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The effects of environmental stressors on oxidant accumulation by ulvoid algae and the impacts of hydrogen peroxide on larval Metacarcinus magister survival and development

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THE EFFECTS OF ENVIRONMENTAL STRESSORS ON OXIDANT ACCUMULATION BY ULVOID ALGAE AND THE IMPACTS OF HYDROGEN PEROXIDE ON LARVAL *METACARCINUS MAGISTER* SURVIVAL AND DEVELOPMENT

By

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MASTER'S THESIS

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Daniel H. van Hees

November 11, 2011

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In Partial Fulfillment
Of the Requirements for the Degree
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ABSTRACT

Current global conditions are leading to increased numbers of harmful macroalgal blooms, which have detrimental effects on the ecosystems in which they occur. Macroalgae produce harmful chemicals such as ROS (reactive oxygen species) and dopamine. These chemicals may be produced in high quantities in response to environmental stresses, such as increasing water temperature, emersion, hypoxia, and then subsequently introduced into the aquatic environment. In Washington State, little work has been done to quantify ROS accumulation by macroalgae, or investigate oxidant impacts on invertebrates. This project investigated the impacts of environmental (emersion, water temperature, hypoxia) and chemical (dopamine) stressors on oxidant accumulation in two bloom forming macroalgae (*Ulvaria obscura* and *Ulva lactuca*) in Washington State. The effects of hydrogen peroxide (H_2O_2) on *Metacarcinus magister* zoeae survival and development were also investigated.

Oxidant concentrations changed in *U. obscura* after exposure to emersion stress. Seawater with previously emersed algae accumulated oxidants at a rate that was one order of magnitude less than seawater with algae that remained submerged. Water temperature and environmental dopamine significantly changed oxidant concentrations in *U. lactuca*, but not *U. obscura.* Seawater with *U. lactuca* that were exposed to 10^{o} C seawater accumulated more oxidants than algae exposed to 15^{o} C and 20^{o} C seawater. Stage I *Metacarcinus magister* zoeae were exposed to hydrogen peroxide levels ranging from no hydrogen peroxide to 6 μM. All zoeae had similar mortality rates; those exposed to hydrogen peroxide had slightly, but not significantly higher mortalities. Zoeae in all treatments also had similar stage durations of 11.6 days. These results suggest that zoeal survival and development rate

are unaffected by accumulated oxidants in the concentrations and exposure times used in this experiment.

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CHAPTER I: INTRODUCTION

Bloom forming algae are a common occurrence in the oceans of the world. Spring algal blooms, driven by increased nutrients and warmer waters, occur annually (Sverdrup 1953). A phytoplankton bloom has the ability to impact many aspects of the ocean ecosystem. For example dimethylsulphide (DMS), produced by plankton such as *Emeliania huxleyi*, oxidizes to form molecules that act as cloud condensation nuclei, which are essential in the creation of ocean weather patterns (Charlson et al. 1987). Hypoxic conditions can result from the sedimentation of decaying algal particles (Gray et al. 2002). Phytoplankton blooms can also produce toxic chemicals, such as domoic acid (Fritz et al. 1992), which may subsequently impact the health of local predators (Baumberger Jr 2009; Wang et al. 2006). Algal blooms also impact humans both physically and economically. In 1991, there were 28 reports of paralytic shellfish poisoning (PSP) in Washington State USA, and in 1992 harmful algal blooms (HABs) did $7 million in damage to the razor clam fisheries (Hoagland et al. 2002).

Relative to phytoplankton blooms, macroalgal blooms tend to be smaller in size and more localized (Valiela et al. 1997). Blooms generally occur in areas with high amounts of nutrient loading (Schramm and Nienhuis 1996). Macroalgal blooms are generally formed by species with both high nutrient uptake as well as high growth rates (Valiela et al. 1997) and many of these species are chlorophytes. Dense aggregations of macrophytes may impact other marine vegetation and animals. For example, seaweed growth on the Great

Barrier Reef has the potential to replace corals (Bell 1992). Chlorophyte macroalgal blooms, or "green tides" often occur on both Pacific and Atlantic Coasts, with America and Asia being affected, as well as Europe (Ye et al. 2011). Due to the association between macroalgal blooms and nutrient loading, many chlorophyte blooms are located near human population centers. This association often results in human interaction with blooms. Generally, macroalgal blooms are thought to be unsightly and, depending on the species composition, can emit strong noxious odors (Frankenstein 2000). Anecdotally, a large chlorophyte bloom in Beijing Harbor in August of 2008 was cause for concern as it almost disrupted the sailing events of the Olympic Games (Liu et al. 2009).

Unlike microalgal blooms, macroalgal blooms are relatively unstudied. Work has been done to investigate the impacts that macroalgae may have on other marine plants, such as eelgrass. Macroalgal blooms have been shown to decrease eelgrass growth through shading and suffocation (den Hartog 1994). Some of the chemicals produced by chlorophyte blooms have also been investigated, both for chemical composition and impacts on marine animals (Van Alstyne and Houser 2003; Van Alstyne and Puglisi 2007). However, there are still many aspects of macroalgal blooms that require investigation, such as the impacts of macroalgal toxins on marine invertebrates. There are a number of studies that investigated the effects of macroalgal exudates on invertebrates (Johnson and Welsh 1985; Magre 1974; Wang et al. 2011) and vertebrates (Engström-Öst and Isaksson 2006). However, there are few studies that have looked at the impacts of components of those macroalgal exudates.

This study was conducted to investigate some of the potential impacts of macroalgal blooms in local Washington State. I studied two bloom-forming chlorophyte species (*Ulva lactuca* and *Ulvaria obscura*), which form blooms throughout the south Salish Sea, in northwest Washington State (Nelson et al. 2003a; Nelson et al. 2008). Both species can be found in the same sites, but occupy different tidal regions. *U. lactuca* is an intertidal alga while *U. obscura* is primarily subtidal, but can extend its range to the low subtidal (Nelson et al. 2010). Ulvoid algae produce a suite of chemicals such as DMS (Van Alstyne and Puglisi 2007), dopamine (Van Alstyne et al. 2011), and reactive oxygen species (ROS) (Collén and Pedersén 1996). Following their production, these chemicals may then be released into the marine environment (Van Alstyne and Houser 2003; Van Alstyne et al. 2006). Using field experiments, I investigated the accumulation of oxidative chemicals under different environmental conditions.

The present project also investigated some of the potential impacts that macroalgal products could have on a marine invertebrate larva. *Metacarcinus magister* is generally found in environments with mud and sand substrates (Strathmann 1987), locations that are often the sites of macroalgal blooms in the south Salish Sea (Nelson et al. 2003a). With the habitat of *M. magister* often coinciding with the occurrence of macroalgal blooms, blooms could have an effect on the ability of the larvae to survive. Therefore, I exposed stage I *M. magister* larvae to oxidant concentrations similar to those found in the algal field experiments.

CHAPTER II: THE EFFECTS OF ENVIRONMENTAL STRESSORS ON OXIDANT ACCUMULATION BY ULVOID ALGAE

INTRODUCTION

Macroalgal Blooms

Macroalgal blooms are natural phenomena in coastal marine ecosystems. However, they are now occurring with greater size and frequency on both the east and west coasts of the United States (Glibert et al. 2001; Kamer et al. 2001). They are usually comprised of species with high nutrient uptake rates and are often dominated by *Ulva spp*. (Rosenberg and Ramus 1984), as well as other related genera (e.g., *Ulvaria*). These blooms can have negative effects on coastal ecosystems. For example, in large blooms, algae can completely cover sediment layers and benthic organisms (Kamer et al. 2001). Macroalgal blooms typically last longer than phytoplankton blooms. Some chlorophyte blooms have existed for over a decade (Gordon and McComb 1989).

Eutrophication is generally thought of as the cause of many algal blooms (Anderson et al. 2002; Nelson et al. 2008; Sfriso et al. 1987) and often allows for the proliferation of marine algae (Van Dolah 2000). The main sources of nutrient inputs associated with eutrophication are sewage and fertilizer runoff. In the Venice lagoon (Italy), *Ulva sp.* proliferated in the presence of high nutrient concentrations (Sfriso et al. 1987). *Ulva intestinalis* biomass increases exponentially with increased nutrients (Lotze and Worm 2002).

Like many areas of the coastal United States, the South Salish Sea, in Washington State, may be experiencing increased algal blooms. In 1999, a majority of the reported algal blooms in the South Salish Sea were located at the southern end of Puget Sound, near the more populated areas (Nelson et al. 2008). Many of the blooms were located in areas exposed to large tidal exchanges as opposed to secluded areas where changes were minimal (Nelson et al. 2003b). In the South Salish Sea, ulvoid algae dominate both intertidal and subtidal blooms (Nelson et al. 2003b), and are generally comprised of one or a few species. *Ulva lactuca* is the predominant species in the rocky intertidal zones while *Ulvaria obscura* is more prevalent in the subtidal zone. Blooms begin to form in the spring and reach maximum biomass levels in the summer. During this time, nutrient inputs come from both oceanic sources and terrestrial runoff (Thom and Albright 1990), promoting increased algal growth.

Along with increased biomass of the bloom forming species, reduction in growth of other aquatic plants and changing oxygen conditions are a common result of macroalgal blooms. Large amounts of macroalgae can cause significant changes in the dissolved gas concentrations of seawater (Gray et al. 2002). Oxygen saturation increases during spring and summer months in intertidal macroalgal habitats (Middelboe and Hansen 2007). At sites where other aquatic vegetation was present, blooms reduced the density of nearby eelgrass beds (Nelson et al. 2003b). Around the South Salish Sea, local residents have complained about sulfurous odors from large macroalgal blooms (Frankenstein 2000; Nelson et al. 2003b).

Changes in seawater temperatures can impact macroalgal blooms. Long term increases in temperatures can increase both the length of the vegetative period and growth rate for algae (Harrington et al. 1999). Longer vegetative periods allow algae to grow more, resulting in larger algal blooms. Water temperatures in the South Salish Sea are seasonal. For example in Ship Harbor, Anacortes WA at 2.6m below mean low low water (MLLW), low temperatures occur in January, and range from 6 to 8 °C (Van Alstyne et al. unpublished data). Temperatures increase to highs between 10 and 13.5 °C in August. In June of 2006, temperatures ranged from 10-13.5 ^{o}C with daily fluctuations up to 3^{o} C.

Production of toxins by bloom-forming macroalgae

Like microalgae, macroalgae release chemicals such as oxidants and dopamine into their aquatic environments (Ross and Van Alstyne 2007; Van Alstyne et al. 2006). Algal chemicals can have toxic effects on environments and organisms. Toxins released by microalgal species worldwide create over 60,000 human sicknesses every year (Van Dolah 2000). However, little research has been done on the effects of macroalgae toxins.

Any cell that photosynthesizes creates oxidants, or reactive oxygen species (ROS) (Lesser 2006). During photosynthesis and respiration, oxygen molecules undergo a univalent reduction, resulting in oxygen intermediates such as superoxide radicals (O_2^-) and singlet oxygen ($^1O_2$) (Figure 1). As oxygen is reduced through cellular pathways in the chloroplasts, mitochondria and endoplasmic reticulum, H_2O_2 is also created (Lesser 2006). Oxidant concentrations under low stress environmental conditions are not high enough to be detrimental to the cell. Enzymatic and nonenzymatic antioxidants react with oxidants,

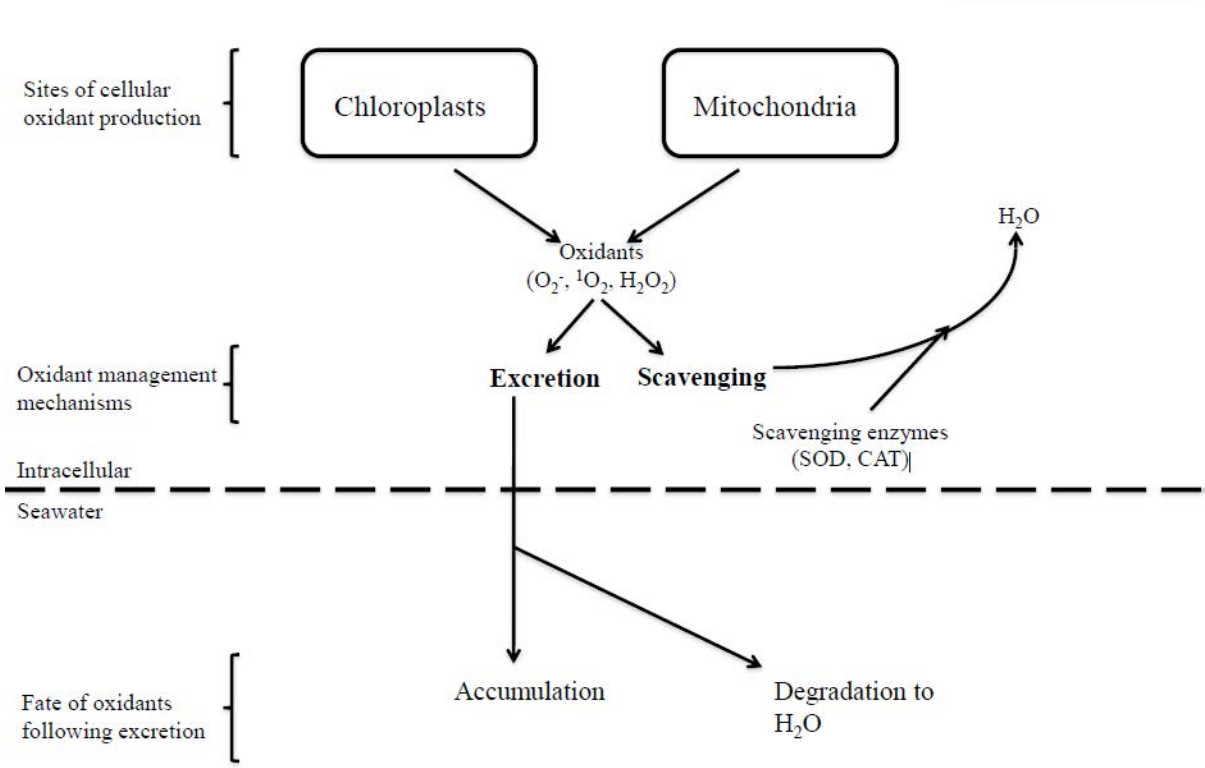

Figure 1. Macroalgal oxidants are produced at sites within algal cells. The fates of those oxidants are scavenging by enzymes or excretion into seawater. Following excretion, oxidants may accumulate or degrade into H_2O (Lesser 2006).

reducing molecules to more neutral compounds, such as H_2O. Superoxide dismutase initially reduces superoxide to H_2O_2. Ascorbate and glutathione peroxidases then convert H_2O_2 to H_2O (Apel and Hirt 2004).

One of the stresses possibly associated with an ulvoid algal bloom is oxidative stress, resulting from the accumulation of seawater oxidants that are released by physiologically stressed algae. Oxidants are produced in cellular organelles that include chloroplasts and mitochondria. When chloroplasts become hyperoxic, oxidants are produced (Lesser 2006). The majority of oxidative stress comes from the creation of OH^-, which is created by combining O_2^- and H_2O_2 (Collén and Davison 1999b). In low stress environments, oxidants are produced constantly, but are scavenged enzymatically and converted to less harmful molecules, like H_2O. In general, as organisms are exposed to increased levels of stress, oxidative stress increases as well. For example, *Ulva rigida* increased the production of H_2O_2 when it was exposed to increased irradiance (Collén and Pedersén 1996) and increased salinity (Collén et al. 1995). However, there are no measurements of the release of oxidants by bloom-forming macroalgae in Washington State.

Increased intracellular oxidant concentrations can affect algal species in negative ways because of their toxic effects on cells, including damage to lipids and DNA, and possible apoptosis (Lesser 2006). *U. lactuca* from Florida releases oxidants after periods of desiccation (Ross and Van Alstyne 2007).

Algae also have mechanisms for scavenging excess oxidants to reduce the harmful effects of ROS. Some of these mechanisms involve enzymes that convert oxidants to less harmful molecules. Antioxidant enzymes, such as catalase (CAT) and superoxide dismutase

(SOD) are present in algae at all times, but are activated when oxidant concentrations increase within the cells. Almost all chloroplasts contain SOD to break down O_2^- to H_2O_2 and O_2 (Kurepa et al. 1997). In algae, H_2O_2 is then scavenged by an ascorbate specific peroxidase (APX) (Asada 1999).

U. obscura is currently the only alga known to produce dopamine (Tocher and Craigie 1966). Dopamine not only functions as an anti-herbivore defense in *U. obscura* (Van Alstyne et al. 2001), but is a possible stressor towards other macroalgae. Compounds released by *Ulvaria* have been shown to inhibit growth in *Fucus* zygotes and growth in *U. lactuca* and *U. obscura* (Nelson et al. 2003b). Dopamine, a chemical released by *U. obscura,* may cause oxidant production (Halliwell 1992), damaging cellular proteins (Graham et al. 1978). Furthermore, dopamine has been shown to have significant genotoxic capabilities by modifying and damaging DNA (Moléus et al. 1983). Dopamine has been shown to be toxic to some marine organisms at naturally occurring concentrations (Van Alstyne et al. unpublished).

When algae are subjected to environmental stressors, oxidant production increases. Stressors shown to increase oxidant production include emersion, changes in temperature, osmotic stress, desiccation and increased UV radiation (Lesser 2006). Like many other algal toxins, the release of dopamine by *U. obscura* increases when the alga is under environmental stress (Van Alstyne et al. 2011). Intertidal algae, such as *U. lactuca*, are subjected to emersion during daily low tides. When the algae are emersed, additional oxidants are created in response to desiccation. Intertidal and subtidal *U. lactuca* have different oxidant release rates. Intertidal *U. lactuca* had greater oxidant scavenging rates

than subtidal *U. lactuca* (Ross and Van Alstyne 2007). While the algae are emersed, oxidants are contained within the algae. When the algae are again submerged in water, oxidants may be released into the seawater (Figure 1).

Goals

The main focus of this research was to determine the effects of environmental stressors on seawater oxidant accumulation by *U. lactuca* and *U. obscura.* Four possible stressors (emersion, increased temperature, environmental dopamine, hypoxia) were tested to discover the effects of the individual stressor on oxidant release. I hypothesized that increased stress levels would elicit an increase in oxidant accumulation. The secondary goal of this project was to compare oxidant accumulation by the two algae in response to the same stress levels. Since *U. lactuca* and *U. obscura* inhabit different tidal regions, I hypothesized that seawater containing *U. lactuca* would accumulate more oxidants than seawater with *U. obscura*.

Study Site

Algae for this project were collected in Ship Harbor, located on Fidalgo Island, Washington (48^{o} 30’ 22.06”N 122^{o} 40’ 24.32”W). The harbor is roughly 660m east to west, and 220m north to south. The harbor bottom has a gentle northern slope, and the bottom is primarily composed of mud and sand, with a few interspersed boulders. An eelgrass bed (primarily *Zostera marina*) occupies the middle of the harbor, and stretches from the east to the west sides. There is often a chlorophyte bloom, composed of *U. lactuca* and *U. obscura* that begins around March. The bloom is located on the west side of Ship Harbor, close to an eelgrass bed.

METHODS

Algal Collections

U. lactuca and *U. obscura* were collected either by hand in the intertidal zone, or by snorkeling in the shallow subtidal zone of Ship Harbor, WA (Figure 2) during July 2010 and transported immediately to Shannon Point Marine Center (SPMC) in Anacortes WA. As *U. lactuca* and *U. obscura* are macroscopically similar, the genus of each individual was determined by microscopic examination at SPMC. The algae were cleaned of dirt and epiphytes, blotted dry and weighed. Weighed algae samples were maintained in an outdoor flow-through aquarium overnight for use the next day.

Algae were subjected to four environmental stresses: 1) emersion 2) high seawater temperatures 3) environmental dopamine and 4) hypoxia. Each experiment was conducted in a single day and on sunny summer days with no clouds in order maintain similar environmental conditions among the four independent experiments. During emersion, all algae were exposed to direct sunlight, but experienced shading later in the day.

Emersion Experiment

U. lactuca and *U. obscura* were exposed to emersion to quantify the release of oxidants following a simulated low tide. On July 8th, 2010 (a clear summer day), 13.8 to 14.2 g of *U. lactuca* and *U. obscura* were placed in each of twenty bowls (29.0 cm diameter) on the ground on the gravel substrate behind SPMC. Algae were emersed for 0 (controls), 1, 2, or 4 hours (N=5 per treatment), representing the range of emersion periods at 1.0 ft below (MLLW) at Ship Harbor. Bowls containing algae were arranged in a

Figure 2. Organisms were collected in Ship Harbor, Fidalgo Island, WA. This map was provided by Google Earth.

randomized block design to standardize shading of the experimental area, which progressed from west to east. All bowls from each treatment were arranged in blocks in the east/west direction and blocks were parallel to each other. Each bowl was randomly positioned within the block on the gravel substrate. The four-hour emersion began at 09:15 when the test area was exposed to full sun. Subsequent treatments were started later in the day to allow immersion of all samples at the same time. At 13:15, to simulate immersion during an incoming tide, 1L of filtered (5μm) seawater (FSW) was added to each bowl.

Beginning immediately after immersion, 100μL water samples were taken from each bowl every ten minutes for the first half hour, and every 30 minutes for the next three hours. To measure oxidants, water samples were transferred immediately to a 96-well Costar black opaque microplate and inserted into a BioTek SynergyMX multiplate reader. Esterase (0.40 U/mL) and dichlorodihydrofluorescein diacetate (25 μM DCFH-DA; Invitrogen #C2938) at 6^{o} C were added to the water samples. Reagent temperatures were not monitored during this experiment. Oxidants were then quantified fluorometrically (excitation: 488nm, emission: 525 nm). Known concentrations (0-50 μM; N=3 per concentration) of commercially obtained H_2O_2 (Fisher UN2014) were used as standards.

Temperatures were monitored throughout the emersion and immersion periods every 10s with temperature probes and recorded on a HOBO Pro Series data logger. One probe was placed in a bowl containing algae while another was placed on the ground outside the bowl.

Repeated measures analyses of variance were used to compare the changes in oxidant concentrations within each bowl over time as a function of the emersion periods.

Oxidant concentrations were compared between species, among emersion lengths and within sampling times after immersion. Family-wise comparisons for species and emersion length were done with a Bonferroni correction to maintain a family-wise alpha < 0.05.

Environmental Dopamine Experiment

On July 16th, 2010, 3.4 to 3.6g *U. lactuca* and *U. obscura* were exposed to FSW containing dopamine to determine its effect on oxidant accumulation. Algae were placed in each of 20 bowls (10 cm diameter), emersed for 2 hours at 11:00 and then submerged in FSW with dopamine concentrations of 0, 200, 400, and 600 μM dopamine (Sigma #H-8502) (*N*=5 per treatment). Thirty minutes later, each bowl containing the algae was put in a larger bowl (29cm diameter) that was then filled with FSW. The large bowl was used to buffer temperature change of the water within the small bowl. Water samples were taken to measure oxidant concentration using the same techniques as those in the emersion experiment. The temperature of the reagents kept near 10° C and adjusted with ice cubes if necessary. These dopamine concentrations were within the range of dopamine concentrations that could naturally occur, given known *U. obscura* densities in the South Salish Sea (Nelson, 2003) and known dopamine release rates (Van Alstyne et al. 2011). Temperatures of bowls containing algae were monitored throughout the experiment with temperature probes and recorded on a HOBO Pro Series data logger.

Repeated measures analyses of variance were used to compare the changes within each bowl over time due to the different treatments. Oxidant concentrations were compared between species, among dopamine concentration, and over time. Family-wise comparisons for species and dopamine concentration were done with a Bonferroni correction to maintain

a family-wise alpha < 0.05. Correlations were used to examine the effects of ambient temperature on oxidant production when algae were exposed to environmental dopamine.

Water Temperature Experiment

On July 27th, 2010 (a clear summer day), 3.3 to 3.6 g of *U. lactuca* and *U. obscura*, were placed in each of 18 bowls (10.0cm diameter), emersed for two hours (starting at 10:50) and then immersed in FSW at either 10°, 15°, or 20° C (*N*=6 per treatment). Bowls containing algae were arranged in a randomized block design similar to the emersion experiment.

After immersion, bowls containing algae were randomly assigned to one of six coolers in the lab for immersion in FSW at the treatment temperature. Three bowls of each species were placed in each of two coolers per temperature. Two liters of FSW at 10, 15, or 20° C were poured into each cooler to maintain the desired temperature within the bowls containing algae. Water temperatures within each cooler were monitored and lowered with ice cubes if necessary.

Water samples were taken as described in the emersion experiment for oxidant analysis. During sample analysis, the esterase and DCFH-DA tubes on the multiplate reader were kept below 10° C by adding ice to the beaker which held the reagent tubes. The temperature of the reagents kept near 10° C and adjusted with ice cubes if necessary.

Repeated measures analyses of variance were used to compare the changes within each bowl over time due to the different treatments. Oxidant concentrations were compared between species, among water temperature and within time. Family-wise comparisons for

species and dopamine concentration were done with a Bonferroni correction to maintain a family-wise alpha < 0.05.

Hypoxia Experiment

On July 29th, 2010, 3.4 to 3.6 g of *U. lactuca* and *U. obscura* were placed in each of 28 bottles and immersed in FSW with a dissolved oxygen saturation of 60% (control) or 29% (low DO) (*N*=7 per treatment) to determine the possible effect of hypoxia on oxidant production. Algae were collected on July 28th, 2010, via snorkeling and processed as mentioned earlier. FSW was bubbled with nitrogen gas the day before the experiment for 6 hours to reduce [DO] to 29% of saturation. The water was added to 250 mL culture bottles and the bottles were capped. The same was done with the FSW control. Water samples were taken every 30 minutes for 4.5 hours and H_2O_2 was measured as described above. Reagent tubes that contained esterase and DCFH-DA were kept at or below 10° C as described in the temperature experiment. Prior to each sampling, bottles were gently agitated to evenly distribute oxidants. All bottles were kept in an incubator at 15° C on a 12 : 12 L : D cycle between samplings.

DO saturation of bottles containing algae was measured with a YSI meter every other sampling time. One randomly selected bottle from each treatment was measured for three minutes to allow equalization of the probe. The bottle was capped after DO saturation was measured.

Ulvaria photosynthesis experiment

The ability of *U. obscura* to photosynthesize after desiccation was examined by comparing to a FSW control the dissolved oxygen saturation of water containing desiccated and undesiccated *U. obscura* after incubation (N=4 per treatment). Algae were collected on August 17th, 2010 via snorkeling in Ship Harbor, WA on the morning of the experiment. Samples were cleaned, weighed and kept in a flow through seawater table until use in the experiment. Algae (3.5g per replicate) for the desiccation treatment were placed in small finger bowls, which were placed on the wood deck behind SPMC for two hours to desiccate.

After desiccation, all algae were placed in biological oxygen demand (BOD) bottles and the bottles were filled with 5µm FSW. BOD bottles filled with FSW with no algae served as controls. All bottles were placed in a large flowing seawater tank outside and incubated for two hours in direct sunlight. The water temperature in the tank was measured throughout the incubation period.

After the incubation period, the dissolved oxygen saturation (DOS) of each bottle was measured. To ensure consistent readings were taken, a small amount of FSW was added to each bottle to completely submerge the probe of the YSI meter. Once inserted, the YSI meter was allowed up to two minutes to equalize.

A univariate analysis of variance was used to analyze the differences between treatments with the algae as the independent variable and percent of dissolved oxygen saturation as the dependent variable. Pairwise comparisons were used to compare the differences between the individual treatment levels while maintaining a family-wise $\alpha < 0.05$.

RESULTS

Emersion experiment

After the algae were immersed, seawater oxidant concentrations increased significantly (ANOVAR, Pillai's Trace, df = 9, F = 704.5, $p<0.001$) over time for both *U. lactuca* and *U. obscura* in all treatments including the controls (Figure 3). The average rate of increase in seawater oxidant concentrations that contained unemersed algae was significantly greater (multiple comparisons, $p<0.001$) than that of emersed algae by at least 9.5 nmol oxidants g^{-1} fresh mass (FM) algae min^{-1} in *U. obscura*.

During the four hours following immersion, the two algal species accumulated significantly different amounts of oxidants (ANOVAR, $F_{3,32} = 32.05$, $p<0.001$). At the end of sampling, maximum seawater oxidant concentrations increased to 3.6 µmol g^{-1} fresh mass (FM) algae in *U. obscura* and 5.3 µmol g^{1} FM algae in *U. lactuca* (Figure 3).

The concentration of oxidants in seawater that contained *U. obscura* increased at significantly different rates over time (ANOVAR, Pillia's Trace $F_{9,8}=269.75$, $p<0.001$) when exposed to different emersion lengths. Seawater containing unemersed (control) algae consistently accumulated more oxidants than seawater containing algae emersed for 1 to 4 hours (multiple comparisons, Bonferroni, $p<0.001$). Oxidant concentrations of seawater with unemersed *U. obscura* were 2.7 times higher than in the three (1, 2, and 4 hour) emersion treatments at the conclusion of the experiment (Table 1). Oxidant concentrations of seawater with unemersed algae accumulated at a rate of 0.0155 µmol g^{-1} FM algae min^{-1}. All seawater with emersed *U. obscura* accumulated oxidants at a rate that was an order of magnitude lower than unemersed *U. obscura* (Figure 3). Seawater that contained *U. lactuca*

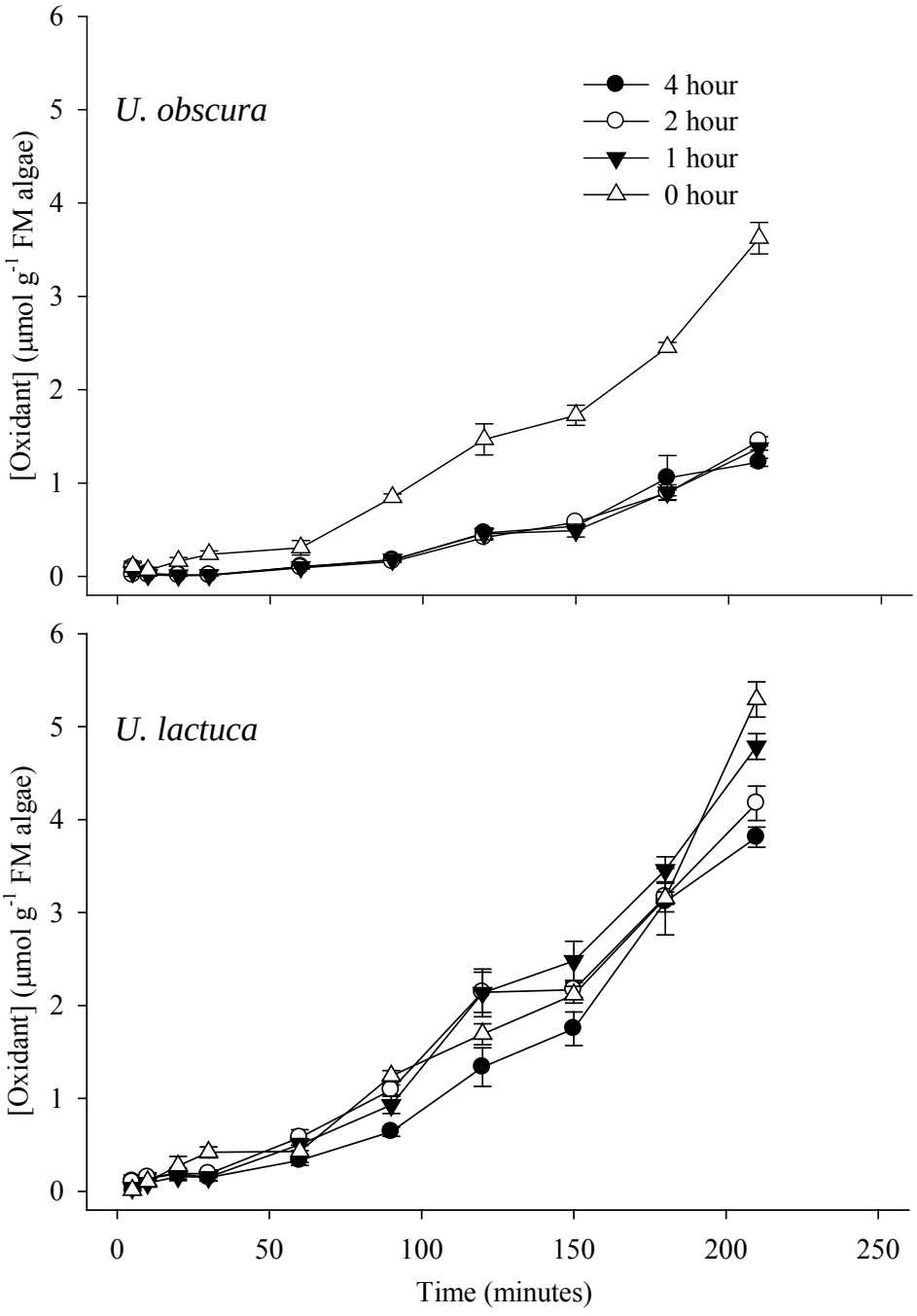

Figure 3. Concentration of oxidants (µmol g-1 FM algae) released by the subtidal *U. obscura* (top) and the intertidal *U. lactuca* (bottom) over time after immersion in FSW, following different periods of emersion. Data are means (*N*=5) +/- 1 SE.

Table 1. Final oxidant concentrations of seawater containing algae from each experiment. Oxidant concentrations and the standard error (SE) were averaged from all replicates in a given treatment.

		Ulva lactuca		*Ulvaria obscura*	
Experiment	Treatment	Final oxidant concentration (μmol oxidants/g algae)	SE	Final oxidant concentration (μmol oxidants/g algae)	SE
Emersion	0 hour	5.29	0.19	3.62	0.17
	1 hour	4.78	0.14	1.37	0.024
	2 hour	4.17	0.19	1.44	0.5
	4 hour	3.81	0.11	1.22	0.44
Dopamine	0 μM	4.09	0.05	1.77	0.31
	200 μM	2.1	0.074	1.43	0.069
	400 μM	1.3	0.047	1.42	0.15
	600 μM	1.32	0.05	0.92	0.14
Temperature	10 degree	5.04	0.49	3.1	0.11
	15 degree	3.89	0.29	3.25	0.081
	20 degree	3.47	0.11	3.25	0.14
Hypoxia	60% DO	5.55	0.078	4.9	0.28
	29% DO	5.07	0.31	5.58	0.21

accumulated similar oxidant concentrations following different emersion periods ($p > 0.05$).

At the beginning of the emersion experiment, the temperature was 37.8° C within bowls containing algae and increased to 47.0° C during 4 hours of emersion (Figure 4). After the algae were immersed, the water temperatures in the bowl dropped to 24.4° C and fluctuated slightly (+/- 3.0 ° C) over the next 3.5 hours. Air temperatures immediately above the gravel dropped to a low of 22.5° C 180 minutes after the algae were immersed.

Dopamine exposure experiment

After algae were immersed in seawater containing dopamine, seawater oxidant concentrations increased over time in both *U. obscura* and *U. lactuca* (ANOVAR, Pillai's Trace, df=9, $F_{9,24}$=136.9, $p<0.001$) (Figure 5). Final oxidant concentrations of seawater lacking dopamine were as much as 2.3 times higher in *U. lactuca* than in *U. obscura* treatments (Table 1).

Seawater containing *U. lactuca* and no dopamine accumulated significantly more oxidants (Multiple comparisons, Bonferroni, $p<0.01$) than seawater containing algae exposed to dopamine. Oxidant accumulations in seawater containing *U. obscura* were statistically indistinguishable between the three dopamine treatments and the control (Figure 5). Oxidant concentrations of seawater that contained *U. obscura* changed quadratically (Contrasts, df = 3, $F_{3,16}$ = 4.06, $p=0.025$) and increased through 150 minutes and then decreased (Figure 5).

Seawater with *U. lactuca* that contained any amount of dopamine accumulated significantly less (ANOVAR, df=3, $F_{3,16}$=16.1, $p<0.001$) oxidants than algae in FSW alone. Final oxidant concentrations of seawater that contained *U. lactuca* exposed to dopamine

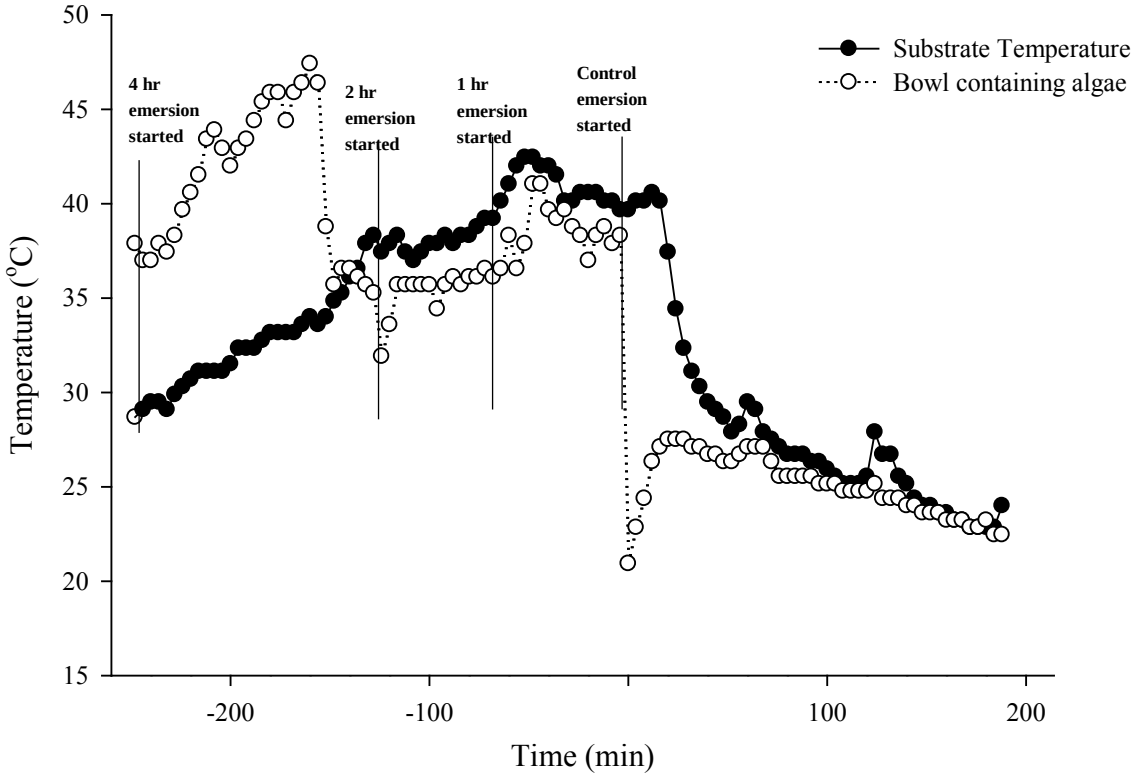

Figure 4. The temperature (in ^{o}C) for both the gravel substrate and emersion bowl environments measured during the emersion experiment. Measurements were taken during the algal desiccation period, which started at 9:15 PST (-248 minutes), and the sample period, which started at 13:15 PST (zero minutes). Vertical lines mark the beginning of an emersion period, with the length of emersion noted above.

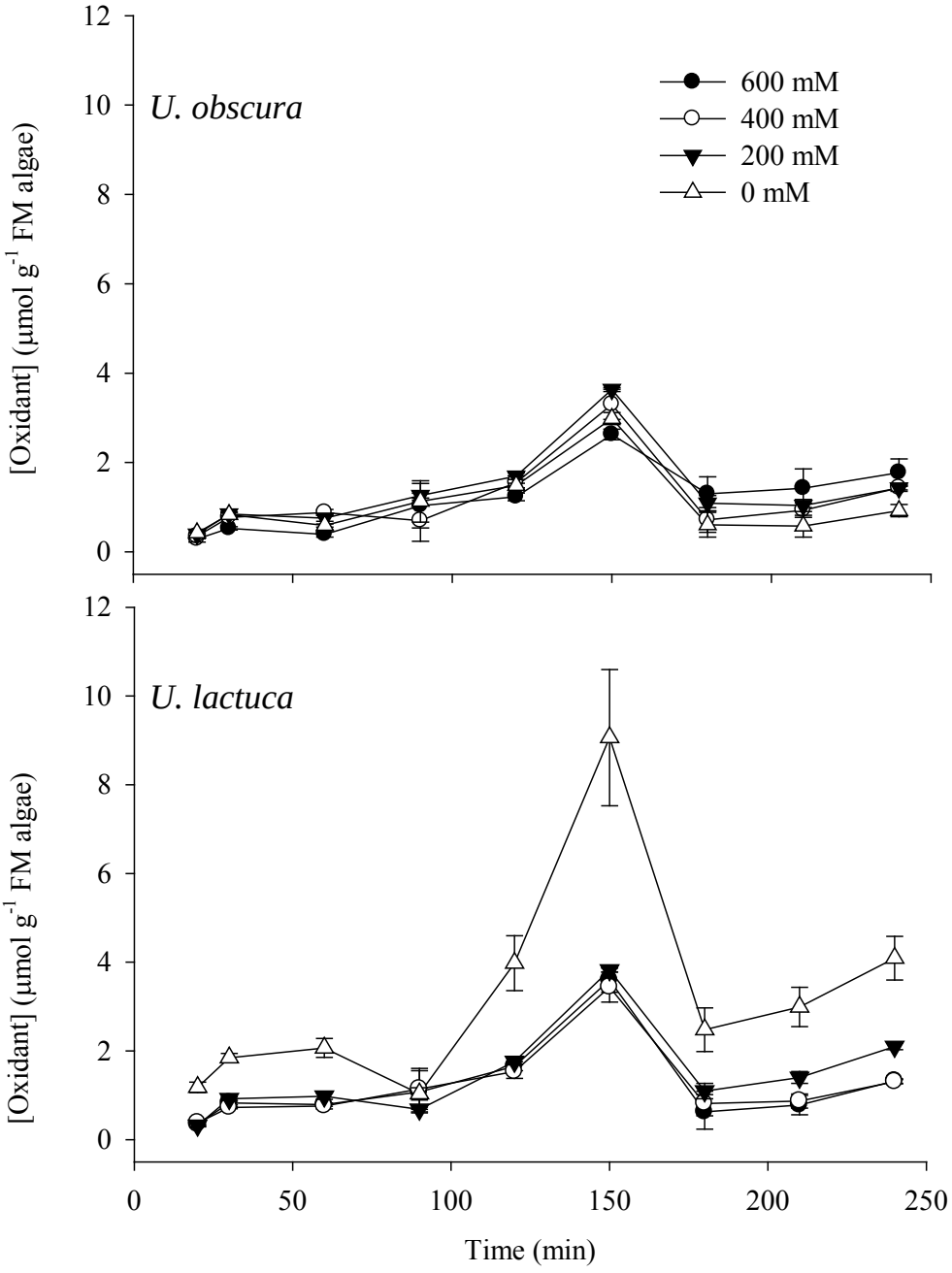

Figure 5. Concentration of oxidants (µmol g^{-1} FM algae) released by the subtidal *U.obscura* (top) and the intertidal *U. lactuca* (bottom) over time after immersion in FSW that contained different dopamine concentrations, following a two hour emersion. Data are means (*N*=5) +/- 1 SE.

were a maximum of 51% (found in the 200 μM treatment) of the control oxidant concentrations (Multiple comparisons, $p<0.001$) (Table 1).

During the dopamine experiment, air temperatures immediately above the gravel during the 2 hour desiccation increased from 28.7° to 33.2° C. Temperatures within the bowls increased from 31.1° to 40.1° C (Figure 6). Following immersion, the water temperature decreased to minimum of 19.8° C after 3.5 hours. During this same time, air temperatures decreased to 17.1° C.

Temperature exposure experiment

After emersed algae were exposed to different seawater temperatures, oxidant concentrations of seawater that contained both *U. obscura* and *U. lactuca* increased significantly over time (ANOVAR, Pillai's Trace, df=9, $F_{9,22}=265.7$, $p<0.001$) (Figure 7). Seawater that contained *U. lactuca* accumulated significantly more (ANOVAR, $F_{1,30}=19.14$, $p<0.001$) oxidants than *U. obscura* for all seawater temperatures. Final oxidant concentrations for seawater with *U. lactuca* were between 1.1 and 1.6 times the concentrations of *U. obscura* (Figure 7, Table 1). There was similar accumulation of oxidants in seawater of different temperatures that contained *U. obscura.*

Seawater with *U. lactuca* accumulated significantly less (ANOVAR, df=2, $F_{2,15}=7.06$, $p<0.01$) oxidants when immersed in seawater of higher temperature (Figure 7). Algae immersed in 10° C seawater accumulated oxidants at a rate of 4.2 μmol g^{-1}FM algae hr^{-1} and algae in 20° C water by 3.3 μmol g^{-1}FM algae hr^{-1}. Oxidant concentrations of seawater that contained *U. lactuca* exposed to 15° seawater averaged 77% of control (100%) oxidants while algae in 20° water averaged 68% (Table 1).

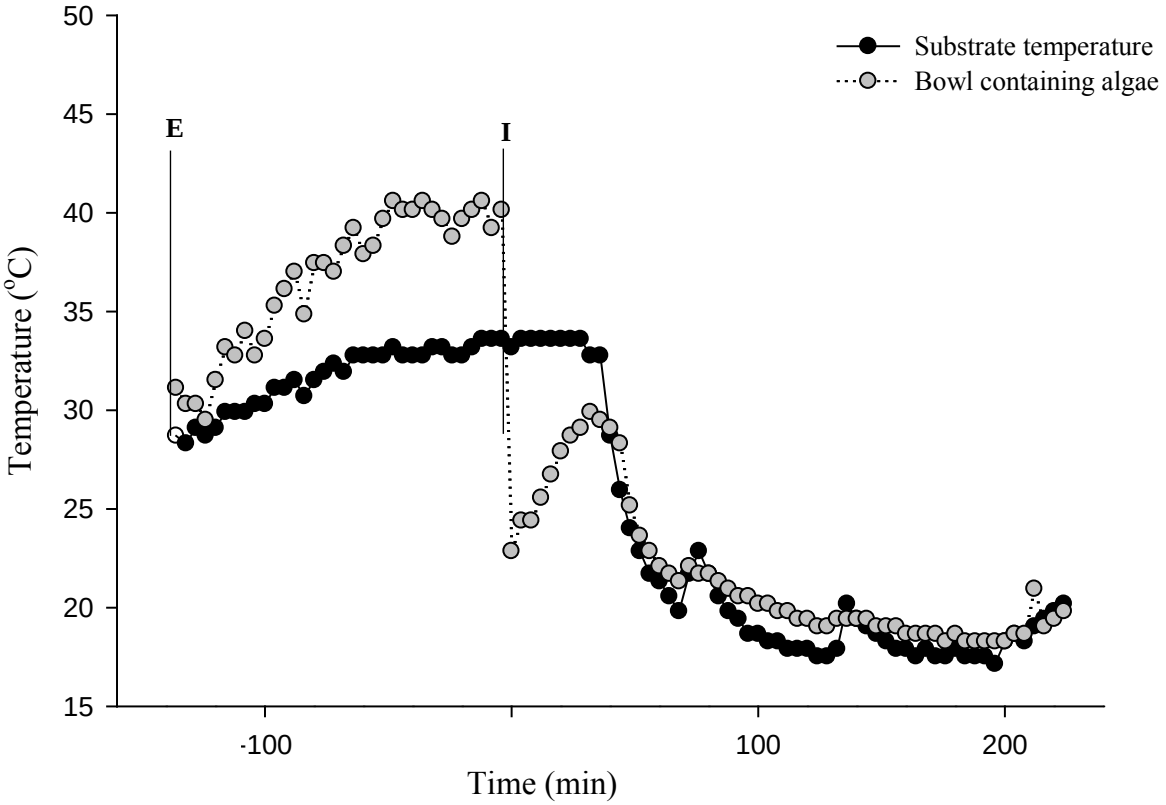

Figure 6. Temperature (in ^{o}C) for both the gravel substrate and bowls containing algae environments measured during emersion and dopamine exposure. Emersion began at 11:00 PST (-136 minutes) (noted with an E) and immersion occurred at 13:00 PST (zero minutes) (noted with an I).

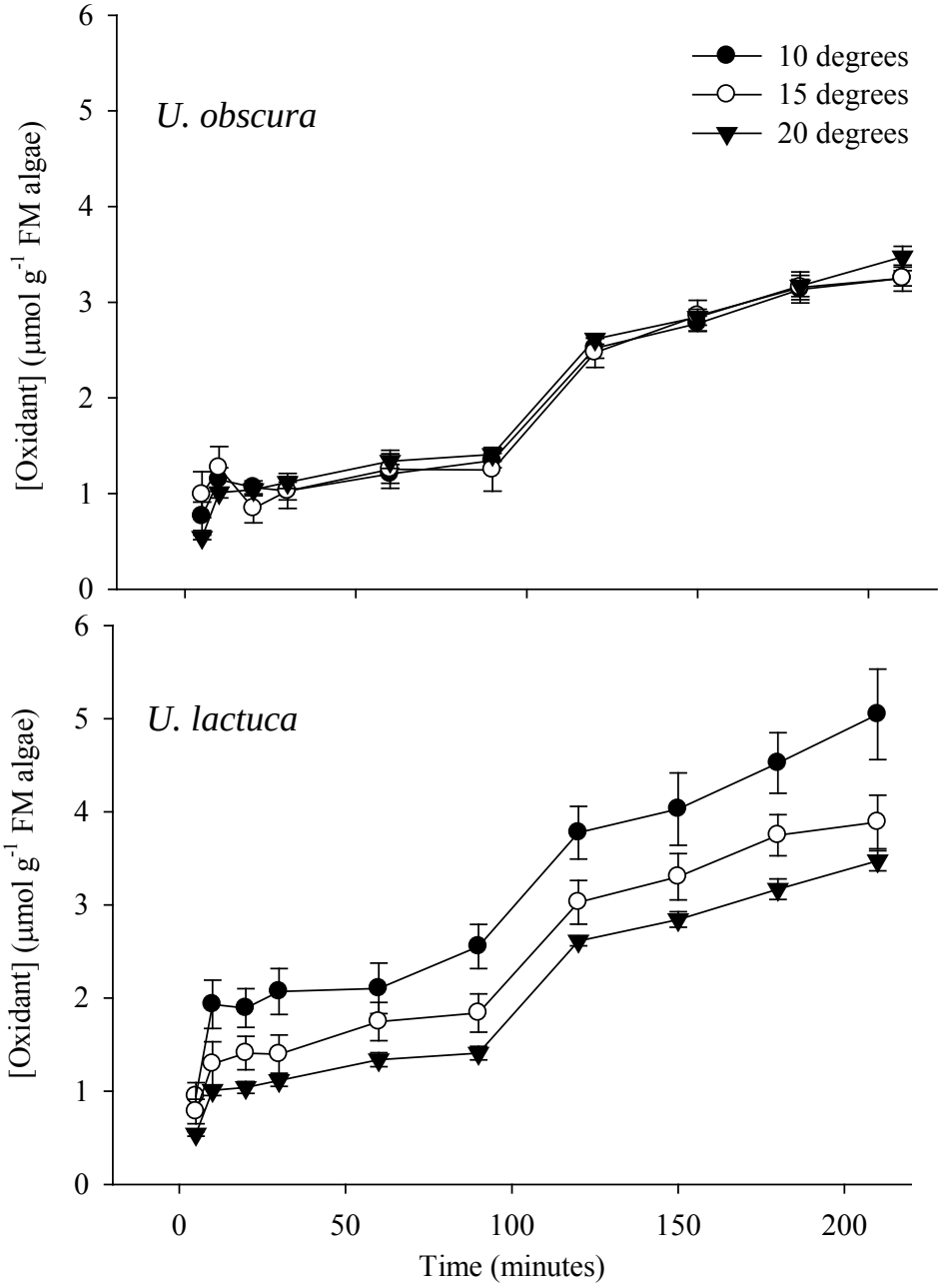

Figure 7. Concentration of oxidants (µmol g^{-1} FM algae) released by the subtidal *U. obscura* (top) and the intertidal *U. lactuca* (bottom), over time following exposure to filtered seawater of three different temperatures. Data are means (*N*=5) +/- 1 SE.

Hypoxia exposure experiment

When algae were immersed during exposure to hypoxic conditions, initial dissolved oxygen saturation (DOS) of the FSW control was 60% and 29% for low DO bottles. Seawater oxidant concentrations increased significantly (ANOVAR, Pillai's Trace, df=9, $F_{9,16}$=380.7, $p<0.001$) over time when both *U. lactuca* and *U. obscura* were immersed in water with different DOS. Seawater with *U. lactuca* and seawater with *U. obscura* accumulated similar amounts of oxidants (Figure 8). Seawater oxidant concentrations increased by 8.0 nmol g^{-1}FM algae min^{-1} in bottles with *U. obscura* and 9.6 nmol g^{-1}FM algae min^{-1} for *U. lactuca* during the first 180 minutes of sampling.

Ulvaria obscura photosynthesis experiment

Bottles containing control algae accumulated more gas bubbles over the 2 hour incubation than bottles with algae that had been desiccated (Figure 9). The water in bottles containing desiccated algae also turned a dark red color while the bottles with control algae showed no color change. The FSW control bottles showed no visual change. Emersion significantly decreased (ANOVA, df=2, $F_{2,9}$=3048.3, $p<0.001$) the DOS of seawater containing *U. obscura*. Bottles that contained emersed algae had a DOS that was 44.3% lower than the FSW control (Figure 10) while the DOS of seawater in bottles with unemersed algae was 155% higher.

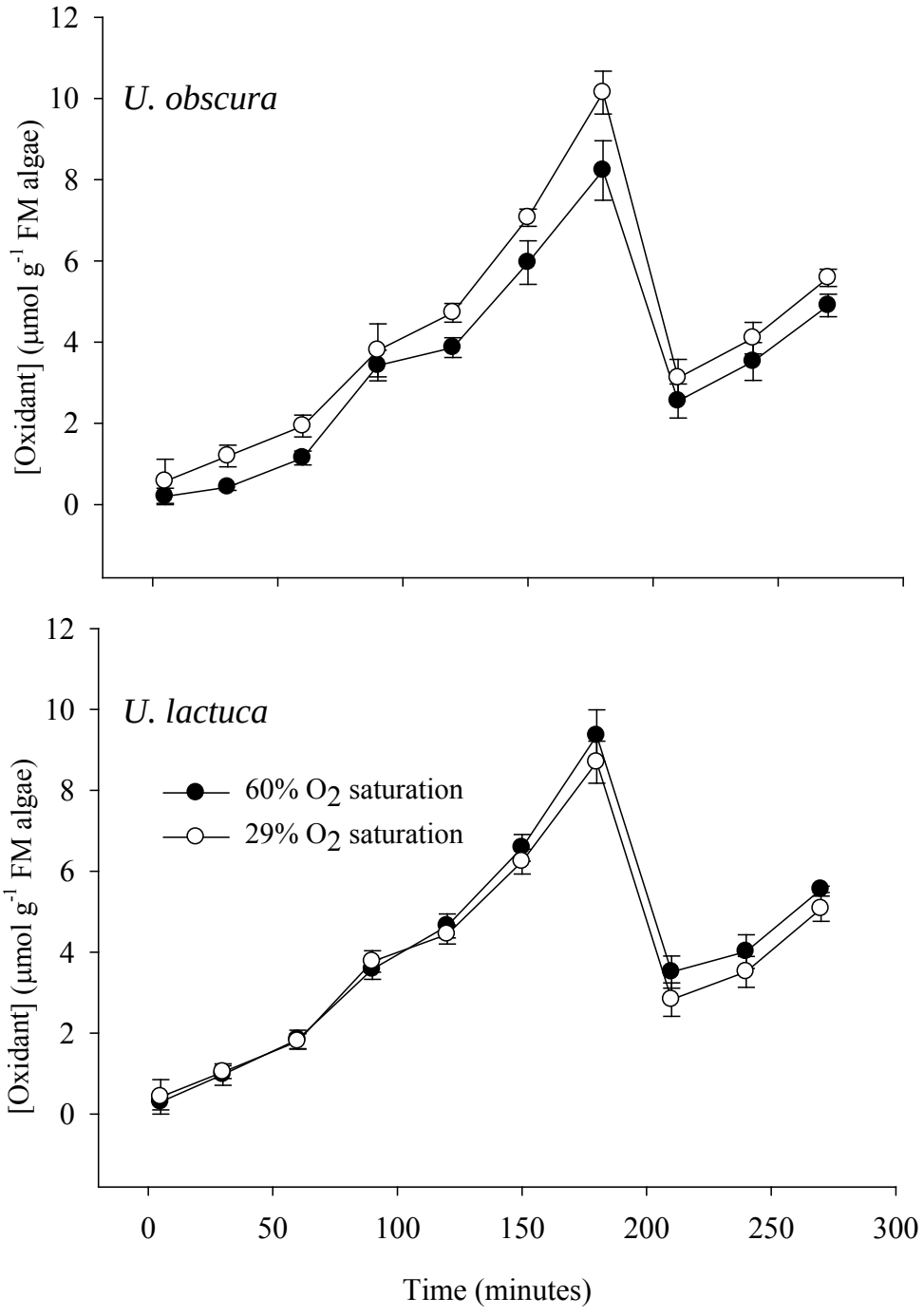

Figure 8. Concentration of oxidants released by the subtidal *U. obscura* (top) and the intertidal *U. lactuca* (bottom) over time, following exposure to seawater with either 29% dissolved oxygen or 60% dissolved oxygen. Data are means (*N*=7) +/- 1 SE.

Figure 9. A picture of biological oxygen demand bottles containing the subtidal *U. obscura* that was emersed for 2 hours (left) and 0 hours (right). Gas accumulation was greater in the bottle that contained unemersed algae, as is visible by the greater amount of bubbles.

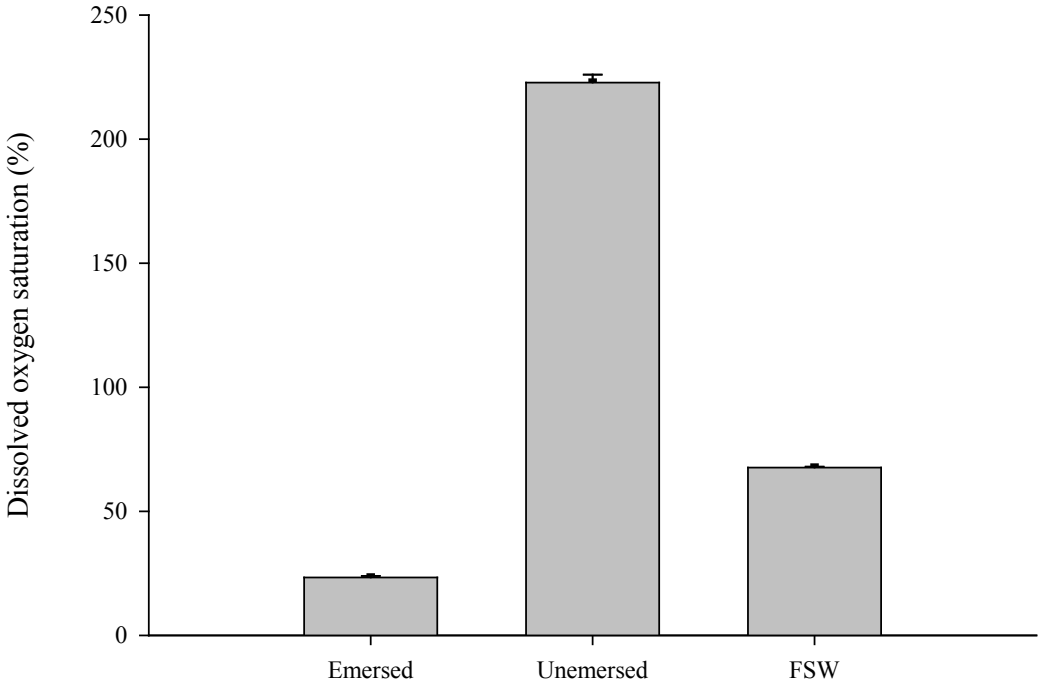

Figure 10. The dissolved oxygen saturation (DOS) in water containing *U. obscura* that was previously emersed for two hours, an unemersed algal control, and FSW with no algae. DOS was measured using a YSI probe after a 2 hour incubation. Data are means +/- 1 SE.

DISCUSSION

The results of these experiments demonstrate that environmental stressors change the accumulation of oxidants that are in seawater that contains *U. lactuca* and *U. obscura*. Another possibility is that algae might produce fewer oxidants under stress. When they responded to environmental stress, the two species of ulvoid algae responded with reduced accumulation of oxidants in seawater compared to controls. Oxidant concentrations in seawater containing *U. lactuca* were significantly lower than controls when algae were exposed to either high water temperatures (Figure 7) or environmental dopamine (Figure 5). Seawater with *U. obscura* had higher oxidant concentrations following emersion, but not when exposed to different water temperatures, dopamine concentrations, or oxygen saturation levels. The accumulation patterns seen in these experiments suggest that there are multiple mechanisms controlling oxidant accumulation in the marine environment.

Seawater oxidant accumulation

The accumulation of oxidants in seawater is determined by production, excretion from cells, intracellular scavenging and extracellular degradation of oxidants. In my experiments, oxidant accumulation in seawater was lower than controls or did not change when algae were exposed to environmental conditions that were expected to be more stressful. In general, excess oxidants diffuse from cells into the environment, reducing oxidative damage to cells. For example, the excretion of oxidants was higher when *Ulva rigida* was exposed to seawater at pH 9.0 compared to lower pH levels (Collén et al. 1995). *Ulva procera* excreted high amounts of H_2O_2 under high UV-B stress relative to controls (Choo et al. 2005). However, in my experiments, *U. lactuca* responded to increased

temperature (Figure 7) and dopamine (Figure 5) and *U. obscura* responded to emersion by accumulating fewer oxidants compared to controls (Figure 3). One explanation for the decrease in oxidant accumulation could be the reduction of released oxidants to less harmful compounds (Lesser 2006). Alternately, the decrease in oxidant accumulation could be caused by a decrease in oxidant production and release by the algae.

Another possible explanation for the decrease in oxidant accumulation in seawater that contained *U. lactuca* is an increase in the activity of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidases (Lesser 2006) or nonenzymatic anitoxidants. Algal species with lower stress tolerances often excrete excess oxidants instead of activating intracellular scavenging (Choo et al. 2004). As stress levels increase, higher activity levels from these enzymes may be required to scavenge the increased levels of cellular oxidants. Previously, the synthesis of catalase was found to increase with increased irradiance in rye (*Secale cereale*) leaves (Hertwig et al. 1992). Regoli et al. (2004) found that oxidant scavenging capabilities of symbiotic cyanobacteria were highest during exposure to high summer seawater temperatures. Since the accumulation of seawater oxidants in my experiments by *U. lactuca* decreased in both high water temperatures and in the presence of dopamine, the activation of the oxidant scavenging enzymes may have been triggered within *U. lactuca*, resulting in an increase in scavenging activity. However, a reduction in photosynthesis due to high stress levels, as shown in my photosynthetic experiment, could also lead to a decrease in the production of ROS (Mehler 1951).

Cellular functions following stress

Oxidants are produced in many metabolic pathways in plants, including photosynthesis and respiration (Foyer et al. 1994). In my experiments, the cellular mechanisms of *U. obscura* (subtidal) that produce oxidants, such as photosynthesis, may have been depressed following stress. Seawater oxidant concentrations increased over time when *U. lactuca* and *U. obscura* were exposed to emersion (Figure 3), dopamine (Figure 5), high seawater temperature (Figure 7) and hypoxia (Figure 8). Under stress, photosynthetic rates can decrease, as does the production of O_2 (Collén and Pedersén 1996). It is possible that oxidants in these experiments were produced through photosynthesis and respiration in controls and respiration in more stressed algae. In my experiment, previously emersed *U. obscura* reduced the oxygen saturation of seawater relative to FSW controls while the presence of unemersed algae increased oxygen saturation (Figure 10). Although *U. obscura* was respiring following emersion in this experiment, there was little evidence of photosynthesis. These pathways could be obstructed or shut down when an organism is exposed to environmental factors such as high water temperature (Hanelt et al. 1993). However, it is unclear whether the emersion period in this experiment was lethal.

Interspecific differences in oxidant accumulation and production

The ability to scavenge oxidants after stress could allow the *U. lactuca* in Washington to withstand higher oxidative stress levels associated with life in the intertidal zone better than *U. obscura*. *U. lactuca* is a mid to low intertidal alga and is more stress tolerant than the low intertidal to subtidal *U. obscura* (Davison and Pearson 1996). From the decrease in oxidant accumulation, it is possible that *U. lactuca* has mechanisms to manage oxidative

stress or produces fewer oxidants under stress whereas *U. obscura* does not show the capabilities to manage the same stress.

Intertidal algae also appear to have different mechanisms for mediating oxidative stress than subtidal species. In my experiments, average oxidant concentrations were 15% higher than controls in bowls that contained the intertidal *U. lactuca* in higher seawater temperatures than those that contained the subtidal *U. obscura* (Table 1). A comparable pattern was found among macroscopically similar filamentous littoral green algae. *Enteromorpha ahlneriana* (low) excreted lower amounts of H_2O_2 when exposed to higher seawater temperatures while *Cladophora glomerata* (high) increased excretion under similar temperatures (Choo et al. 2004). While intertidal algae may have the ability to scavenge oxidants following stress, subtidal algae may respond to the same stress by excreting excess oxidants (Figure 1). The decrease in oxidants found in *U. lactuca* may also be a result of an increase in oxidant scavenging enzymes, which is common among intertidal algae (Collén and Davison 1999a; Collén and Davison 1999b).

While oxidant accumulation in seawater that contained *U. lactuca* was lower in high water temperatures than controls (Figure 7) and dopamine concentrations (Figure 5), no difference was seen in oxidant accumulation in FSW that contained *U. obscura* exposed to similar conditions. A similar experiment found that the annual *C. glomerata* (stress tolerant) may conditionally express H_2O_2 scavenging enzymes whereas the perennial *E. ahleneriana* (stress susceptible) excretes H_2O_2 to combat oxidative stress (Choo et al. 2004). The patterns seen in my experiments could be a result of *U. obscura* lacking the scavenging capabilities of *U. lactuca*, and can only excrete oxidants. However, differences in oxidant production could

also have produced these differences. In laboratory experiments, net photosynthesis following desiccation, measured by oxygen evolution, dropped further in *U. obscura* than in *U. lactuca* (Nelson et al. 2010). With reduced photosynthetic capabilities, it is possible that oxidant accumulation would be lower in seawater that contained *U. obscura* compared to *U. lactuca.*

Contributions of environmental factors to stress

Algae are subjected to multiple stressors during emersion (Schonbeck and Norton 1979). A similar combination of multiple stressors in my experiments may have constituted a stress level high enough to either increase the oxidant scavenging activity or reduce photosynthesis and respiration of *U. lactuca*. Following a period of emersion, but no subsequent stress, seawater that contained *U. lactuca* accumulated similar oxidant concentrations in both emersed and unemersed algae. It is possible that emersion alone is manageable by *U. lactuca*. Another possibility is that the control conditions were stressful as well as the treatment conditions.

Dopamine in naturally occurring concentrations may have provided enough stress to trigger the mechanisms for managing oxidative stress by *U lactuca* in this experiment. Oxidant accumulation in seawater by *U. obscura* was unaffected by the presence of dopamine (Figure 5). Released dopamine from *U. obscura* could possibly act as an allelopathic chemical by increasing the oxidative stress level of *U. lactuca*. Marine macrophytes have previously been shown to impact other photosynthesizes. For example, *U. obscura* extracts reduced germination frequency and germ tube length in *Fucus garneri* (Nelson et al. 2003b). Dopamine released by *U. obscura* may similarly act as a stressor of

other nearby macrophytes, such as *U. lactuca*, given high enough dopamine concentrations. *U. obscura* may be unaffected by external dopamine because it is the alga that produces the chemical.

As bloom-forming macroalgae create and persist in local hypoxic conditions (Valiela et al. 1997), they may be adapted to dealing with low O_2 conditions. Changes in oxygen concentration could have little impact on the production of oxidants by *U. lactuca* and *U. obscura*.

Oxidant release patterns

The increase in seawater oxidants by *U. lactuca* in these experiments may have been a result of an oxidative burst, a rapid production of oxidative compounds within the alga. Oxidative bursts are often associated with responses to attacks on, or injury of an alga (Potin 2008). They have been found in a few land plants (Doke 1983; Lamb and Dixon 1997) and algae. Oxidative bursts are generally thought to be mechanisms for mitigating damage from wounding and invasion or inhibiting growth of both endophytes (Bouarab et al. 1999) and epiphytes (Küpper et al. 2001). In the unicellular chlorophyte *Dasycladus vermicularis*, a sharp increase in H_2O_2 was observed 40 minutes following mechanical injury to the algae, which may indicate the presence of an oxidative burst (Ross et al. 2005).

The oxidant release patterns seen in this project may also be partially a result of temperature changes to the esterase and DCFH-DA reagents. Ice baths that surrounded the reagents were kept near 10^{o} C during the dopamine and hypoxia experiments but the actual reagent temperatures may have increased throughout sampling. When new reagent mixtures were introduced to the sampling around 150 minutes in the dopamine experiment and 180

minutes in the hypoxia experiment, the reagent temperatures were 6^{o} C. The reagents may have been fluorescing at lower rates when at lower temperatures. The new reagent solutions would have decayed less than the previous solutions, and would therefore measure lower oxidant concentrations. This may partially explain the rapid drop in oxidant concentrations found in the dopamine (Figure 5) and hypoxia (Figure 8) experiments.

Although oxidants accumulated in either linear or logarithmic patterns in my experiments, there was accumulation throughout sampling for both *U. lactuca* and *U. obscura* after exposure to each stress. In photosynthetic organisms, oxidants are produced steadily by primary cellular functions such as photosynthesis and respiration (Apel and Hirt 2004; Mehler 1951). This could result in a constant diffusion of oxidants from the alga into seawater, resulting in oxidant accumulation. Similarly, the increases in oxidant concentrations seen in my experiments were likely due to accumulation throughout sampling rather than an actual increase in oxidant release. However, the rate at which oxidants accumulated depended on the level of environmental stress to which the alga was exposed. In these experiments, sampling periods were 3-3.5 hrs long with nowhere for oxidants to disperse. "Green tides" generally occur in protected areas and oxidants could theoretically accumulate for a period of this length as an incoming tide continues to high water.

Conclusions

Oxidant concentrations were generally higher in *U. lactuca* than *U. obscura.* Although the accumulation of oxidants over a given time was gradual, there appears to be potential for accumulation in the marine environment. Further investigations should be made to determine the cellular mechanisms behind the reduction in oxidant accumulation seen

here. The reduction in oxidant accumulation found in these experiments may be similar among the chlorophytes, but not other algal phyla. As macroalgal blooms continue to increase in size and number, the environmental impacts of these oxidant levels needs to be investigated. It will not only be necessary to determine the mechanisms behind the production of these chemicals, but also if the concentrations of chemicals produced by intertidal macroalgal blooms are sufficient to impact other marine organisms. This will provide a better understanding of the mechanisms behind the impacts of green tides, and their impacts on local environments.

CHAPTER III: THE IMPACTS OF HYDROGEN PEROXIDE ON THE SURVIVAL AND DEVELOPMENT OF FIRST STAGE ZOEAL *METACARCINUS MAGISTER*

INTRODUCTION

The brachyuran crab *Metacarcinus magister* is commonly found along the west coast of North America, from central California to the Aleutian Islands (Hart 1982). Adults are often found in coastal areas with sand and mud bottoms (Strathmann 1987), similar to the locations of macroalgal blooms in Washington State (Nelson et al. 2003a; Nelson et al. 2003b). In the San Juan Islands, WA, mating occurs from May-June (Strathmann 1987). Females become ovigerous around November-December and burrow in sand or mud until hatching occurs from February-May (Mayer 1973). After hatching, zoeae require favorable conditions to survive, namely salinity levels from 25-30 $^{0}/_{00}$ (Buchanan and Millemann 1969) and temperatures from 10-13.9^{o} C (Reed 1969). The pelagic larval period of *M. magister* lasts 80-160 days (Strathmann 1987), during which time the larvae could be exposed to a variety of environmental conditions.

Chemicals found in seawater can impact the survival of recently hatched zoeae. Decreased zoeal survival occurred following acute (96 hr) exposure to the fertilizer Methoxychlor (Armstrong et al. 1976). Zoeae exposed to heavy metals such as copper, mercury, and zinc showed reduced survival by 50%, following a similar 96 hr exposure (Martin et al. 1981). Biotic chemicals, such as algal toxins, can also impact zoeal survival and development. For example, consumption of the toxic alga *Alexandrium andersoni* resulted in accelerated larval mortality (Garcia et al. 2011). However, the impacts of specific chemicals produced by algae forming macroalgal blooms, or "green tides" on zoeal *M. magister* survival and development are not currently found in the literature.

Macroalgal blooms, or green tides, have been increasing in size and number worldwide over the past two decades. On the west coast of the United States, in the South Salish Sea, Washington State, there have been increasing chlorophyte blooms, often located near population centers (Frankenstein 2000). Ulvoid blooms in the Salish Sea generally occur in areas of low tidal exchange, such as shallow coves or broad bays (Frankenstein 2000). *Ulva lactuca* generally dominates intertidal blooms while *Ulvaria obscura* is dominant in subtidal blooms (Nelson et al. 2003a), with both species often appearing in the same bloom location. These locations have mud and sand substrate and experience low tidal flushing, which can be prime *M. magister* habitat (Hart 1982).

Reactive oxygen species (ROS) are produced and released by green tide-forming ulvoid algae (Figure 1). ROS are a result of oxygen metabolism, and are produced in chloroplasts, mitochondria, and the endoplasmic reticulum of macroalgal cells (Lesser 2006). In biological systems oxygen becomes reactive through a univalent reduction, creating many intermediate chemicals, such as a superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and eventually water. Although ROS are important in certain cellular functions, they can damage lipids and proteins (Freeman and Crapo 1982; Gutteridge and Halliwell 1990), as well as DNA (Imlay and Linn 1988) if in high enough concentrations.

While these compounds are produced within macroalgal cells, both *U. lactuca* and *U. obscura* have been shown to excrete ROS in seawater (Chapter II). Although many ROS have relatively short lifetimes, H_2O_2 can remain in seawater anywhere from minutes to an hour (Lesser 2006). This accumulation of ROS may have impacts on other marine organisms. However, the impacts of ROS on invertebrate larvae are not well known. Exposure of larvae of the nudibranch *Phestilla sibogae* to H_2O_2 induced velar loss in

concentrations of 10^{-1} M (Pires and Hadfield 1991). Chlorophyte macroalgae can have negative impacts on marine invertebrates, such as the barnacle *Balanus balanoidies* (L.) (Magre 1974) and the abalone *Haliotis discus hannai* (Wang et al. 2011).

Zoeae hatched near an ulvoid bloom may be subjected to the environmental conditions created by that bloom such as hypoxia (Gray et al. 2002) and algal produced chemicals (Van Alstyne et al. 2011). The Brachyuran crab *M. magister* can be found in the same locations as local green tides. During a flood tide, zoeae could be transported over a macroalgal bloom. During this time, zoeae would be exposed to any chemicals (such as ROS) that would have accumulated on the ulvoid bloom during that tide.

To determine the specific impacts of macroalgal blooms on invertebrate communities, individual products of green tide macroalgae need to be examined. The purpose of the present study was to determine the effects of H_2O_2 on larval *M. magister* survival and stage duration. By examining survival and duration of the first larval stage of *M. magister*, my study provides new information on the impacts that an ulvoid algal blooms have on this particular crab species.

METHODS

Larval collection

Adult *M. magister* ovigers were collected from Ship Harbor, Anacortes, WA (48^{o} 30' 22.06"N 122^{o} 40' 24.32"W) via SCUBA on March 9th, 2011 (Figure 2). Following collection, ovigers were maintained individually in separate aquaria in a flow-through seawater system at the Shannon Point Marine Center. They were checked daily for evidence of hatching to ensure all zoeae used in this experiment were less than 24 hours old. On the first day of the experiment, zoeae were collected from three different ovigers that had hatched their larvae on the same day to account for any brood variation that might be present. Zoeae were removed using a 5 μm filter from the tanks that contained ovigers and held in a bowl of 5μm filtered seawater (FSW) until the experiment began.

Hydrogen Peroxide Exposure

To determine the effects of oxidants released by ulvoid algae, day 1 *M. magister* zoeae were exposed to 0 (control), 1, 3, or 6 μM H_2O_2 for one hour to simulate being swept into a ulvoid algal bloom on an incoming tide. Laboratory grade H_2O_2 (Fisher UN2014) was added to 20 bowls containing 200 ml of 5μm filtered seawater (FSW) to create the H_2O_2 treatments (*N*=5 per treatment). One hundred to 200 zoeae, drawn from the pooled mass collection of day 1 zoeae, were haphazardly placed in each bowl and held in the four treatments for one hour to simulate a one-time exposure to H_2O_2 in a macroalgal bloom.

After exposure to H_2O_2, individual zoeae were transferred to 12-well culture plates (5 plates per treatment) to determine larval survival and the time to the first zoeal molt (Stage 1). Zoeae were selected haphazardly from the bowls and transferred individually to wells

that each contained 3 mL of 5µM FSW. Upon transfer to culture plates, all larvae were alive, based on visual observation. After zoeae were transferred to culture plates, they were fed *Artemia sp.* nauplii that were obtained daily by incubating cysts overnight in 0.2 µm FSW under a heat lamp. All culture plates were then randomly arranged in an incubator set at 15^{o} C on a 12 : 12 L: D cycle.

Each day, following the initial exposure to H_2O_2, the survival and molt status of each zoea was examined under a dissecting microscope. Larvae were considered alive if there was even the slightest movement observed. If the zoea was alive, it was transferred via Pasteur pipette to a new culture plate with fresh FSW and *Artemia sp.* nauplii (20-30 per well). After all live zoeae were transferred to new plates, they were placed back in the incubator.

Zoeae were maintained in this fashion until each either died or molted to the second zoeal stage. A zoea was considered molted when it was fully free of its previous carapace. Zoeae were removed from the experiment following either death or molt.

Data Analysis

The daily survival for each plate was determined as the number of surviving larvae from each plate divided by the number of zoeae (12 per plate) that started the experiment. These values were then averaged for the five culture plates in each treatment, with each plate representing one replicate. The values were arcsin transformed to achieve normality. Means were compared using a one-way ANOVA, with H_2O_2 as the independent factor, and stage survival as the dependent factor.

Stage duration was determined by averaging the day of molt for all zoeae in a tray. Zoeae that died prior to molting were not factored into the stage duration values. These averages were then compared using a one-way ANOVA, with H_2O_2 as the independent factor and stage duration as the dependent factor.

RESULTS

Zoeae exposed to H_2O_2 had at least a 60-70% higher mortality than zoeae exposed to no H_2O_2, but this difference was not significant ($F_{3,16} = 1.955$, $p = 0.16$) (Figure 11). No exposure to H_2O_2 of any concentration affected the stage duration for *M. magister* zoeae. The average stage duration for zoeae from all treatments was 12 days (+/- 0.32 days (SD) for the control and 11.6 (+/- 0.25 days (SD)) for H_2O_2 treatments ($F_{3.16} = 0.905$, $p = .461$) (Figure 12). Numbers of exposed zoeae surviving initially dropped quickly to 50-60% surviving while control numbers remained above 80% until day 9 (Figure 13) when molting began. Molting began around day 10 for all treatments and was mostly finished by day 14 (Figure 13).

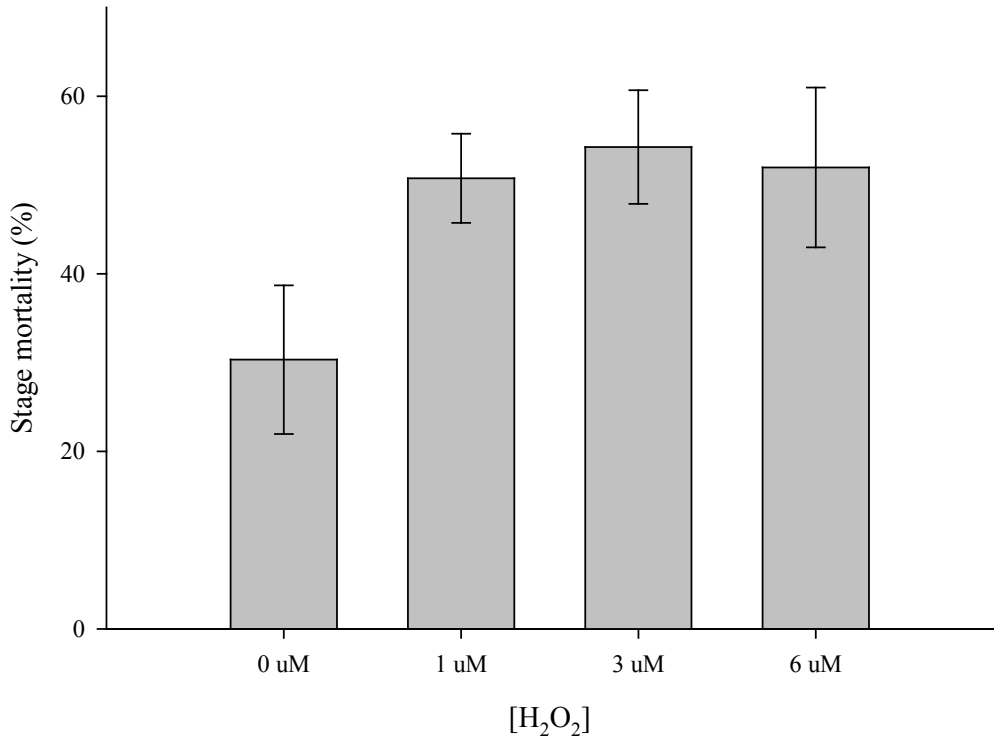

Figure 11. Stage mortality of first stage *M. magister* zoeae exposed to different concentrations of hydrogen peroxide. Zoeae were initially exposed to hydrogen peroxide for one hour and then monitored for stage survivorship. Error bars are means (*N*=5) +/- 1 SE.

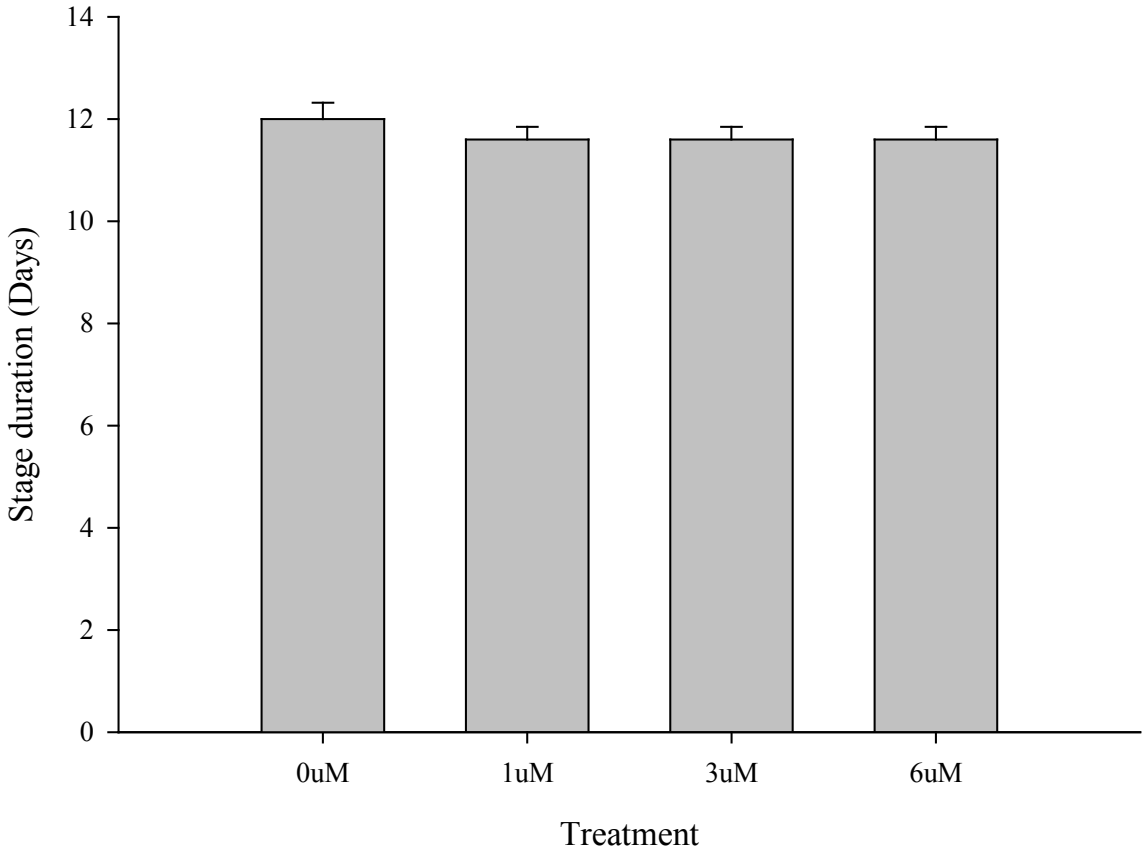

Figure 12. Stage duration of first stage *M. magister* zoeae that were exposed one of four different H_2O_2 concentrations. Data are means (*N*=5) +/- 1 SE.

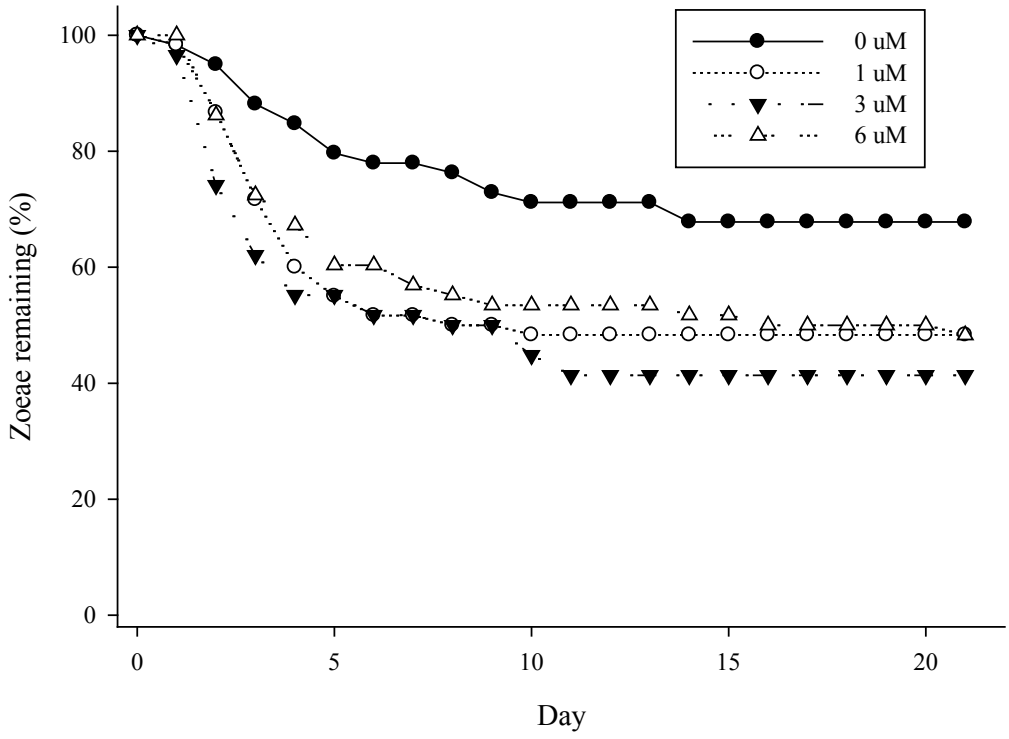

Figure 13. The percent of *M. magister* zoea surviving in each treatment, following a one hour exposure to 1, 3, or 6 µM hydrogen peroxide, and a no hydrogen peroxide control.

DISCUSSION

The mortality and duration of stage one *M. magister* zoeae were unaffected following exposure to H_2O_2. A variety of factors can affect the survival of crab larvae to metamorphosis, including the environmental conditions that are encountered during larval development. During the pelagic development of *M. magister* zoeae, the chemistry of the water can impact stage duration and mortality. Hydrogen peroxide has been shown to reduce survival and development in other invertebrate larvae. Velar loss was seen in the nudibranch *Phestilla sibogae* when larvae were exposed for seven hours to H_2O_2 concentrations of $0.6x10^{-4}$ to $2.0x10^{-4}$ M (Pires and Hadfield 1991). However, the concentrations used by Pires and Hadfield (1991) were one order of magnitude higher than those of my experiment.

"Green tide" forming algae produce a suite of chemicals such as DMS, dopamine, and oxidants (including H_2O_2) (Ross and Van Alstyne 2007; Van Alstyne and Puglisi 2007; Van Alstyne et al. 2006; Van Alstyne et al. 2011). Previous studies have seen a decrease in the survival of invertebrates upon exposure to Chlorophyte macroalgae. The abalone *Haliotis discus hannai* showed increased mortality when exposed to seawater cultured with decomposing *Ulva prolifera* (Wang et al. 2011). In an experiment using water incubated with *U. lactuca*, no larvae from five crab species (*Callinectes sapidus*, *Carcinus maenas*, *Eurypanopeus depressus*, *Nepoanope texana savi*, *and Rhithropanopeus harissii*) survived to the megalopa stage (Johnson and Welsh 1985). However, H_2O_2 and other oxidative chemicals comprise only a portion of the chemicals released by an ulvoid algae bloom. It is possible that other chemicals released by *U. lactuca* act individually or synergistically with

H_2O_2 to increase the mortality of zoeae. Exposure to only one of the chemicals released may not be enough of a stress to reduce survival.

Exposure to conditions involving oxidative stress can impact internal cellular mechanisms rather than external characteristics. Antarctic limpets exposed to H_2O_2 and increased temperature showed increased oxidant scavenging enzyme activity (Abele et al. 1998). Zoeae in this experiment may have responded to H_2O_2 exposure by mediating the oxidants internally as well, but no conclusions can be made based on these results.

It is also possible that a zoea could be swept onto a macroalgal bloom multiple times, on multiple tides, instead of the single exposure tested in this experiment. In previous experiments, zoeae were constantly exposed to mercury (DeCoursey and Vernberg 1972) and *U. lactuca* exudates (Johnson and Welsh 1985) until mortality occurred. Had a chronic exposure to H_2O_2 been used in this experiment, the difference between the control and treatment mortality may have been more pronounced.

Future research on the effects of these H_2O_2 concentrations on other organisms with pelagic larval stages should be conducted. Although there was no response from first stage *M. magister* zoeae, other larvae are associated with macroalgal bloom locations. The impacts of these chemicals on other larvae should be investigated to determine any possible impacts these blooms may have on an ecosystem.

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