, 2008). This points to a need to evaluate species of Pseudo-nitzschia not currently thought of as toxigenic in order to determine is all Pseudo-nitzschia species have an innate capacity to biosynthesize DA, and the environmental factors that are most favorable to toxin production. It is still not entirely clear why Pseudo-nitzschia make DA, or what, if any physiological function, it serves in these diatoms or in the rhodophyte macroalgae where it was originally characterized (Ramsdell, 2007). Proposed roles for DA in diatoms include: metal chelation (Rue, 2001; Ladizinsky, 2003; Wells, 2012), grazer avoidance (LeLong, 2012), as a response to fluctuating irradiance (MacIntyre, 2011) or simply an additional sink for photosynthetic reductant as a compatible amino acid (Smith et al. 2001); though, possibly as a consequence of intraspecific variability, conflicting reports arise for all scenarios (reviewed in LeLong 2012).

Pseudo-nitzschia blooms are associated with an increased nutrient load; both from anthropogenic inputs and coastal upwelling (reviewed in Anderson et al. 2008). For a Pseudo-nitzschia bloom to be a true HAB, the bloom must consist of toxigenic species and strains, actively producing DA. Years of field and laboratory experiments have revealed a pattern describing the key elements that determine whether or not a bloom will be a DA positive, toxic bloom (reviewed in Bates, 1998; LeLong, 2012; Mos 2001; Pan, 1998; Trainer, 2012). Namely, greatest DA accumulation most consistently occurs when cells are in stationary phase, silica or phosphate limited, iron limited or subject to increased copper or higher irradiance. Because it is
an amino acid, nitrogen sufficiency is required for DA biosynthesis. In addition, the presence of bacteria or associated metabolites seems to have some role in promoting elevated DA in culture, though it has been shown that bacteria themselves are incapable of producing the toxin (Bates, 1995). In short, production of DA is hypothesized to be triggered by some kind of stress, and is often negatively correlated to growth rate, and more generally to potentially growth modulating factors. The physiological role of DA is still an open question and the following summary of available observations provides some insights onto shared factors stimulating cellular production.

**Hypotheses regarding the physiological roles of DA**

The presence of bacteria in cultures of *Pseudo-nitzschia* has an additive effect on DA levels, but it has also been confirmed that associated bacteria themselves do not produce DA independent of *Pseudo-nitzschia* (Bates, 1995, 2004, Stewart 2008). One thought, is that the bacteria and *Pseudo-nitzschia* exist symbiotically in a recycling and sharing of nitrogen and carbon sources. Bacterial transfer experiments have proven unsuccessful in conferring a DA phenotype to non-toxic isolates and vice versa (Sisun-Mangus et al. 2014, LeLong 2014). However, co-culture with bacterial isolates from DA naïve *Pseudo-nitzschia pungens* cultures did result in deleterious, algicidal, effects on DA producing *Pseudo-nitzschia australis* isolates (Sisun-Mangus, 2014). Previous studies demonstrated similar results, in that toxin positive isolates grown in axenic media had high mortality rates and lethargic growth, and concurrently lower DA yields overall (Bates, 1995). Thus, current evidence points to an innate ability of at least some *Pseudo-nitzschia* strains to produce DA, but that it can be modulated by external biological interactions.
Secondary phytometabolites sometimes act as allelopathic molecules or grazing deterents. Domoic Acid has been considered for its use as an allelopathic agent and its aid in giving competitive advantage to *Pseudo-nitzschia* in a mixed community. Though some studies have found a positive correlation between DA and reduced grazing by copepods (Bargu, 2006), which might provide an advantage through reduced grazing pressure, the fact is that even toxigenic cells are still consumed and are frequently major components of fecal pellets. As such, alternate explanations have been offered for why the presence of DA positive *Pseudo-nitzschia* may suppress the growth of co-occurring organisms. Aside from the molecule DA itself having allelopathic properties, alternate theories relating to growth rate (Olson, 2010), iron chelation by DA (Prince, 2013), or even the presence of *Pseudo-nitzschia* metabolites other than DA (Xu, 2015) have all been proposed to explain enhanced competitiveness by *Pseudo-nitzschia*. Recent reports describe an active lipoxygenase metabolism (d’Ipolito, 2009) and a species specific lipoxygenase profile in the genus (Lamari, 2013), alluding to the existence of differing strategies to enhance survival and competitiveness among the species. Direct efforts to find allelopathic effects of DA on other organisms have garnered little to no evidence to support DA as the chemical responsible for allelopathy in *Pseudo-nitzschia* (Lundholm, 2005, Xu 2015).

Another theory as to the physiological function of DA pertains to the association between DA biology and trace metal acquisition, or chelation (Wells, 2012; Rue, 2001 Ladizinsky, 2003; reviewed in LeLong, 2012). An early study on causative agents of DA toxicity found a 1.7X fold increase in cellular DA, but unaltered growth rate, after inoculation with high (above environmental) concentration of Lithium (Rao, 1998). This was hypothesized to be a factor in the 1987 Prince Edward Island DA positive *Pseudo-nitzschia multiseries* bloom that was fed off a fresh water input downstream from a waste dump. Though the lab study found lithium was able
to alter DA accumulation rates, the authors did not ascribe a role for lithium in DA metabolism. An excess of aqueous copper has repeatedly been shown to increase cellular DA (Rue 2001, Ladizinsky 2003). Ladizinsky (2003) found environmental correlates of higher copper in coastal upwelling regions associated with higher *Pseudo-nitzschia* populations and DA positive blooms. These initial studies also confirmed a strong binding affinity in DA:Cu complexes. Maldonado (2002) explored the influence of iron on DA levels in *Pseudo nitzsizia* and found that Fe limited conditions induced higher rates of DA secretion and increased concentrations of extracellular dissolved DA. Continuing in this vein, further research (Maldonado, 2005) elucidated a potential role for DA in an iron uptake system. Cultures transferred from iron replete to low iron conditions, ramped up both intracellular production and extracellular secretion of DA to match the need for iron. These experiments are complemented by iron enrichment experiments in high nitrogen low chlorophyll (HNLC) regions, where seed populations of *Pseudo-nitzschia* are able to quickly bloom and persist after other diatoms have died off following iron enrichment (Trick, 2010; Silver et al., 2010). Taken together, the data suggest that DA is used both to sequester excess concentrations of metals, as a protective mechanism, and as a means to chelate and concentrate metals like iron for biological use, when found in scarcity. Conversely, a study on exogenous addition of DA to a toxin negative *Pseudo-nitzschia delicatissima* isolate, found no protective measures were achieved when that culture was grown in the presence of high concentrations of copper. Mortality of DA inoculated culture matched that of the control (LeLong, 2012). The commonality among the metal acquisition experiments, may be the stress brought on by either a nutritional limitation or a toxicity of excess.

Irradiance fluctuation is another environmental trigger that may cause stress to un-acclimated photosynthetic cells. Like cellular growth, DA production is ultimately driven by
photosynthetically derived energy, therefore at minimum, irradiance is a requirement for toxin production. There have not been many lab studies on the effects of irradiance saturation and DA production (El-Sabaawi, 2006). As a secondary metabolite, DA is a product derived from existing carbon pools and not the product of a single gene. Among speculation for potential roles of DA, a generalized view holds, that as a compatible amino acid derived as a product of isoprenoid metabolism and from amino acid synthesis pools, DA is formed as an additional reductant sink under circumstances, such as in the presence of excess irradiance, favoring secondary metabolism. This view supports the hypothesis that DA is a general stress response product. In order to explore the role of DA metabolism as a general stress response, we must first explore and define, what “stress” means to an algal cell and further what metrics can be used to assess stress in the cell. Following is a brief overview on algal stress physiology and its manifestations.

**Algal Stress Perceptions and Responses**

All life forms must sense and respond to their environment and metabolically acclimate as needed. Broadly speaking, this equates to sensing and responding to the chemical signals that translate both biotic and abiotic events. Fluctuations in the external environment often manifest in the organism as the universal experience of cellular stress. In the following discussion, biotic stress refers to stress initiated by other biological forms, i.e. allelopathic compounds, viral infections or other pathogens. Abiotic stress in the context of photoautotrophs, refers to all the exogenous factors a cell relies on for primary production. These include access to micro- and macro-nutrients, irradiance, temperature and salinity. Events that perturb the current metabolic
state, if not lethal, are felt on a cellular level as a signaling pressure that will lead to some physiological response on the part of the cell.

An example of a perturbed metabolic state with oft times dire consequences, may be found in the chemical reactions involving molecular oxygen. A consequence of an oxygenated atmosphere is the common existence of oxygen radical species. Known collectively as Reactive Oxygen Species (ROS), these are byproducts resulting from chemical interactions involving molecular oxygen (O$_2$). An imbalance of intracellular generation of ROS and antioxidant capacities results in a state of oxidative stress, leading to damage or death in the cell. Beyond their potential to cause damage, oxidative stress signaling is also known to regulate a variety of metabolic networks in plants (Reviewed in Apel, 2004), and there is growing evidence to support the presence of such highly regulated networking in micro-algae as well (Falciatore, 2000; Sunda, 2002). Differing sensitivities and resistance to oxidative stress have been used to explain differences in phytoplankton response among isolates and the physiological outcomes elicited by external pressures (Chankova, 2014; Janknegt, 2009; McGinty, 2012).

To avoid the lethal aspects of ROS production, cells initiate an antioxidant response in order to mitigate ROS accumulation. Oxidative stress mitigation is achieved through an enzymatic response as well as a chemical response with carotenoids (ie xanthophyll cycle), GSH, ascorbate and amino acids such as proline and taurine providing additional protection. A summary of some common antioxidant defense enzymes and small molecules is found in Table I. Reduced (or reactive) oxygen species vary by half-life and redox potential with ·OH (hydroxyl) being the most reactive and therefore most damaging, followed by O$_2^-$ (superoxide) and H$_2$O$_2$ (hydrogen peroxide). Robust and redundant defense mechanisms exist for superoxide
and hydrogen peroxide. No such scavenging activity exists for hydroxyl, as it is so highly reactive that it reacts at its diffusion rate and leaves no time for cellular defense to take place.

As the second most reactive oxygen species (second to hydroxyl) and with a half-life that exceeds its diffusion rate, superoxide is a prime target of reduction within the cell. The first line of defense against superoxide is the enzyme Superoxide Dismutase (SOD). Four metallic isoforms exist of SOD, distinguished by their metal cofactor (Iron, Manganese, Copper/Zinc and Nickel) and at least one isoform of SOD can be found in most eukaryotic and prokaryotic organisms. SOD catalyzes the dismutation, or breakdown, of superoxide into molecular oxygen and the less reactive hydrogen peroxide. Diatoms favor the Mn and Fe forms of SOD, though examples exist of other metal cofactor SODs in diatoms (Peers 2004).

Hydrogen peroxide, \( \text{H}_2\text{O}_2 \), is a slightly less reactive form of oxygen and is predominately produced following the dismutation of superoxide by SOD. \( \text{H}_2\text{O}_2 \) is degraded by the enzymes catalase (CAT) and other peroxidases, sometimes using the molecule ascorbate as reductant. Evidence also exists of externally associated bacteria being used as “helper” organisms in oceanic phytoplankton (Morris, 2011), helping by reducing \( \text{H}_2\text{O}_2 \) in the phycosphere of marine phytoplankton. In fact, oceanic profiles of hydrogen peroxide show a light and time of day periodicity suggesting photo- production of \( \text{H}_2\text{O}_2 \) by phytoplankton and subsequent extracellular quenching by bacteria. As \( \text{H}_2\text{O}_2 \) is easily applied exogenously, many studies exist demonstrating the effects on phytoplankton physiology. Differing sensitivities to \( \text{H}_2\text{O}_2 \) have been found among species and isolates of common genera. This may be the result of species or isolate specific adaptations in antioxidant defense mechanisms. Further, reports on \( \text{H}_2\text{O}_2 \) levels throughout a mesocosm bloom cycle demonstrate that self-shading reduces \( \text{H}_2\text{O}_2 \) production (Barros, 2003).
As a less damaging and readily quenched ROS, H$_2$O$_2$ may act as a signaling molecule in plants, sending the message throughout the organism to increase radical defenses.

Although, ROS generation in photosynthetic organisms occurs as a result of normal chloroplast and mitochondrial metabolism (Halliwell, 2006), increased ROS production will also occur at PSI and PSII as a consequence of reduced photosynthetic efficiency (Niyogi, 2000). In the aquatic environment, photosynthetic organisms must respond to a dynamically shifting light regime. Adaptability to both excess and light deficiency is key to maintaining cellular stasis. When faced with excessive light, an imbalance can occur such that the energy absorbed through the light harvesting complex (LHC) exceeds that which can be dissipated or transduced by photosystem II (PSII). Immediate relief may be found through heat dissipation, also called non-photochemical quenching (NPQ). Continued exposure to excess photon energy, left unmitigated will result in an accumulation of ROS. Outside of alterations to photosynthetic rates and over longer terms, composition of the light harvesting antennae, strategies may also include alternate sinks for excess photons. However, increasing total PAR quantity is not the only parameter that can induce oxidative stress. The imbalance of photon energy with light harvesting capacity will be exacerbated under any condition of biotic or abiotic stress that reduce the ability to incorporate photosynthetically derived reductant for downstream processes. Unbalanced growth in phototrophic cells arises when photon energy is in excess of its rate of use by carbon (CO$_2$) assimilation (Asada, 2006). As photosynthetic apparatus is catabolized or damaged, excess ROS accumulates in the PS II reaction centers (Falkowski and Raven, 2007). Photoautotrophic organisms will ultimately experience the spectrum of unbalanced growth conditions through the common consequence of reduced photosynthetic efficiency resulting in an excess of reactive oxygen species (Asada, Karpinski).
A minimal requirement of trace metals exists for sustained growth in primary producers. Key elements are required for many bio-molecules and reactions in photosynthetic organisms. Iron (Fe) and is essential for electron transporters and chlorophyll (Chl) biosynthesis, manganese (Mn) plays a part in chlorophyll functionality and photosystem activity, copper (Cu) and zinc (Zn) are both components of enzyme structure, like SOD. At standard coastal oceanic conditions, trace metals are found in viable concentrations. However, at surplus concentrations, metals can cause cellular damage and even loss of viability. Excess metals cause damage through direct protein and lipid damage (blocking of thiol groups), displacement of essential metal cofactors from enzymes and protein structures, and finally through the generation of ROS via Fenton and Haber-Weiss reactions (Pinto, 2003). As a first line of defense against excess metal quotas, exclusion or sequestration mechanisms are employed to avoid metal induced damage. But at higher concentrations, free metal cations induce the evolution of persistent radical oxygen species. The effects of metal-induced reactive oxygen species on phytoplankton have been studied extensively (Bertrand, 2005; Okamato, 2001; Szivak, 2009). Key metal deficiencies can similarly lead to an influx of radical oxygen species, though the result stems from a mechanism akin to macro-nutrient starvation. It is thought that a limitation in Fe decreases efficiency in the electron transport rate, resulting in an increase in electron acceptance by molecular oxygen (O$_2$). These newly radicalized oxygen molecules coupled with a lack of Fe-requiring ROS quenching enzymes, yield oxidative stress. Evaluation of an iron (Fe) starved *Phaeodactylum tricornutum* transcriptome, showed down regulation of SOD and other Fe requiring gene products concurrent with induction of alternate antioxidant ROS quenching systems (Allen, 2008).

Numerous groups have demonstrated that nutrient limitation causes a decline in photosynthetic efficiency. Wykoff et al. (Wykoff, 1998) showed similar results of decreased
electron transport, increased heat dissipation and reduction in total chlorophyll content occurred in _Chlamydomonas reinhardtii_ cultures subjected to either N or P starvation. The dinoflagellate _Prorocentrum donghaiense_ responded to phosphate limitation with an overproduction of ROS and initiation of caspase and PCD activity, though not leading to cell death (Huang et al. 2016), demonstrating stress regulation in response to nutrient limitation is felt as a ROS pressure in this toxin producing phytoplankton.

Be it from a perturbation in metal concentration, nutrients or photon flux; a common consequence of unbalanced growth in photoautotrophic organisms is the manifestation of a preponderance of radical oxygen species. The strategies employed by a phytoplankton to mitigate the production of ROS and oxidative stress will dictate its success to carve out a niche in an environment rife with potential stress inducing conditions. Numerous groups have shown an eco-physiological niche for stress tolerance. An objective of this thesis is the assessment of whether and which _Pseudo-nitzschia_ species have specific niches in stress tolerance.

Little information is available on the potential parallel stress responses elicited by the diverse array of toxin enhancing signals in nature. The host of biotic and abiotic factors most closely associated with DA accumulation, share the common underlying consequence of an induced cellular stress. The following questions can be posed: 1) _is exacerbated DA accumulation the result of a unique acclimation to each unique parameter outlined above, or 2) is DA accumulation a generic response to the common internal consequence shared by this set of external pressures._

Though DA itself has not been implicated as an anti-oxidant, one proposed role is that, by providing a sink for photosynthetic reductant, it may serve as a mechanism to eliminate excess photosynthetic energy under conditions of imbalanced growth (MacIntyre, 2011, LeLong 2012).
Enhanced resilience to oxidative stress, regardless of the particular environmental source, would give species of the *Pseudo-nitzschia* genus an advantage over other algae in a mixed community and offer niches unavailable to more stress sensitive cells.

The association of induced stress and toxin production is presumed to be a factor in HAB development, yet little work has been done to directly link Domoic Acid to associated biomarkers of physiological stress. DA levels vary by strain and even within a near mono-specific bloom, isolates will produce DA to varying degrees. Aspects of physiological diversity beyond the DA phenotype have been explored less extensively. In light of the information presented above; the common consequence of myriad parameters being a ROS spike, as well as the information provided that DA blooms are often associated with a general ecological stress, this thesis was undertaken to examine the association of stress physiology and DA production in a range of species within the *Pseudo-nitzschia* genus.

**Hypothesis**

Domoic Acid is produced in some members of the *Pseudo-nitzschia* genus as a general stress response and thus DA production will positively correlate with ROS production and other biomarkers of algal stress.

**Questions**

1. Do low to high irradiance transitions induce DA?
2. Will an artificial stress, mimicking the ROS mediated photo-oxidative physiological stress instigated by high light, induce DA?
3. Do species of *Pseudo-nitzschia* respond similarly to oxidative stress?
Methods

Isolate Identification

*Pseudo-nitzschia* cultures were generated from single cell picks isolated from natural sea water. Isolates were subsequently identified to the species level by LSU rDNA sequencing, Table II. (Holly Bowers, personal communication)

Algal Culture Growth and Maintenance

Isolate stock cultures were maintained at 15 °C on a 16:8h light:dark cycle. Light was maintained at 100 umole quanta m$^{-2}$ s$^{-1}$ with fluorescent lamps. Cultures were grown in 0.2um filtered and autoclaved sterilized sea water and enriched with Guillard’s f/2 media (Sigma). Cultures were serially transferred into fresh media while in exponential growth phase. Culture growth was routinely assessed by cell counts of aliquots of culture preserved in 1% Lugols’s solution using a gridded Sedgewick-Rafter chamber. At least 200 cells were counted for each sample. For all experiments, log phase stock cultures were used to inoculate at least triplicate freshly prepared culture media 1:100 v/v dilution as indicated.

High Light Stress Experiments

High Light (HL) stress experiments were conducted by exposing test cultures from standard culture irradiance conditions to at least 400 umol quanta m$^{-2}$ s$^{-1}$ provided by introducing LED full spectrum white lights, as measured by a Licor LI-185A photometer PAR sensor. Cultures used in high light experiments were conditioned at standard light levels before introduction to high light regime.
Electron Transport Rate (ETR)

Rapid Light Curves were generated using a Walz water PAM. Curves were fitted to ETR vs PAR using the equation:

\[ \text{ETR} = \text{ETR}_{\text{max}} \left( 1 - e^{-\alpha \text{PAR} / \text{ETR}_{\text{max}}} \right) e^{-\beta \text{PAR} / \alpha} \]

Where \( \alpha \) is the initial slope of the light curve and \( \beta \) is the slope at photoinhibition.

After the initial curve fit, final values for \( \text{ETR}_{\text{max}} \) and \( E_k \) (saturating light value) were calculated following the method derived by Jassby and Platt (Jassby, 1976), using the following equations:

\[ \text{ETR}_{\text{max}} = \frac{\text{ETR}_{\text{max}} \left[ \alpha / (\alpha + \beta) \right] \left[ \beta / (\alpha + \beta) \right]}{\alpha / \alpha} \]

\[ E_k = \frac{\text{ETR}_{\text{max}} / \alpha \ln(\alpha + \beta)}{\beta} \]

Protein Extraction and Quantification

Algal samples were collected onto 1.2 um polycarbonate filters (Millipore) and archived at -70° until further use. Filter samples were resuspended into protein extraction buffer (50mM KHPO4, pH 7.0, 0.1% Triton-X detergent and 0.1mM EDTA). Resuspended cells were sonicated at low pulse (30%) for approximately 10 seconds and then rested on ice for 5 minutes. Samples were
pelleted via centrifuge for 20 minutes at 5k RPM and clarified supernatant was used in assay. Protein concentration was determined using Thermo-Scientific BCA Protein Assay kit (Prod# 23225).

SuperOxide Dismutase (SOD)

A modified version of the assay developed by Fridovich and Beauchamp was used to measure SOD activity. The Xanthine- Xanthine Oxidase system is used to generate the oxygen radical super oxide (Fig. 2), which then reacts with the color forming nitro-blue tetrazolium (NBT) salt, a purple dye that absorbs at 560nm. Addition of SOD quenches the superoxide generated by Xan/XOD and inhibits dye formation. SOD activity is determined by the amount of inhibition of absorption at 560nm as compared to a SOD-free, uninhibited blank. One unit of SOD is defined as the amount needed to achieve 50% reduction of color formation in the blank aliquot. The % inhibition is determined using the equation:

\[
\text{EQ. 4 % Inhibition } = (1 - (\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{blank}}) \times 100
\]

The sample volume required for 50% inhibition is designated V50 and is determined using the equation:

\[
\text{EQ. 5 V50 } = \text{volume in assay (0.05mL)} \times (50/\text{%Inhibition in assay})
\]

The specific activity of an enzyme is calculated in units of activity per mg protein.
EQ. 6 Specific Activity (Units SOD/mg protein) = V50/protein concentration (mg/mL)

In order to perform bulk assays on large sample numbers, the assay was further modified for use in a high throughput microplate format, following the protocol of Janknegt 2007. Briefly, a 50uL aliquot of either sample or blank was added to a total volume of 200uL assay buffer containing 50mM KPO₄, 0.1mM EDTA, 0.01mM NBT and 0.05mM Xanthine. The plate was read before the reaction is initiated in order to have a “blank” reading of the pigment containing extracts. This will later be subtracted from the final values in order to account for any pigments absorbing at 560nm. The reaction is initiated upon addition of 0.01Units Xanthine Oxidase and color is allowed to develop at room temperature for twenty minutes. A bovine SOD standard was generated as a positive control and as proof of principle (Fig. 3).

Domoic Acid ELISA

Domoic Acid (DA) was analyzed by enzyme linked immunosorbant assay (ELISA) kit from Mercury Bioscience (DAK-36). Absorbance of the horseradish peroxidase reporter product was read at 450nm on a Molecular Devices Thermomax microplate reader.

Hydrogen peroxide quantification

Extracellular Hydrogen peroxide (H₂O₂), was measured by passing an aliquot of culture through a 2um syringe filter and collecting the effluent. Hydrogen peroxide reacts with Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), (Invitrogen product#A22188) in a 1:1 stoichiometry to
produce highly fluorescent resorufin in the presence of horseradish peroxidase. Fluorescence of resorufin was excited at 544 nm, and the emission was recorded at 590 nm by a f-max Fluorescence Spectrophotometer (Molecular Devices).

*Methyl Viologen as an Agent for Photochemical Stress Induction*

Phytoplankton in native environments are constantly exposed to fluctuations in physical factors and especially fluctuations of light intensities, as during mixing of subsurface layers to near surface. Reactive oxygen species (ROS) are generated as a consequence, especially when the light intensities reach heights that drive electron transport at rates greater than that of reducing power during CO2 fixation. To provide a standardized proxy of ROS build up, as would be elicited during periods of heightened electron transport, *Pseudo-nitzschia* cultures were grown in the presence of methyl viologen (MV) (Fig. 4). MV acts as an artificial electron acceptor that elicits a strong oxidative stress in cells as it intercepts electrons from photosystem I, diverting the electrons flow from the electron chain to produce superoxide, a very potent ROS. MV can participate in cyclic reduction-oxidation reactions in biological systems. Upon receiving a single electron reduction, a free radical is formed, which is immediately oxidized by molecular oxygen, generating a superoxide anion radical. MV is reoxidized and capable of accepting another electron, continuing the electron transfer process. High levels of MV will generate levels of superoxide that become damaging and/or lethal to the cell as well as halting the photosynthetic process. Lesser amounts of superoxide are reduced to the less damaging ROS, hydrogen peroxide, H2O2.

A precedent for use of MV as an oxidative stress inducer in photo-active cells exists in the literature. MV, known by the commercial name Paraquat, is used in the field as an herbicide.
Examples exist in both plants and phytoplankton for experimental use (Table III). Numerous studies of mycrocystin toxin induction in the freshwater cyanobacteria _Mycrocystis aeruginosa_ exist to test the relationship between light (light fluctuation has been demonstrated to attenuate toxin levels in this species) and ROS levels, using MV as an artificial inducer of ROS (Sevilla, 2012, Kaebernick, 2000, Ross, 2006). The manufacture and release of dimethyl sulfide as a response to oxidative stress has been studied in the coccolithophore _Emiliania huxleyi_, where again, MV was used as the artificial ROS inducer (Fernandes, 2012, Evans, 2006). In addition to experiments that have surveyed oxidative stress induction and toxin production, MV has been used to evaluate stress responses in mutant strains of the picoplanktonic marine cyanobacteria _Synechococcus_ (Stuart, 2013) and the role of ROS signaling in transcriptional regulation of siderophore production in the cyanobacteria _Anabaena_ (Jeanjean, 2008). In addition to algae, MV is used sometimes used to evaluate stress tolerance in plants (Noctor, 2016).

The dose of MV used varies widely depending on the target organism and the intent of the experiment (Table III). For the current study, the goal was utilize MV to induce a non-lethal oxidative stress in a range _Pseudo-nitzschia_ species of varying cell sizes.

**Methyl Viologen Stress Experiments**

Internal cellular ROS stress was generated using Methyl viologen (N,N′-dimethyl-4,4′-bipyridinium dichloride, C12H14Cl2N2){\textregistered}(Sigma, M-2254). MV dose response was assessed in a pilot study and 10uM was determined to be an optimal amount for an acute stress induction that was not lethal. Additionally, a low dose 1uM concentration was chosen to assess a longer term, low dose acclimated response. For the high dose acute experiments, cultures were grown in replicate of n = 4 each, control and MV treatment. Samples were collected and matched at 0 and
24hr timepoints. For the acclimated low dose experiment, cultures were grown in replicate of n = 3 each, control and MV treatment and sampled at various timepoints along an entire growth curve.

**Taxonomic survey of stress management**

Baseline SOD activity and general antioxidant capacity were assessed in a sampling of 42 isolates comprised of six unique species, isolated from Monterey Bay, CA in 2015. A larger sampling of individuals was initially screened for DA content and the subset of 42 was selected to represent both toxin positive and toxin negative isolates. After selection, single isolates were grown in triplicate 250mL culture under standard conditions. Aliquots of 50mL subsamples were collected onto 1.2um polycarbonate filters which were then flash frozen in liquid Nitrogen and stored at -70°C until further use.

**ABTS- An Assay for General Antioxidant Capacity**

A microplate-adapted ABTS/Trolox assay was used to measure the total antioxidant activity of cell extract (Re, 1999). 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS, is oxidized by potassium persulfate, K₂S₂O₈, to yield a blue/green chromophore. Addition of antioxidant causes the reduction of the radicalized ABTS and a decreased absorption at the chromophore absorption maxima (405nm).

\[ \text{EQ 7. } ABTS + K_2S_2O_8 \rightarrow ABTS^* + (\text{blue/green}) \]

\[(ABTS^* + ) + \text{antioxidant} \rightarrow ABTS (\text{reduced absorption 405 nm})\]
ABTS was added to \( \text{K}_2\text{S}_2\text{O}_8 \) in a 1:0.5 stoichiometry. 50 uL sample aliquots were added to microplate wells containing ABTS/ \( \text{K}_2\text{S}_2\text{O}_8 \) reagent and allowed to incubate at room temperature for 20 minutes before final absorption values were read at 405nm. Trolox, a water soluble vitamin E analogue, is used as a reference of antioxidant activity. A 6-point standard curve of Trolox, ranging from 0 to 0.42 mM, was generated and assayed alongside samples (Fig. 5). The antioxidant capacity of samples is reported in units of Trolox Equivalents and normalized on a per cell basis.

_Domoic Acid Certified Reference Material_

Pure Domoic Acid was spiked into either a SOD assay or a solution of hydrogen peroxide to test the potential for DA antioxidant properties. NRC-CRM-DA-e, Domoic Acid reference standard was obtained from the National Research Council of Canada, Certified Reference Material Program.
Results

*Excess photon energy increases DA production and induces an Oxidative Stress Response*

Simple irradiance shift-up experiments were conducted to assess whether DA production is in-part regulated by photochemical stress. Cell abundance and growth rate were not affected by an increase in irradiance over a 48 hour time period as compared to cultures grown under standard light conditions (Table IV). Subjecting toxin positive *Pseudo-nitzschia australis* culture to 4-fold increased irradiance resulted in a roughly 5-fold increase in cellular DA production at both 24 and 48 hour time-points (127 fg DA cell⁻¹ Control, 652 fg DA cell⁻¹ High Light and 86 fg DA cell⁻¹ Control, 418 fg DA cell⁻¹ respectively). There was a significant irradiance effect on the amount of DA produced (ANOVA, F₁,₁₄ = 5.504, P = 0.0342) (Fig. 6). Increased toxin production under increased irradiance was consistent with induction of oxidative stress metabolism as exhibited by a concurrent, though non-significant, rise in SOD activity (T₂₄Con = 79.12 Units SOD/mg protein +/- 27.2 T₂₄HL = 146.05 Units SOD/mg protein +/- 55.50, T₄₈Con = 51.60 Units SOD/mg protein +/- 16.27, T₄₈HL = 113.90 Units SOD/mg protein +/- 56.89) (Fig. 7) and a decreased electron transport rate (ETR) in those cultures subjected to high light conditions (T₂₄Con = 45.74 µmol photons m⁻² s⁻¹ +/- 7.14 T₂₄HLs = 9.90 µmol electrons m⁻² s⁻¹ +/- 8.24, T₄₈Con = 61.08 µmol electrons m⁻² s⁻¹ +/- 5.25, T₄₈HL = 28.56 µmol electrons m⁻² s⁻¹ +/- 7.29) (Fig. 8, 9, 10, 11). There was a significant effect of ETR produced for the light conditions tested (ANOVA, F₂,₁₃ = 20.9698, P < 0.0001). Applying the ETR curve fit from Eq. 5, the expected electron transport rate of a culture grown under standard conditions (100 µmol photons m⁻² s⁻¹) when pulsed with 400 µmol photons m⁻² s⁻¹ irradiance, will be 40.85 µmol photons m⁻² s⁻¹ (Fig, 9, 11). However, using the observed data of the cultures grown under 400 µmol photons m⁻² s⁻¹
for a period of 24 hours, and fit to the same curve, the actual value was an average of 9.9 µmol photons m⁻² s⁻¹ (Fig. 10, 11). This is a 4-fold reduction in electron transport efficiency, corresponding to a 4-fold excess of irradiance. This confirms that the increased irradiance was in fact, an excess of irradiance. Saturating irradiance was calculated for all treatments and time points (Fig. 12, 13, 14). There was a significant effect of saturating light intensities produced for the light conditions tested (ANOVA, F₂,₁₃ = 4.4605, P = 0.0335). Saturation irradiance, Eₖ increased during culture growth at standard control irradiance (521.64 +/- 70.46 µmol photons m⁻² s⁻¹ and 737.35 +/- 144.73 µmol photons m⁻² s⁻¹ for 24 hours and 48 hours respectively) (Fig. 12, 14). Following 24 h exposure to the high irradiance treatment, Eₖ was significantly reduced (216 +/- 152.32 µmol photons m⁻² s⁻¹ although by 48 hours Eₖ of high light cultures increased to (635.16 +/- 196.14 µmol photons m⁻² s⁻¹) not significantly lower than the control value, suggesting an irradiance induced lag in photoacclimation (Fig. 13, 14).

Chemically Induced oxidative stress: Methyl Viologen Effects on Domoic Acid production and Pseudo-nitzschia physiology

Initial experiments revealed a 5-fold increase in cellular DA production using a 10uM MV dose with Pseudo-nitzschia australis (Fig. 15). This MV dose level, though growth limiting relative to controls (31%), was sub-lethal over a 48hour time scale and was selected for its ability to induce a stress without complete loss of viable culture.

10uM MV Acute (24 hour) experiments
The pre-determined 10uM MV dose was applied to a range of *Pseudo-nitzschia* species and effect assessed after 24 hours of exposure to the herbicide. The immediate response of cells was measured 4 hours after illumination in the presence of the MV treatment. Hydrogen peroxide production was significantly enhanced after MV treatment in two out of six species. The hydrogen peroxide production was paralleled with a marked decrease in photosynthetic efficiency at the same time point. Though growth was repressed in all species tested, only three species; *Pseudo-nitzschia* pungens, *Pseudo-nitzschia* fraudulenta and *Pseudo-nitzschia* hemii; showed an actual decline in cell abundance (Fig. 16, 17). Accumulation of cellular protein content was not inhibited by treatment with MV (Fig, 16, 18). All MV treated isolates of *Pseudo-nitzschia* australis showed a 24hr increase in ng/cell protein concentration on par with increases seen in control cultures. *Pseudo-nitzschia* fraudulenta showed a significant increase in protein content per cell after 24 hour MV treatment, while *Pseudo-nitzschia* deli and *Pseudo-nitzschia* pungens responded to MV treatment with a slight decline in cellular protein content. SOD activity presented as low in almost all cases, and when it did rise in response to MV treatment, the effect was not consistent across isolates of the same species (Fig. 19, 20). In DA positive isolates, cellular DA content did not increase after treatment with MV but production did not entirely cease (Fig. 16, 21). For those species isolates that had no or non-detectable DA accumulation in standard culture conditions, *Pseudo-nitzschia fraudulenta, Pseudo-nitzschia hemii* and *Pseudo-nitzschia delicatissima*, DA levels were all at or below the limit of the ELISA assay detection. *Pseudo-nitzschia pungens* baseline production was above the limit of detection (assay limit of detection = 0.3 ppb Domoic Acid), but low. In these experiments, confirmed DA producing isolates were represented by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. In only one isolate of *Pseudo-nitzschia australis* could an increase in cellular DA,
(cDA) be detected after treatment with MV, but even here the effect was non-significant.
Samples were taken for dissolved DA (dDA) in all experiments, but it was never detected above
the limit of the ELISA assay detection (data not shown) indicating little export of available cDA
in response to MV treatment. Statistical tests and results are summarized in Table V. Samples of
the six species were tested at 0, 4 and 24 hour timepoints for extracellular hydrogen peroxide
generation. *Pseudo-nitzschia pungens* and *Pseudo-nitzschia hemii* generated a dramatic spike in
extracellular H$_2$O$_2$ production 4 hours after inoculation with 10 uM MV (2,000-3,000nMoles/L)
(Fig. 22), that decreased by an order of magnitude when measured at the 24 hout time-point. All
species except for *Pseudo-nitzschia delicatissima*, had generated small (<1000 nmoles/L) but
significant quantities of H$_2$O$_2$ when measured at the 24 hour time-point. Additionally, a post 4
hour treatment, dark adapted PAM fv/fm reading was taken (Fig 23). All species except *Pseudo-
nitzschia delicatissima* had a significant reduction in fv/fm values, with *Pseudo-nitzschia
pungens* and *Pseudo-nitzschia hemii* having the greatest reduction, likely associated with H$_2$O$_2$
generation.

*Acclimated response to chronic oxidative stress*

In order to compare the acute response of a non-lethal but high MV dose to a low but prolonged
doze of MV, cells were given 1/10$^{th}$ (1uM MV) the chemical dosage, and allowed to grow over a
full exponential to stationary phase cycle. Cell growth was not statistically different between
cells grown under standard conditions and cells treated with 1 uM MV (Fig. 24). Although cDA
trends in control and treated cells were in opposition (Fig. 25), they were not statistically
significant. Treatment with 1uM MV actively repressed SOD activity at 24 hours and then
showed no difference between control cells at further time points (Fig. 26). At culture irradiance, 1 uM MV treated cells exhibited a reduced ETR (Fig. 27), with increased significance at longer exposure times. Saturating light, Ek, similarly trended toward reduction after treatment (Fig. 28).

Species Specificity in Stress Capacity

Basal stress management capacity was tested among a pool of field isolates representing six unique species. A broad range in DA toxicity phenotypes was present among the six species studied, and of those species who tested toxin positive, an additional range of DA production levels existed within those groups as well (Fig. 29). Stress levels were assessed using two spectrophotometric assays; one to assess total antioxidant capacity (ABTS) and one to assess super-oxide dismutase (SOD) activity. The assays were applied to cells harvested from nutrient replete, exponential phase cultures, grown under standard light conditions (100 µmol electrons m\(^{-2}\) s\(^{-1}\)). An assay for general antioxidant capacity of the soluble fraction did not show an inherent species specific difference (Fig. 30) in antioxidant abilities, though a slight positive trend did exist for a correlation between DA content and inherent antioxidant potential (Fig. 31). When a linear fit model was applied to individual groups of toxin producing species, *Pseudo-nitzschia australis* and *Pseudo-nitzschia multiseries* exhibited a positive and significant correlation between antioxidant capacity and cellular DA content (*Pseudo-nitzschia australis*: ANOVA, F\(_{1,17}\) = 4.85, P = 0.04, n = 19, *Pseudo-nitzschia multiseries*: ANOVA, F\(_{1,5}\) = 7.96, P = 0.04, n = 7) (Fig. 32). Among the 42 isolates tested, there was a species specific statistical difference in baseline SOD activity (ANOM, F\(_{5,36}\) = 5.55, P = 0.0007, n = 42) (Fig. 33), but it did not correlate with DA content (Fig. 34, 35).
Based on the observation that antioxidant capacity and DA cellular content were positively correlated, two experiments were performed to test the function of DA as an antioxidant. Purified reference Domoic Acid was spiked in to a SOD standard. Neither an additive nor a synergistic effect was observed upon addition of DA to SOD (Fig. 36). Additionally, DA was spiked into a solution of hydrogen peroxide. No H$_2$O$_2$ quenching was observed as measured by resorufin fluorescence (Fig. 36).

**Discussion**

In order to exploit shared resources in a seemingly homogenous environment, phytoplankton have evolved distinct traits to gain advantage over co-occurring species. Margalef, in his seminal 1979 paper, defined a performance index of survival adaptations in phytoplankton based on key environmental and physical variables, (namely: response to advection and turbulence, presence or absence of vertical transport and functional morphology of the phytoplankton). The functional traits that make up a phenotype complement the niche for that organism to occupy. Reynolds expanded on this line of reasoning, in freshwater algae, to include the defining of ‘functional groups’ of algae, the grouping of algae based on functional survival adaptations (Reynolds, 1980, 2002). Key phytoplankton specializations are identified in the areas of motility, nutrient acquisition, nutrient requirements and light harvesting adaptations (response to light). Based on this model, environmental variables can be assessed and best estimates can be made as to what population of species are most adapted and therefore most likely suited to inhabit that environment. In the marine environment, near homogenous blooms of HAB forming species are found to employ adaptations from across the full scope of these categories, and it is
hard to define a potential HAB occurrence by species functional traits alone. Future oceans are predicted to be warmer, more acidic, more prone to eutrophication and to face continued exposure to anthropogenic influence in the form of agricultural and urban effluent (Hoegh-Guldberg and Bruno, 2010). It is possible and even likely that increased HAB events will become the new-normal for future oceans (Moore, 2008). It will become increasingly important to interpret and predict the response of HAB forming species to environmental stresses (Hallegraeff, 2010). This study identified enhanced photochemical stress tolerance in toxin producing species of the HAB forming diatom, *Pseudo-nitzschia*, and implicates the presence of a physiological survival adaptation in this genus favoring the toxic phenotype under conditions of environmental stress.

Multiple theories exist to explain the physiological role of DA and the environmental factors that regulate its production in *Pseudo-nitzschia* species. In this project, I have attempted to unify disparate regulatory models under their common endpoint: ROS generation, testing its role as a general inducer of DA metabolism. To that end, a series of experiments were undertaken in order to initiate an artificial cellular oxidative stress in cultures of *Pseudo-nitzschia* and test the hypothesis that cDA will rise proportional to a cellular stress response. High irradiance shift-up manipulations were used to establish a connection between photo-oxidative stress and increased DA production. The results of that study demonstrate that excess irradiance will concurrently induce both an oxidative stress response and an increase in cellular DA. Methyl Viologen (N,N’-dimethyl-4,4’-bipyridinium dichloride, C_{12}H_{14}Cl_{2}N_{2}) a chemical ROS promoter, was used as a model of stress induction to assess the same parameters devoid of external influence beyond ROS induced stress. With regards to DA production, excess irradiance yielded a significant positive response from *Pseudo-nitzschia* cells while the chemical model did
not. Interestingly, the results of a taxonomic survey suggest that, among those *Pseudo-nitzschia* species tested, an innate enhanced resilience to stress exists among the genus *Pseudo-nitzschia*, which is positively correlated with their DA toxicity phenotype.

Acute, sublethal application of MV to cultures was expected to produce an oxidative stress response. Surprisingly, in no case did SOD activity rise above the control level activity. In fact, in some cases, SOD activity was diminished following MV treatment. As SOD was measured after 24 hours of MV treatment, it is possible that, even in this acute time frame, exposure to superoxide radicals caused damage to SOD at a rate that outpaced the enzyme reducing power and ultimately enzyme damage overcame nascent ability to quench ROS. Likely, so long as SOD activity is sufficient to mitigate superoxide at the site of production, survival in treated cells is equal to survival in control cells. But, as observed in this set of experiments, when damage outweighs repair, cells succumb to mortality at high rates in the acute time frame. Ultimately, SOD did not act as an ideal index of oxidative cell stress status in these experiments.

Hydrogen peroxide generation provided the clearest signifier of an oxidative stress response in at least two out of the six species monitored, *Pseudo-nitzschia pungens* and *Pseudo-nitzschia heimii*, that was paralleled by non-photochemical fluorescence quench in PAM fluorometry. *Pseudo-nitzschia pungens* and *Pseudo-nitzschia heimii* appear to be especially sensitive to MV treatment. This is independent of cell volume as *Pseudo-nitzschia pungens* belongs to the larger seriata size class and *Pseudo-nitzschia heimii* is of the smaller delicatissima size class of *Pseudo-nitzschia*. (A description and summary of cell size classes is found in Lelong, 2012, where the smaller delicatissima size class is defined by a cell width <3 µm and seriata as those species with a cell width >5 µm.) Relationally, the other small cell species of
*Pseudo-nitzschia* tested here, *Pseudo-nitzschia delicatissima*, did not show similar sensitivity to MV, affirming that cell volume was not a primary predictor of response to ROS load.

Lower MV dose (1uM vs 10 uM) challenges, also did not stimulate DA accumulation, even though a disruption of photosynthetic ETR was apparent. Cell growth was comparable between treatment and control, and cellular DA levels remained low in both groups as well, though control cells demonstrated a characteristic accumulation of cDA in the stationary phase while MV treated cells showed a decline in cDA near growth termination. Here as well, the SOD activity did not prove to be a robust stress marker. SOD activity in both groups followed a curve paralleling rates of growth and decline. The only difference in SOD activity occurred at the 24-hour time point where again, contrary to expectations, the MV treated group actually showed a significant decline, as compared to the control value, in protective activity. This is perhaps an indication of MV generated H₂O₂ inhibition of SOD, but H₂O₂ was not measured in this experimental set, so that remains a speculation. Similar to observations in the high-dose acute series of MV experiments, this could be the result of direct damage to the enzyme, even though the onslaught of ROS was lesser. The only real evidence that MV treated cells experienced a stress, similar to the high dose acute experiment series, came from the decrease in photosynthetic activity. ETR rates of MV treated cultures were marked with a non-significant decreasing trend and saturating light levels of the same group significantly decreased. By using a low-DA producing isolate, this experiment demonstrated that non-lethal enhancement of ROS does not induce increased DA production. Though capable of DA metabolism, no additional protective qualities were conferred through DA accumulation in this isolate.

It is possible that though MV is a strong generator of superoxide and subsequent cellular stress, it is a poor model to use in assessment of secondary metabolite regulation. Methyl
Viologen’s role in cyclic redox reactions in PSI is enabled by its ability to act as an alternate electron acceptor to ferredoxin. Although the dose of MV used in these experiments was attenuated so as not to completely inhibit photosynthesis, the presence of any MV in the system will necessarily compete with the flow of electrons to ferredoxin and thereby limit the yield of NADPH generated in subsequent steps. Proposed models of DA biosynthesis require both sufficient ATP and NADPH pools to support precursor biosynthesis (Pan, 1998). The observations obtained in this study indicated that toxigenic *Pseudo-nitzschia* isolates did not block DA accumulation under a MV induced stress, gives credence to the hypothesis that DA metabolism is in some way associated with stress biology. However, it may be that the choice of MV as the initiator of oxidative stress, inhibits the full capacity of the cell to manufacture DA. If DA were truly superfluous to stress acclimation, one would expect synthesis to halt completely. Further, there was no observed increase in dissolved DA pools in these experiments, thereby demonstrating that DA is not excreted in response to stress.

In lab studies, a growth limiting stress of some kind (enumerated in introduction) is often applied to cultures of *Pseudo-nitzschia* in order to illicit DA accumulation. Yet, naturally occurring blooms of *Pseudo-nitzschia* often consist of healthy appearing (and as confirmed by fv/fm), actively reproducing chains of *Pseudo-nitzschia*. Toxic blooms are often, but not always associated with a key nutrient limitation, or conditions that stray from predicted Redfield ratios (Anderson 2008, Gilbert 2011). It would seem a paradox that though toxin may be induced through stress, occurrences of DA bloom events are not limited to stress inducing conditions. For this reason, I sought to explore differences in physiological phenotypes of *Pseudo-nitzschia* species present during a recent bloom event in Monterey Bay, CA. Could it be that toxin positive species of *Pseudo-nitzschia* possess traits that enhance their fitness and allow them to out
compete other species within the genus and thereby come to near mono-specific bloom conditions? Further, as an inhabitant of the mixed layer, *Pseudo-nitzschia* benefit from rapid adaptability to a changing environment.

Field expeditions in the Monterey Bay undertaken in the spring of 2015, paralleled the course of a *Pseudo-nitzschia* HAB event. The succession of *Pseudo-nitzschia* species outlined the favoring of a non-toxic, high diversity population pre-bloom, followed by a near homogenous bloom of toxic *Pseudo-nitzschia australis* (Bowers, 2015). A library of *Pseudo-nitzschia* isolates from this bloom was generated and used here in a phenotypic survey that revealed significant differences in potential stress mitigation strategies. *Pseudo-nitzschia pungens*, isolated during pre- and early bloom conditions exhibited significantly higher baseline SOD activities, while toxin positive strains of *Pseudo-nitzschia australis* and *Pseudo-nitzschia seriata*; species that came to numerically dominate the bloom at later dates; presented a lower baseline SOD, but overall higher antioxidant capacity. The ABTS assay used here to evaluate antioxidant ability is a generic assay and so it is not clear what compounds or activities were specifically responsible for the ROS quenching seen in assay results. The potential antioxidant function of DA itself, was ruled out in several bioassays meant to clarify this possibility (Fig. 36). Further, it is unclear what advantage a high baseline SOD activity might connote, whether this is an indication of a cell that is more equipped to alleviate accumulating ROS or whether this cell is experiencing standard conditions as “stress”. The series of MV experiments clearly demonstrated that despite a higher SOD baseline, the species who most readily succumbed to induced superoxide production were the ones presumably most outfitted to quench it.

Other studies have indicated species effects on fitness and stress physiology. A toxic strain of *Pseudo-nitzschia* was found to be tolerant of UVB while non-toxic strains were highly
sensitive to the same treatment (Hargraves, 1993), though there was no rise in DA levels following UV treatment. This parallels findings presented here, where non-toxic *Pseudo-nitzschia pungens* and *Pseudo-nitzschia heimii* showed extreme sensitivity to herbicide, while toxic strains response ranged from slight to tolerant, again with no associated alteration in DA. Findings along this trend are further bolstered by a field study conducted in the Santa Barbara Chanel (Mengelt, 2005), showing *Pseudo-nitzschia* members of a DA toxic bloom had adapted enhanced resilience to UV inhibition and correspondingly, gained an ecological advantage. None of these findings directly links toxicity to antioxidant capabilities or ascribes a new role to DA, yet the association underlies most, if not all observations of DA accumulations. These observations indicate a closer look at species specific eco-physiological differences driving observed diversity and homogenous bloom patterns may be warranted. It is unknown how the occurrence of HABs will respond to the changing climate. Current thinking suggests that warming waters and increased acidification of the oceans may create conditions that favor more stress tolerant, and if findings here hold true, more toxic ecotypes (Wells, 2015).

**Conclusion**

The stated hypothesis of this thesis was that DA production is a general response to cellular stress and thus DA could be induced or attenuated through a direct ROS signal. Up regulation of DA production was induced when toxin positive cultures were grown in excess irradiance. Biomarkers of oxidative stress accompanied the increase in DA, supporting the hypothesis that excess photon pressure generates a cellular stress response. The second question asked if an artificial chemical induction of oxidative stress could mimic the physiological response of excess irradiance. The data shown here do not support that outcome. Additional data collected, suggests a correlation in enhanced cellular resilience to stress among toxic species.
References


acid in northern anchovies and California sea lions associated with an unusual mortality event. 

*Natl. Toxins* 7, 85±92.


Harmful Algae, 9, pp. 374–383


Mengelt, C and Prezelin, BB. (2005). UVA enhancement of carbon fixation and resilience to UV inhibition in the genus Pseudo-nitzschia may provide a competitive advantage in high UV surface waters. Marine Ecology Progress Series, 301: 81–93.


United States Food and Drug Administration, Fish and Fishery Products Hazards and Controls Guidance. Fourth Edition – April 2011


Table I. Enzymatic activities and gene products associated with mitigation of oxidative stress. Adapted from: Snoeijs (2011)

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<td><strong>SOD</strong> EC 1.15.1.1</td>
<td>Superoxide dismutase</td>
<td>Superoxide radical (O$_2^-$) $\Rightarrow$ O$_2$ and H$_2$O$_2$</td>
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<td><strong>CAT</strong> EC 1.11.1.6</td>
<td>Catalase</td>
<td>H$_2$O$_2$ $\Rightarrow$ H$_2$O + O$_2$</td>
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<td><strong>APX</strong> EC 1.11.1.11</td>
<td>Ascorbate peroxidase</td>
<td>ascorbate + H$_2$O$_2$ = DHA + 2 H$_2$O</td>
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<td><strong>DHAR</strong> EC 1.8.5.1</td>
<td>Dehydroascorbate reductase (DHA reductase)</td>
<td>2 glutathione + DHA $\Leftrightarrow$ glutathione disulfide + ascorbate</td>
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<td>Glutathione reductase (GSH reductase)</td>
<td>glutathione disulfide + NADPH + H$^+$ $\Leftrightarrow$ 2 glutathione + NADP$^+$</td>
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Table II. Isolates Used in Experiments. Isolate 13b, *Pseudo-nitzschia australis*, was collected in San Pedro, California. All other isolates were collected in Monterey Bay, California.

**Isolates used in SOD and ABTS survey**

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**Table III.** Examples of Methyl Viologen used in algal experiments to induce oxidative stress.

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<td>1mM</td>
<td>DMS stress induction</td>
<td>Decrease in DMS ROS stain reacts positively to addition of MV</td>
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<td>Evans (2006)</td>
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<td>Stuart (2013)</td>
<td><em>Synechococcus</em></td>
<td>5, 50, 100nM</td>
<td>Ascertain stress tolerance in genetic mutants</td>
<td>MV tolerance increased in mutants over control</td>
</tr>
<tr>
<td>Jeanjean (2008)</td>
<td><em>Anabaena</em></td>
<td>0-0.6 uM</td>
<td>Siderophore induction as a response to oxidative stress</td>
<td>Elevated ROS in siderophore lacking mutants subjected to MV</td>
</tr>
<tr>
<td>Sevilla (2012)</td>
<td><em>Microcystis</em></td>
<td>1 and 3 uM</td>
<td>Test hypothesis that myc transcripts will increase, and therefore mycrocystin will increase, with addition of MV induced ROS</td>
<td>mycD and mycrocystin decrease</td>
</tr>
<tr>
<td>Kaebernick (2000)</td>
<td><em>Microcystis</em></td>
<td>1mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ross (2006)</td>
<td><em>Microcystis</em></td>
<td>1mM</td>
<td></td>
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</tbody>
</table>
Table IV. Change in cell concentration (cells/mL) in Pseudo-nitzschia australis over 48 hours of growth as a function of growth under either standard or increased irradiance conditions. Values are mean values (+/- SE).

<table>
<thead>
<tr>
<th></th>
<th>cells/mL 24hour</th>
<th>cells/mL 48hour</th>
<th>%change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5229 +/-333</td>
<td>7216 +/-1440</td>
<td>138</td>
</tr>
<tr>
<td>High Light</td>
<td>6826 +/-3070</td>
<td>7242 +/-2020</td>
<td>106</td>
</tr>
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</table>
Table V. One-way ANOVA results for the effects of 10uM MV acute experiments on each species’ change in protein content, cell abundance, DA content and SOD activity.

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>%ngProt/cell</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pungens</td>
<td>24.597</td>
<td>1,6</td>
<td><strong>0.0026</strong></td>
</tr>
<tr>
<td>heimii</td>
<td>1.8723</td>
<td>1,6</td>
<td>0.2202</td>
</tr>
<tr>
<td>fradulenta</td>
<td>14.8207</td>
<td>1,6</td>
<td><strong>0.0085</strong></td>
</tr>
<tr>
<td>deli</td>
<td>2.272</td>
<td>1,6</td>
<td>0.1825</td>
</tr>
<tr>
<td>australis200</td>
<td>1.2756</td>
<td>1,6</td>
<td>0.3018</td>
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<tr>
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<td>2.7697</td>
<td>1,6</td>
<td>0.1471</td>
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<tr>
<td>australis229</td>
<td>1.8293</td>
<td>1,6</td>
<td>0.225</td>
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<tr>
<td>multi-series</td>
<td>4.3789</td>
<td>1,6</td>
<td>0.0813</td>
</tr>
<tr>
<td><strong>cell %yield</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pungens</td>
<td>2.6293</td>
<td>1,6</td>
<td>0.156</td>
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<tr>
<td>heimii</td>
<td>5.032</td>
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<td>0.0661</td>
</tr>
<tr>
<td>fradulenta</td>
<td>23.6276</td>
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<td><strong>0.0028</strong></td>
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<td>deli</td>
<td>2.1302</td>
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<td>0.0526</td>
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<td>0.8261</td>
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<td>3.1249</td>
<td>1,6</td>
<td>0.1275</td>
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<tr>
<td>australis229</td>
<td>2.2358</td>
<td>1,6</td>
<td>0.1855</td>
</tr>
<tr>
<td>multi-series</td>
<td>0.0906</td>
<td>1,6</td>
<td>0.7736</td>
</tr>
<tr>
<td><strong>%cDA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>df</td>
<td>P</td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td><strong>pungens</strong></td>
<td>12.5899</td>
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<td>0.0121</td>
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<td>0.4278</td>
</tr>
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<td>1.9683</td>
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<td>0.2102</td>
</tr>
<tr>
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<td>0.0933</td>
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<td>1,6</td>
<td>0.1928</td>
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<tr>
<td><strong>multi-series</strong></td>
<td>0.494</td>
<td>1,6</td>
<td>0.5085</td>
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% SOD yield

<table>
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<tbody>
<tr>
<td><strong>pungens</strong></td>
<td>0.001</td>
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<td>0.9758</td>
</tr>
<tr>
<td><strong>heimii</strong></td>
<td>0.6249</td>
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<td>0.4593</td>
</tr>
<tr>
<td><strong>fradulenta</strong></td>
<td>3.1158</td>
<td>1,6</td>
<td>0.128</td>
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<tr>
<td><strong>deli</strong></td>
<td>0.4702</td>
<td>1,6</td>
<td>0.5185</td>
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<tr>
<td><strong>australis200</strong></td>
<td>0.8835</td>
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<td>0.3835</td>
</tr>
<tr>
<td><strong>australis207</strong></td>
<td>1.0884</td>
<td>1,6</td>
<td>0.337</td>
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<tr>
<td><strong>australis229</strong></td>
<td>1.0978</td>
<td>1,6</td>
<td>0.3351</td>
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<tr>
<td><strong>multi-series</strong></td>
<td>2.9929</td>
<td>1,6</td>
<td>0.1344</td>
</tr>
</tbody>
</table>
Figure 1. Structure of Domoic Acid (commons.wikimedia.org)
Figure 2. Mechanism of Xanthine-Xanthine Oxidase mediated SOD assay (image from www.rndsystems.org).
Figure 3. Example of a % color inhibition curve generated using a bovine SOD standard.
Figure 4. Chemical structure of Methyl Viologen, MV (N,N'-dimethyl-4,4'-bipyridinium dichloride, C12H14Cl2N2).
Figure 5. Example of a typical Trolox standard curve used in antioxidant capacity assay.
Figure 6. Change in Domoic Acid concentration (fgDA/cell) in Pseudo-nitzschia australis as a function of growth under either standard or increased irradiance conditions. Horizontal Bars are median values; length of bars represent high/low of range. Time point is indicated by color and texturized bar fill represents HL treatment. Letters indicate LS Student’s t-Test comparisons. Means not sharing the same letter are significantly different.
Figure 7. Change in SOD activity (Units SOD/ mg protein) in Pseudo-nitzschia australis as a function of growth under either standard or increased irradiance conditions. Horizontal Bars are median values; length of bars represent high/low of range. Time point is indicated by color and texturized bar fill represents HL treatment.
Figure 8. Rapid Light Curves were generated following 24 and 48hrs growth under standard or High Light conditions.
Maximal Electron Transport Rate (\(\mu\text{mol electrons m}^{-2}\text{s}^{-1}\)) in *Pseudo-nitzschia australis* as a response to growth under standard irradiance conditions at 24 and 48hrs.

*Figure 9.*
Figure 10. Reduction in maximal Electron Transport Rate (µmol electrons m\(^{-2}\) s\(^{-1}\)) in Pseudonitzschia australis as a response to growth under increased irradiance conditions at 24 and 48hrs.
Figure 11. Change in maximal Electron Transport Rate (µmol electrons m−2 s−1) in Pseudo-nitzschia australis as a response to growth under either standard or increased irradiance conditions. Bars are mean values (+/− SE). Letters indicate LS Student’s t-Test comparisons. Means not sharing the same letter are significantly different.
Figure 12 Saturating Light levels ($E_k$) ($\mu$mol electrons m$^{-2}$ s$^{-1}$) in *Pseudo-nitzschia australis* as a response to growth under standard irradiance conditions at 24 and 48hrs.
Figure 13. Saturating Light levels ($E_k$) (µmol electrons m$^{-2}$ s$^{-1}$) in Pseudo-nitzschia australis as a response to growth under increased irradiance conditions at 24 and 48hrs.
Figure 14. Change in saturating light levels (µquanta electrons m\(^{-2}\)s\(^{-1}\)) in *Pseudo-nitzschia australis* as a response to growth under either standard or increased irradiance conditions. Bars are mean values (+/- SE). Letters indicate LS Student’s t-Test comparisons. Means not sharing the same letter are significantly different.
Figure 15. Pilot study to assess dose response of MV and effects potential effects on Domoic Acid production in a toxin positive *Pseudo-nitzschia* culture.
Figure 16. Raw data and summary of species growth (cells/mL and ng protein/cell) and cDA content evaluation following acute MV treatment by species at 24hrs. Bars are mean values (+/- SE).
Figure 17. Change in percent yield cell abundance (cells/mL) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours. Calculation is change in cell abundance of control or treated sample as a percentage of T₀ untreated culture. Bars are mean values (+/− SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 18. Change in percent yield protein content (ng protein/cell) in six species of Pseudo-nitzschia as a function of growth with or without 10uM MV for 24 hours. Calculation is change in protein concentration of control or treated sample as a percentage of T₀ untreated culture. Bars are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 19. Raw data and summary of species SOD activity evaluation following acute MV treatment by species at 24hrs. Bars are mean values (+/- SE).
Figure 20. Relative change in SOD activity (units SOD/ mg protein) for six species of Pseudo-nitzschia as a function of growth with or without 10μM MV for 24 hours. Calculation is change in activity of control or treated sample as a percentage of T₀ untreated culture. Bars are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 21. Change in percent yield cellular DA (fg DA/cell) in six species of *Pseudo-nitzschia* as a function of growth with or without 10uM MV for 24 hours. Calculation is change in cDA concentration of control or treated sample as a percentage of T₀ untreated culture. Bars are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 22. Change in hydrogen peroxide generation (nMoles H$_2$O$_2$/L nM H$_2$O$_2$) in six species of *Pseudo-nitzschia* as a function of growth with or without 10uM MV for 24 hours. Bars are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 23. Change in photosynthetic efficiency (fv/fm) in six species of *Pseudo-nitzschia* as a function of growth with or without 10uM MV for 24 hours. Bars are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 24. Cell abundance of control or 1μM MV treated cultures over a 7-day growth cycle of *Pseudo-nitzschia australis*. Circles are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 25. Cellular DA concentrations of control or 1μM MV treated cultures over a 7-day growth cycle of *Pseudo-nitzschia australis*. Circles are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 26. SOD activity (Units SOD/ mg protein) of control or 1uM MV treated cultures over a 7-day growth cycle of *Pseudo-nitzschia australis*. Circles are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 27. Change in maximal electron transport rate (µmol electrons m\(^{-2}\) s\(^{-1}\)) of control or 1uM MV treated cultures over a 7-day growth cycle of *Pseudo-nitzschia australis*. Circles are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 28. Change in saturating light intensity (µquanta electrons m\(^{-2}\) s\(^{-1}\)) of control or 1uM MV treated cultures over a 7-day growth cycle of Pseudo-nitzschia australis. Circles are mean values (+/- SE). An (*) indicates a significant difference between control and treatment conditions assessed using ANOVA comparison.
Figure 29. Cellular Domoic Acid concentration of field isolates collected from Monterey Bay, CA, 2015, n = 42.
Figure 30. Assay for naive antioxidant capacity (nmol Trolox Equivalents/ cell) of field isolates collected from Monterey Bay, CA, 2015, n = 42.
Figure 31. Linear fit analysis of cellular DA content vs Trolox equivalents, a proxy for cellular antioxidant capacity, in field isolates collected from Monterey Bay, CA, 2015, n = 42.
Figure 32. Linear fit analysis of cellular DA content vs Trolox equivalents, a proxy for cellular antioxidant capacity, in only the toxin positive species field isolates collected from Monterey Bay, CA, 2015, *Pseudo-nitzschia australis* n = 19, *Pseudo-nitzschia multiseries* n = 7, *Pseudo-nitzschia seriata* n = 5.
Figure 33. Assay for naïve SOD activity (Units SOD/ mg protein) of field isolates collected from Monterey Bay, CA, 2015, n = 42. Letters indicate ANOM (analysis of means) comparisons. Means not sharing the same letter are significantly different.
Figure 34. Linear fit analysis of cellular DA content vs SOD activity, in field isolates collected from Monterey Bay, CA, 2015, n = 42.
Figure 35. Linear fit analysis of cellular DA content vs SOD activity in only the toxin positive species field isolates collected from Monterey Bay, CA, 2015, *Pseudo-nitzschia australis* n = 19, *Pseudo-nitzschia multiseries* n = 7, *Pseudo-nitzschia seriata* n = 5.
Figure 36. Domoic Acid has no associated anti-oxidant properties when assayed by ability to (A) quench \( \text{H}_2\text{O}_2 \) radicals or (B) synergistically or additively enhance SOD inhibition of superoxide radicals.