Effects of Climate Change on Two Species of Foundational Brown Algae, *Nereocystis luetkeana* and *Fucus gardneri*, Within the Salish Sea

by

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B.Sc., Simon Fraser University, 2016

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Spring 2021

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Abstract

Ocean acidification and warming have large-scale impacts on marine organisms and ecosystems. To evaluate effects of these stressors, two foundational algal species in the Salish Sea were chosen, *Fucus gardneri* and *Nereocystis luetkeana*. Using *Fucus*, we evaluated how a wide range of pH levels (8.0-6.0) impacts embryonic development. During exposure to acidic conditions, embryos were capable of germination and forming a rhizoid on time. However, rhizoid elongation was significantly reduced. In a second study, we found that *Nereocystis* zoospores developed normally when incubated at 10 or 15°C. However, significant reductions in germination were observed when zoospores were exposed to 17.5°C while many lysed at 20°C. In addition, more of the *N. luetkeana* sampled from a population growing in the warmer region (Stanley Park) were able to maintain low levels of reactive oxygen species (ROS) when exposed to 17.5°C than *N. luetkeana* collected from a population living at a cooler site (French Beach).

Keywords: ocean acidification; warming; bull kelp: *Nereocystis*; *Fucus*; early development; climate change

Acknowledgements

First and foremost, I want to acknowledge the tremendous support that I have received from my friends, family, and loved ones throughout this process. Without their encouragement and confidence in me, this Masters would not have been possible. In particular, my parents and sister, who have believed in me since day 1. Your unwavering support and guidance have allowed me to create my own path that has made me the person that I am today. I can't imagine going through life's challenges without you, and ultimately I know that I'll never have to. To my loving girlfriend Raven, who has stood by my side through thick and thin and was always there for me when I needed reassurance or just someone to lean on. I cannot thank you enough for putting up with me during my times of struggle. I am forever grateful and I look forward to what our upcoming years have in store for us. To my supervisor Sherryl, your guidance has not only allowed me to complete this degree but has changed how I approach my daily life. The knowledge of knowing when to dig deeper and question the finer details is something that I will carry forward with me in all my future endeavours. Lastly, to all my colleagues, labmates, and friends who contributed to my success throughout the past few years, I thank you. This project had more people involved than I can begin to mention and was very much a collaborative effort.

Adapt and Overcome!

Braeden Schiltroth, Master of Science

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List of Acronyms

ASW	Artificial seawater
AF	After fertilization
SST	Sea surface temperature
SD	Standard deviation
ROS	Reactive oxygen species
HSP	Heat shock proteins
MUR	Multi-scale ultra-high resolution
BC	British Columbia
gu	Greyscale units
EST	Expressed sequence tag

Chapter 1. Introduction

1.1. Climatic Change in Our Oceans

Since the late 20th century, our global climate patterns have changed drastically, largely due to the increased levels of anthropogenic carbon dioxide. As CO_2 and other greenhouse gases collect within the earth's atmosphere, they trap heat and warm the planet's surface. This has led to global warming that has already forced the world's climate 1°C above pre-industrial levels. Additionally, overwhelming evidence has shown that our oceans are warmer, more acidic, and less productive (Bindoff et al., 2019). When carbon dioxide is absorbed by our oceans it produces carbonic acid (H_2CO_3) as it reacts with water, releasing hydrogen ions into the seawater and increasing acidity (Doney et al., 2009). These changes are having increasingly profound effects on our oceans and the marine species that occupy them. Both ocean warming and ocean acidification have forced organisms to adapt to long term changes or relocate themselves to avoid living outside their physiological limits (Wootton et al., 2008; Harley, 2011). As these environmental changes continue to affect the distribution and abundance of marine life, coastal communities and those that rely on oceanic resources are at risk.

Global ocean pH has dropped from 8.2 to 8.1 since pre-industrial times, largely due to the vast absorption of 20 to 30% of anthropogenic carbon dioxide emissions in that timespan (Bindoff et al., 2019). Since the pH scale is logarithmic, this corresponds to approximately a 30% increase in acidity. If emissions continue to increase as they have been, projections show that acidity in our ocean's surface waters could increase by 150% by the end of the century (Kerr, 2010). Given that we have already seen numerous impacts on aquatic life due to ocean acidification, there will undoubtedly be a growing list of species that are negatively affected as pH levels continue to decrease.

The effects of ocean acidification on marine organisms are extensive and impact a variety of trophic levels. Studies have shown that acidic seawater conditions with increased CO_2 concentrations generally increase growth of primary producers and decrease taxonomic diversity (Hall-Spencer et al., 2008; Fabricius et al., 2011; Enochs et al., 2015). As water chemistry is altered with this influx of CO_2, more hydrogen ions become available to react with carbonate ions and create bicarbonate (HCO_3-). This

removal of carbonate ions from the seawater leaves less available for calcifying organisms such as echinoderms, molluscs, corals, and calcareous algae that need it to build calcium carbonate skeletal structures and shells. Additionally, as carbonate levels drop below a certain concentration, calcium carbonate structures will even begin to dissolve, leaving these marine species defenseless (Doney et al., 2009; Gazeau et al., 2013). This change in ocean composition does not only affect calcifying organisms, but impacts a wide variety of species growth, development, and abundance. Since the intracellular pH of heterotrophs is typically lower than seawater (Hochachka and Somero, 2002) and many metabolic processes are altered by minor changes in pH, organisms must modulate their internal pH. Although many organisms can cope with these changes or buffer intracellular pH with homeostatic mechanisms, the metabolic cost of these changes can sometimes lead to decreased growth or fitness (Wood et al., 2008). Elevated levels of CO_2 can however be favorable for photosynthesis and vegetative growth of primary producers like seagrasses and algae (Koch et al., 2013; Graiff et al., 2015; Al-Janabi et al., 2016). This increased production can make its way through multiple trophic levels, impacting entire ecosystems.

The oceans also provide an irreplaceable heat sink for the planet, taking up more than 90% of excess heat from our climate to date. If global warming is limited to 2°C by the end of the century, projections show that our oceans will take in 2-4 times more heat than it had between 1970 and now. This absorption could reach 5-7 times if emissions continue to increase. Additionally, the frequency of marine heatwaves has doubled since the 1980's, with their intensity, duration, and extent also expected to increase. With 2°C of warming, this frequency would reach 20 times higher than they were during the pre-industrial era (Bindoff et al., 2019). These projections emphasize how the world's oceans are changing in the long term, but when focusing on regional environments these pH and temperature conditions can range more widely than global averages, which can have long lasting effects on local ecosystems and communities.

Like ocean acidification, ocean warming not only impacts individual organisms but also can have ecosystem-level effects. Studies have shown prolonged decreases in productivity following warming events, having a large impact on marine food webs (Behrenfeld and O'Malley, 2006; Yao and Somero, 2014). Additionally, when research focused on three globally important foundational species, marine heatwaves correlated strongly with increased coral bleaching, decreased seagrass density, and decreased

kelp biomass (Smale et al., 2019). Given the large role of these integral habitat forming species, their decline could lead to a negative bottom-up cascade in ecosystems where they reside. Estimates have put global losses of kelp around 2% per year over the past 50 years, in large part due to ocean warming and extreme heatwaves (Wernberg et al., 2019). Although the effects of ocean warming are highly variable based on location and species, the impacts on kelp populations have been observed throughout regions all around the world, in places like North-Central California, Maine, Nova-Scotia, British Columbia (BC), Ireland, Norway, and Australia (Wernberg et al., 2019). For example, off the coast of Western Australia, marine heatwaves in 2010/2011 led to a mean loss of 43% of kelp forests along that coastline (Wernberg et al., 2016). Organisms redistributed towards cooler southern waters, making previously temperate communities become more tropicalized, with new species fish being introduced as currents shift and conditions change (Wernberg et al., 2016). Similarly, a massive kelp die-off occurred in Northern California after a marine heatwave, referred to as “the blob” by many scientists, rolled through in 2014/2015 and persisted into 2016 (Leising, 2015). In this time, sea surface temperatures (SST) were 1-4°C warmer than any recorded temperatures in the past century and this coincided with a 90% loss of kelp in the area (Kintisch, 2015).

1.2. Organismal Response to Effects of Climate Change

For organisms to survive the effects of climate change, they may be forced to acclimate or adapt if conditions are outside their tolerable limits. Extreme abiotic stressors can disrupt homoeostasis between an organism and its environment as physiological functions begin to deteriorate, but individuals can initiate a defensive response through acclimation. Acclimation is when organisms make physiological changes that help to minimize the effects of environmental stressors (Ownby, 2002). One way that these changes can be made is through epigenetic modifications, when molecular alterations are made to the DNA which regulate how genes are expressed. Complexes of DNA and proteins called chromatin can be manipulated through methylation of DNA or histone modifications. These changes influence the accessibility of transcription factors which turn genes on or off by binding to the DNA. Studies have linked the amount of altered DNA methylation with thermal stress in different invertebrates (Marsh and Pasqualone 2014) and fishes (Anastasiadi et al., 2017) (Burgerhout et al., 2017) and this has also been observed in corals exposed to ocean

acidification (Putnam et al., 2016; Liew et al., 2018). Since these epigenetic adjustments can take place within a single generation, these modifications may be the key to rapid physiological changes that allow an organism to acclimate to extreme conditions. Although evidence has shown that epigenetic marks can be heritable and passed to the next generation, (Jablonka and Raz, 2009), the correct genes must be present for the appropriate adjustments to take place. If epigenetic modifications are not enough to cope with drastic environmental changes, new mutations or genotypes may be required, through adaptation.

Adaptations are accumulated through generations, as beneficial mutations and genes are preserved in successful individuals and passed onto offspring. Generally, high genetic diversity will increase the occurrence of beneficial traits and thus, the adaptive potential of a population. Genotypic diversity can be influenced by population size, dispersal, and connectivity between neighbouring populations (Hoffmann et al., 2017; Razgour et al., 2019). For example, isolation or lack of gene flow between small populations could lead to selection of a few adaptive alleles, potentially forcing a bottleneck or a founder effect (Dlugosch and Parker, 2008; Banks et al., 2013). Although the factors that contribute to genetic variation can be complex, it is ultimately an organism's genetic makeup that will determine its ability to adapt to extreme abiotic conditions.

When exposed to environmental stressors, organisms have evolutionarily conserved mechanisms that aid in a cellular stress response. Stress proteins such as molecular chaperones and DNA repair enzymes which are involved in sensing and repairing molecular damage, are upregulated and activated during exposure to stressful conditions (Kültz, 2005). This response is triggered by damage to macromolecules such as membrane lipids, proteins, or DNA (Kültz, 2003). Another way that cells respond to abiotic stress is through an oxidative burst, or increased production of ROS (Visch et al., (2019). ROS are oxygen intermediates that form through various metabolic processes inside the chloroplast, mitochondria and peroxisomes. They are important signal transduction molecules that regulate pathways during a stress response (Choudhury et al., 2017). Although low levels of ROS are required for everyday processes to occur, excessive production leads to oxidative stress and can be detrimental to cell function. When ROS production exceeds the amount that can be removed, oxidative stress can lead to various deleterious events such as cell damage to proteins, DNA, lipids, and

signal transduction pathways (Das and Roychoudhury, 2014; Narayanan, 2018). ROS scavengers and antioxidants attempt to establish an oxidative equilibrium by removing excess ROS inside cells. The ability of organisms to remove or stabilize reactive forms of oxygen will be a major determinant in their ability to cope with environmental changes. Thus, if organisms can effectively upregulate the production of ROS scavengers and antioxidants, control over ROS levels could be improved during a response to environmental stress.

The race between improved stress response and climate change is common, but for certain foundational species the repercussions could have impacts on entire ecosystems. These organisms play a major role in establishing community structure and creating environments that support rich biodiversity. Seaweeds commonly create suitable habitat that supports numerous organisms, making them valuable along many coastlines, and a prime example of foundational organisms. In fact, the term was first used by Paul K. Dayton in 1972 to describe certain marine algal communities. The more time it takes for climatic shifts to take hold, the better chance these integral species have to accommodate physiological changes.

1.3. Foundational Seaweeds Along the BC Coast

The coastlines of BC are home to a diverse array of seaweeds, some of which are considered foundational species. On the outer coast of Vancouver Island, subtidal zones consist of both low-lying turf algae as well as large mixed kelp canopies, whereas intertidal habitat is covered in a wide range of red, green, and brown algae. Here, conditions are heavily influenced by cool upwellings originating from the deep Pacific Ocean. Whereas, in the inner Salish Sea fresh surface water flows in from mountain snowmelt and numerous watersheds from the mainland of BC. These inner regions are also home to highly populated cities and heavy boat traffic, making this area a concoction of ever-changing abiotic factors and a challenging area for certain seaweeds to reside long-term. As a result, a smaller diversity of species dominate the inner Salish Sea compared to the West Coast.

In both the outer coast and inner Salish Sea, brown seaweeds (Phaeophyceae) make up a large proportion of the algal biomass found in the intertidal and upper subtidal, predominantly of the orders Fucales (the fucoids) and Laminariales (the kelps).

Fucoid species of rockweed dominate the rocky intertidal in both these regions and are known for their hardiness across a broad range of environmental factors including salinity, temperature, and desiccation (Schagerl and Möstl, 2001; Lago-Leston et al., 2010; Takolander et al., 2017). *Fucus* sp. are foundational, providing a base to the ecosystem that a large array of fish and invertebrates rely on for food and shelter (Schiel, 2006). *Fucus gardneri* and *Fucus distichus* have commonly been used as synonyms in the Pacific Northwest (Guiry & Guiry, 2021; AlgaeBase). In deeper subtidal zones of the Salish Sea, three-dimensional habitats are predominantly formed by kelp forests. While canopy-forming kelp beds along the outer coast are comprised of both *Macrocystis pyrifera* and *Nereocystis luetkeana*, the inner Salish Sea is primarily made up of *Nereocystis luetkeana.* Being the only major canopy-forming kelp here means that it is integral for creating expansive habitats for a wide array of marine species including migrating salmon, a culturally and economically valuable resource on BC's coast. They also help prevent coastal erosion, act as a natural carbon sink, and provide a food source for marine organisms as well as people. This makes *Nereocystis* an important foundational species in the Salish Sea, without which many other species may not flourish.

1.4. Reproduction and Early Development

While all seaweed's developmental stages are exposed to similar environmental stressors, it is often these early stages that are most vulnerable (Fredersdorf et al., 2009; Roleda et al., 2012; Capdevila et al., 2019). Initial early zygotes and zoospores typically exist for short periods of time and are often unicellular, small, and relatively defenseless compared to adults. As a result, exposure to extreme conditions can often be fatal for early life stages, whereas adult stages will often have a chance for recovery as stressors subside. By evaluating the progression of early life stages under stressful conditions, we can better understand how development is affected and how populations may respond to future environmental changes.

1.4.1. Fucus

Fucus gardneri reproduces sexually and contains specialized cavities called conceptacles that house eggs and sperm. While some fucoid species are diecious, with

sperm and eggs found on separate individuals, the species *F. gardneri* is monecious which means that sperm and eggs are housed within the same conceptacle (Figure 1.1). It is thought that this close proximity helps ensure a high rate of fertilization; up to 90-95% when conditions are optimal (Pearson and Brawley, 1998). However, this mode of reproduction can also lead to a high level of self fertilization or inbreeding, which can result in reduced genetic diversity within the population.

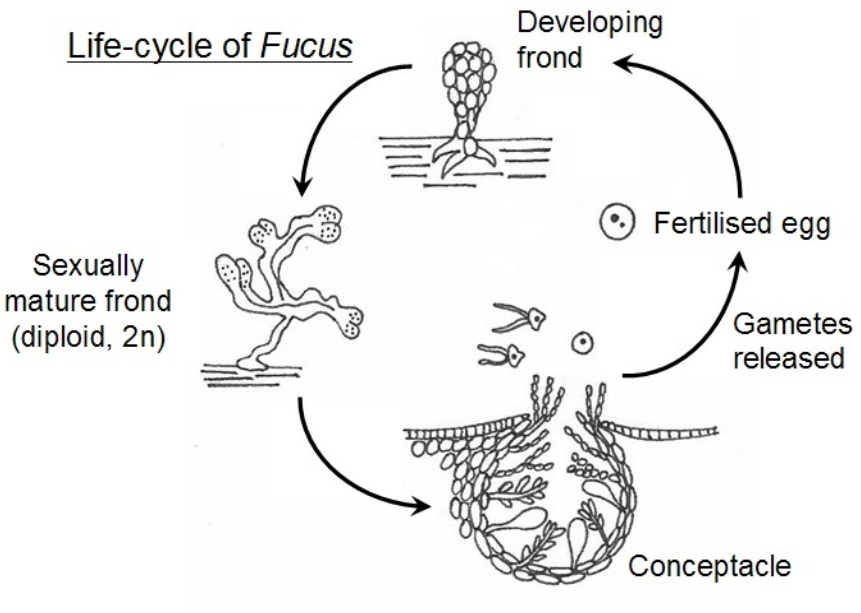


Figure 1.1. Life cycle of *Fucus gardneri*. The mature adult is diploid and houses fronds that contain small bumps or conceptacles when reproductive. *Fucus gardneri* is monecious which means that male and female gametes (haploid, 1n) are released from the same conceptacle. Once the egg becomes fertilized zygotes settle and begin early development, including germination and attachment to the ocean floor via a holdfast.

Image Courtesy: https://cronodon.com/BioTech/Seaweeds.html

Fucoid zygotes have been used as a model system for studying early development for many years since they are easy to manipulate under laboratory conditions (Bisgrove and Kropf, 2007). Zygotes are relatively large, up to 100μm in diameter (Figure 1.2A), and once released, environmental cues (predominantly unidirectional light) influence the orientation of the axis by a process known as photopolarization. At this stage the cell has polarity, with a rhizoid pole forming on the shaded side of the zygote and thallus pole located on the lighted side. Following

establishment of a developmental axis, zygotes proceed with germination. The cell wall is weakened at the rhizoid pole and turgor pressure builds to form a protrusion there (Hable, 2014) (Figure 1.2B). Next, the first asymmetric cellular division occurs, creating 2 cells with different developmental fates (Figure 1.2C). The thallus cell, which ultimately produces the reproductive and photosynthetic fronds faces upwards towards incoming light, while the rhizoid cell on the shaded side eventually becomes the holdfast that anchors the organism (Goodner and Quatrano, 1993). The bulge that forms on the rhizoid pole elongates via tip growth, a highly conserved mechanism that occurs in a wide range of taxa, including: fungal hyphae, algal rhizoids, and root hairs and pollen tubes of plants (Gow et al., 1984; Cárdenas, 2009; Bascom et al., 2018). This mechanism of growth relies on an internal pH gradient that is maintained along the rhizoid via proton fluxes in and out of the cell (Kropf, 1997). Cell division continues in both the globular thallus and elongated rhizoid (Figure 1.2D) to create a small germling that anchors to the substratum (Figure 1.2E).

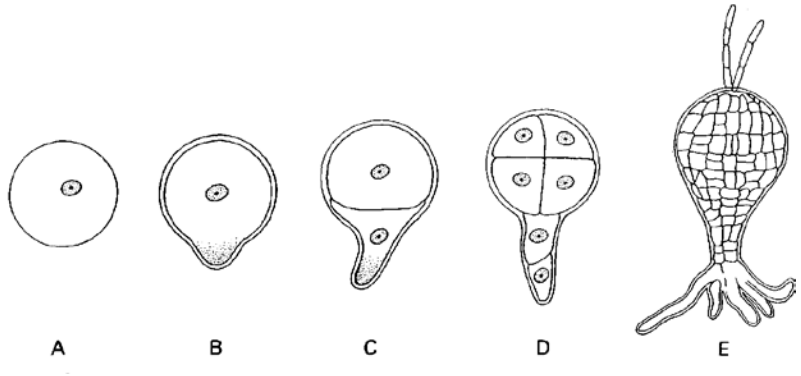


Figure 1.2. Embryogenesis of *Fucus gardneri*. A) Large spherical apolar zygote, up to 100µm in diameter B) Initial establishment of cell polarity with thallus and rhizoid pole followed by formation of rhizoid (germination) (approx 10-12 hours after fertilization) C) First cell division (approx 24 hours after fertilization) occurs perpendicular to axis of rhizoid and creates thallus and rhizoid cells D) Further cell divisions occur, starting parallel to the first division and followed subsequently by a perpendicular division E) Cells continue to divide and form a small germling with multiple rhizoids that will become the holdfast.

Image courtesy: Goodner and Quatrano, 1993

1.4.2. Kelp

Bull kelp's (*N. luetkeana*) life cycle alternates between haploid and diploid phases throughout a life cycle known as an alternation of generations. Haploid zoospores are produced between the months of June and November by meiosis within a small patch called a sorus near the distal end of the fronds on the diploid sporophytes. These sori abscise from the blade, releasing massive amounts of motile zoospores which move to the ocean floor. There can be 20 or more fronds per individual and each of these can have about ⅓ of their surface area made up of fertile sori. Given that these fronds can grow up to 14 feet long, this means that one individual may produce upwards of 3.7 trillion zoospores in a given season (Scagel, 1961). After settling on the ocean floor, these haploid zoospores germinate and go through a series of cellular divisions which form male and female gametophytes. Eggs located on the female gametophyte secrete a pheromone, lamoxirene, which induces the release of sperm from male gametophytes. This chemoattractant attracts the sperm to eggs (oogonia) on the female gametophyte where fertilization occurs (Maier et al., 2001). This fusion of egg and sperm goes on to form a small diploid sporophyte anchored to the ocean floor by a holdfast. This stage grows quickly, up to 25cm in one day, and reaching lengths of up to 36m (Foreman, 1970; Druehl and Clarkston, 2016). Elongated blades attached to a gas-filled pneumatocyst ascend towards the surface where they form a surface canopy. Large sporophytes make up dense underwater forests and become reproductive as adults so the cycle can begin once more (Figure 1.3). *Nereocystis* is viewed as an annual since each year the majority of adult sporophytes are washed away by winter storms. How *N. luetkeana* beds are capable of recolonizing the same area the following year is not well understood, but researchers believe that some gametophytes or early sporophytes remain dormant on the ocean floor over winter, developing into adult sporophytes the following year (Chenelot et al., 2001).

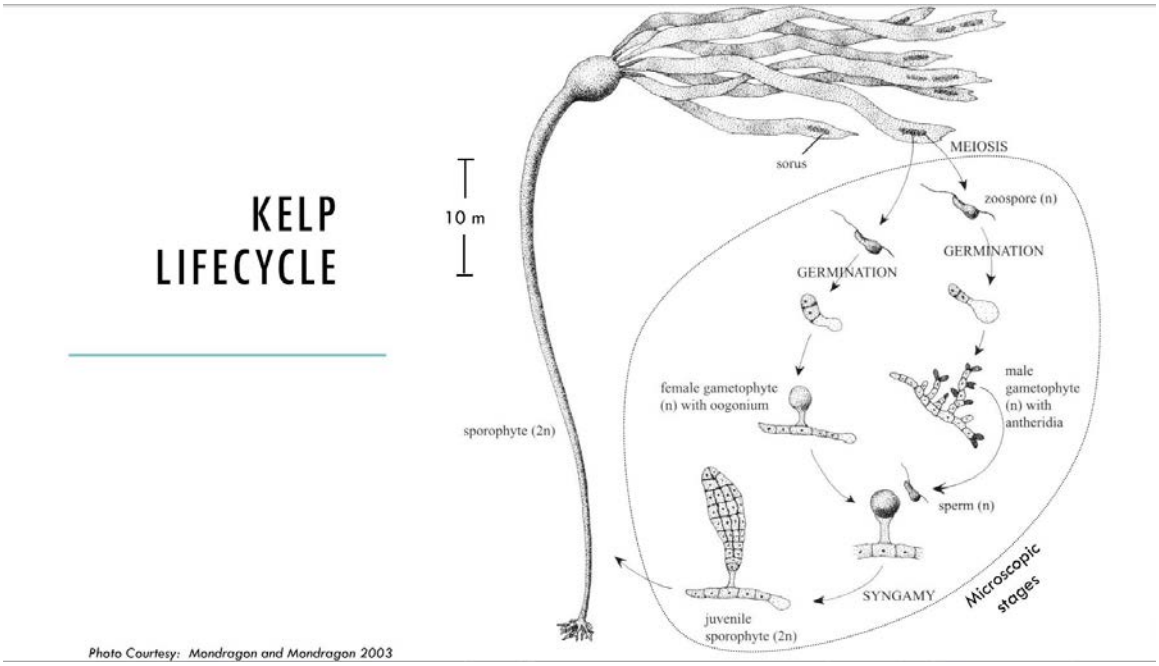


Figure 1.3. ***Nereocystis luetkeana*** **life cycle. A large reproductive sporophyte produces sori along its fronds which release male and female zoospores (haploid, 1n). The zoospores germinate on the ocean floor and develop into male and female gametophytes through a series of cell divisions. Oogonia on the female gametophyte attract sperm from male gametophytes and fertilization occurs. This fusion creates a small juvenile sporophyte (diploid, 2n) which grows into a large adult sporophyte.**

Image Courtesy: Mondragon and Mondragon 2003

1.5. Research Questions

This thesis investigates the effects of two climate change parameters, ocean acidification and warming, on propagule development of two foundational species of brown algae, *Fucus gardneri* and *Nereocystis luetkeana*. We hypothesize that acidic seawater conditions will impact the early development of *Fucus* since zygotic germination and embryonic growth rely on maintenance of an internal pH gradient. The findings relating to this research are in chapter 2 of this thesis, titled “Impacts of Acidic Seawater on Early Developmental Stages of *Fucus gardneri* from Burrard Inlet, British Columbia”. This work was published in the Frontiers in Marine Science (Schiltroth et al., 2019).

We also predict that two different *N. luetkeana* populations that had been exposed to distinct temperature conditions will respond differently to temperature stress. To evaluate this, we determined what temperatures *Nereocystis* populations in the Salish Sea were exposed to and then tested what effects these ranges of temperatures have on early developmental stages. We also assessed which temperature were lethal for zoospores and compared both populations response to temperature by measuring levels of ROS. These findings are outlined in chapter 3, titled “Temperature Tolerance of Developing *Nereocystis luetkeana* Spores from Two Populations in the Salish Sea”. This work will be submitted as a manuscript to a scientific journal in the upcoming months.

Chapter 2. Impacts of Acidic Seawater on Early Developmental Stages of *Fucus gardneri* from Burrard Inlet, British Columbia

2.1. Abstract

Increases in stressors associated with climate change such as ocean acidification and warming temperatures pose a serious threat to intertidal ecosystems. Of crucial importance are the effects on foundational species, such as fucoid algae, a critical component of rocky intertidal shorelines around the world. The impact of climate change on adult fronds of fucoid algae has been documented but effects on early developmental stages are not as well understood. In particular, ocean acidification stands to impact these stages because zygotes and embryos are known to maintain internal pH and develop a cytosolic pH gradient during development. To assess the effects of seawater acidification on early development, zygotes of *Fucus gardneri* were exposed to artificial seawater (ASW) buffered to conditions that approximate current global averages and extend largely beyond future projections. Exposure to acidic seawater had significant effects on embryonic growth. Specifically, rhizoid elongation, which occurs by a process known as tip growth, was significantly reduced with each 0.5 unit drop in pH. When pH was decreased from 8.0 to 7.5, which is similar to levels that have been observed in Burrard Inlet, there was reduction in rhizoid growth rate of almost 20%. Under more extreme conditions, at pH 6, rhizoid growth rates were reduced by 64% in comparison to embryos exposed to seawater at pH 8.0. On the other hand, acidic seawater had no effect on earlier processes; zygotes became multicellular embryos with well-formed rhizoids on a similar time course within the first 24 h of development, even when exposed to pH 6, an extreme pH well below what is expected in the future. This suggests that zygotes can maintain an internal pH that allows germination and cell division to occur. Tip growth, however, depends on the extended maintenance of an internal pH gradient. It is therefore possible that disruptions to this gradient could account for the observed reductions in rhizoid elongation. Under acidic conditions proton influx into the cell becomes energetically more favorable than at pH 8, and expulsion would be more difficult. This could disrupt the cytosolic pH gradient and in turn affect rhizoid growth.

2.2. Introduction

Fucoid algae are found on rocky intertidal shores around the world. They are foundational species in the ecosystems they occupy as they provide food, shelter, and habitat for many other organisms (Schiel, 2006). They are generally known as ecosystem engineers because they form extensive canopies that provide refuge for other organisms, especially during low tides. Their presence is associated with increases in species diversity and abundance at a variety of sites including locations impacted by industrial activities or contaminants, making them indicator species for the habitats they occupy (Watt and Scrosati, 2013; Bellgrove et al., 2017; Lauze and Hable, 2017; Scrosati, 2017).

To persist in the intertidal zone, algal populations must be able to survive, grow, and reproduce despite fluctuating and often stressful conditions. Mature fronds of fucoid algae are well-known for their ability to tolerate dehydration and heat; in the Summer months they can recover after hours of exposure when tides are low during the day (Ferreira et al., 2014). Increases in the stress imposed on intertidal species are expected as our climate changes in coming years. In addition to warmer temperatures, ocean waters are also becoming more acidic, a phenomenon that is driven by increasing levels of anthropogenically produced carbon dioxide (CO_2). As atmospheric CO_2 levels rise, gas exchange between the sea surface and the air causes a corresponding increase in the amount of CO_2 in the water. Once dissolved, CO_2 reacts with water to produce carbonic acid which dissociates into hydrogen, bicarbonate, and carbonate ions. The increase in hydrogen acidifies the seawater and reduces the amount of carbonate ions present [reviewed in Doney et al. (2009)]. Globally, the pH of ocean waters has fallen from roughly 8.2 in pre-industrial times to 8.1 and predictions estimate further decreases of up to 0.32 pH units by the end of the century if CO_2 production continues unabated (Orr et al., 2005; IPCC, 2014). Regional ocean pH can also deviate substantially from global averages. As part of the California Current System, the southern coast of BC is subject to periodic penetration of acidified waters from offshore upwelling events followed by downwelling episodes that have the opposite effect (Johannessen and Macdonald, 2009; Chan et al., 2017) These patterns produce dynamic fluctuations in intertidal seawater pH, causing it to reach levels as low as 7.3 at some sites (Marliave et

al., 2011). In future decades, further declines in seawater pH are expected as atmospheric CO_2 levels continue to rise.

Ocean acidification that comes from an influx of CO_2 can have a wide range of impacts on marine organisms. The increase in CO_2 in the seawater can be beneficial for primary producers like eelgrass and algae, since they can utilize the carbon for photosynthesis and growth (Zimmerman et al., 1997; Olischläger et al., 2012; Koch et al., 2013). However, as CO_2 levels rise, the ocean's carbonate chemistry also changes, making it difficult for calcifying seaweeds and invertebrates to build calcium carbonate skeletal structures or shells because carbonate ions in the seawater are less prevalent (Gazeau et al., 2013; Hofmann and Bischof, 2014). Studies on fucoid algae evaluated the effects of increased CO_2 on vegetative growth of germlings (greater than 8 weeks old) and adult fronds with conflicting results (Gutow et al., 2014; Graiff et al., 2015; Al-Janabi et al., 2016), possibly arising from differential effects of increased photosynthetic activity resulting from higher CO_2 levels and the negative effects of reduced pH on cellular activities. As hydrogen ions become more concentrated in the external environment, the ability to maintain cytoplasmic pH at normal levels within cells may become more difficult. This is particularly important for algal species related to *Fucus* which also depend on the formation and maintenance of pH gradients in the cytoplasm during embryonic growth and development (Gibbon and Kropf, 1994).

Zygotes of fucoid algae are initially spherical in shape and over the first few hours of development there is a cytoplasmic reorganization that results in the formation of a protrusion, the rhizoid, from one pole. This process is called germination; it is turgor driven and occurs at a site where the cell wall has been weakened. The first cell division is an asymmetric one that bisects the zygote into two cells with different shapes and developmental fates. The spherical thallus cell is cleaved into a ball of cells that eventually gives rise to most of the mature alga, including the reproductive and photosynthetic fronds. The rhizoid cell, on the other hand, contains the protrusion that formed at germination; it elongates by extension of the tip apex in a process known as tip growth, eventually developing into the holdfast that anchors the alga to the substratum. The rhizoid also undergoes several rounds of cell divisions, but in this case the cell plates are all oriented parallel to one another and perpendicular to the growth axis. As development proceeds, the rhizoid continues to elongate via tip growth of the

apical-most rhizoid cell, producing a long file of cells [reviewed in Kropf (1997), Bisgrove and Kropf (2007), and Hable (2014)].

Tip growth is a turgor-driven process that also involves focused delivery of cell wall and membrane materials to the growing tip. It occurs in specific cells of organisms from a range of taxa. In addition to brown algal rhizoids, fungal hyphae, as well as pollen tubes and root hairs of plants all elongate via tip growth (Gow et al., 1984; Cárdenas, 2009; Bascom et al., 2018). This type of cell expansion correlates with gradients of Ca^{2+} and H+ ions that form in the cytoplasm parallel with the direction in which the cell is elongating, with the highest concentrations of each ion found in the apical regions near the growing tip. These gradients are thought to have an important role in tip growth by driving the activities of different regulatory proteins and processes along the cell and restricting growth to extension from the apical-most region [reviewed in Obermeyer and Feijó (2017), Bascom et al. (2018)].

In tip growing cells an internal pH gradient is established and maintained within the cytoplasm by controlling proton fluxes into and out of the cell. Protons flow in at the tip and are used in metabolic processes or expelled in basal regions of the cell, creating a gradient that is most acidic at the apex [Takeuchi et al., 1988; reviewed in Obermeyer and Feijó (2017)]. In fucoid algae the first detectable cytosolic pH gradient forms at the presumptive rhizoid pole early in zygotic development, well before rhizoid growth begins. The magnitude of this gradient is initially small, with a difference of less than 0.1 pH units, but it increases as development proceeds. At the time of germination, the emerging rhizoid is approximately 0.1 units more acidic than the cytoplasm in the thallus which is maintained close to pH 7.5 (Gibbon and Kropf, 1993, 1994; Kropf et al., 1995b). The gradient continues to intensify; at the 2-celled stage the rhizoid tip has acidified to a pH of 7.2 generating a differential of 0.3–0.5 pH units between the tip and the cytoplasm further back at the base (Gibbon and Kropf, 1994). Dissipation of the gradient by treatment with H+ buffers inhibits rhizoid growth, indicating that the gradient is important for embryonic development (Kropf et al., 1995a). Since rhizoid formation and tip growth depend on creating and maintaining an intracellular pH gradient, these stages of the life cycle stand to be impacted by ocean acidification if zygotes and embryos cannot adequately cope with higher proton concentrations in their environment. In this study, the effects of seawater acidification on early development of *Fucus gardneri* at Burrard Inlet were assessed. Zygotes and embryos were cultured in artificial seawater (ASW)

solutions buffered to a pH value that ranged from 8 to 6. These conditions were chosen to approximate current global averages, encompass levels that have already been observed at the Barnet Marine Park, and include more extreme conditions. Three key aspects of early development were examined: the ability of zygotes to form a rhizoid, the timing of rhizoid formation, and the rate of rhizoid growth. The goal of this study was to examine whether seawater pH would affect early developmental processes and determine whether embryos from this population could withstand extreme acidity.

2.3. Materials and Methods

2.3.1. Algal Collection and Culture Conditions

Early developmental stages of fucoid algae were sourced from a population of *F. gardneri* growing at the Barnet Marine Park located on Burrard Inlet in Burnaby, BC (Figure 2.1). Burrard Inlet lies in an urban region with several large municipalities and industrial sites situated along its shorelines. A sawmill and several export terminals which include facilities for petroleum and sulfur are located near the Barnet Marine Park and marine biota within its boundaries are exposed to contaminants associated with occasional releases from these facilities. In addition to chemical contaminants, inlet waters are also subject to increasing levels of stressors associated with climate change. SSTs fluctuate throughout the year, dipping to approximately 5°C in the winter and often reaching highs of 20°C or more during the Summer months (NASA JPL, 2019). Furthermore, fluctuations in the pH of seawater collected from the park at the time of sampling ranged from 8.2 down to 7.4. Despite these fluctuating conditions and the presence of multiple types of contaminants, the intertidal zone at this site harbors a robust population of *F. gardneri*.

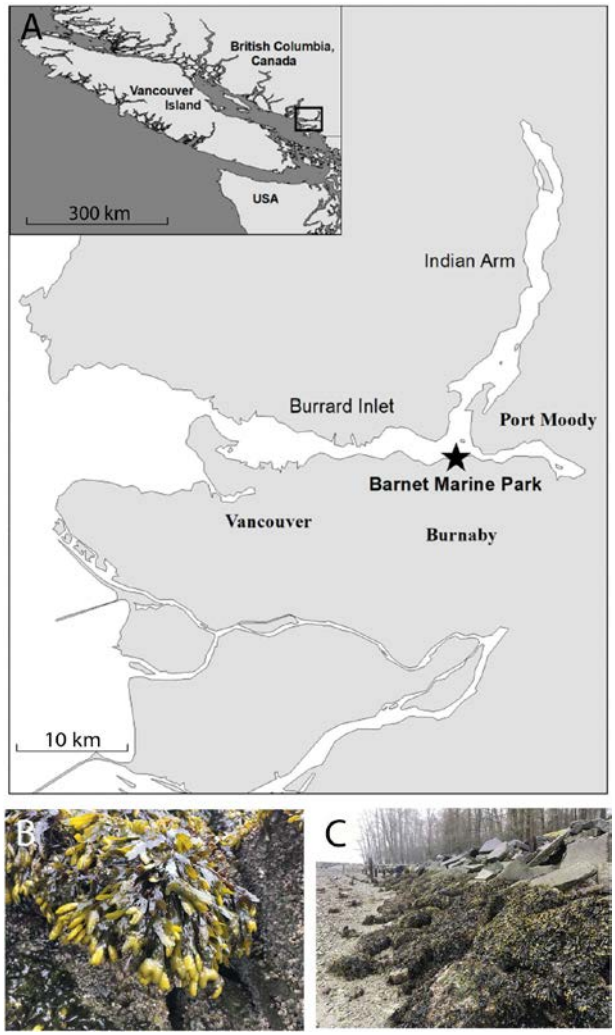


Figure 2.1. The Barnet Marine Park harbors a robust population of *F. gardneri*. Reproductive fronds were collected from a site within the Barnet Marine Park (49°17′31.9″N 122°55′34.1″W), located in an industrialized area in the city of Burnaby, approximately 15 km east of Vancouver (A). Inset shows the general location of the park with respect to Vancouver Island. A population of healthy *F. gardneri* covering discarded pavement blocks and boulders in the intertidal zone at the site as viewed at low tide (B,C). Scale bars are 10 and 300 km in the map and inset, respectively; star in A denotes collection site.

Sexually mature fronds were collected at low tide and stored in the dark at 4°C for at least 3 days until use. Zygote release was induced by incubating receptacles under approximately 115 μmol/m2/s light for 1 h at 15°C in buffered ASW (10 mM KCl, 9mM $CaCl_2$ (Fisher Scientific), 0.45 M NaCl (ACP Chemicals), 16 mM $MgSO_4$ (Caledon Laboratories), 10 mM tris base (Invitrogen), 0.04 mg/ml chloramphenicol (Sigma-Aldrich); Bisgrove and Kropf, 2001). The pH was adjusted to 8.0, 7.5, 7.0, 6.5, or 6.0

using concentrated HCl. This method was chosen so that pH levels remained constant in our buffered ASW while avoiding unwanted increases in photosynthesis that could arise with bubbling of CO_2. These pH values were chosen to approximate current conditions and extend well beyond the 0.32-unit reduction projected to occur over the next 80 years (IPCC, 2014). On the coast of BC, ocean pH dips to levels that are substantially lower than global averages of 8.1 (Marliave et al., 2011; IPCC, 2014). We observed fluctuations down to 7.4 at Barnet Marine Park; if conditions at this site follow global projections, pH could drop to levels as low as 7.0 by the end of the century. We extended our analysis to include more extreme conditions, down to pH 6.0, to fully assess sensitivities and determine how resilient zygotes were to low pH.

Zygotes were collected by rinsing fronds with seawater at the respective pH then filtering the solution containing the zygotes through a 300 µm nylon mesh to separate them from the fronds and other debris. Zygotes were rinsed twice with fresh ASW at the appropriate pH, distributed into three separate petri dishes per pH treatment, and incubated at 15°C in unidirectional light (115 µmol/m^2/s). The time of fertilization was considered to be 30 min after fronds were placed in ASW.

2.3.2. Experimental Design and Analysis

To assess the effects of seawater pH on germination, four separate trials, each using a different batch (collection) of fronds, were conducted as follows. Receptacles from a single algal collection were induced to release zygotes in seawater of the appropriate pH (described above). For each of the five pH treatments, zygotes were aliquot into three petri dishes and incubated until 24 h after fertilization (AF). Each petri dish was divided into four equal sections, and a picture was taken from a random location within each section, for a total of four pictures per dish. The percent of zygotes that germinated in each petri dish was calculated by determining the proportion that had well-formed rhizoids and an average was taken from the three replicate plates. The four treatment averages from each trial were averaged together to determine the overall germination percentage at each seawater pH.

The effects of seawater pH on the timing of germination were assessed by averaging the results from three trials. Each trial consisted of three replicate plates, exposed to two different pH levels, pH 8 or the more extreme pH 6. These two pH levels

were initially chosen to see whether there were different effects on the timing of germination. Since zygotes in both treatments followed the same time course there was no reason to test pH levels between 8 and 6. Since germination was expected to begin around 10 h AF in zygotes exposed to pH 8, a window of 10–14 h AF was chosen for analysis. Four photos, one from each section of the plate, were taken at 10, 12, and 14 h AF. Averages for the three trials were determined by averaging germination percentages at each timepoint for each pH treatment, as described above.

Rhizoid growth rates were assessed between 1- and 6-days AF in four separate trials. Each trial consisted of five pH treatments, each with three replicate plates. Ten germinated zygotes were randomly selected and measured from photographs taken of each of the four sections on every plate for a total of 40 rhizoid lengths per plate. Lengths were measured from the base of the thallus to the tip of the rhizoid on day 1 and day 6 from the same plates. The difference in lengths were used to calculate growth rates in μm/day by the following formula: (average length 6 days AF – average length 1 day AF)/5 days. Growth rates from three replicate plates were averaged together and the four averages from each trial were averaged to determine the average growth rates for each pH treatment.

In all experiments, photographs were taken using an Olympus SZX16 microscope equipped with a Retiga 4000R digital camera and Q-Capture Pro 6.0 software. Rhizoid lengths were measured using ImageJ software1. Statistical analyses were performed using JMP 14 software. One- or two-way ANOVA analyses followed by post hoc Tukey tests were used to test for differences between means. Levene's test and normal quantile plots were also used in JMP to assess ANOVA assumptions of normality and homogeneity of variance.

2.4. Results

The effects of reduced pH on three key aspects of early development were examined; the ability of zygotes to form a rhizoid, the timing of rhizoid formation, and the rate of rhizoid growth.

2.4.1. Germination Occurred in Zygotes Exposed to Acidic ASW

To determine whether early developmental processes could occur in acidified seawater the percent of zygotes that were able to germinate was assessed. Zygotes were allowed to develop until 24 h AF which is several hours past the time when these events typically occur. This provided ample time for rhizoid formation in all pH treatments. Embryos cultured in seawater at pH 8, which approximates the global average, developed from spherical shaped cells into multicellular embryos with well-formed rhizoids (Figure 2.2). Embryos cultured in more acidic seawater were similar in appearance. The majority of embryos were multicellular, had germinated, and had rhizoids that appeared to be similar in size, regardless of seawater pH. Zygotes, therefore, were able to form an axis and germinate even in seawater as acidic as pH 6. To assess whether acidic conditions affected the ability of zygotes to germinate, the percent of zygotes that formed rhizoids was determined in each pH treatment. At pH 8, an average of 90.6% (±4.71 SD) of embryos had rhizoids. At lower pH levels, the average percent of embryos with rhizoids ranged from 88.2 to 94.3% (±2.71 to 5.67 SD). This level of germination is high and statistically indistinguishable from the levels observed for embryos developing at pH 8 ($p \geq 0.339$; one-way ANOVA with post hoc Tukey's test). We therefore conclude that zygotes were able to form a developmental axis and initiate rhizoid growth, regardless of the pH of the seawater.

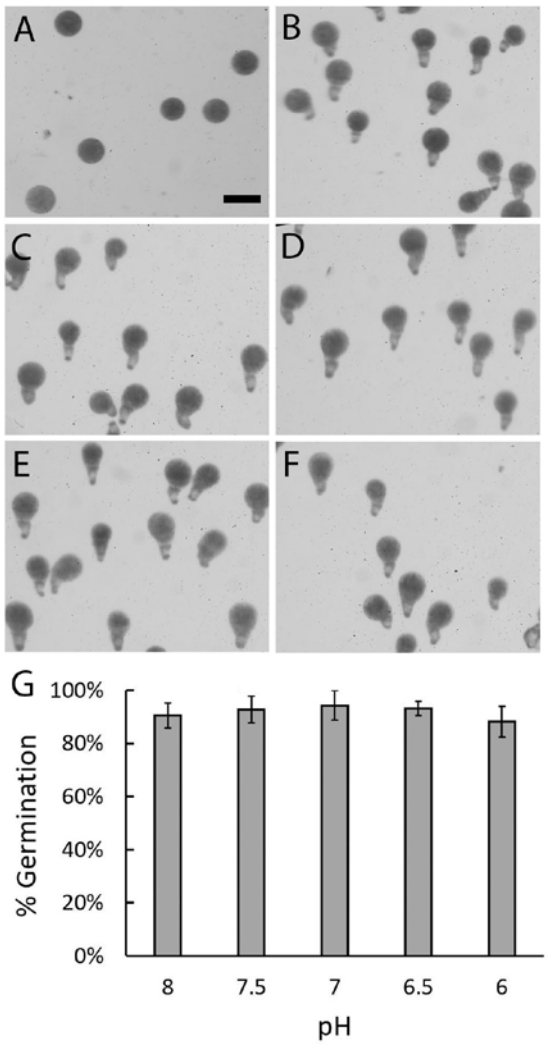


Figure 2.2. **Zygotes can form rhizoids under acidic conditions. Most zygotes were spherical in shape at 10 h AF, regardless of pH (A). At 24 h AF, most embryos cultured in ASW at pH 8 (B), 7.5 (C), 7 (D), 6.5 (E), or 6 (F) had well-formed rhizoids. Quantification of zygotes/embryos that germinated by 24 h AF revealed similar percentages in all five pH treatments (G). Bars depict the average percent of embryos with rhizoids with standard deviations (SDs). Averages represent data from four trials (N = 1910–2334 zygotes per trial). Size bar in (A) is 100 µm and applies to all photographs.**

2.4.2. Zygotes Exposed to Basic or Acidic Seawater Formed Rhizoids at the Same Time

The above analysis revealed that rhizoid formation could occur under acidic conditions. However, since zygotes were analyzed several hours after germination, any developmental delays could have been overlooked. Therefore, the effects of acidic seawater on the timing of germination was assessed. Zygotes were incubated under two

different conditions, pH 8 and a highly acidic treatment, pH 6. When examined 10 h AF, most zygotes in both treatments were still spherical in shape (Figure 2.3). Only a few had small protrusions, indicative of the earliest signs of germination. At 12 h AF, the majority of zygotes had formed small rhizoids in both treatments and by 14 h AF, rhizoids on germinated zygotes appeared longer than they were at 12 h AF These visual observations suggested that most zygotes germinated between 10 and 12 h AF.

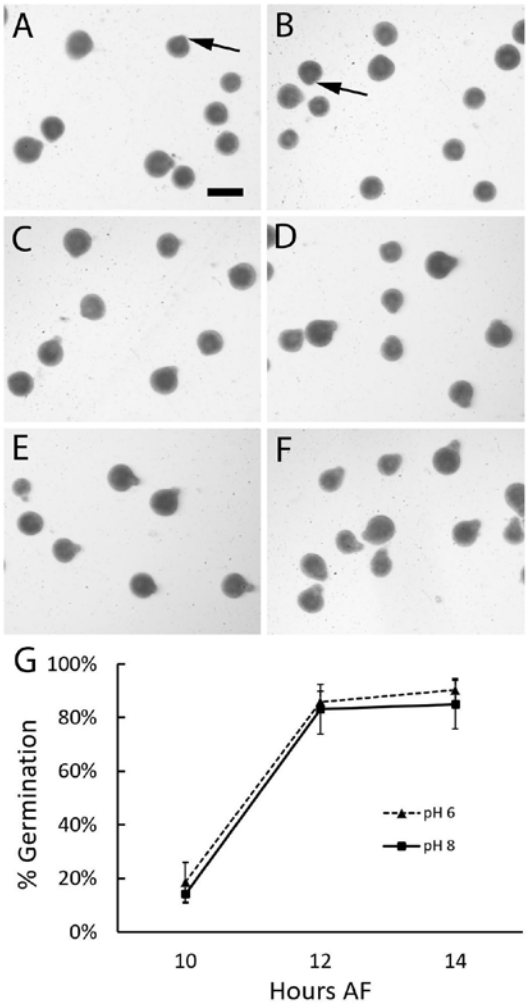


Figure 2.3. **Zygotes germinate at the same time regardless of seawater pH. At 10 h AF small bulges consistent with the onset of rhizoid formation (arrows) were found on a few zygotes developing in ASW at pH 6 (A) and pH 8 (B). Longer rhizoids were observed on more of the zygotes examined 12 (C,D) and 14 (E,F) hours AF at pH 6 (C,E), and pH 8 (D,F). On average the percent of zygotes that had formed rhizoids at 10, 12, or 14 h AF were similar at pH 6 and 8 (G). Data points depict the average percent of zygotes with rhizoids and bars represent SDs. Averages represent data from three trials (N = 2967-4968 zygotes per trial). Size bar in (A) is 100 µm and applies to all photographs.**

To determine whether there were any differences in the timing of germination between treatments, we calculated the percent of zygotes that had formed rhizoids at each timepoint. At 10 h AF an average of 18.6 and 14.2% (±7.34 and 3.47 SD) of zygotes had germinated in pH 6 and 8, respectively, indicating that germination had begun in both treatments. Two hours later these numbers increased to 85.9 and 83.2% (±3.96 and 9.25 SD), indicating that most zygotes had germinated by 12 h AF. Subsequent increases in the number of individuals with rhizoids were small; at 14 h AF an average of 90.3 and 85.0% (±4.37 and 9.06 SD) of zygotes were germinated at pH 6 and 8, respectively. At each timepoint, a comparison of the average percent of zygotes that formed rhizoids revealed no difference between the two pH treatments (two-way ANOVA with post hoc Tukey's test; $0.546 < p < 0.952$). Thus, germination occurred at the same time regardless of whether zygotes were cultured in seawater at pH 8 or the more acidic pH 6.

2.4.3. Seawater Acidity Correlates With Reductions in Embryonic Growth Rates

Once formed, rhizoids elongate by tip growth, a process that could be affected by low extracellular pH since it involves the formation of an intracellular pH gradient. To evaluate this possibility, rhizoids were assessed 6 days AF and at this time embryos exposed to different pH levels were all multicellular with long rhizoids. This indicates that normal developmental processes like cell division and tip growth still occurred, regardless of pH (Figure 2.4). However, embryos exposed to acidic seawater had rhizoids that were visibly shorter than those cultured in ASW at pH 8.0, suggesting that reductions in external pH influence tip growth.

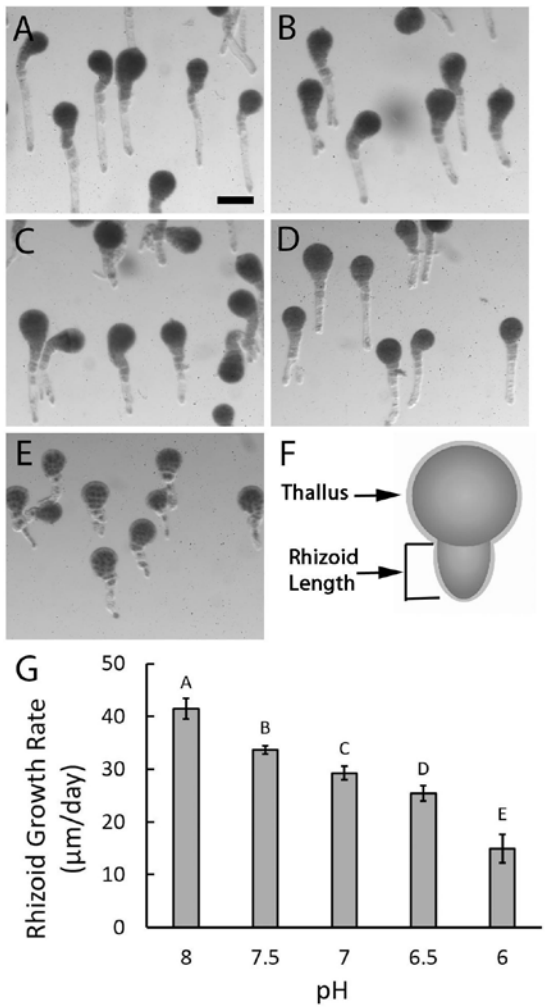


Figure 2.4. **Rhizoid elongation rates are reduced in acidic seawater. Embryos growing in ASW at pH 8 (A) had long rhizoids when observed 6 days AF while those cultured in ASW at pH 7.5 (B), 7.0 (C), 6.5 (D), and 6.0 (E) were, respectively, shorter. Rhizoid lengths were measured from the base of the thallus to the tip of the rhizoid (F) on randomly selected embryos 1- and 6-days AF. Growth rates were determined by dividing the amount of growth that occurred between day 1 and day 6 by the 5 days. Quantification of rhizoid growth rates revealed progressive decreases with each 0.5 unit decrease in pH (G). Bars depict the average rate of rhizoid growth with SDs. Average growth rates represent data from four trials (N = 600 embryos on day 1 as well as day 6, for each trial). Different letters above the bars denote means that are statistically different (one-way ANOVA with post hoc Tukey's test; $0.001 \leq p \leq 0.046$). Size bar in (A) is 100 µm and applies to all photographs**

Embryos developing in ASW at pH 8.0 had rhizoids that elongated 41.48 µm/day (±1.95 SD), whereas embryos developing in ASW at pH 7.5, grew approximately 20% slower at 33.64 µm/day (±0.78 SD). At pH 7.0 growth rates dropped to 29.3 µm/day

(±1.28 SD), almost a 30% reduction from pH 8.0. Further reductions in growth rates were observed with each 0.5 unit drop in pH to a minimum of 14.93 µm/day (±2.71 SD) in embryos developing at pH 6.0, a 64% reduction from pH 8.0. Statistical analyses confirmed that the decreases in growth rates observed with each incremental decline in pH were significantly different (one-way ANOVA with post hoc Tukey's test, $0.001 \leq p \leq 0.046$). Taken together, these results indicate that acidic conditions impair embryo development by reducing rhizoid elongation rates.

2.5. Discussion

Fucoid algae are integral components of the intertidal ecosystems that they occupy, providing food and shelter for many other species. Seaweeds growing in this environment are exposed to constantly changing and often stressful conditions, which are predicted to intensify with climate change. In coming years oceans are expected to acidify; global averages are predicted to decrease 0.32 units by the end of the century (Orr et al., 2005; IPCC, 2014). Any negative impacts of these changes on foundational species, like fucoid algae, could have cascading effects for entire intertidal ecosystems if their ability to persist in the long term is threatened (Schiel, 2006; Watt and Scrosati, 2013). In a thriving population adult stages must be able to produce viable offspring that can cope with future conditions. In fucoid algae, the importance of maintaining internal pH during early development has been shown (Gibbon and Kropf, 1994). In the current study, the effects of seawater acidification on embryonic growth and development were investigated.

Zygotes and embryos were grown in seawater buffered to conditions that approximate current global averages, encompass levels that have already been observed in Burrard Inlet, and include more extreme conditions. During the first 10–12 h AF zygotes of fucoid algae must maintain internal pH at a level compatible with cytosolic processes and generate a pH gradient in the cytoplasm along the rhizoid/thallus axis. Seawater acidification and the concomitant increases in H+ concentrations outside the cell could impact these processes since internal pH is regulated, at least in part, by controlling proton fluxes across the plasma membrane. If seawater acidification had interfered with cellular regulation of internal pH, we would expect to have seen an effect on the ability of zygotes to form a rhizoid/thallus axis and germinate. We found that seawater pH had no effect on zygotic development prior to germination. This conclusion

is based on two lines of evidence. First, germination rates were equivalent and high for zygotes developing in all five pH treatments. Secondly, rhizoid formation occurred on the same developmental time-course, regardless of whether zygotes were incubated in seawater at pH 6 or 8. This suggests that when the pH of the external medium is reduced, zygotes are either able to regulate internal pH or can develop under a range of cytoplasmic pH conditions, at least prior to germination.

In a previous study, it was shown that zygotes of this stage were able to maintain a stable cytosolic pH of about 7.5 (within 0.2 units) when exposed to seawater with pH as low as 6.2 (Gibbon and Kropf, 1993). Consistent with these previous findings, we found zygotes were able to germinate on time (10–12 h AF) even in seawater with pH as low as 6.

At later developmental stages, reductions in external pH had negative impacts on growth. After germination, there were significant declines in rhizoid growth rate as pH was incrementally decreased. Even in ASW with a pH as high as 7.5, the growth rate was reduced by 19% compared to zygotes developing at pH 8. Rhizoid elongation continued to decline with further decreases in pH, culminating in a 64% reduction in growth at pH 6 when compared to zygotes growing in ASW at pH 8. At these stages of development, embryonic growth occurs in large part through polarized extension of the rhizoid tip, via a process known as tip growth. This type of cell expansion, which is conserved across diverse eukaryotic phyla, depends on the presence of a pH gradient parallel with the axis of elongation which is maintained by controlling proton concentrations in the cell [Gow et al., 1984; Gibbon and Kropf, 1994; Cárdenas, 2009; reviewed in Obermeyer and Feijó (2017) and Bascom et al. (2018)]. Previous studies using zygotes of a fucoid algae (*Silvetia compressa*, formerly known as *Pelvetia fastigiata*) have shown that the internal pH gradient is required for rhizoid elongation and that when the gradient is abolished, subsequent tip growth is inhibited. In addition, there is a correlation between the magnitude of the gradient and rate of growth (Gibbon and Kropf, 1994). Since we found that reductions in external pH also impaired rhizoid elongation, we propose that acidic seawater affects the ability of the embryo to maintain an internal pH gradient.

In tip growing cells, a pH gradient is formed when H+ ions move in at the growing tip where cytoplasmic pH is lowest and are either used in metabolic processes or

expelled further back toward the base of the cell [Gibbon and Kropf, 1994; reviewed in Obermeyer and Feijó (2017)]. In embryos of fucoid algae growing in seawater at pH 8, H+ ions are more concentrated in the cytoplasm than outside the cell (Figure 2.5A). In this scenario, protons entering the cell at the tip move inward against their concentration gradient and passively flow outward further back along the rhizoid or are metabolized in the cytoplasm. As the external pH decreases from 8 to 7 or lower, the proton concentration in the surrounding medium would become higher than inside the cell (Figures 2.5B,C). It is now energetically favorable for protons to enter at the tip as they would be moving down a concentration gradient. Removal near the base, however, is more difficult because it would be unfavorable to expel excess protons that are not used up in metabolic processes within the cell. If external pH levels are too low, an excess of protons inside the cell could build up and affect the magnitude of the pH gradient, which would in turn inhibit rhizoid elongation (Gibbon and Kropf, 1994). In this report, significant reductions in rhizoid growth were observed with each incremental increase in seawater acidity, consistent with the idea that under acidic conditions it is more challenging for embryos to maintain a pH gradient in the rhizoid. To regulate proton flow in acidic seawater, a change in transporter activity at different regions in the rhizoid may be required if metabolic activities are insufficient to remove excess protons. Although, the mechanisms that regulate internal pH in fucoid algae are not well understood, studies using pharmalogical agents have implicated H+-ATPase and Na+/H+ antiporter activities as contributors (Gibbon and Kropf, 1993). Seawater acidification could also impact tip growth in additional ways. For example, the activity of enzymes in the cell wall could be affected by external pH, as each enzyme has a pH optimum where its activity is maximal. If enzymes involved in cell wall synthesis are not working efficiently, turgor driven growth may become slightly de-localized, allowing rhizoids to expand laterally as well as at the tip. This could result in shorter, slightly wider rhizoids. Regardless of the mechanisms, it appears to be difficult for embryos to adjust when the proton concentration is high in the external seawater, given that we observed reductions in rhizoid elongation rates under acidic conditions.

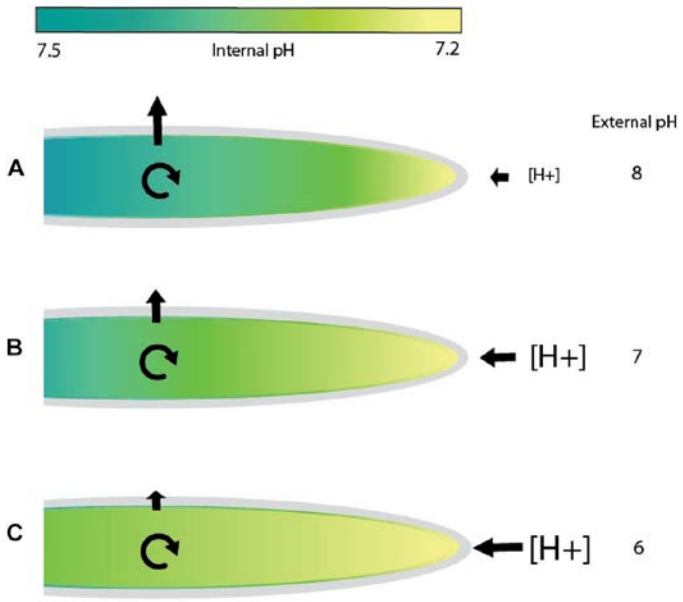


Figure 2.5. **Model of proton flow in *F. gardneri* rhizoids exposed to seawater of differing pH values. In seawater at pH 8, tip growing rhizoids of fucoid algae maintain an internal gradient with a magnitude of 0.3–0.5 pH units (Gibbon and Kropf, 1994). Diagrams depict examples of cells with a pH of 7.2 at the growing tip and 7.5 further back at the base (indicated by differences in the color of the cytoplasm). (A) In seawater at pH 8, proton concentrations are higher inside the growing rhizoid than outside. Hydrogen ions would be transported into the rhizoid at the tip against a concentration gradient and are either metabolized or flow passively out of the cell further back near the base of the rhizoid. (B) At an external pH of 7, the concentration of protons would be slightly higher outside the cell than in the cytoplasm at the rhizoid tip, a condition that favors a passive influx of protons. However, expelling H+ions near the base would be energetically unfavorable as the proton concentration is higher outside the cell. (C) At an extremely acidic pH, such as 6, proton flow into the tip will be highly favorable, perhaps flooding the cytoplasm with H+ ions. Maintaining the internal pH gradient at normal levels would be more difficult as proton expulsion is now occurring against a large concentration gradient. This is shown in (B) and (C), where internal gradients would be disrupted by excess protons. Cyclic arrows indicate proton removal via metabolic processes and straight arrows represent proton movement across the plasma membrane. The colored bar indicates the magnitude of the cytosolic pH gradient. Increasing font sizes of [H+] symbolizes higher hydrogen ion concentrations in the external seawater.**

Whether populations of fucoid algae can adapt or acclimate to more acidic conditions over the long term will depend on their ability to optimize enzyme activity, alter proton transport, or adjust metabolic activities enough to sustain optimal rhizoid growth.

As it stands now, embryos are best adapted to growth in seawater at pH 8 and they cannot maintain optimal growth under more acidic conditions, since significant decreases in rhizoid elongation were observed with each incremental drop in seawater pH. As seawater pH decreases and H+ efflux becomes more difficult, embryos must alter their physiology to more efficiently remove excess protons. Currently pH fluctuates between a high of 8.2 down to 7.4 at the Barnet Marine Park. Given these fluctuations, it may be difficult for embryos to adjust to acidic conditions if changes in the localization patterns of the relevant transporters are required. It would be interesting to assess whether preexposure to acidic conditions of fronds with developing eggs and sperm, affects the sensitivity of subsequent embryonic development to low pH. However, this is difficult to address as reproductive fronds contain eggs at different stages of development and the fronds may be exposed to field conditions that change with weather and discharges from nearby industries in the weeks preceding collection, including fluctuations in pH levels. An alternative possibility would be to assess the effects of acidic seawater on embryos of fucoid algae from other sites with more stable pH levels closer to 8, and/or less impacted by anthropogenic inputs.

Regardless of their ability to adjust, embryos are still able to grow, even at the most acidic pH, which is well below projections for global ocean acidification by the end of the century (IPCC, 2014). Under current conditions the assemblage of fucoid algae at the Barnet Marine Park is surviving. This suggests that the impacts on embryonic growth from fluctuations in pH at this site have not yet been enough to have a major impact on the population as whole, although whether it has been regressing over time is unknown. This study, however, shows that future declines in seawater pH will impair embryonic growth. This shift could compromise the ability of the population to persist in the long term since embryos are thought to be more vulnerable to predation and other environmental stressors than mature algae and other stressors such as temperature are also expected to increase in the coming years.

Chapter 3. Temperature Tolerance of Developing *Nereocystis luetkeana* Spores from Two Populations in the Salish Sea

3.1. Abstract

Kelp forests provide important three-dimensional habitat in ecosystems around the globe but are threatened by environmental changes like increased ocean warming. Reports of kelp declines following marine heatwaves have raised concerns regarding changing temperature conditions along the BC coast and the impacts they may have on kelp populations. *Nereocystis luetkeana* is a dominant canopy forming kelp along the coast of BC that has been in decline in recent decades, notably within the Salish Sea. This brought us to question what temperatures these *N. luetkeana* populations are exposed to in this region. Two different temperature regimes (approx. 4-6°C difference) were found in the Central Strait of Georgia and Strait of Juan de Fuca in the summer months, when *Nereocystis* sporophytes are reproductive. To evaluate how temperature impacts the early development of *N. luetkeana*, we exposed zoospores from both locations to a range of temperatures (10, 15, 17.5, and 20°C). We determined that temperatures of 17.5°C were detrimental for spore germination and growth in both populations, while temperatures of 20°C or higher killed zoospores almost immediately. We also assessed the amount of ROS produced in zoospores, which served as a measure of oxidative stress at each temperature. Zoospores collected from the warmer site had low levels of ROS following exposure to 17.5°C compared to those collected from cooler sites. This suggests that populations with prior exposure to warm temperatures may be better equipped to respond to heat stress.

3.2. Introduction

As ocean temperatures continue to rise, marine organisms are being pushed beyond their limits of tolerance. The effects of rising temperatures on marine life can range widely depending on the magnitude or rate of warming, and the varying levels of thermal tolerance between different populations and species. However, some common effects of heat stress occur across a wide range of taxa, such as: suppression of the ability to synthesize or properly fold proteins, increasing membrane fluidity, inactivation

of enzymatic function, and initiation of programmed cell death (Narayanan, 2018). Organisms can use highly conserved defense mechanisms to deal with these effects of temperature by initiating a stress response. Through the adjustment of various physiological characteristics, individuals can attempt to minimize the negative impacts of high temperature stress. One common mechanism is to upregulate heat shock proteins (HSPs) that aid in protein quality control processes (Shankar and Mehendale, 2005; Henkel and Hofmann, 2008; Leggat et al., 2020). Additionally, as temperatures rise and heat stress imposed on an organism builds, so does electron transport activity in mitochondria and chloroplasts, increasing the production of ROS. This response has also been shown to occur during cold stress (O' Kane et al., 1996). ROS are oxygen intermediates that form as by-products of regular metabolic activities in all aerobic and photosynthetic cells. Although these processes are required for cell function, an overproduction of ROS can lead to oxidative stress involving various deleterious events such as damage to proteins, DNA, lipids, and signal transduction pathways (Das and Roychoudhury, 2014; Narayanan, 2018). Cells have a complex array of mechanisms for maintaining a healthy balance of ROS. When the production of ROS exceeds normal levels, signalling pathways are triggered that call upon ROS scavengers or antioxidants. These substances can bind to different forms of reactive oxygen, making new, more stable compounds. However, these proteins and antioxidants can only extract ROS to a certain concentration. Thus, if the imposed stress is enough to form more ROS than can be used or removed, programmed cell death is initiated (Das and Roychoudhury, 2014). How ROS levels and temperature stress impact marine life is an important area of study for the conservation of aquatic ecosystems, particularly when foundational organisms such as kelp are involved.

Kelp forests are dynamic ecosystems that provide critical shelter and nourishment for a diverse array of organisms (Duggins, 1988; Shaffer, 2004). They are found along nearly one quarter of the world's coastlines, typically in cool, nutrient-rich waters, occupying shallow subtidal rocky zones (Dayton, 1985; Wernberg et al., 2019). These large majestic macroalgae represent foundational species as they form dense forests that influence the distribution and diversity of other marine organisms, many with commercial and/or recreational value (Dayton, 1985; Lamb et al. 2011). Also, kelp, through their high levels of primary productivity, provide large amounts of carbon to the base of the nearshore marine food web, as well through spatial subsidies to deep water

and terrestrial ecosystems (Wheeler, 1990). In recent years, concerns have been raised regarding the ability of kelp to persist in changing environmental conditions attributed to anthropogenic activity and climate change. Multiple interacting stressors including ocean warming, ocean acidification, storm activity, increases in grazer abundance, and pollution all have impacts on kelp populations, both directly and indirectly.

Large declines in kelp abundance and changes in distribution have been attributed to ocean warming in particular, with losses reported around the world following distinct heat waves. In Southeast and Southwest Australia, which have been recognized as global hotspots for ocean warming (Hobday and Pecl, 2014), range contractions of kelp populations have occurred, redistributing these underwater forests towards cooler waters. A heat wave in 2010/2011 off the coast of Western Australia led to the loss of *Ecklonia radiata*, the dominant kelp in this region, as well as *Scytothalia dorycarpa* across ≈100km of coastline (Andrews et al., 2014; Layton et al, 2020). In Tasmania, over 95% of surface canopy kelp, including *Macrocystis pyrifera*, has been lost in recent decades, largely attributed to more frequent influence from the warm, nutrient-poor waters of the East Australian Current (Oliver et al., 2018; Mabin et al., 2019). Similarly, In Northern California, around 90% of kelp cover was lost between 2014 and 2016 following a marine heat wave with unprecedented duration and magnitude, referred to as “the blob” (Leising, 2015). This hydrological phenomenon increased local ocean temperatures between 1 and 4°C, hotter than any warming recorded since the late 1800’s (Cavole et al., 2016). After another recent spike in ocean temperatures in 2018, there is concern that the frequency of these heat waves will increase as climate change continues (Cheng et al., 2019).

Within the Pacific Northwest, concerns have also been raised regarding the decline of kelp populations of the Salish Sea. *Nereocystis luetkeana* (In Haida – Ihqyaama) is the dominant canopy forming kelp found in this region and it has important cultural and economic significance. Since the only other large, surface canopy-forming kelp, *Macrocystis pyrifera,* is mainly found on the outer more-exposed, western-facing coastlines, *Nereocystis* is integral within the inner reaches of the Salish Sea as it is the major species that provides three-dimensional habitat in this region. Here, we define the Salish Sea to include Puget Sound in the USA, the Strait of Juan de Fuca bordering both USA and Canada, as well as the Central Strait of Georgia in Canada. Although each of these areas within the Salish Sea have populations of *N. luetkeana* residing within them,

changes in environmental conditions are contributing to their decline (Pfister et al., 2017; Berry et al., 2020). The vast nature and complex hydrology of this oceanic region means that each piece of coastline has a unique subset of conditions, dependent on seasonality and location. Due to the varying conditions found in different areas of the Salish Sea it is possible that *N. luetkeana* found at separate sites will be adapted to different conditions or ranges of temperatures.

N. luetkeana has an annual life cycle, meaning it is crucial for each stage to withstand the abiotic conditions of that year in order to supply recruits for the next generation (Fig 1.3). The cycle appears to be obligatory, i.e. there has to be an alternation of the haploid and diploid thalli. During late Spring and early stages of Summer, *Nereocystis* grows rapidly towards the surface as a sporophytic adult. A patch known as a sorus will form on the distal end of the blade, containing millions of zoospores. These sori will abscise from the frond with distinct diel periodicity, falling to the ocean floor where motile zoospores are released into the water column (Amsler and Neushel, 1989). If a spore does successfully settle, it forms a small protrusion, or germ tube within 24 hours in a process known as germination. The cytoplasm moves from the zoospore into the germ tube. This germ tube will continue to elongate and then goes through a series of cellular divisions, creating filamentous gametophytes. Half of the zoospores develop into male gametophytes, the other half into females. If early zoospores are hindered by extreme temperatures and are unable to form viable gametophytes, recolonization of the next generation is unlikely to occur.

This study investigates the temperature limits of *N. luetkeana* zoospores and how temperature stress may impact separate populations differently. We hypothesize that zoospores collected from a warmer site will be more temperature tolerant than those from a site with cooler conditions. This was evaluated by measuring zoospore production in sori from each site and by assessing the thermal limits of collected zoospores. In addition, the fluorescent staining of ROS was used to evaluate the amount of stress in zoospores and germlings during development.

3.3. Materials and Methods

3.3.1. Algal Collection

Mature reproductive dark sori were collected at low tide from fronds of 10 or more separate sporophytes of *N. luetkeana* near Lumberman's Arch in Stanley Park (Vancouver, BC) as well as French Beach (60km west of Victoria, BC). Sori were placed in a cooler with layers of ice and paper towel and immediately transported back to Simon Fraser University (Burnaby, BC). Sori were cut free from surrounding non-reproductive tissue, scraped free of any bryozoans or contaminants and rinsed in artificial salt water (ASW) (ASW was made according to Bisgrove and Kropf, 2001 (10mM KCl, 9 mM $CaCl_2$ (Fisher Scientific), 0.45 M NaCl (ACP Chemicals), 16mM $MgSO_4$ (Caledon Laboratories), 10mM tris base (Invitrogen), 0.04 mg/ml chloramphenicol (Sigma-Aldrich). Next, the sori were immersed for 30 seconds in an antiseptic iodine solution (3% Betadine® in 97% ASW), rinsed again in ASW, rubbed dry with paper towel and laid out on trays in a growth chamber to dry overnight.

3.3.2. Experimental Design and Analysis

The amount of zoospore release from a given area of sori was calculated using 7 trials from Stanley park and 6 trials from French Beach. After 12-24 hours of drying, uniform circles (3.80cm diameter) were excised from the sori and placed in a measured volume (between 300 and 600ml) of ASW for 1 hour. Shed zoospores were filtered through a nylon mesh to remove any leftover debris prior to counting and plating. Approximately 5ml of the spore suspension was then pipetted into 3 (or 6) separate petri dishes. Small (0.0001ml) samples were taken from each dish and placed into a hemocytometer which was used to count how many zoospores were in each milliliter of ASW. From this, the number of zoospores per area of sori could be calculated by finding the average number of zoospores/ml, multiplying by the volume (ml) of ASW that zoospores were shed into, dividing by the number of sori circles that zoospores were shed from, and lastly, dividing by the area of a sori circle ($11.34cm^2$) to give the number of zoospores/cm^2 of sori.

To evaluate whether zoospores could germinate in different temperature conditions, germination percentages were counted for 6 trials from French Beach and 4

from Stanley Park (due to lack of shedding early in the season from Stanley Park). For each trial, two petri dishes containing approximately 5ml of filtered zoospores were evaluated per temperature. The zoospore suspension in the dishes was not diluted any further, leaving spore densities ranging from approximately $10x10^6$ - $50x10^6$ zoospores per dish. Dishes were incubated in the dark for 24 hours in 4 separate growth chambers at 10°C, 15°C, 17.5°C, and 20°C. After 24 hours, 4 photographs were taken from different quadrats on each petri dish. These photographs were then used to count how many zoospores had germinated on average per temperature. Germling lengths were also measured from the same dishes 24 hours AF. 12 germinated zoospores were randomly selected from each image and lengths were measured by drawing a segmented line through the center of the spore from the base to the tip of the germ tube. The sex ratio of propagules was assumed to be 50:50 (male:female) since zoospores were not grown long enough to determine their sex. Measurements were made using ImageJ software.

ROS production was measured in kelp zoospores using 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; 100 mM stock in DMSO; Invitrogen). Protocols for a closely related Phaeophycean alga (*Fucus serratus)* were used (Coelho et al., 2008). 24 hours AF and following temperature treatments, zoospores were incubated for 30 minutes in ASW containing 100µM of CM-H_2DCFDA. Zoospores were then rinsed for another 30 minutes in ASW before images were taken. After CM-H_2DCFDA enters the cell it is cleaved by intracellular esterases to create CM-H_2DCF. It must then be oxidized by specific ROS (primarily H_2O_2 and hydroxl radicals) in order to produce a fluorescent adduct (CM-DCF) that remains inside the cell. The same line used to measure germling length in ImageJ was used to gather a mean greyscale value for that spore.

In all experiments, photographs were taken using an inverted Zeiss microscope and Hamamatsu 1394 ORCA-ERA 13 camera. Zoospore germination was counted and germ tube lengths were measured using ImageJ® software (https://imagej.nih.gov/ij/). Statistical analyses, including two and three-way ANOVAs followed by tukey's tests and creation of the box plot in Fig 3.3H were performed using JMP 14 software (https://www.jmp.com/en_us/software/data-analysis-software.html).

3.3.3. Sea Surface Temperature Data Collection

SST data was sourced from NASA's Jet Propulsion Laboratory (JPL) and obtained using multi-scale ultra-high resolution (MUR) satellite imagery (NASA JPL, 2019). Data was collected from 2003-2018 using coordinates from a chosen area that represents the Strait of Juan de Fuca (48.095 - 48.595, -124.405 - -122.805) and the Central Strait of Georgia (48.775 - 50.305, -125.195 - -122.775). Similar datasets were collected for the months of June, July, and August in areas that encompassed French Beach (48.285 - 48.485, -124.055 -123.855) and Stanley Park sites (49.215 - 49.375, -123.295 - -123.135). SSTs that make up the dataset were collected daily for every 0.01° of latitude and longitude. These SSTs were averaged together from each chosen area to obtain monthly average SSTs. Accuracy of SSTs was verified with a thermometer in the field at both sites.

3.4. Results

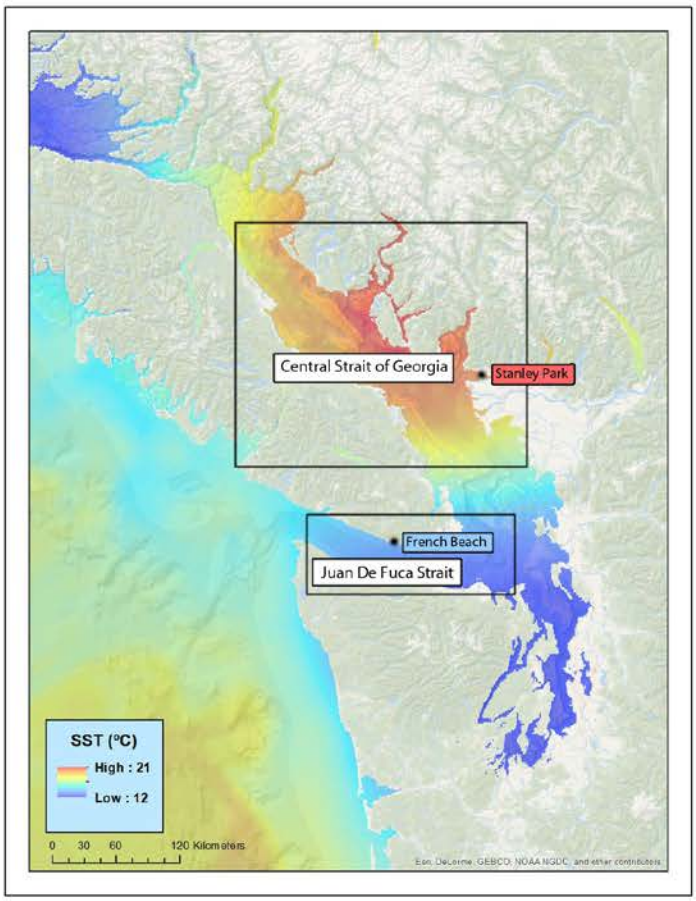
3.4.1. Temperature Regimes Within the Salish Sea

The Salish Sea's estuarine hydrology is heavily influenced by mainland riverine inputs as well as the outer Pacific Ocean marine influx, and its complex topography of basins, sills, channels, etc., creating unique environmental parameters in different areas of this region. We used datasets from MUR satellite imagery to assess the SST profiles that occur in the Salish Sea. A map was made using GIS software and SSTs collected from July 31, 2015. This revealed surface temperatures as high as 21°C in the Central Strait of Georgia, in contrast to highs of only 12°C in the Strait of Juan de Fuca (Fig 3.1). To determine how consistent these temperatures were in each region, average monthly SSTs over 15 years were plotted (Fig 3.1B). Over this time, the Strait of Juan de Fuca had average SSTs in the Summer months (June, July August) that ranged between 9.9 and 15.2°C. Whereas, the SSTs in the Strait of Central Strait of Georgia were between 14.0 and 19.2°C, around 4-6°C warmer. Temperatures in both areas dropped during the Fall, reaching lows between 6 and 8°C during winter months, with the Central Strait of Georgia registering 0.5-2°C cooler on average.

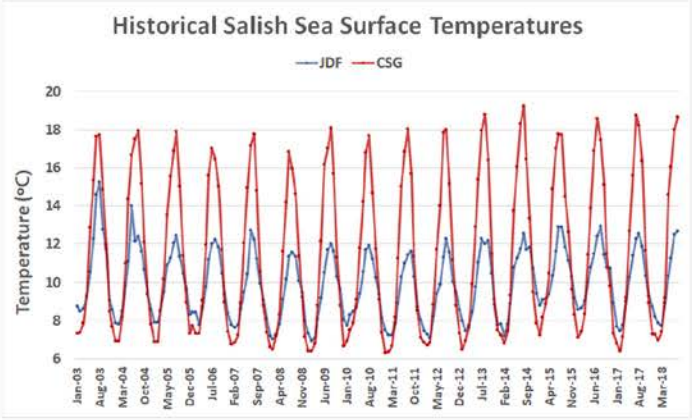
Stanley Park and French Beach were chosen as sites for further analysis since they are found within the regions described above and also harbor populations of *N.*

luetkeana. In the Summer months from 2003 until 2018, SSTs at Stanley Park averaged between 15.8-17.8°C while those at French Beach ranged from 10.5-13.4°C (Fig 3.1C). With respect to SSTs, the trends observed at these sites are similar to those documented for the larger regions corresponding to the Central Strait of Georgia and Strait of Juan de Fuca.

A


Central Strait of Georgia
Stanley Park
French Beach
Juan De Fuca Strait
SST (°C)
High : 21
Low : 12
0 30 60 120 Kilometers

B


Historical Salish Sea Surface Temperatures
JDF
CSG
Temperature (°C)
20
18
16
14
12
10
8
6
Jan-03
Aug-03
Mar-04
Oct-04
May-05
Dec-05
Jul-06
Feb-07
Sep-07
Apr-08
Nov-08
Jun-09
Jan-10
Aug-10
Mar-11
Oct-11
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Apr-15
Nov-15
Jun-16
Jan-17
Aug-17
Mar-18

C

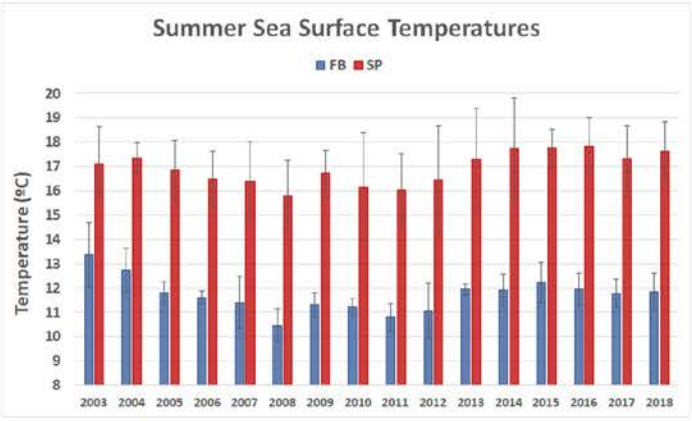
Summer Sea Surface Temperatures
FB
SP
Temperature (°C)
20
19
18
17
16
15
14
13
12
11
10
9
8
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018

Figure 3.1. Map of the Salish Sea with SSTs from a Summer day (A). Cooler temperatures (12°C minimum) are shown in blue while hot temperatures are shown in red (21°C maximum). Scale bar is 120km. Black boxes indicate approximate areas chosen for SST data collection. SSTs for the Strait of JDF and Central Strait of Georgia have varied in the past 15 years by about 4-6°C in the Summer, while dropping to similar levels in the winter (B). SSTs at French Beach and Stanley Park, also show approximately 5-6°C difference on average in the Summer months (June, July, August).

3.4.2. Effects of Temperature on Spore production

Since Stanley Park and French Beach are exposed to different temperature regimes throughout the Summer months, this raises the question of whether zoospore production is impacted by temperature. Zoospore production at each of the two sites was monitored during the Summer and Fall of 2017 along with the corresponding SSTs (Figure 3.2). In July and August, French Beach was sampled four times, over a period when daily SSTs ranged from 10°C to 14°C. On the earliest sampling date (July 13th), 1.69 x 10^6 zoospores/cm^2 were released from collected sori. This number increased to 9.26, 7.74, to 12.94 x 10^6 zoospores/cm^2, respectively, for the following collections (July 25th, August 10th, and 28th). Comparatively, Stanley Park was sampled twice in July, during a time period when SSTs were consistently above 17°C. The density of zoospores produced by sori collected on July 10th and 29th were 1.6x 10^5 and 4.1x10^5 zoospores/cm^2, or approximately 10 to 20-fold lower than the amounts produced by sori from French Beach sampled on similar dates, July 13th and July 25th (1.69 and 9.26 x 10^6 zoospores/cm^2 respectively). However, on September 6th, spore production from Stanley Park sori increased by over ten-fold from 4.1x10^5 to 5.38 x 10^6 zoospores/cm^2, even though SSTs were still above 18°C. Sori sampled from French Beach a week earlier (August 28th) produced 12.9 x 10^6 zoospores/cm^2, nearly 2.5 times more. Temperatures at French Beach in the weeks preceding these collections were consistently 5-7°C lower than at Stanley Park.

Over the next two weeks, SSTs fell to 15°C at Stanley Park, coinciding with a large increase in zoospore production on September 19th, to 14.0 x 10^6 zoospores/cm^2. SSTs continued to decline at Stanley Park reaching 12°C on October 11th when the next collection was made. These sori produced 3.79 x 10^6 zoospores/cm^2 which was a little lower than the number of zoospores produced at French Beach from sori collected five days later (4.58 x 10^6 zoospores/cm^2). Temperatures remained below 11°C at both sites

for the remainder of October and November and the number of zoospores produced by sori on each of the collection dates were 6.75 x 10^6 zoospores/cm^2 or greater. In summary, spore production was highly variable at both sites, regardless of temperature. The biggest discrepancies between the two sites were early in the season during the month of July. At this time, SSTs were 5 or 6°C higher at Stanley Park than at French Beach and zoospore densities were substantially lower. However, later in the season Stanley Park spore densities returned to high levels even when SSTs were still high. Due to natural fluctuations in SSTs and high variability of spore production throughout the season, impacts of temperature on zoospore production is difficult to assess, although production of zoospores in Stanley Park appeared to be hindered early in the season when temperatures were above 17°C.

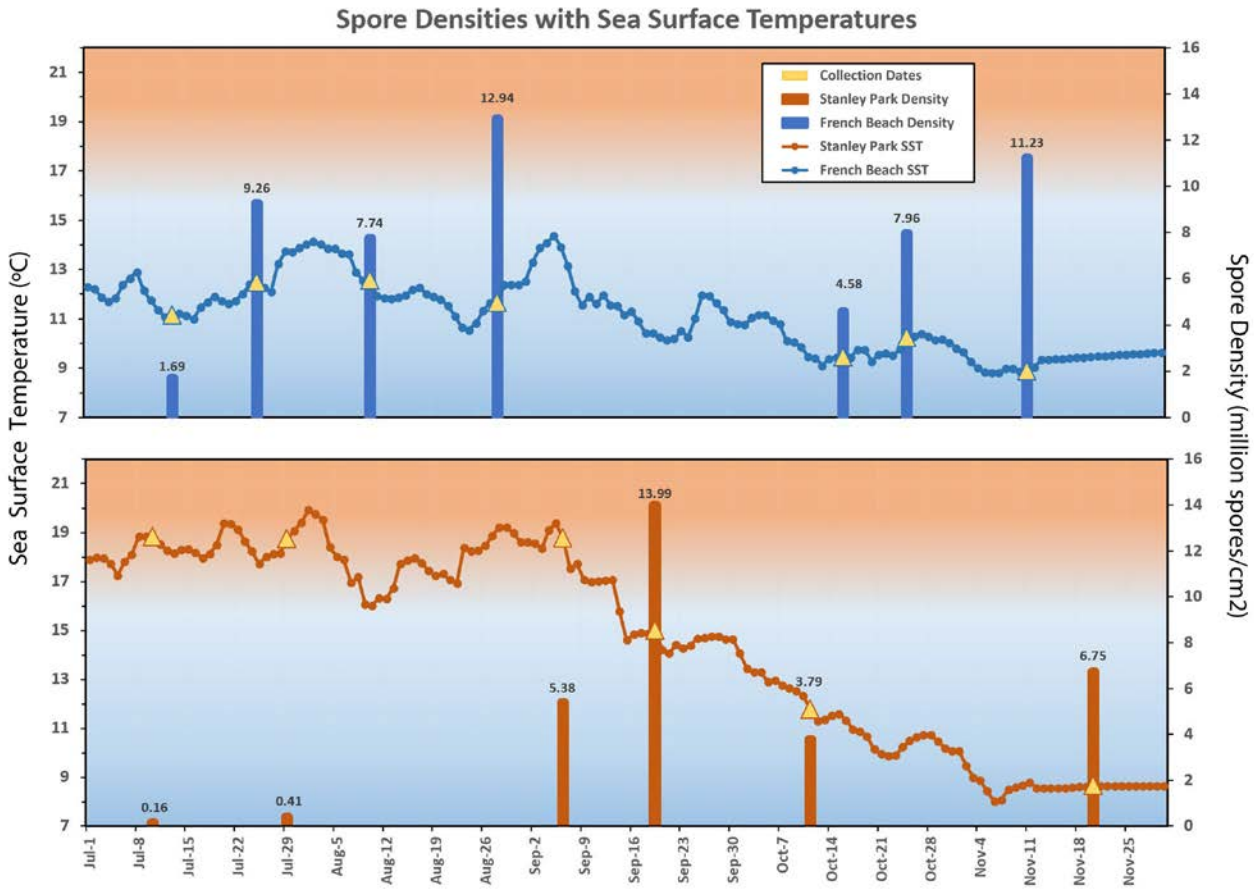

Figure 3.2. ***N. luetkeana*** **zoospore densities measured from Stanley Park (orange) and French Beach (blue) populations were highly variable throughout the reproductive season (July-November). SST data is shown as a line graph and is overlaid with spore densities displayed as bar graphs. SST units are shown on the left axis while millions of zoospores per cm^2 of sori is on the right axis. Yellow triangles denote collections dates.**

3.4.3. Effects of Temperature on Zoospore Germination and Germ Tube Growth

We evaluated zoospore development following exposure to a range of temperatures in the lab. Two variables were measured: the percent of zoospores that were able to germinate and germling growth. At 10 and 15°C, the majority of zoospores appeared to germinate, or form germ tubes. Occasionally, fragments of cells and cellular debris was observed, indicating that a few cells had lysed (Figure 3.3A, B). When exposed to 17.5°C, development was impaired and those that did develop had visibly shorter germ tubes. Fewer intact zoospores were observed along with increased cellular

debris, indicating an increase in the number of lysed zoospores (Figure 3.3C). At 20°C the amount of cell debris was extensive, and further development of the few remaining intact zoospores was severely impaired, indicating that temperatures of 20°C or higher are extremely stressful (Fig 3.3D). Temperatures of 25°C and 30°C were also tested but appeared to be intolerable as intact zoospores were not present.

To quantify the effects of temperature on spore development, the ability to germinate was assessed. After exposure to 10 and 15°C respectively, 60.8% and 62.3% of zoospores collected from French Beach germinated (Figure 3.3E, F). However, at 17.5°C, the number of zoospores that germinated dropped to 35.9% on average; At 20°C, only a small portion of zoospores were intact and very few of those zoospores germinated (0.1%). In comparison, Stanley Park zoospores responded to temperature treatments in the same way with respect to germination. At 10 and 15°C, 65.2% and 65.7% of zoospores germinated, and these averages dropped to 41.9% and 0.4% at 17.5°C and 20°C, respectively. These numbers are likely overestimates as extensive lysis was also observed and the number of cells that lysed could not be counted. These germination percentages were similar between populations, regardless of temperature. There was variability in the amount of germination between some trials when zoospores were incubated at the lower temperatures. For example, germination of zoospores incubated at 10 or 15°C ranged from 38.4 to 79.0% amongst all trials. Regardless of this variability, zoospores from both populations had germination rates decrease at 17.5°C when compared to 15°C ($p \leq 0.0268$; two-way ANOVA with post hoc Tukey's test).

Germling growth was also assessed by measuring germling lengths 24 hours after zoospores were shed. In germlings from French Beach, average lengths were 12.4μm (±1.2 SD) and 13.0μm (±1.0 SD) at 24 hours, at 10 and 15°C respectively. Following exposure to 17.5°C, germling lengths were shorter, measuring 10.1μm (±0.8 SD) on average. Stanley Park zoospores responded in a similar way, with lengths decreasing from an average of 13.2μm (±3.0 SD) and 13.7μm (±2.4 SD) at 10 and 15°C, respectively, to 8.5μm (±0.5 SD) at 17.5 °C (Figure 3.3G, H). At 20°C germling growth was not measured since there were few intact cells and those that were present had not germinated. These results show that, like germination percentages, germ tube lengths in both populations decreased at temperatures of 17.5ºC ($p \leq 0.0001$; two-way ANOVA with post hoc Tukey's test), with the highest growth occurring at 10 and 15°C.

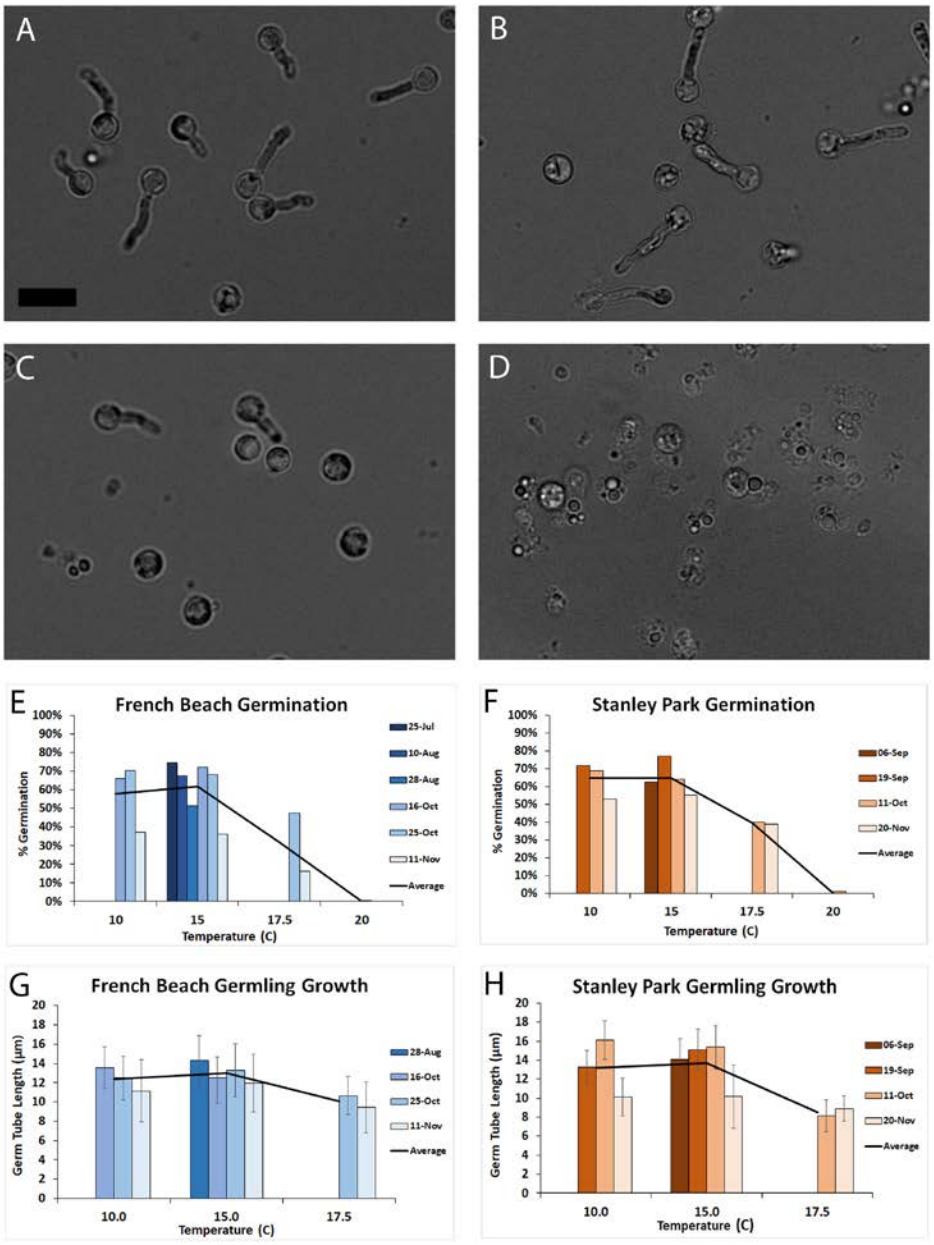

Figure 3.3. **Zoospores of *N. luetkeana* at 10°C and 15°C (A , B) appeared healthy with many zoospores germinated. At 17.5°C (C), more zoospores were round or ungerminated. With exposure to 20°C (D) extensive cell debris was observed, with few intact cells remaining. Size bar indicates 20um. Average germination rates per trial were quantified and showcase the decline in germination percentage at 17.5°C and 20°C in both populations (E, F) (N=105-1175 intact zoospores per bar for 10, 15, and 17.5°C). A similar trend was observed with rhizoid lengths, with a decrease in growth at 17.5°C and 20°C (G, H). Each bar denotes one trial while the black lines show average germination and germling lengths at each temperature. (N=14-48 per bar)**

3.4.4. Levels of Reactive Oxygen Species in Zoospores and Germlings

From the results presented above, temperatures of 17.5°C or higher were shown to negatively impact the number of zoospores that germinate and grow. However, under these warm conditions some zoospores from both sites were still able to germinate and form growing germ tubes. With the remaining intact zoospores and germlings we aimed to determine how stressed individual zoospores were following exposure to different temperatures. The fluorescent dye, CM-H_2DCFDA, was used to assess the amount of ROS present in germinated and ungerminated zoospores because increased levels of ROS are responsible for the negative effects that come with oxidative stress. As this dye enters the cell it is cleaved by intracellular esterases and becomes oxidized by various forms of ROS to produce a fluorescent product that becomes trapped inside the cell and can be viewed using an epifluorescent microscope. The observed fluorescence intensities serve as quantitative measures of the amount of ROS present in cells.

Following exposure to a range of temperatures, healthy germinated zoospores were expected to have low levels of fluorescence since they should be capable of scavenging and removing excessive ROS. For zoospores that were unable to germinate, stress levels were expected to be higher, coinciding with an increased production of ROS and higher fluorescence intensities. In all temperature treatments, germinated zoospores appeared very dim and were only slightly higher than background levels measured in areas of the slide adjacent to the zoospores, suggesting effective management of ROS. Comparatively, some ungerminated zoospores appeared bright while others displayed less intense fluorescence (Figure 3.4A-F). This means that some ungerminated zoospores could manage ROS levels sufficiently while others could not.

To evaluate stress in zoospores at each temperature, the amount of ROS in each zoospore was quantified. The mean fluorescence intensity was measured in photographs along a line drawn from the base of each germinated zoospore to its growing tip, or directly across the middle of ungerminated zoospores. These measures showed that all germinated zoospores from both populations had consistently low levels of fluorescence throughout, as expected for zoospores that were growing and developing. Fluorescence intensities ranged from 0-432 greyscale units (gu) (Figure 3.4G). This confirms that they were able to effectively manage levels of ROS following

exposure to all temperatures. Ungerminated zoospores however, exhibited a range of fluorescence intensities (1-3718gu), indicating some zoospores contained high levels of ROS while others had low levels. Although variability in fluorescence was high amongst all ungerminated zoospores, the average fluorescence intensities for ungerminated French Beach zoospores at 17.5°C were higher than they were at 10 and 15°C ($p \leq 0.0018$; three-way ANOVA with post hoc Tukey's test). Whereas, for ungerminated Stanley Park zoospores, average fluorescence intensities were lower at 17.5°C when compared to 10 and 15°C ($p \leq 0.0001$; three-way ANOVA with post hoc Tukey's test).

To determine whether there was a difference in the number of stressed zoospores between populations, a box plot was used to show the proportion of zoospores with different fluorescence intensities at each temperature (Figure 3.4H). This showed that many zoospores from the French Beach population had high levels of fluorescence at 17.5°C, with 25% having intensities over 2958gu, with a mean fluorescence intensity of 1159gu. At 10°C, 25% of measured zoospores were over 236gu and had mean of 508gu, while at 15°C, 25% were measured over 185gu with a mean of 457gu. Since the proportion of zoospores with high fluorescence intensities was much higher at 17.5°C, this shows that more zoospores from French Beach had increased levels of ROS or were stressed at 17.5°C. Comparatively, zoospores collected from Stanley Park had the largest proportion of zoospores with high fluorescence intensities following exposure to low temperatures, with 25% of sampled zoospores above 1875gu and a mean of 1018gu at 10°C. Whereas at 15°C, zoospores in the highest 25 percentile were above 574gu and had a mean of 721, and at 17.5°C, 25% of zoospores were above 290gu with a mean of 699gu. Since more zoospores from Stanley Park had high fluorescence intensities at low temperatures, this suggests that more zoospores were stressed with high levels of ROS at 10°C than at 15°C or 17.5°C. Thus, the proportion of zoospores with high levels of ROS was largest at 17.5°C for French Beach zoospores and at 10°C for Stanley Park zoospores.

To assess whether ROS was variable in ungerminated zoospores at each temperature, the proportion of ungerminated zoospores with high and low levels of ROS is shown in Table 3.1. The table outlines the percentage of ungerminated zoospores showing fluorescence levels higher than the maximum fluorescence measured for a germinated spore (>432gu), or in other terms, the proportion of ungerminated zoospores with high levels of ROS. These percentages show that more ungerminated French

Beach zoospores (65.6%) showed high fluorescence intensities at high temperatures (17.5°C), whereas more ungerminated zoospores from Stanley Park (75.8 and 74.0%) did so at lower temperatures (10 and 15°C, respectively). Thus, zoospores from French Beach had a higher proportion of ungerminated zoospores with high levels of ROS at high temperatures compared to low temperatures. Stanley Park zoospores on the other hand had more ungerminated zoospores with high ROS production at low temperatures compared to high temperatures.

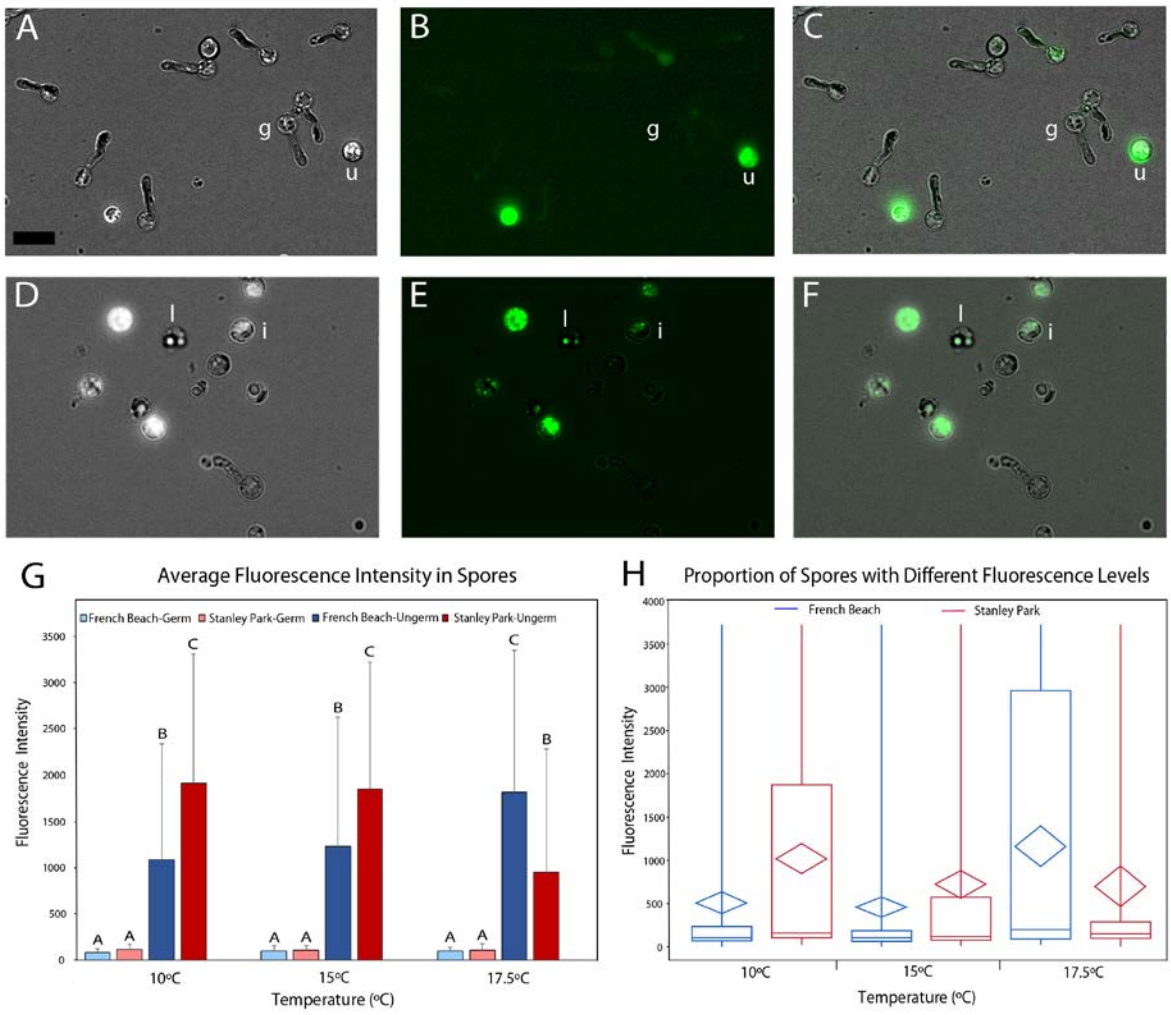

Figure 3.4. **Images of *N. luetkeana* zoospores at 15°C captured under brightfield settings (A), showing fluorescent ROS in zoospores marked by CMFDA (B), as well as the two images overlaid one another (C). Similarly, zoospores are shown at 17.5°C in (D, E, F). "g" denotes germinated zoospores while "u" is next to examples of ungerminated zoospores. "l" indicates a lysed spore whereas "i" showcases an intact spore. Size bar indicates 20um. Quantification of ROS levels in germinated and ungerminated zoospores showed that germinated zoospores had very low levels of ROS (below 432 greyscale units) while ungerminated zoospores, although variable, showed much higher levels of ROS on average (G). The number of zoospores that showed high levels of ROS was higher in the French Beach population when exposed to 17.5°C. Whereas Stanley Park had more zoospores with high levels of ROS when at 10°C (H). Box plots show upper and lower quartiles (75th and 25th percentile respectively) as well as maximum and minimum measures indicated by whiskers. The confidence diamond contains the mean, with top and bottom points showing the upper and lower 95% of the mean. The middle line shown within the box is the median. (N=101-268 zoospores per temperature from both populations).**

Table 3.1. Zoospore ROS Levels

Site	French Beach			Stanley Park		
Temp (°C)	10	15	17.5	10	15	17.5
Spores w/ High ROS	20.18% (N=223)	15.67% (N=268)	40.38% (N=156)	37.92% (N=240)	26.15% (N=218)	21.78% (N=101)
Ungerminated Zoospores w/ High ROS	47.9% (N=45)	49.4% (N=42)	65.6% (N=63)	75.8% (N=91)	74.0% (N=57)	31.0% (N=22)
Ungerminated Zoospores w/ Low ROS	52.1% (N=49)	50.6% (N=43)	34.4% (N=33)	24.2% (N=29)	26.0% (N=20)	69.0% (N=49)

Note: High ROS levels are described as any greyscale value over 432, the highest greyscale value measured in a germinated spore

3.5. Discussion

We found that 17.5°C is a restrictive temperature for early *N. luetkeana* development in both populations. While some zoospores can survive at 17.5°C, they will have a decreased chance at proceeding with early development. Evidence of this comes from our findings that average germination rates in both populations decreased from between 60.8-65.7% at 10 and 15°C to between 35.9%-41.9% at 17.5°C. Additionally, fewer intact cells were present at temperatures of 17.5°C due to increased lysis. Since the number of lysed cells could not be measured following exposure to high temperatures, it is difficult to determine how many cells had already died. This means that germination percentages are likely lower than the calculated percentage at 17.5°C. This is because when there were many cells that lysed in a trial, our calculated germination percentages were higher than they would be if the total number of zoospores, including lysed zoospores, were counted. This unknown amount of lysis contributes to a disproportionate germination percentage at high temperature compared to low temperatures since percentages cannot be calculated with the total number of original zoospores. Germling growth also decreased when exposed to 17.5°C, reaching lengths of 8.5-10.1μm compared to 12.4-13.7μm at lower temperatures.

The detrimental impacts of high temperature on spore development were similar in both Stanley Park and French Beach populations, however, ROS measurements exhibited differences between populations based on the number of zoospores that were stressed at each temperature. Stanley Park zoospores appear better equipped to

manage stress that comes with exposure to high temperature (17.5°C) since fewer zoospores had high levels of ROS compared to the French Beach population. Whereas fewer zoospores from French Beach had high levels of ROS at low temperatures (10 and 15°C) when compared to zoospores from Stanley Park. The number of zoospores with high ROS levels likely differed between populations because of variable ROS production in ungerminated zoospores, since those that germinated all produced low levels of ROS and the proportion that germinated was similar. Healthy germinated cells have likely reached a balanced equilibrium in which ROS scavengers or antioxidants have worked to remove excess reactive oxygen inside the cell.

Ungerminated zoospores exhibited a wide range of fluorescence measures in all temperature treatments. This could be the result of some zoospores being in a stage of dormancy producing little to no ROS. This would suggest that French Beach had more dormant zoospores when at 10 or 15°C, while more Stanley Park zoospores were dormant at 17.5°C, leading to less ROS production. Another explanation for variable ROS levels in ungerminated zoospores is that some zoospores are at different stages of progression, approaching thermal limits where lysis or death would occur. If the French Beach population had more stressed zoospores that are closer to death than Stanley Park at 17.5°C, this could be due to different levels of stress-induced expression of ROS scavengers. Bromoperoxidases have been shown to be involved with oxidative stress regulation in Laminariales (Wiencke and Bischof, 2012), thus, varying levels of scavengers like bromoperoxidases between populations could alter a spore's ability to discard reactive oxygen. Regardless of the mechanisms involved in ROS maintenance, the differences in number of zoospores with increased ROS show that the population of sporophytes of *N. luetkeana* from Stanley Park may have a head start in terms of acclimating to warmer temperatures in the future.

Given our measured zoospore densities and the temperatures at time of collection, zoospore production appeared to be limited at the beginning of the reproductive season, coinciding with temperatures that were above 17.5°C. Given that temperatures above 17.5°C were also restrictive for zoospore germination and growth, this means that the small number of zoospores that are released will also have a difficult time proceeding through development. This, in effect creates a shortened season for effective reproduction at warm sites like Stanley Park, by limiting the number of viable

zoospores early in the summer. Since French Beach was never exposed to temperatures exceeding 15°C, differences in the populations ability to produce zoospores during high temperatures could not be determined. Even though less zoospores were produced at Stanley Park early in the season, higher densities were measured just over a month later when temperatures were just as high. Thus, more trials from multiple field seasons will be needed to ensure if increased temperature is a determining factor for decreased spore density, rather than temporal or spatial differences. We know that variations in spore density may not only depend on the time of year, but also time of day. Amsler and Neushel, 1989 showed that 80% of sori were released 2 hours before and 4 hours after sunrise. Release of sori has also been linked to spring and neap tides cycles. They also showed that 50% of zoospores were released from abscised sori within 1 hour, and 95% within 4 hours. This quick release tactic likely aids in keeping zoospores nearby the parent sporophyte rather than getting swept away in the current. However, during collections one could not accurately tell how mature sori were, although only darker reproductive patches were used for shedding zoospores.

Past studies have estimated that a single plant may produce 3.7 trillion zoospores throughout a reproductive season (Scagel, 1961), suggesting that the impacts of temperature will not likely impact spore production on a scale that would be detrimental for long term success. However, studies on closely related species of Laminarians have shown that a minimum density of 1 spore/mm^2 is needed for successful recruitment (Reed, 1990), likely because as gametophytes grow, male and female thalli must be in close proximity for fertilization to occur. Although a chemical pheromone, lamoxirene, aids in this process, currents can be strong and disperses zoospores far away from one another. These variable currents and conditions mean that a high density of zoospores is required for fertilization to occur. Additionally, limited suitable substrate and the presence of grazers like sea urchins can decrease the chance for successful settlement and early growth.

In summary, these two kelp populations have been exposed to a wide range of conditions within their respective regions of the Salish Sea. Although they have shown resilience to these shifting environmental conditions in recent decades, our oceans are changing at an unprecedented rate. If incidents such as “the blob” or strong El Nino events continue to occur more frequently as expected, these integral kelp species may be in danger. On the other hand, if we are able to slow ocean warming to a rate where

these populations can improve ROS mediation and adjust their thermal range through acclimation, these species increase their chances of surviving long into the future.

Chapter 4. Discussion

In this thesis, the impacts of ocean acidification and warming on early development in two brown algal species, *Fucus gardneri* and *Nereocystis luetkeana,* are reported. In chapter 2, the effects of acidic seawater on zygotes and embryos of *F. gardneri*, a foundational species for intertidal ecosystems on the BC coast, was investigated. Rhizoid growth of embryos was found to be reduced following exposure to seawater with lowered pH, while early zygotic development proceeded normally. This suggests that zygotes can regulate early internal pH to sustain levels required for germination. Following germination however, acidic seawater impacts the rate at which rhizoid tip growth proceeds. This is likely due to an inability to maintain the internal pH gradient that is required for tip growth during this stage of development (Gibbon and Kropf, 1994). In chapter 3, studies on the effects of temperature on spore development and stress levels in *N. luetkeana* are reported. Zoospores from two populations residing at different sites in the Salish Sea were investigated. One population has been exposed to SSTs 5-6°C warmer in the Summer months than the other. We evaluated how a range of temperatures affects early development in zoospores from both populations and discovered that germination and germling growth was hindered by temperatures of 17.5°C or higher in zoospores from both sites. We also found that surviving zoospores from kelp growing at the site exposed to warmer Summer temperatures had lower levels of ROS after exposure to 17.5ºC than those collected from the site with cooler temperatures. This finding indicates that more of the zoospores collected from the warmer site are better equipped to withstand stressfully warm temperatures.

The effects of ocean acidification on *Fucus gardneri* in the Salish Sea are important to assess since the early development of fucoid algae relies on the maintenance of internal pH (Kropf et al., 1995). When early life stages were evaluated under a range of pH conditions, we established two major findings. The first was that early development of zygotes proceeded normally, even in pH conditions well below those currently found in the Salish Sea. During exposure to these various pH levels, germination rates remained high (88-94%) and zygotes germinated on time (between 10 and 12 hours). Since we saw these stages develop normally in all trials, this shows that zygotes are capable of withstanding a wide range of pH levels prior to germination. Our second finding was that embryonic rhizoidal tip growth following germination was

significantly affected by pH. With each 0.5 incremental decrease in external pH levels, we saw rhizoid growth rates significantly decrease, likely due to an inability to maintain an internal pH gradient during rhizoid tip growth. Embryos with inhibited growth may have difficulty settling or attaching to certain substrates, particularly when competing with other species. Additionally, if embryos take longer to develop they may be more vulnerable to grazing by small invertebrates that inhabit the intertidal zone or more susceptible to other stressors such as warming temperatures or pollution.

To evaluate *N. luetkeana*'s resiliency to heat stress in the Salish Sea we set out to find what temperatures zoospores could tolerate, or what their thermal limits are. We found that 20°C is deadly for nearly all zoospores and that 17.5°C negatively impacted germination and growth. At 20°C there was a large amount of cell debris and a small proportion of cells left intact, whereas at 17.5°C the number of zoospores that germinated was about half of the amount (35.9%-41.9%) observed during cooler temperatures (60.8%-65.7% at 10 and 15°C). More cell debris or cell lysis was also observed at 17.5°C compared to 10 and 15°C. Since warmer sites like Stanley Park can reach temperatures of 17.5°C or higher, this means that *N. luetkeana* zoospores are already exposed to stressful temperatures, likely reducing the number that are able continue their development and form germlings. Additionally, over the last century SSTs in parts of the Salish Sea have increased at a rate that exceeds global averages (Mote, 2003; BC Ministry of Environment, 2016). This means that as our global oceans increase by 1-2°C in the next century, portions of the Salish Sea such as the Central Strait of Georgia may exceed temperatures of 20°C for extended periods throughout the Summer months, effectively killing of zoospores of *Nereocystis* during that time.

We also questioned whether zoospores collected from local *Nereocystis* populations exhibit different levels of thermal tolerance since kelp growing in the Central Strait of Georgia was found to be exposed to warmer temperatures (4-6°C) in the Summer months compared to kelp growing in the Strait of Juan de Fuca. We found that zoospores from a cooler site (French Beach) had more zoospores with high levels of ROS present in high temperature treatments when compared to zoospores collected from a warmer site (Stanley Park). This suggests that more of the zoospores from Stanley Park have mechanisms in place for efficient removal and management of ROS

at high temperatures. Whether this effective intracellular ROS maintenance arose over years of selection and adaptation or short-term acclimation is unknown.

The ability of *F. gardneri* or *N. luetkeana* to effectively acclimate or adapt to environmental stressors like ocean acidification and warming depends heavily on the genetic makeup of individual populations or their ability to acquire beneficial genes from other populations. We know that monecious *Fucus* species frequently self-fertilize since both male and female gametes are released from the same conceptacle, often during periods of low water movement (Müller and Gassmann, 1985; Coleman and Brawley 2005). This inbreeding can lead to a lack of genetic diversity (Perrin et al., 2007), which over time could decrease a population's adaptive potential (Wernberg et al., 2018). Dispersal, however, can still play an important role in connecting gene pools from neighbouring populations. For *Nereocystis*, the co-ancestry and genetic diversity of populations in the Salish Sea was evaluated in a recent study (Gierke, 2019). This research found that *N. luetkeana* in the Salish Sea has low levels of genetic diversity, with a decreasing gradient in allelic richness from the outer coast into the inner Central Strait of Georgia and Puget Sound. Patterns in co-ancestry between populations from Alaska to California revealed that this reduced diversity is likely due to a founder's effect that could have occurred during the recolonization that followed glaciation events around 25, 000 years ago. (Gierke, 2019). Additionally, since millions of zoospores from the same sori are released in close proximity to one another, individuals must rely on currents and dispersal via rafting for outcrossing with neighbouring sporophytes. However, while the Strait of Juan de Fuca is subject to heavy currents and influence from the Pacific West Coast, up to only 42% of this water gets flushed into the Central Strait of Georgia (Khangaonkar et al., 2017). The lack of water exchange from the outer coast and high levels of pollutants and temperatures in the inner Salish Sea create environments that may require localized adaptation or acclimation for survival. Low levels of genetic diversity in *Fucus* and *Nereocystis* populations means that while acclimation is possible, adapting to environmental changes may be less likely. If populations can be found that exhibit a high range of tolerance because of their genetic makeup or previous acclimation, they can be selected for future rehabilitation and conservation work.

To better understand what genetic modifications would assist *Fucus* and *Nereocystis* acclimation, expressed sequence tag (EST) libraries can be developed to

represent what genes are expressed following exposure to abiotic stresses. Until entire genomes are sequenced for these species, the development of these libraries can aid in gene discovery and understanding of expression levels during environmental stress.

Multiple studies with *Fucus* sp. have measured an upregulation of HSPs in response to abiotic stressors, along with other transcripts that call upon light harvesting genes involved with photoprotection (Li and Brawley, 2004) (Pearson et al., 2010). Using this approach, EST libraries can be identified that contain genes or expression patterns responsible for the production of these protective proteins. Although this type of research has begun for species of *Fucus*, genetic studies on most brown algae are still in their infancy. Future studies can use these EST libraries as a benchmark when evaluating different stressors or ecotypes of a given species. For species of kelp like *N. luetkeana* that have been relatively understudied, other closely related algae can be used as a comparison for studies on gene expression and adaptation.

Recent research on another kelp species, *Saccharina latissima*, evaluated the sex-dependant impact of temperature (Monteiro, 2019). Gene expression measured in haploid gametophytes at a range of temperatures (4, 12, and 20°C) showed that gametophytes shift developmental and sex differentiation processes to focus on heat stress protection at 20°C compared to 4 and 12°C, suggesting low temperatures are more suitable for gametophytic growth and development. More studies like this on different brown algal species will be needed to better understand comparative gene expression through various life stages and what that means for acclimation during early development. A separate study evaluated the reproductive success in different populations of *Macrocystis pyrifera* from California and Chile under warming and acidification conditions (Hollarsmith et al., 2020). Researchers observed a reduced production of eggs and sporophytes when haploid zoospores were exposed to increased temperatures (20°C) and an increased production of eggs with low levels of pH (7.5). However, differences in reproductive success were established between populations that came from differing climatic regimes. Only zoospores from low-latitude California populations matured into diploid sporophytes during high temperature treatments, while zoospores from Chile produced no eggs. Also, gametophytes that came from sites with highly variable pH tended to produce more eggs in low pH conditions. Together these results show that reproductive bottlenecks have formed in certain *Macrocystis* populations and that responses to warming and acidification can be population specific.

Even research focused on thermal tolerance in corals and their algal symbionts have provided important evidence of how related brown algae may be able to acclimate or adapt to future warming. One study showed that prior exposure to high temperatures within a certain range can increase survivability during subsequent warming (Howells et al., 2011). This tolerance is largely influenced by the algal symbionts (Symbiodiniaceae) found within corals. By manipulating which populations of these dinoflagellates were present in corals exposed to warming, studies showed that Symbiodiniaceae from warm reefs increased survivorship of corals exposed to high temperature stress, compared to those containing algal symbionts from cooler reefs (Howells et al., 2011). Symbiodiniaceae are part of a sister-group (Alveolates) to stramenopiles, which both belong in the SAR (Stramenopile, Alveolata and Rhizaria) supergroup (Burki et al., 2007). Given the relatedness between brown algae and dinoflagellates, more could be learned about the ability of kelp and fucoid algae to modulate temperature from studies like this one rather than those focused on the much more distant relatives like plants, animals, and fungi. Thus, it is possible that prior chronic exposure to high temperatures may increase survivorship of brown algae in future warming conditions as they did for closely related dinoflagellates found in corals. These findings suggest that selecting for populations previously exposed to warming temperatures can aid in the restoration of coral reefs and may also be an effective strategy for other related algae in the race against ocean warming.

In summary, this thesis investigates how early development of *Fucus gardneri* and *Nereocystis luetkeana* is impacted by ocean acidification and warming, respectively. Although these foundational species have existed in the Salish Sea for several decades, there is no knowing how rapidly these environmental changes will take place or how long it will take to push these organisms outside their tolerable limits. As conditions in the Salish Sea continue to increase in acidity and temperature these organisms may be required to acclimate or adapt to survive. For *F. gardneri*, their level of tolerance to acidification in the future appears sufficient for survival throughout early development. Although embryonic growth decreased in low pH conditions, prior development through integral early life stages progressed normally, even in extremely acidic conditions. *N. luetkeana* on the other hand, appears to face a more immediate threat as some populations are already exposed to stressful temperatures. Thus, increased warming may limit effective spore production early in the season and reduce the ability of early

zoospores to germinate and grow. This also raises the question of how early zygotes and embryos of *F. gardneri* in the Salish Sea will cope with increased warming into the future. Scientists may be able to aid in the conservation of these foundational species by finding and protecting populations that display stress tolerant traits or have high genetic diversity that could aid in future acclimation and adaptation.

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