Correlating multiple players in the mass-wasting of seagrass

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Abstract

Seagrasses provide many critical ecosystem services including habitat for commercial fisheries, shoreline stabilization and primary production. Global seagrass wasting events in the 1930s and 1940s sparked fundamental research into the causal agents of seagrass decline. The marine pathogen *Labyrinthula zosterae* has been linked to disease symptoms in seagrass, and is capable of spreading leaf-to-leaf, causing necrotic lesions, which may eventually cause death of seagrass meadows on a global scale (Muehlstein 1988). Many scientists have investigated the infamous disease epidemics of the 1930s and 1940s, yet many questions and uncertainties remain. The possibility exists for a complex set of interacting forces coming together to cause catastrophic loses to seagrasses around the world. In this research study, a series of pilot experiments were undertaken with the intent to improve efficiency and accuracy of identifying, isolating and monitoring for disease outbreaks. We found the frequency of pathogen isolation varies by site, and so does the frequency of strain isolation. Also, *Labyrinthula spp*. are able to survive outside of seagrass beds in flotsam and beach wrack, which demonstrates a possible vector for *Labyrinthula* transfer between seagrass meadows. *Labyrinthula* can be isolated more frequently from green and green/brown tissue than from brown tissue alone, challenging the notion that the brown, necrotic tissue is the hub of pathogen activity. *Labyrinthula* is also easier to isolate during processing after samples have been refrigerated for a week. This finding allows scientists to culture material from meadows that are farther from laboratories and eases the burden of investigators who are in the field and in the lab. Finally, we found that *Labyrinthula* transfers from agar to agar in new cultures more effectively when the samples are left in the light when compared to samples that were transferred and placed in total darkness. All of these findings further support the theory that there are certain key environmental conditions which could affect the likelihood the pathogen will tip the scale in seagrass meadows from a state of infection towards a state of disease. Understanding the implications of this link between disease ecology and global climatic conditions increases our ability to assess the causes and risks of the so-called ‘wasting disease’ and other threats of extinction and mass-wasting in seagrass communities into the future, where climate change is predicted to negatively affect many coastal biological communities.

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Glossary

Eelgrass: Marine eelgrass; Zostera marina; a marine flowering angiosperm

Ecotoplasm: Exterior cytoplasm of the *Labyrinthula* spp. cells, otherwise known as ectoplamsodic nets, ectoplasmodic pseudopods and slimeways characteristic

Eutrophication: Increase in water column nutrient concentrations, leading to dense 'blooms' of phytoplankton and hypoxia. Common sources of excess nutrients in the nearshore marine environment include farms and leaky septic systems.

Hotspot: While this term is often used to refer to places of high biodiversity, here the term refers to places that are most likely to incur extinctions due to chronic and acute environmental stress. Common stressors are physical, chemical, biological or anthropogenic in origin.

Hypoxia: depletion of dissolved oxygen in the water column or substrate sediments to levels that are toxic to aquatic organisms

Infection: Colonization of a host (in this case, seagrass species) by another organism

Labyrinthula spp.: This term is used to describe the genus of *Labyrinthula*, regardless species or subspecies.

Labyrinthula zosterae: The pathogenic form of *Labyrinthula*

Lacunae: Spaces between the leaf blade cells that transport gases such as oxygen and sulfur

Outbreak: Related a stage in seagrass disease where the organism is rapidly spreading

Pathogen: Any biological organism, in this case often *Labyrinthula*, that is capable of infecting an organism and causing disease

Phenolic acids: phenolcarboxylic acids; type of organic compound found in plants and thought to inhibit infection of *Zostera marina* from *Labyrinthula spp*.

Phytotoxin: Any substance that is toxic to a plant

Pseudopods: 'False feet' of *Labyrinthula* cells thought to aid in motility of the cells through the ectoplasmodic nets

Seagrass: Any submerged, marine flowering plant

Sulfide: An anion of sulfur that is toxic to eukaryotic organisms, such as seagrass, in certain concentrations

Parenchyma: the cellular tissue, typically soft and succulent, found chiefly in the softer parts of seagrass leaves

Wasting-disease: A seagrass loss phenomenon, such as occurred in the 1930's in the North Atlantic Ocean, where extensive, simultaneous losses to seagrass meadows are caused by *Labyrinthula zosterae*.

Wrack: Seagrass, seaweed and other flotsam that arrive at the shoreline over a large body of water through wave action and tides, leaving piles in lines that form shore normal along the beach.

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DEDICATION

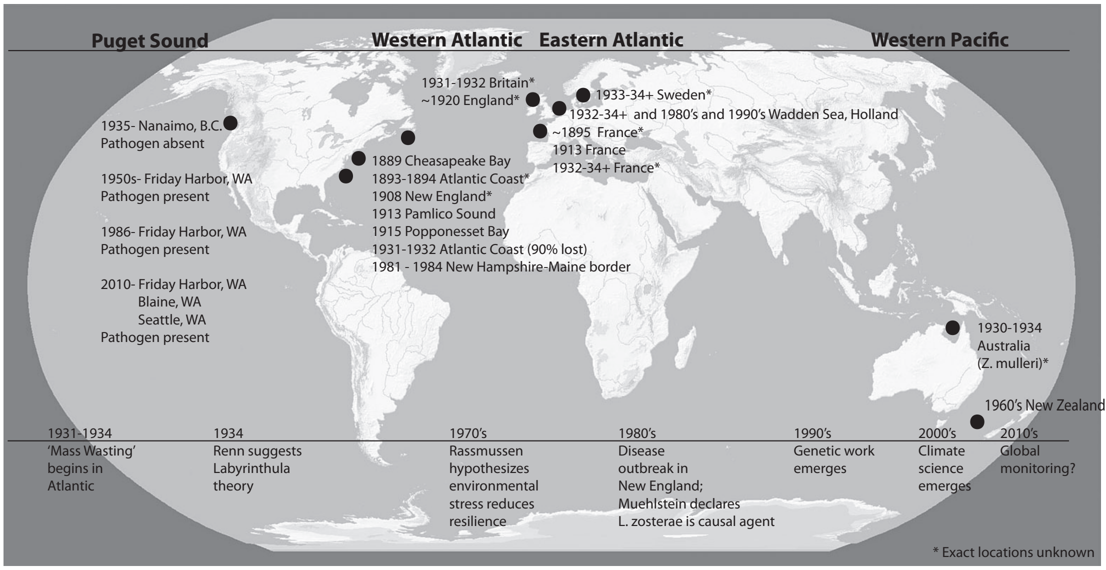
For the seagrasses

No matter what I say,
All that I really love
Is the rain that flattens on the bay,
And the eel-grass in the cove;
The jingle-shells that lie and bleach
At the tide-line, and the trace
Of higher tides along the beach:
Nothing in this place.

– Eel-grass by Edna St. Vincent Millay

Background

Reported losses to seagrass beds have occurred throughout the world and are still being recorded today (Short and Wyllie-Echeverria 1996). Severe losses to seagrass worldwide are reported as early as the 1889 in Chesapeake Bay as depicted in Figure 1 below. Reported losses from a so-called ‘wasting disease’ in the 1930s spanned two continents; North America, and Europe. Major losses to seagrass, occurring as a result of other anthropogenic and environmental causes, are known to have occurred in the United States, France, England, Australia, Africa, Mexico, India, (Short and Wyllie-Echeverria 1996). Although many articles have been written about acute loss of seagrass, and many theories have been proposed, there is no irrefutable causal agent in mass die-off events of seagrasses worldwide. A close analysis of the research provides evidence that multiple factors, acting alone or in concert, play a role in seagrass losses, predicting a persistent threat of acute seagrass loss into the future.



Atlas and timeline of *Zostera marina* ‘wasting’ events

Mapped based on reports from Cottam (1935), Short et al. (1986) and Short and Wyllie-Echeverria (1995) by Brooke K. Sullivan 2010 -2011

Figure 1: Atlas and timeline of seagrass mass-wasting events attributed to disease

Previous Research and Reports

Catastrophic loss of seagrass beds on both the eastern and western seaboards of the Atlantic coasts in the early 1930’s spurred the earliest wave of documented studies regarding acute loss of seagrasses. The losses were immediately attributed to a ‘mass-wasting’ disease, that some theorized had traveled across the Atlantic from the United States towards Western Europe. Mass seagrass wasting events observed in the 1930’s prompted many scientists to maintain records of the newly recognized disease phenomenon and join a search for the etiological agent of the disease.

Cottam (1935) reports losses from wasting events at various localities as early as 1889. Written theories about the causative agents of mass-wasting events developed after synchronized observations of localized eelgrass decline revealed widespread, acute loss of entire eelgrass beds along the western Atlantic Ocean (Huntsman 1932, Cottam 1933, 1935, Lewis and Taylor 1933, Mounce 1933, Stevens 1933, Taylor 1933, Renn 1934, 1935, 1936). One or two years following these early reports, subsequent reports of loss in the eastern Atlantic began surfacing as well; first in France (Fischer-Piette 1932) and then throughout the British Isles (Cotton 1933), Sweden (Lonnberg and Gustafson 1933, Blegvad 1933), Holland (Spierenburg 1933), Denmark (Petersen 1934, Blegvad 1934), and Germany (Wohlenburg 1935). Acute losses of eelgrass in coastal waters of the Pacific Ocean were not observed during that same period.

Symptoms

Several scholars reported symptoms of the 'wasting disease' at the time, in several locations around the world. While Butcher (1934) found it difficult to distinguish diseased plants from those dying at the end of the season, Den Hartog (1989) revealed that a key way to determine the cause of decline is to examine the young tissue. Because normal die-off at the end of each growing season does regularly not present itself in the youngest tissue, dark lesions in young tissue could be an indicator of advanced disease. Short et. al. (1986) found only dead matted rhizomes remained after a disease episode, with only a few remaining reproductive shoots or expanses and only a few scattered shoots where productive beds had occurred previously. In that study, field samples did not reveal an abundance of half-dead shoots or shoots covered in heavy epiphytic growth, as would be expected in beds suffering from increased turbidity, reduced light levels or eutrophication. Losses were not uniform within the beds; those in lower salinity and higher temperatures did not suffer losses to the same extent as those in higher salinity and lower temperatures.

Scientists observing mass-wasting events around the globe developed similar descriptions of the symptoms and progression of the disease during known 'wasting' events (Lewis 1932, Tutin 1934, Moffitt and Cottam 1941). Reports of the 'wasting disease' often included detailed descriptions of physical symptoms, including initial appearance of small brown dots, which then spread along the leaf blades and became larger black and brown streaks, often leading to loss of entire shoots and sometimes resulting in decimation of a total bed area. Scientists also observed discolored rhizomes following an outbreak, though discoloration did not occur as early as it did on the leaves. Further, rhizomes frequently survived for a year or more after observation of disease symptoms in the stem (Tutin 1938).

No reports of disease symptoms appear prior the 1930's epidemic. However, a subsequent review of herbarium samples in the British Isles and the Netherlands confirmed the presence of 'wasting' disease symptoms in seagrass blades prior the 1930's (Den Hartog 1989). The number of herbarium samples further appeared to decline after reported losses. Only a small portion of samples filed in the herbaria showed evidence of widespread disease in the populations sampled, yet substantial evidence of wasting disease symptom presence in eelgrass ecosystems prior to the 1930's, and as early as 1840, could be observed at the time of the study. Due to the selectivity of a given collector at the time of sampling, herbarium specimens should not be considered an accurate indication of actual field conditions. Collectors are often looking for exemplary specimen, and the samples are often further cleaned, removing dead and dying material prior to pressing for storage.

Recurrence

Observation of a disease outbreak at the border of New Hampshire and Maine in the 1980's led to a resurgence of scientific interest in the causes of such catastrophic seagrass declines. The disease symptoms observed in the 1980's were analogous to the accounts of loss from the 1930's. Studies of the mass wasting event of the 1980's in New Hampshire, were undertaken in both field and mesocosm experiments. From these observations a sequence key emerged to assess the wasting disease. First, small black spots and streaks appear, spreading longitudinally in the leaves of plants, causing air-filled lacunae to fill with water. After a few weeks, the leaves may become mostly or totally blackened, detach and float to the surface or sink to the bottom. Some blackened leaves may not detach from the rhizomes, but remain on the mud surface. After repeated defoliation, rhizomes may become discolored, and die. Only a few reproductive stems remained in the beds (Short et al. 1986, 1988). A Wasting Index was finally developed to help monitor progression of the disease in lab experiments, and in the field (Burdick et al. 1993); however, it has not been widely used or repeated in research since it was developed.

Recovery

Following the wasting-events of the 1930's Cottam (1935) reported that recovery began within a few years in many beds, however variation in recovery time could be shown along the entire western Atlantic coast. An expedition to determine recovery status in the Northeastern United States was undertaken by Cottam in 1934, and he found some locations were already recovering, while others were stable but not increasing in coverage. He observed and recorded that disappearances occurred slower and recovered faster in areas of lower salinity, suggesting an environmental component to recovery. As early as 1935, it appeared to many observers that the eelgrass was making a marked recovery in the United States and Europe, though some areas were not recovering (Cottam 1935; Martin 1954).

The role of microorganisms, environmental and anthropogenic stressors, phenolic acids and genetic variability in seagrass disease

A thorough review of literature pertaining to the wasting disease of eelgrass reveals seemingly contradictory information regarding causal agents, environmental and anthropogenic stressors and experimental results. Numerous scientists have studied potential causal agents, effects from and recovery following acute loss to seagrass by 'wasting disease' symptoms.

Role of Microorganisms

Disease as a causal agent in the mass-wasting of eelgrass gained immediate popularity (Nienhuis 1994). During the flood of research that followed the 1930's epidemic, many theories emerged to explain the wasting disease symptoms and observed losses of seagrass beds occurring simultaneously around the world. Early on, multiple potential pathogens were isolated from diseased tissue by various scientists. As mentioned previously, Den Hartog (1989) reviewed herbarium samples of eelgrass and found that symptoms of the wasting disease were present in the historical collection in Holland. Since he was not able to find correlations between the diseased specimen samples and outbreaks of the disease, he concluded that if an organism was causing the wasting disease, it was most likely ever-present in the eelgrass and smaller losses from disease events were a part of the regular ecology of the eelgrass plant.

Bacteria

Fisher-Piette (1934) reported the discovery of a bacterium in the diseased tissue, but did not prove its pathogenicity. Charles Renn (1935) quickly discounted this work because he was unable to isolate a pathogenic bacterium from eelgrass.

Fungi

Another potential pathogen, *Lulworthia halima (*previously *Ophiobolus halimus*), was isolated from *Zostera marina* by a number of scientists in Canada, the United States, Denmark and the British Isles (Mounce and Diehl 1934, Petersen 1934, Tutin 1938). Petersen (1934) was able to show that the parasitic fungus was able to invade healthy tissue. The fungal organism responsible for wasting disease was characterized by Petersen as a mycelium occurring in abundance in the cortex of the rhizomes. Petersen measured the hyphae as 2-5 microns broad, much branched, and divided by transverse septa, which penetrate and kill the seagrass cells. He noted that the peritheca development begins in late winter and early in spring and continues for a time into the summer. Petersen discovered the organism was capable of transmitting disease symptoms from rhizome to rhizome by infection through the hyphae, or by spores.

Oomycete

Charles Renn (1934, 1935, 1936) found a species of *Labyrinthula* could always be isolated in samples of diseased eelgrass tissue sent to him from across the United States of America and Europe. *Labyrinthula* has undergone many phylogenic changes since first being described and discussions continue today (Pokorny 1967; RaghuKumar 1996). RaguKumar (1996) states, "these organisms are swimming in ocean of benign neglect". Currently, *Labyrinthula* is described as a eukaryotic organism of the Labyrinthulid family of the Labyrinthulomycetes stramenophiles (TOL 2011). This particular reference lists a total of nine species, three of which (*L. cienkowski, L. macrocystis, and L. zosterae*) have been described as a similar pathogenic organism by various authors (Renn 1935, Pokorny 1967, Muehlstein 1988). The phylogeny and taxonomy of this genus is still under debate. However, *Labyrinthula* species are generally characterized as a distinct spindle-shaped cell, which develops a colony of cells that are surrounded by a common extra-cellular matrix.

Early on, Renn (1935) found *Labyrinthula* difficult to isolate, however he was able to observe the organism invading the living tissue of the plant and causing spotting on the leaves. He hypothesized for this reason that *Labyrinthula* was the most likely cause of the 'wasting disease'. Tutin (1938) also found *Labyrinthula* in diseased eelgrass and noted that once the diseased patch disappeared, the hypothesized pathogen could no longer be isolated from leaf blades in the meadow, and did not appear to be spreading any further to other patches. Furthermore, Tutin reports that seeds were always free of pathogens and theorized that areas of higher seed production would result in higher rates of regeneration during and after disease outbreaks. He substantiated this idea by the fact that seagrasses in the Mediterranean Sea, where conditions are right for heavy seed production each year, have reported no disease, and the Atlantic Coast of the United States, which suffers greater variation in temperature, results in reduced seedling recruitment and more severe disease outbreaks. In the south, regeneration from seed would occur more frequently, while the northern reaches may require translocation of rhizomes and shoots from other areas to recolonize lost beds. Due to these factors of ecological variability, Tutin believed recovery would occur more rapidly in the South, than in the North. No studies could be found that either supported or refuted this assertion. Young (1943) also made some very detailed observations of *Labyrinthula*, and interactions of the organism with eelgrass. Working under the guidance of Renn, Young was able to develop successful methods of culturing *Labyrinthula*. He also provides early drawings and diagrams of the individual cells and colony morphology. He appears to be the first to identify and describe a particular spore-like phase in *Labyrinthula* that he observed directly invading eelgrass tissue. These results suggested that at least two parasites might be capable of decimating seagrass beds globally, however the theory of *Labyrinthula* as the causative agent in the mass-wasting events gained immediate popularity and support. Scientists of the time hypothesized that a new pathogenic species of *Labyrinthula* might be capable of causing acute loss to eelgrass in coastal meadows and further, it was capable of being transported throughout the world.

Recurrence of *Labyrinthula*

Between years 1981 and1984, a new era of eelgrass loss emerged. A 'wasting-disease' recurrence at Great Bay Estuary at the New Hampshire and Maine state border exhibited similar symptoms to the epidemic observed by biologists in the 1930's. Studies, both in the field and in experimental mesocosms (Short 1985), reintroduced the theory that a pathogen from the genus *Labyrinthula* was the cause of 'mass-wasting' disease in seagrass (Short et al. 1986). Short found that eelgrass in culture flasks showed no signs of disease prior to exposure to *Labyrinthula*. Following inoculation experiments, eelgrass leaf blades were observed with 100 percent infection and resulted in 75 percent mortality after exposure to infected leaf tissue gathered from a tank showing evidence of wasting disease-like symptoms. Eelgrass in control culture flasks, showing no signs of disease, remained healthy and continued growing. During these experiments, temperature remained constant, ruling out temperature as the primary cause of eelgrass wasting event. They theorized that the protist in question was either not ubiquitous in the environment or that eelgrass itself may vary in its susceptibility to the disease (Short et al. 1986). Using Koch's Experiment, Short collaborated on another study with Lisa Muehlstein and David Porter (1987) in which they conclude that *Labyrinthula* is indeed the causative agent in the eelgrass 'wasting disease' phenomenon. The pathogen was determined to have two phases: infection and mass wasting. The initial infection phase, characterized by Short, Muehlstein and Porter (1991), included the presence of a pathogenic strain of *Labyrinthula*, causing dark, necrotic lesions on young and old tissue. The second phase occurs when there is a mass mortality event. In order to test for pathogenicity, strains of *Labyrinthula*, isolated in field samples, were cultured to analyze for variability in structure and appearance. Transplanting leaves from infected tissue onto uninfected specimen, showed symptoms of disease in infected plants. Within one week, 100 percent of the replicates observed suffered infection from the pathogenic strain and lesions spread to 3-4 cm in length. Controls and non-pathogenic tissue showed no signs of infection. Subsequently, Muehlstein and Porter renamed the pathogenic strain from *Layrinthula macrocystis* Cienkowski to *Labyrinthula zosterae*.

In contrast to the conclusions of Muehlstein and Porter, Den Hartog (1996) was unconvinced that *Labyrinthula* was the causative agent of the wasting disease. He observed that *Labyrinthula* had been isolated from nearly all eelgrass populations sampled, however a direct link between the pathogen and the mass wasting events did not exist. Life stages and genetics in the *Labyrinthula* organisms themselves are currently being explored in greater depth, which may confirm a spore-like phase in the lifecycle of certain *Labyrinthula* as was noted by Young in 1943 (Martin, personal communication 2010). Development of this research may help to answer some of the remaining questions about taxonomy and phylogeny, along with a better understanding of how *Labyrinthula* may have evolved in relation to seagrass, and add to further understanding its potential ability to induce mass-wasting events.

Role of Genetic Variability

A growing body of research suggests that genetic variability within eelgrass could affect the pathogenicity and disease resistance to pathogens (Wyllie-Echeverria, Talbot and Rearick 2010). A recent project undertaken in the San Juan Archipelago (WA) characterized the amount of genetic variation in eelgrass and *Labyrinthula* within and between beds (Gaydos et al., unpublished). Based on morphological variation, isolation cultures show at least four possible strains of *Labyrinthula* that could vary in their associated pathogenicity (Muehlstein 1988). Variation in pathogenicity and genetics of these strains could help explain variation in observed effects around the world (Gaydos et al., unpublished).

Role of Phenolic Resistance

More recent work also assesses the ability of eelgrass to release secondary metabolites, such as phenolic acids, that help a plant resist disease following infection. Vergeer and Develi (1997) observed phenolic acid production and release in *Z. marina* following infection. They observed that *Z. marina* contained a marked increase of phenolic acids in the older brown and streaked tissues of its leaves. Greener tissues also contained phenolic acids. Vergeer (1995) attempted to show a marked increase of the phenolic acids following infection by *Labyrinthula*. They propose that eelgrass, already stressed physiologically, may not be able to produce the levels of phenolic compounds necessary to ward off disease when infection occurs. These findings provide evidence for the importance of studying phenolic acids as a way of understanding physiological defense mechanisms. It is clear that more work is needed to understand the role of phenolic compounds and the role they could play as biomarkers of resilience to environmental stressors and disease.

Role of Environmental and Anthropogenic Stress

During the large outbreak of the wasting disease in the 1930's and 1940's, other stressors were identified as potential causes of acute loss of seagrasses. A theory of environmental pollutants as causative agents of the wasting disease, studied by a few scientists of the time, presented evidence of eelgrass loss occurring as result of oil waste (Duncan 1933).

Although early on, two potential pathogens were found to exhibit similar 'wasting disease'-like symptoms in the eelgrass, Tutin (1938) theorized that since these organisms were found in widespread populations under different climatic variables, the losses were more likely due to the ecology of the eelgrass itself, rather than a specific microbe. He theorized that limited light and turbidity could substantially stress a plant, making it more susceptible to disease outbreaks from omnipresent *Labyrinthula*. This theory was in conflict with the prevailing theories of the time.

Stevens (1936, 1939) was not convinced that Renn's discovery of *Labyrinthula* was the causative agent of the 'wasting disease'. Stevens believed the widespread, simultaneous loss of eelgrass suggested an environmental agent. Shortly after Stevens' paper, Martin (1954) found that precipitation extremes were strongly associated with the loss of eelgrass beds. By analyzing reported losses and precipitation events in the New England, Middle Atlantic and Virginia-North Carolina areas, he was able correlate losses with variations in known precipitation events. Along the Atlantic coast overall, 1930 was a period of extreme drought. In addition to the drought, temperatures and salinities in the bays were higher than normal for two consecutive years. The timings of the greatest loss of eelgrasses and the greatest droughts in the western Atlantic correlated in both 1898 and 1930. While this association could be explanatory, Cottam (1935) reports the eelgrass wasting disease began in 1931 and progressed into 1932, so occurrence of the drought may not have entirely coincided with the losses. Martin (1954) shares that recovery of the eelgrass from the 1930's drought was so slow that losses from the next drought reported in 1941 went either unnoticed or unreported. He reports that the most severe drought in Europe occurred in 1921.

In Europe, Butcher (1941) reported that accounts of eelgrass loss began long before the losses noted in the 1930's, stating that decline in the eelgrass was first noticed in the period of 1920-1922. Further losses were reported between 1931 and 1932; however no drought is recorded in Europe for that period. This report presents strong circumstantial evidence of the role that environmental factors could play in the outbreak of the 'wasting disease'. Discussions recorded at the end of the paper suggest that Cottam found these theories interesting, and he would not rule out the possibility of environmental factors or genetic variability in the eelgrass itself.

Lewis theorized that the Pacific eelgrass communities may have co-evolved with the *Labyrinthula spp.* allowing eelgrass to evolve resistance to the pathogen. He suggested that recent introduction of the pathogen to the Atlantic via of ballast water transport through the recently completed Panama Canal could account for the impacts of the pathogen there (Martin 1954).

In the 1980's scholars studied the impacts of environmental pollution and anthropogenic stressors as causes of major loss of eelgrass, and determined that such effects were capable of decimating an entire bed (Orth and Moore 1983). Impacts from nutrient loads, anoxic sediments ((Nienhuis 1983), industrial expansion, alteration of circulation patterns (Cambridge and McComb 1984), herbicide run-off, and turbidity (Jones and Tippie 1983) were studied to determine effects on existing eelgrass populations. Availability of light may also directly impact the host-pathogen interaction. Studies of the impact *Labyrinthula* has on seagrass photobiology show photosynthesis decreases and can be halted in areas of infection and disease (Ralph and Short 2002).

It is clear that environmental conditions play some role in the severity of epidemics and recovery. More detailed discussion of the role climate and environment could play in wasting disease events is given in the next chapter.

Role of Climate

Global patterns of changing climatic and environmental stressors to terrestrial and aquatic systems have received increasing attention by scientists over the last 10-15 years, though they are largely absent from historical research. Studies regarding the effects of global climate change have become more widely represented and better understood, so predictions about specific and localized effects have been closely examined for some regions, and discussions of bleak futuristic scenarios including mass-die off and extinctions are making their way into scientific research. The effects of global climate change on seagrass communities has been explored by several researchers (Brouns 1994; Platt et. Al. 1994; Edwards 1995; Bijlsma et. al 1996; Short and Neckles 1999). However, an analysis of global climate change on marine plant disease is lacking. There is still a good deal of research to be conducted regarding the relationship between changes to global climate patterns and the occurrence of the eelgrass wasting disease.

Short and Neckles (1999) explored and updated known and potential effects from global climate change, including increased temperature, sea level rise, increased carbon dioxide, and increased UV-B radiation. They found that the primary effects of temperature increases are alteration of growth rates and other physiological functions of the plant. Indirect effects of temperature changes are community shifts driven by the increased rate of eutrophication and changes in the frequency and magnitude of storm events. Furthermore, distribution of species in the ecosystem could change due to increased temperature stress and change in reproductive strategies. Change in sea level is predicted to result in other effects, such as increased water depth, shift in intertidal patterns, altered current patterns, and increased seawater intrusion into estuaries and rivers. Increased salinity could further alter distribution patterns and sexual reproductive success due to altered seed germination rates, propagule formation, photosynthesis, growth and biomass. Increased salinity may also lead to increased occurrence of acute losses from wasting disease (McCone and Tanner 2009). Increased water depth will limit light availability to deeper plants and reduce photosynthesis. Water movement pattern alterations will enhance turbidity and exacerbate the growth of epiphytes. Increased CO_2 could be expected to increase competition among species, as well as between various populations, including algae. Enhanced UV-B radiation could inhibit photosynthetic activity and increase resources from the plants being converted to UV blocking compounds. It is clear that many possibilities exist for alteration of seagrass ecology through global climate pattern changes.

Interintertidal Heat Stress

One thing Short and Neckles (1999) did not report was the variable effects of climate change to interintertidal species. Due to predictions of warming intensifying at the equator, many predictions in the last decade seem to have focused on a shift of sensitive species towards the cooler poles. Helmuth et al. (2002) changed the discussion about heat and desiccation stress by showing that patterns of heat intensity were not as simple as being strongest at the equator, and reduced father north. In fact, they found that higher latitude interintertidal ecosystems in the Eastern Pacific ocean, especially in Washington State, were at a much greater risk of being exposed to summer season mid-day intertidal lows, and therefore can be expected to suffer increased stress from global temperature increases when compared to sites closer to the equator where the lowest summer tides often occur in the night or morning rather than mid-day. Due to these findings, they predict that a number of extinction "hotspots" will be revealed at a local scale, especially in the north.

Hypoxia and Hydrogen Sulfide Intrusion

Another area of research into climate change that has gained momentum is the effect of hydrogen sulfide intrusion in eelgrass plants. Pedersen, Binzer and Borum (2004) looked explicitly at the effects of sulfide intrusion to eelgrass by analyzing levels of oxygen and sulfide simultaneously within the eelgrass plants. Sulfide, whose gaseous state equilibrium is controlled by pH levels, was shown to be a phytotoxin for *Z. marina.* Eelgrass produces oxygen, which it releases from root nodes, thereby oxidizing sediments in the substrate. Because photosynthesis cannot occur in the dark, risk of sulfide intrusion is greatest when production of oxygen is halted and oxidation of sulfide is limited. Sulfide is highly toxic to eukaryotic cells by inhibiting cytochrome oxidase at even very small concentration. The entire root may become anoxic after only one-hour of reduced oxygen in the water column, showing that storage of oxygen in the lacunae is not very efficient. This suggests oxygen is rapidly consumed by plant respiration or lost to the sediment. Once sulfide intrusion occurs, even after oxygen levels are restored, it can take many hours before the sulfide is re-oxidized and the plants return to a stable state. Sudden die-off of seagrasses have been observed and attributed to instances of hypoxia, hyper-salinity, and unusual periods of warm and calm weather. It is clear more research into the possible link between hydrogen sulfide toxicity and eelgrass-wasting disease is warranted.

When eelgrass plants are stressed, they have decreased ability to perform photosynthesis, so they cannot produce sufficient amounts of O_2 or phenolic acids which protect them from sulfide intrusion and disease. As yet, these findings have not been considered in the literature regarding impacts of global climate change to seagrasses or what the implications of these findings could be for the risk of disease epidemics therein.

Ocean acidification

Another major predicted effect of global climate change is gradual lowering of the pH of the ocean. The occurrence of ocean acidification has already been documented and is known to reduce the ability of calcifying organisms to produce shells and utilize calcium carbonate and calcium ions in metabolism (Hoffman 2010). *Labyrinthula* is further postulated to require calcium ions for development of ectoplasmodic pseudopods (Nakatsuji and Bell 1980). In an ocean with reduced calcium carbonate, this may be a sign of hope for seagrass communities facing global climate change.

Alteration of Host-Pathogen Interactions

In many cases, Harvell et al. (2002) have found climate warming will alter host-pathogen interactions by 1) increasing pathogen development rates, transmission, and number of generations per year, 2) relaxing overwintering restrictions on pathogen lifecycle and 3) modifying host susceptibility to infection. The question here is, what will alteration of host-pathogen relationships mean for *Labyrinthula* and seagrass?

Many studies have been undertaken in the last century or more to determine causal agents in the acute loss of seagrasses worldwide, however no conclusive findings explain all seagrass mass-wasting events that have been observed. A great deal is still unknown about the pathogenicity of *Labyrinthula spp*. and the roles outside environmental, anthropogenic, chemical and genetic factors have on the ability of *Labyrinthula* to shift from a phase of infection to a phase of disease and mass-wasting. A series of experiments was conducted to improve understanding of the ecology of *Labyrinthula* and to help guide the development of efficient sampling and culture methods.

Combined role of *Labyrinthula*, environmental and anthropogenic stress, genetic variability and phenolic compound production

Short et al. (1987) stated that both anthropogenic stressors and natural environmental factors could account for the loss of seagrasses globally. It has been suggested that the combined effects of these conditions could devastate eelgrass populations (Short et al., 1987; Den Hartog, 1989). Den Hartog (1989) further surmised that, due to variability in the local environmental conditions during the various outbreaks of the 1930's, no definitive cause for the eelgrass mass-wasting disease could ever be determined. While studies have measured key environmental factors for successful eelgrass growth and the effects of *Labyrinthula* on eelgrass under a variety of environmental scenarios, no key predictor has emerged to suggest that a single environmental condition could be solely responsible for elevated occurrences of the disease symptoms in seagrass meadows.

In Denmark, Rassmussen (1973, 1977) showed that elevated summer temperatures and mild winters could be associated with disease outbreaks in the 1930's. In the Dutch Wadden Sea, limited light penetration and increased turbidity over consecutive growing seasons was more closely associated with losses than temperature (Hartog 1977, Geisen et al. 1990). Following nearly a decade of decline, the Wadden Sea suffered total extinction of *Z. marina* in 2004. Reports indicate that cover by macroalgae in the eutrophic Wadden Sea may have restricted production of seeds, leading to unrecoverable losses in seagrass meadows. For this reason, eutrophication could be considered a contributing factor in the final extirpation of an already stressed population of eelgrass (Kawijk et al. 2010).

While many theories have been presented about the individual roles environmental, anthropogenic, microbial and chemical stressors on seagrass populations, the combined roles of these factors remain largely untested. Following the 1930's epidemic, there was still considerable controversy as to the actual cause of the eelgrass 'wasting-disease' (Muehlstein 1989). Scientists report more recent disease outbreaks and losses since the well-recorded events of the 1930's and the detailed disease work of the1980's (Kawijk et al. 2010, Short and Wyllie-Echeverria 1996). Although nearly a century of research has taken place on environmental requirements and limitations to both eelgrass and *Labyrinthula*, scientists are yet, still unable to determine exactly what triggers *Labyrinthula* to proliferate and cause acute losses of seagrasses.

When disease does result in mass mortality, losses of eelgrass significantly affect coastal ecosystems and economic systems that rely on them (Table 1). Eelgrass and other seagrass ecosystems provide important habitat for a variety of ecologically, economically and culturally significant resources, and loss of those resources may be critical to the function of coastal marine ecosystems and ecosystem services to human beings (Orth et al. 2006).

Table 1: Key ecological services and functions seagrasses provide for coastal ecosystems and human well-being. Ecological services and functions modified from the MEA (2005) and Phillips (1984).

Ecological Services to Humans	**Ecological Functions to Coastal Ecosystems**
Water purification	Nutrient cycling
Food production	Alter water flow
Fiber and fuel alternatives	Habitat
Sense of place	Primary productivity
Cultural heritage	Food for mega-herbivores
Aesthetic value	Sediment stabilization
	Gas cycling

There remain many unanswered questions about *Labyrinthula* and its role in seagrass decline. There is clear evidence to support more research of disease in the field of seagrass conservation and biology. Considerable variation among ecological conditions around the world suggests multiple factors may be acting in concert to trigger the favorable and parasitic growth of *Labyrinthula*, which could result in mass-wasting events around the world, similar to those that occurred early in the 20th century. With growing climatic variation, including the effects of temperature increases, sea level change, hypoxia, ocean acidification, interintertidal heat stress and eutrophication in coastal and nearshore ecosystems, more research is needed to clarify the relationship between possible causal mechanisms of loss to critical ecosystems that are shown to be vulnerable to extreme conditional changes.

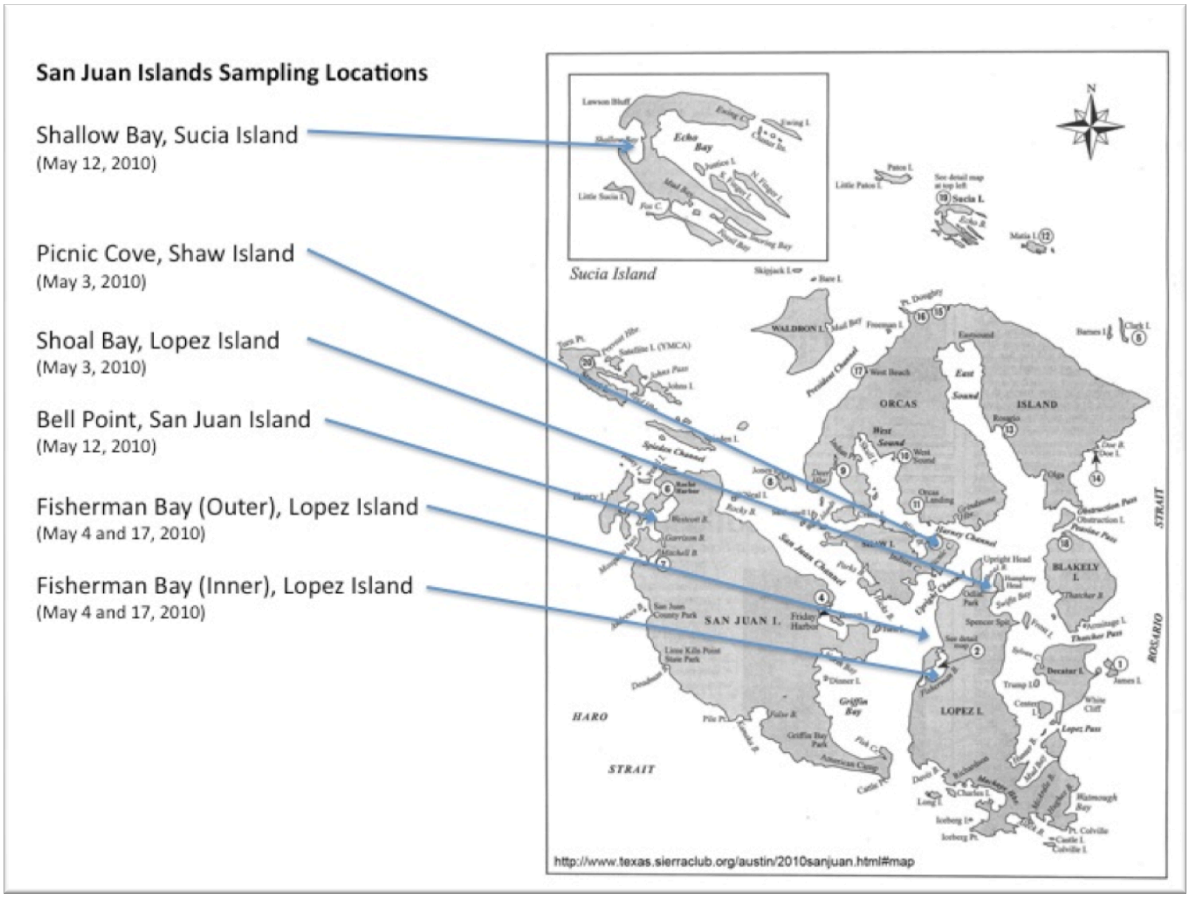
Presence of the pathogen in and of itself is not an indicator that mass-wasting will occur. In order to protect seagrasses, the biodiversity of seagrass-dependent species and the variety of ecosystem services seagrasses provide, research should focus on continued understand this poorly-understood organism, *Labyrinthula*. The combined roles of disease, environmental and anthropogenic stress, genetics and chemical resistance needs should be given priority in coastal marine research so that a conclusive explanation for the causal agents in the mass-wasting events of seagrass can be explored empirically. Seagrass beds, already physiologically weakened by environmental and anthropogenic stressors could be less resilient to infection by *Labyrinthula* so that under certain conditions, specific strains of *Labyrinthula* may be capable of causing acute and extensive mass-wasting events. However, a thorough investigation of these complex relationships is needed to demonstrate such connectivity. An attempt is made in the last chapter of this thesis to describe a possible framework for assessing interacting environmental effects on both *Labyrinthula* and *Z. marina* to begin developing analytical tools for assessing risk and identifying potential extinction hotspots at local and regional scales.

Summary

An analysis of literature pertaining to the causal agents in mass wasting events reveal differing and sometime contradictory results. Specifically, disagreements exist regarding the role of anthropogenic stressors, and other environmental variables such as climate, geography, chemistry, geology and biological agents in mass wasting events of seagrass meadows. In order to better understand the pathogen, a series of pilot studies were conducted to explore the potential to expand existing theories and challenge some of the assumptions that pervade growing body of research regarding the 'wasting disease'. There is a great deal of interest in understanding the role of ecological and climatic factors that can affect the ability of *Labyrinthula* to jump from an infection state to that of a fully fledged pandemic and epidemic phase of disease in seagrass. Targeted studies that approach these questions can build the framework for larger mesocosm and field studies that address these pertinent questions. Current sampling methods require a long and relatively complicated method of gathering and processing samples in order to isolate *Labyrinthula* from seagrass tissue. Being able to store the samples prior to processing could be very helpful for collections that occur in remote areas and could ease daily workloads of future research endeavors. Previous studies found *Labyrinthula* is ubiquitous in *Zostera marina* and other seagrass meadows (Vergeer and Den Hartog 1993), but the spatial distribution and frequency of occurrence of the pathogen within and between sites has not been reported in the literature. Using field and laboratory data collected from the San Juan Archipelago, Seattle area beaches and Drayton Harbor, multiple hypotheses could be tested related to the culture and ecology of *Labyrinthula zosterae*.

Methods

Seagrass samples obtained for all experiments were collected from northern and central Puget Sound. In northern Puget Sound, samples were collected from the San Juan Archipelago and Drayton Harbor near Blaine, Washington. In central Puget Sound, samples were taken from Golden Gardens Beach and Carkeek Beach, located in north Seattle, Washington.



http://www.texas.sierraclub.org/austin/2010sanjuan.html#map

Figure 2: Map of San Juan Archipelago subintertidal sampling locations. Samples were collected in partnership with the SeaDoc Society.

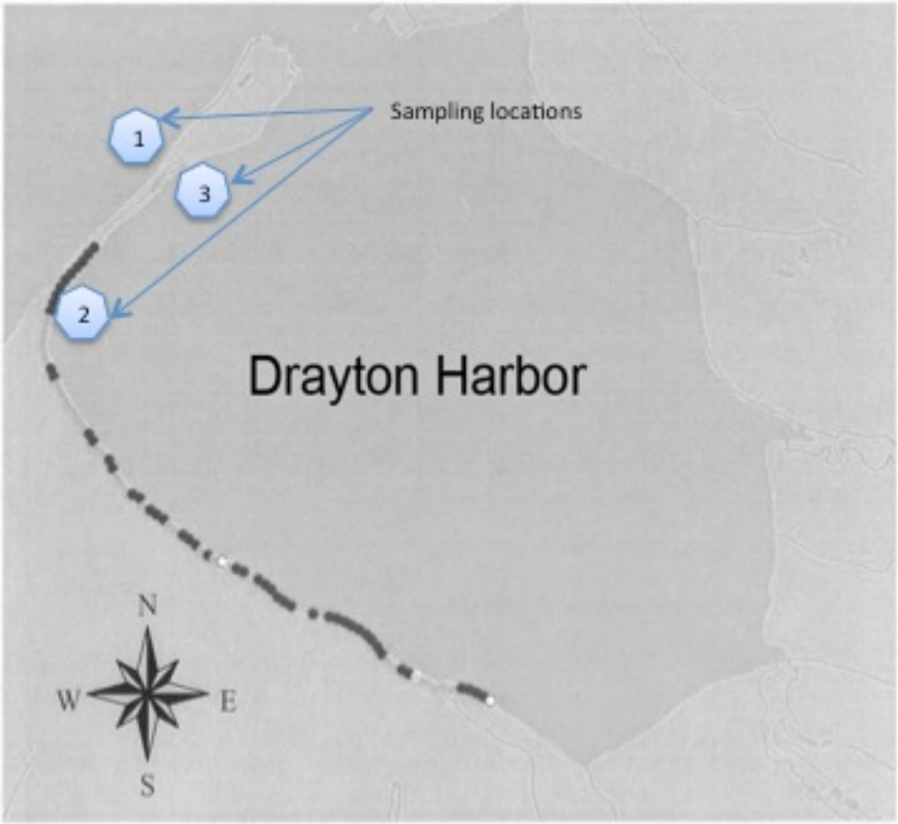


Figure 3: Map of sampling locations in Drayton Harbor, near Blaine, Washington. Samples were collected September 12, 2010.

In order to gather seagrass shoots for *Labyrinthula* isolation and analysis, two methods of sample collection were employed; intertidal and subintertidal. Subintertidal collections were obtained by diving for subintertidal samples along transects in nearshore waters of the San Juan Archipelago (Figure 2) and interintertidal collections were obtained by collecting samples along terrestrial shoreline transects in beach wrack located in Eagle Cove and Cattle Point on San Juan Island, Drayton Harbor in Blaine Washington, and Carkeek Park and Golden Gardens Beaches in Seattle, Washington (Figure 3).

Subintertidal Sampling

	A			MLLW		B		Shoreline			C
	0					0					0
	5					5					5
D	0/10	10	20	30	40	50/10	60	70	80	90	100/10
	15					15					15
	20					20					20
	25					25					25
	30					30					30
	35					35					35
	40					40					40

Figure 4: Typical transect sampling grid for subintertidal specimen (in meters). Samples were collected at 10 meter intervals from three transects that intercept one shore-normal baseline transect perpendicularly every 50 meters.

Subintertidal *Labyrinthula* samples were isolated from *Zostera marina* shoots collected in the summer of 2010. Samples originated in six nearshore *Z. marina* meadows within the San Juan Archipelago (Figures 2 and 4). The six sites were selected for sampling based on reported trends in their total areal coverage (declining or stable/increasing) as determined by the 2008 Department of Natural Resources Submerged Vegetation Monitoring Project report.

Photo 1: Diver prepares to enter the water with pre-numbered sampling bags and a data board

Subintertidal sampling, performed over the course of two weeks by four divers, was conducted by entering the water from the SeaDocII research vessel (Photo 1). Sampling at Shoal Bay on Lopez Island and Picnic Cove on Shaw Island occurred on May 3, 2010. Sampling of Fisherman Bay Inner and Fisherman Bay Outer on Lopez Island occurred on May 4, 2010. Sampling at Bell Point on San Juan Island and Shallow Bay on Sucia Island occurred on May 12, 2010. Final sampling at Fisherman Bay Inner and Fisherman Bay Outer occurred on May 17, 2010.

Photo 2: Plastic Ziploc™ numbered sampling bags

In order to collect the individual samples, plastic Ziploc™ bags, turned inside out around the sampler's hand were used like a glove to scoop the *Z. marina* specimen out of the substrate. Divers then pulled the bag right-side-out over the sample and sealed it from the outside, effectively isolating the plant until it could be taken to the lab for processing (Photo 2). This method ensured that microorganisms were not transferred between samples and by way of the diver during sampling efforts. Each *Z. marina* sample collected in the field was kept isolated from other samples by encasement in an individually numbered plastic bag and then stored in a cooler during transport to Friday Harbor Labs.

Table 2: Dive transect collection summary

	Subintertidal Collection Sites					
	Picnic Cove (PC)	*Shoal Bay (SB)*	*Fisherman's Bay Inner (FBI)*	*Fisherman's Bay Outer (FBO)*	*Bell Point (BP)*	*Shallow Bay (SHB)*
Trend	Declining	Stable/ increasing	Declining	Stable/ increasing	Declining	Stable/ increasing
Collection Date	5/3/2010	5/3/2010	5/4/2010 5/17/2010	5/4/2010 5/17/2010	5/12/2010	5/12/2010
Total samples	25	35	10+ 19 = 29	10+ 23 = 33	6	28

For each of the six sites, one 100-meter shore-normal transect was intersected with a 40-meter transect every 50 meters for a total of three transects per *Zostera marina* population. Each of the three transects was sampled at 2 meter intervals for 40 meters, leading to a total of 20-30 samples per site (See Table 2). However, only twelve plants could be located at the Bell Point site, of which six plants were sampled. In cases where there were no shoots at the sampling point on the transect grid, samples were taken along the baseline transect at 10 meter intervals to ensure enough samples were collected to generate desired power in our statistical analysis.

Intertidal Sampling

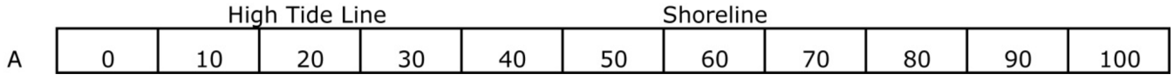


Figure 5: Typical beach wrack sampling transect

Samples and isolates that were not collected from underwater dive transects were collected from Eagle Cove and Cattle Point, May 2010, Carkeek and Golden Gardens Beaches October 7, 2010 and the Drayton Harbor shoreline on September 12, 2010. A 100 meter transect was established along the high tide line as determined by the presence of beach wrack (Figure 5). Subsequently, transects were sampled every 10 meters for a total of 10 samples per transect. As in subintertidal sampling, individual Ziploc™ bags were turned inside out and used like a glove to collect the plants and then pulled around the tissue in order to avoid contamination by the sampler and cross-contamination during transport to the lab. Samples were kept cool by storing in a cooler until they could be processed.

Table 3: Beach Wrack Collection Summary

	Collection Site			
	Drayton Harbor South (DHS)	Drayton Harbor West (DHW)	Drayton Harbor Outer (DHO)	Drayton Harbor Totals
Trend	Increasing	Increasing	Increasing	Increasing
Collection Date	9/12/2010	9/12/2010	9/12/2010	
Total Samples	5	5	10	20

Drayton Harbor intertidal sampling totals are shown in Table 3.

Processing Subintertidal and Intertidal Samples

Photo 3: Typical sample processing station

Photo 4: Post-collection samples ready for processing

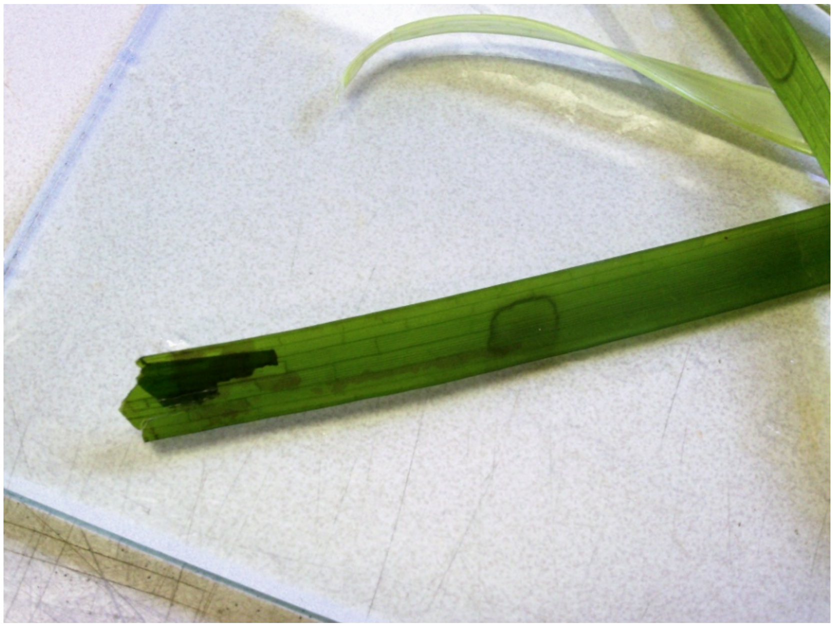


Photo 5: *Z. marina* leaf rinsed and ready for plating

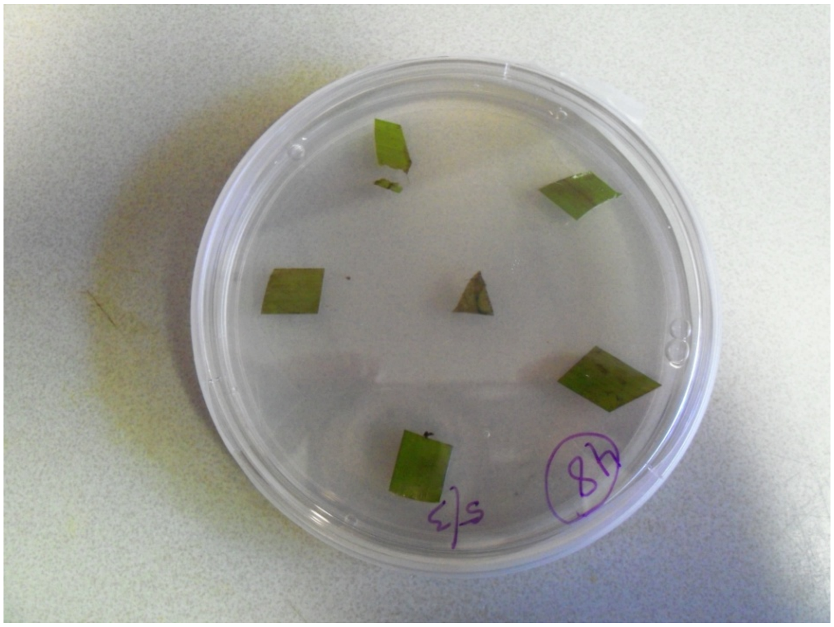


Photo 6: Plated 1-3 cm *Z. marina* leaf blades

The processing work stations were set up with EtOH, scalpels, scissors, filtered sea water, pre-plated sterile agar mediums, Parafilm™, glass plate, lighter, Bunsen burner, gloves, pens, pencils, camera and a light microscope (Photo 3). Once field samples arrived at the lab they were removed from coolers and placed in plastic holding trays for processing (Photo 4). Samples were then removed from the bags and processed individually, allowing for sterilization of equipment prior to processing subsequent samples. If the samples contained heavy amounts of mud, rinsing with filtered seawater removed excessive mud (Photo 5). Technicians then cut the second rank leaf blades of each specimen with a metal razor blade on a sterilized glass surface in order to obtain six to ten 1-3 cm leaf sections from each sample. Next, technicians placed the cut leaf blade sections onto agar culture medium (Watson and Ordal 1956, Porter 1990), which was mixed and plated on sterile disposable plastic Petri dishes the night before (Photo 6). Between samples, working surfaces, tools and hands could be sterilized with 95% EtOH to prevent cross-infection among samples. Each dish was sealed with Parafilm™ immediately after the blade sections were plated. Technicians processed samples individually and all tools and surfaces were sterilized with 95% EtOH between samples to avoid cross-contamination. All specimens were plated or treated for experimentation within 24 hours of sampling.

Variation in Frequency of *Labyrinthula* Isolation and Strains Between Sites

In order to assess whether variation in the frequency of *Labyrinthula* occurs between sites, seagrass samples were collected from six subintertidal meadows in the San Juan Archipelago during the summer of 2010. I reasoned that some sites would have more isolations based on variation in the presence of *Labyrinthula* over each site. Identification of the organism as it was isolated from plated leaf blades produced a spatial record of *Labyrinthula* infection by strain type for each site. Preliminary strain identification followed Muehlstein's (1988) characterizations of colony formations. *Labyrinthula* colonies were characterized as thick, medium or thin. Total numbers of samples in which *Labyrinthula* was present (P) or absent (A) were recorded for each site. The study was designed to use an A X B contingency table to categorize known values, where the site of the original sample was compared with the total amount of samples where *Labyrinthula* was either present or absent in the processed cultures and which strain was present.

Variation in frequency of isolation and proximity to shoreline

I tested whether or not the frequency of *L. zosterae* varied with respect to distance from shore. I reasoned that *Z. marina* growing farther from the shore would have higher frequency of *Labyrinthula* isolation because early studies of the wasting disease suggested deeper beds and those farther from the shore suffered greater losses during the wasting events of the 1930s. The data collected along the dive transects followed a standard grid originating from the Mean Low Lower Water mark (see Subintertidal Sampling above), allowing me to preliminarily test whether the frequency of *L. zostera* was associated with a known distance from shore. Data were analyzed to determine where on the subintertidal transect grid the samples originated and whether the sample resulted in an isolated colony of *Labyrinthula*.

Variation in Frequency of *Labyrinthula* Isolation and Parent Tissue Type

At the time of processing, we recorded the color and condition of the *Z. marina* leaf tissue from which *Labyrinthula zosterae* was isolated. Data from subintertidal sampling and processing were reviewed for notes regarding the type of tissue (green = g, brown = b, or green and brown = g/b) from which *Labyrinthula* was originally isolated. Instances where the parent tissue color was not noted were not included in the data set. I reasoned that *Labyrinthula* would be more likely to be cultured from green tissue because previous studies have shown that photosynthesis may be reduced in the green tissues surrounding dark lesions and that the advancing edge of infections is where the most rapid growth of *Labyrinthula* cells occurs.

In order to answer this question the culture data from subintertidal samples was ordered based on 1) the resulting *Labyrinthula* strain that was cultured, 2) the type of leaf tissue the sample was isolated from, 3) the sampling site location (Shallow Bay (SB), Fisherman's Bay inner (FBI), Fisherman's Bay outer (FBO), Picnic Cove (PC), Bell Point, Wescott Bay (WB), or Shoal Bay (SHB)), and 4) as either no strain present (N), thick strain isolated (Th) or other strain isolated (O). When more than one strain was isolated from a single leaf blade sample, two data entries were made. Finally, data were recorded as originating from a green, green/brown or brown leaf tissue.

Ability to Culture *Labyrinthula* from Seagrass Beach Wrack

The mechanism of spread of *L. zostera* between beds is not known. Leaf-to-leaf contact has been fairly well documented in the literature (Muelstein 1988). I reasoned that infected material coming off the living plants was a potential source of infection for *Z. marina*. Since the organism can grow without liquid solution in the laboratory, it seemed possible that beach wrack collections of lost tissue may contain living *Labyrinthula*. Testing seagrass that has washed ashore could prove beach wrack and flotsam are a possible vector of *Labyrinthula*. To address this question, we tested whether live *L. zosterae* could be isolated from beach wrack.

Photo 7: Typical sampling location in Drayton Harbor, Sept. 12, 2010. Beach wrack was 15-20 feet wide and contained approximately 99% eelgrass shoots according to a qualitative assessment of 1-meter square plots.

Zostera marina beach wrack samples were collected using intertidal sampling methods described above. Each sample was given a unique number so its original location would be known. Leaf blades, cut into 1 cm pieces and placed on an agar medium, were analyzed to determine whether *Labyrinthula* could be isolated from material that washes up on the tide line. Along with all the Drayton Harbor samples, six *Phyllospadix scouleri* samples isolated from Cattle Point, San Juan Island, and four from Eagle Cove, San Juan Island were also included in the analyses. All plates were observed daily during the experiment to observe *Labyrinthula* colony formations. Plates were kept sealed and viewed through a light microscope to ensure that small colonies were not overlooked. *Labyrinthula* status was recorded for each plate as either present (P) or absent (A). When a plate was overtaken with fungus, the plate was no longer observed. Relative cover by fungi and bacteria were also recorded from the Drayton Harbor samples at the end of processing. Samples were observed for a total of two weeks and scored as present when *Labyrinthula* was detected, and as absent when it was not.

Variation in *Labyrinthula* Strain Identification Through Colony Morphology Characterization

Current research suggests there may be multiple strains of *Labyrinthula* that vary in their pathogenicity (Muehlstein 1988, Martin et al., unpublished). If so, colony morphology could be an indicator of the strain present. We tested whether colony morphology is a consistent characteristic for strain identification by determining the morphology of isolated cultures, then re-plating the culture and re-determining their morphology.

Dive transect samples collected using subintertidal sampling methods were processed and plated on agar as describe above. Observation of *L. zosterae* colony formation and morphology were made daily. Presence of *Labyrinthula* was confirmed through X200 phase contrast microscopy. Once presence of the pathogen was confirmed, characterization of colony morphology was recorded to attempt to differentiate potentially different strains of *Labyrinthula zosterae* through cell and colony morphological characterization. At the time of isolation, morphological characteristics of the colony formation were recorded as thick, medium or thin. After recording this information, the isolated sample was cut from the agar and placed on a new agar plate. The new sample was observed daily and a second observer assessed colony formation type and recorded their findings. Variation in morphological assessment of each colony was used to determine the usefulness of colony formation in determining the potential presence of a particular strain of *Labyrinthula* and the likelihood of error in reporting by different observers.

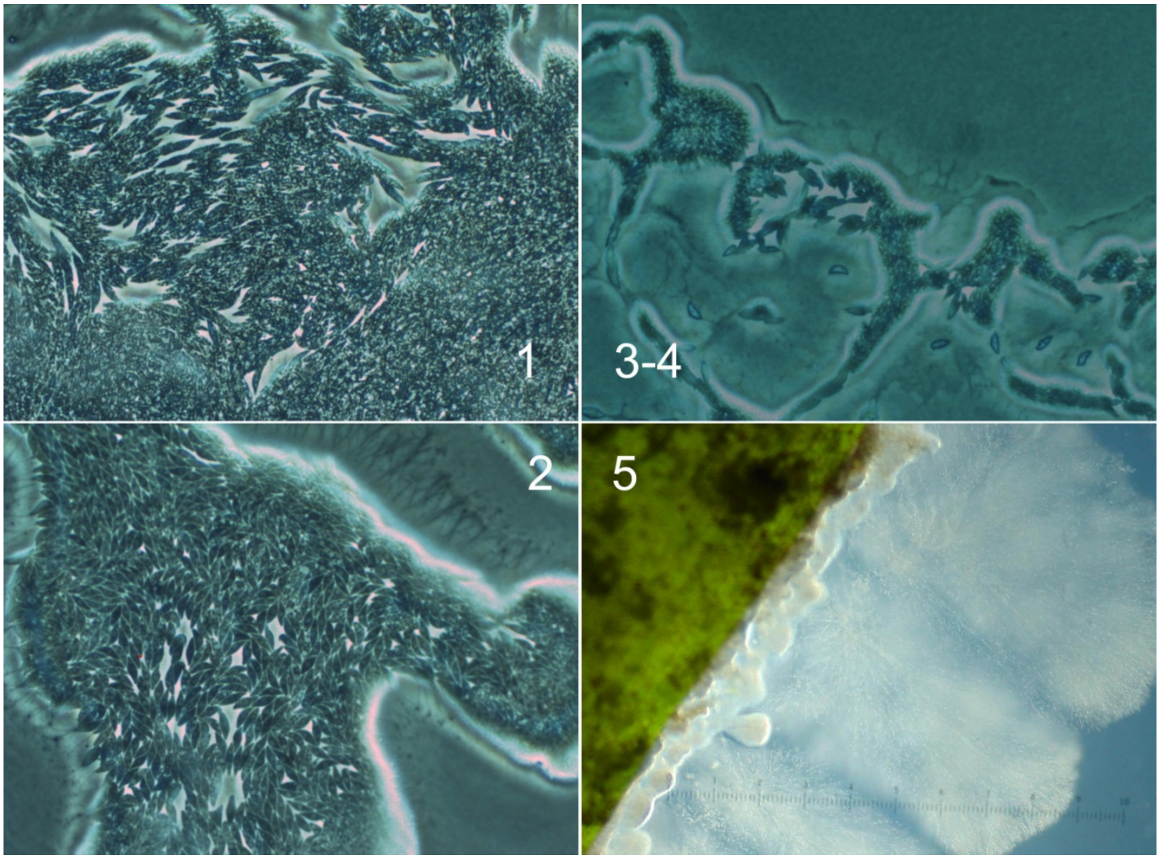


Figure 6: Variation in *Labyrinthula* cell morphology (Phase contrast x200, with the exception of Frame 5 which is x10). Frame 1 shows the cells of the thick strain, Frame 2 shows cells of the medium strain, Frame 3-4 is shows cells of the thin strain, and Frame 5 is showing a clover formation which we considered a thick strain as well (Photo by Dan Martin).

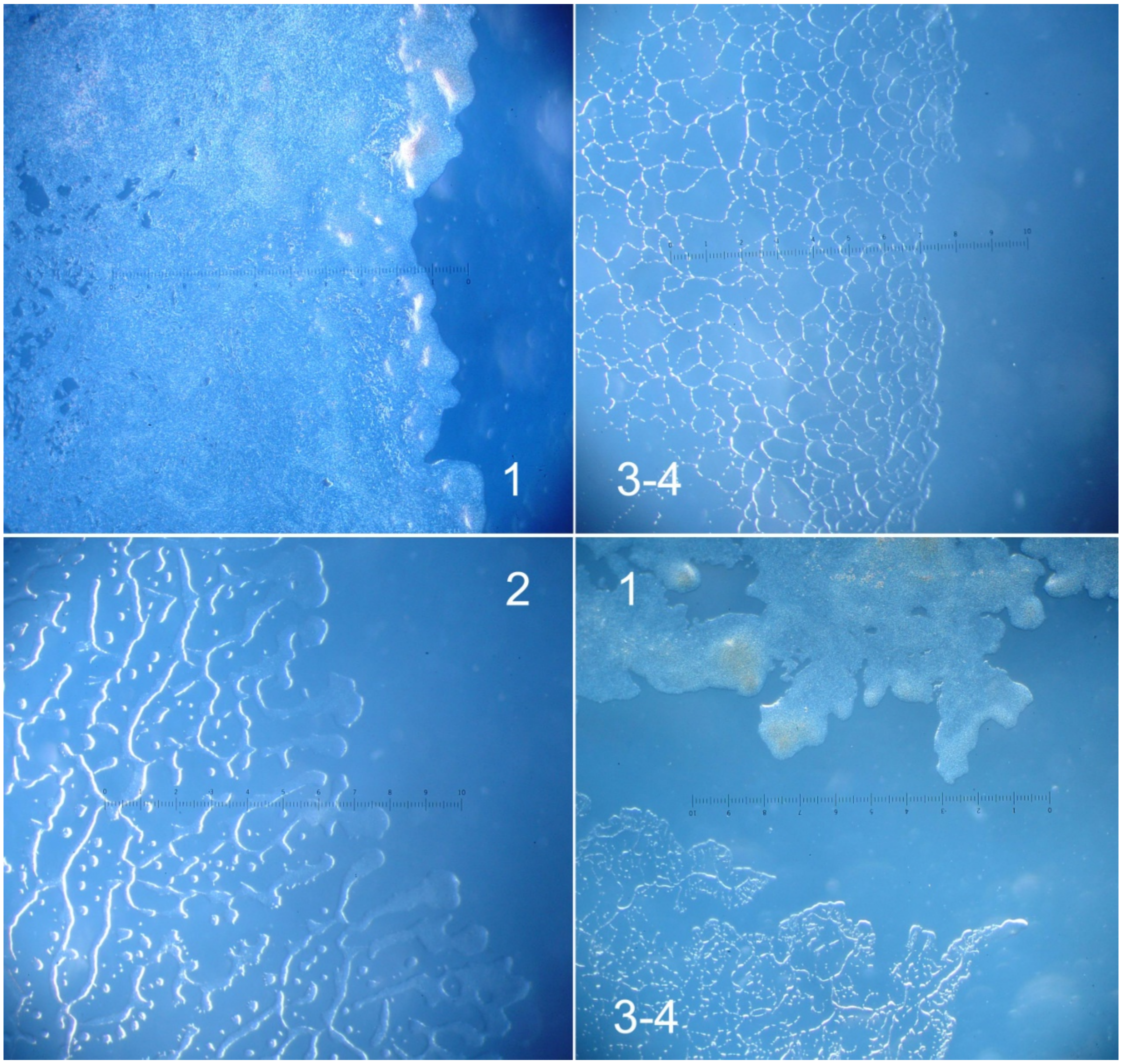


Figure 7: ***Labyrinthula*** **colony morphology (taken with a micron camera through a phase contrast microscope at x10 magnification). Number 1 is the thick colony, Number 2 is the medium colony, and Numbers 3-4 are the thin colony. The bottom right frame shows colony type 1 in the same medium as colony type 3-4 (Photo by Dan Martin).**

For both assessments, the colonies were analyzed at 10 X magnification to determine whether they should be characterized as thin, medium or thick colony growth (Figures 6 and 7). Notes on morphology further describe other characteristics, including the formation of a slick/continuous edge, fingered or branching patterns, orange color, and formation of what are known as "sori". Sori have been hypothesized to be reproductive stages in the Labyrinthulid and Thraustochytrid organism development (RaghuKumar 1996).

Effects of Refrigeration and Freezing on the Ability to Culture *Labyrinthula*

The hypothesis that sampled seagrass could be collected and stored prior to processing, resulting in the same success rate of isolation as fresh samples, was tested by gathering 50 samples using intertidal sampling methods. Samples were split into three groups, so that a third of the samples were refrigerated, one third of the samples were frozen and one third were plated fresh in order to determine the effects of freezing and refrigeration on the process of isolation. Samples were kept in the freezer and refrigerator for two weeks and then plated and observed daily for colony formation. Notes were also recorded regarding the presence and relative coverage of fungi and bacteria.

Effect of Light on *Labyrinthula* Culture Transfer Success

The role of light on the ability of *Labyrinthula* to grow from transferred agar was analyzed in the lab. Technicians transferred isolates of *Labyrinthula* collected from subintertidal sampling by cutting live cultures out of existing agar medium and re-plated onto fresh agar plates. Individual plates were placed in full light and full darkness for 7 days. Presence or absence of *Labyrinthula* was recorded on day 7. Samples that were removed from the dark treatment were then moved under growing lights (24 hour light period) for two weeks and new growth was recorded. Variation in presence was analyzed to determine any effect a treatment of total darkness may have on the ability of *Labyrinthula* to grow and spread. Samples that became overgrown with fungus during the experiment were not included in the results became it could not be ascertained whether *Labyrinthula* had grown in the agar or not.

Results

Variation in Frequency of *Labyrinthula* Isolation Between Sites

Figure 8: Subintertidal sampling isolation results (Prepared by Dan Martin). Red indicated a thick strain was observed, orange indicates a medium strain was observed, light orange indicates a thin strain was observed, green indicates that no Labyrinthula was observed, and white means no sample was taken at that point in the transect sampling grid.

Table 4: Summary of presence and absence data organized by distance

Site	Present		Absent		Total
SB	17	48.57%	18	51.43%	35
FBI	29	96.67%	1	3.33%	30
FBO	30	88.24%	4	11.76%	34
PC	13	52.00%	12	48.00%	25
BP	6	100.00%	0	0.00%	6
SHB	15	65.22%	8	34.78%	23
Total	110		43		153

The results from the transect grids above were broken down and analyzed to assess whether significant variations in frequency of isolation occurred by site. Bell point, Fisherman's Bay Inner, and Fisherman's Bay Outer resulted in the highest percentage of *Labyrinthula* isolation by site, followed by Shallow Bay, Picnic Cove and Shoal Bay respectively (Table 4).

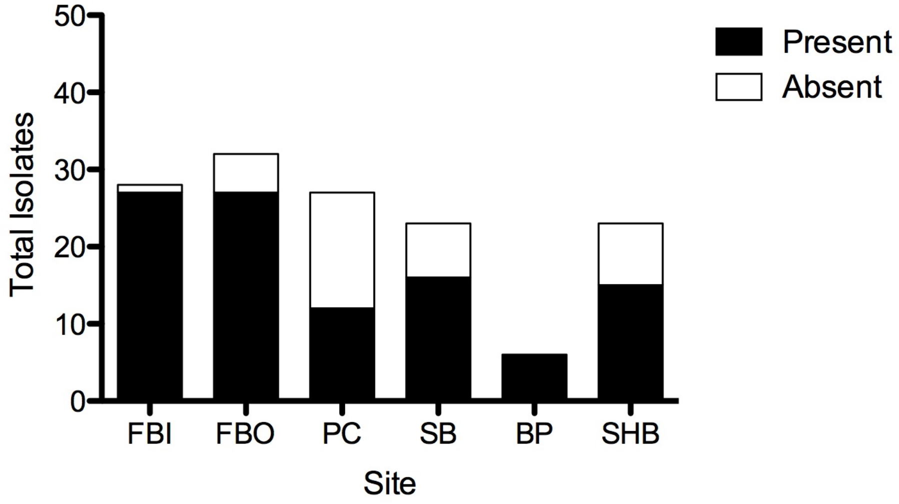


Figure 9: Frequency of isolation by site. $X^2_{.10,5}$ = 24.70, P (X^2=24.70) = .0002.

A bar chart was created to display the results of site the isolation study (Figure 9). All the sites, with the exception of Shoal Bay, had greater than 50-percent frequency of isolation. Bell Point and Fisherman's Bay Inner had greater than 90-percent positive isolation from subintertidal sampling. The Chi-squared test suggests we can be confident that significant differences in infection are present in at least one of the sites, leading to rejection of the null hypothesis.

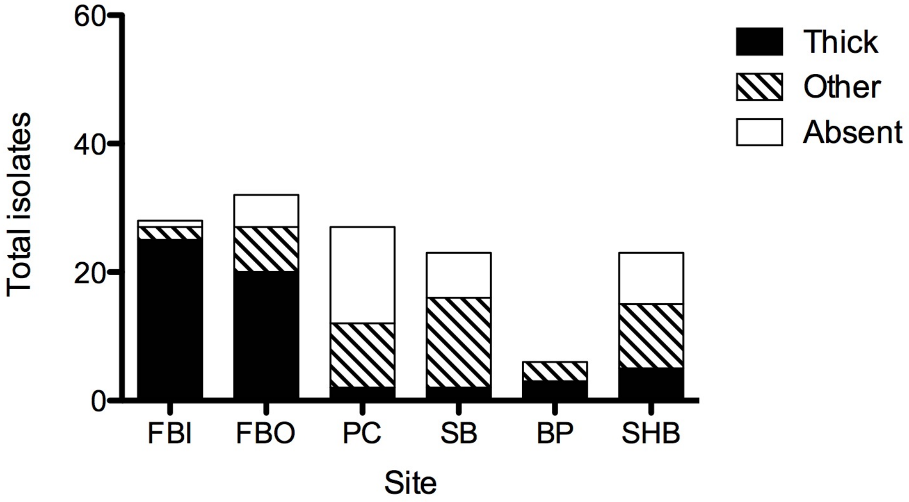


Figure 10: Frequency of isolation by site and strain. $X^2_{.10,10}$ = 66.98, P (X^2=66.98) < .0001.

Further break down of the site isolation by colony formation demonstrate that Fisherman's Bay Inner and Fisherman's Bay Outer resulted in the largest isolation by percent of the thick strain *Labyrithula* (Figure 10). The thick strain has been hypothesized by Muelstein (1988) to be the pathogenic form of *Labyrinthula*.

Tissue type and *Labyrinthula zosterae* isolation

In order to determine the likelihood of successfully culturing the microorganism from brown versus green tissue, all positive isolations of *Labyrinthula* from subintertidal sampling efforts were analyzed for differences in parent tissue materials. Data were analyzed for frequency of *Labyrinthula* isolation from each of the three conditions of parent material (green, green/brown and brown). A frequency analysis was conducted to test for significant differences in *Labyrinthula* isolation from green, brown and green/brown tissue. The data for this experiment can be found in Tables 5 and 6 and Figures 11 and 12.

Table 5: Total isolates organized by parent tissue color

Parent Material	Green	Green/ Brown	Brown	
Total Isolates	69 (33.5%)	92 (44.66%)	45 (21.84%)	206

The highest number of positive isolations came from green/brown parent material, followed by green and finally brown parent materials.

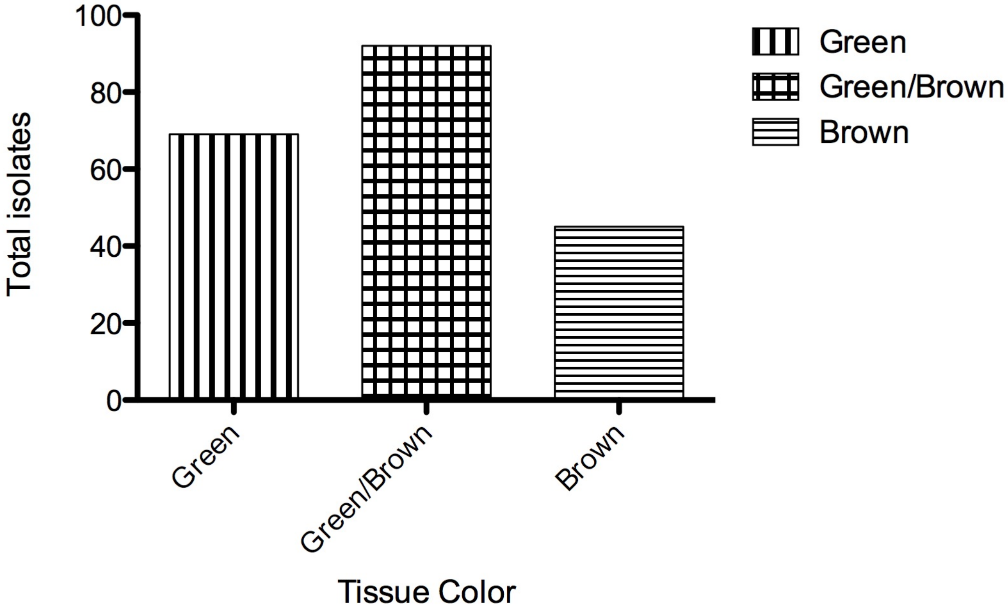


Figure 11: Bar chart of frequency of isolation by tissue color

A bar chart was used to visually demonstrate the variability of the data. When the total number of isolates cultured from each sample are considered in a Chi-Square Goodness of Fit test (Figure 11), there were more occurrences of isolation from green and brown tissue, followed by green tissue and lastly in brown tissue.

Frequency of Labyrinthula zosterae isolation with respect to distance from shore

Table 6: Observed occurrence of isolation and the proximity of samples to shoreline

		Present		Absent		Total
Distance	0	9	75.00%	3	25.00%	12
	5	7	58.33%	5	41.67%	12
	10	7	53.85%	6	46.15%	13
	15	11	78.57%	3	21.43%	14
	20	12	80.00%	3	20.00%	15
	25	9	69.23%	4	30.77%	13
	30	8	61.54%	5	38.46%	13
	35	6	54.55%	5	45.45%	11
	40	10	90.91%	1	9.09%	11
Total		79		35		114

Sampling results were organized into a table demonstrating the total number of samples taken, percent present and absent, if the sample resulted in isolation of *Labyrinthula* or not and how far from the shoreline the sample was taken (Table 6). The highest number of positive isolations occurred 40 meters from the shore, followed by 20 meters, 15 meters and 0 meters.

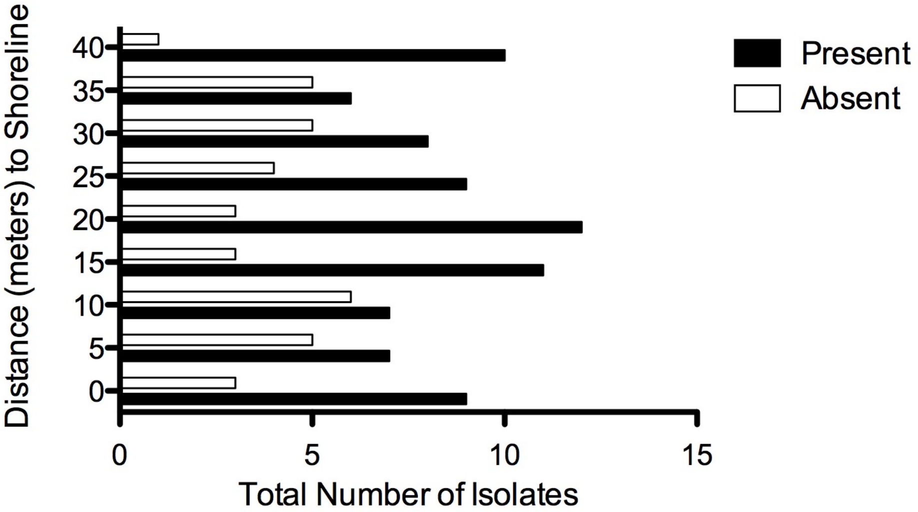


Figure 12: Variation in frequency of isolation and proximity to shoreline. $X^2_{.10,1}$ = .2853, P (X^2=.2853) = .5932

A bar graph was created to demonstrate the frequency of isolation at each distance on the transect grids. A Chi-squared analysis revealed that there was no significant variation in the frequency of isolation at the distance intervals tested (Figure 12). Our analysis suggests we accept the hypothesis that frequency of *L. zostera* isolation is unassociated with distance from shore.

Culturing *Labyrinthula* from beach wrack

Results from intertidal sampling were analyzed to determine the ability of *Labyrinthula* to survive in materials washed ashore. Data are shown in Figure 13.

Table 7: Beach wrack sampling results by site

Site	Present	Absent
1	0	1
2	1	0
3	0	1
4	0	1
5	0	1
6	0	5
7	1	4
8	1	4
9	1	4
10	2	3
11	6	0
12	4	0
Total	16	24

In this experiment, 16 out of 40 total samples (40%) tested positive for infection while 24 out of 40 samples (60%) were unsuccessful (Table 7).

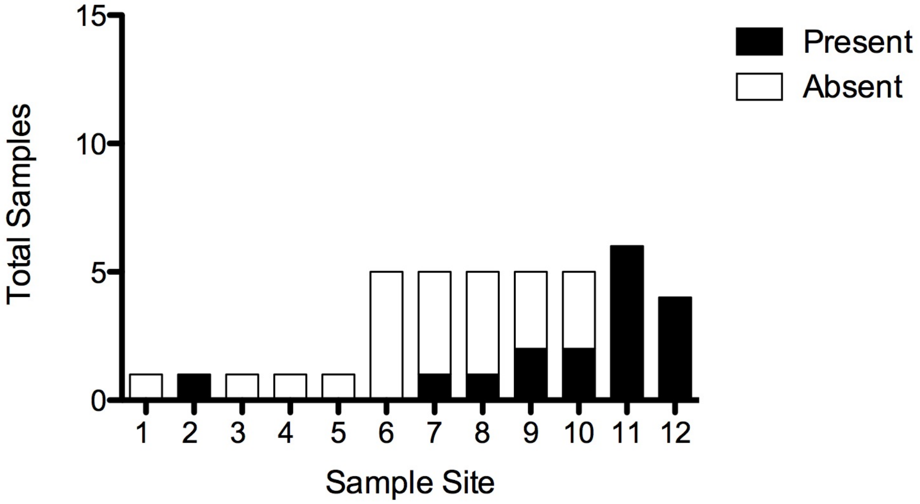


Figure 13: Isolation frequency from intertidal sampling. $X^2_{.10,\,11}$ = .23.63, P (X^2=.23.63) = .0144

Results indicated that it is possible for *Labyrinthula* to survive on beach wrack of both *Zostera marina* and *P. scoulerii.* Chi-square analysis reveals significant variation in frequency of isolation based on collection site. There are multiple morphologies of *Labyrinthula* noted for these results.

Labyrinthula **Identification and Colony Morphology**

In order to compile these data, records of morphological characterization of individuals cultured from material collected using subintertidal sampling methods were reviewed alongside morphological characteristics recorded for re-plated cultures. We counted each occurrence of individual strain isolations, and a comparison of lab notes was conducted to determine if differences due to changes in morphology were evident and whether observations were consistent between observers.

Table 8: Morphological variation found in data for *Labyrinthula* survey

Total entries surveyed	Number of entries where determination of thick, medium and thin colonies were recorded the same	Total number of entries where the colony type or at least one other visual characteristic, including presence of a continuous slick, or fingered/branching, orange, or sori were noted differently
59	35	24

The majority of samples showed no changes in morphology between plated generations and observers (Table 8). However, visual characteristics did vary between plated generations and observers in 40% of samples. Our methods do not allow us to confidently partition this variation between true morphological changes and observer bias.

Effects of temperature on samples storage prior to *Labyrinthula* isolation

Table 9: Total counts of *Labyrinthula spp.* isolation results and chi-squared goodness of fit test

	Present		Absent		TOTAL
Freezer	2	20.00%	8	80.00%	10
Refrigerator	5	50.00%	5	50.00%	10
Fresh	6	20.00%	24	80.00%	30
	13		37		50

Data show that refrigerating the samples prior to processing resulted in double the frequency of successful *Labyrinthula* isolation versus fresh plating and freezing samples prior to processing (Table 9).

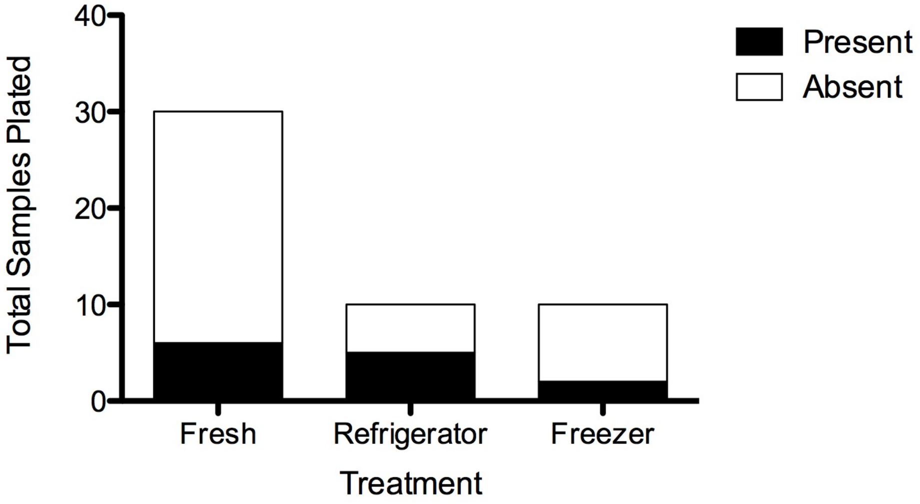


Figure 14: Effects of refrigeration and freezing on frequency of *Labyrinthula spp.* Isolation. $X^2_{.10,2}$ = 3.742, P (X^2=3.742) = .1540

However, Chi-square analysis reveals no significant difference in the frequency of isolation from fresh, refrigerated or frozen samples (Figure 14). The results of this pilot study suggest refrigeration and freezing for two weeks have no significant negative effect on the probability of successfully isolating *Labyrinthula* from leaf blades compared to fresh-plated samples.

Effects of light on *Labyrinthula* culture and isolation

Table 10: Data results from light experiment

	Present		**Absent**		**Total**
Light	24	92%	2	8%	26
Dark	10	26%	29	74%	39

Only 10 out of 39 samples (~25%) transferred positively to the agar media in the dark during the 2-week period (Table 10). Data reveal a 30-percent increase in the frequency of isolation from samples transferred and left in the light, versus samples transferred and left in the dark. It should be noted that all samples that grew in the dark were thick colony formations.

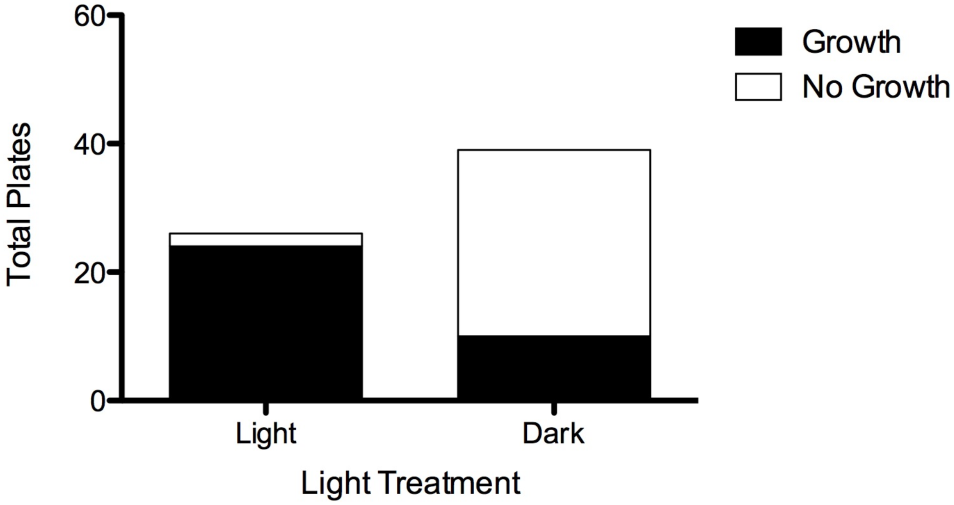


Figure 15: Effect of light on the growth of *Labyrinthula spp*. transfers. Fisher's Exact test P=.0001.

Fisher's exact frequency test shows that there is a significant variation in the isolation frequency between samples plated and left for one week in the light versus in the dark at a constant temperature of 22 degrees Celsius.

Discussion

Variation in Frequency of *Labyrinthula* Isolation Between Sites

Variation in infection by site reveals that although *Labyrinthula* is ubiquitous in seagrasses, the frequency of infection by *Labyrinthula* is variable. This finding may lead to discoveries about what environmental conditions favor an outbreak of disease by *Labyrinthula spp*. Future analysis may look at paired sampling of sites with treatments that are theorized to expand the density or range of *Labyrinthula* infection and contrasts in treatment effects can be inferred. The importance of developing theories and analyzing risks of disease and other catastrophic losses from climate change and other stressors cannot be over emphasized.

Variation in Frequency of *Labyrinthula* Isolation and Parent Tissue Type

There appear to be significantly more successful isolations from green and green/brown tissue, suggesting the darkened spots, lesions and streaks are not the center of active disease but rather where the pathogen has already struck. The patterns of growth and the longevity of *Labyrinthula* cells in culture further suggest that the leading edge of the colony is the place of advancing growth and cell activity. These findings suggest sampling efforts aimed as gathering as much *Labyrinthula* as possible should target sampling to green and green/brown tissue.

This finding is also consistent with other studies (Renn 1935), which suggest the microbe is present prior to the dark stains and streaking tissue characteristic of the wasting disease and may be absent from the dark lesion tissues. This naturally leads to questions about the effect of advancing and deteriorating ectoplasmodic nets on the seagrass leaf blades, and keeps the possibility open for alternative explanations of the wasting disease agents as was suggested by Neinhuis (1994).

Variation in isolation and distance from Shoreline

The data suggest that frequency of infection of *Z. marina* by *L. zosterae* is not dependent on the distance from shore, at least over the range of distances we tested. This was not the result I expected. A more complete spatial analysis would increase our confidence in this result, but data collected in this study revealed no spatial association with respect to distance from the shoreline. Further work in the ecology of this disease can consider the role of elevation in the ability to predict *Labyrinthula* infections. As was suggested by Vergeer and Den Hartog (1993), *Labyrinthula* appears to be ubiquitous in the nearshore meadows of San Juan Island Archipelago.

Ability to Culture *Labyrinthula* from Seagrass Beach Wrack

Our results indicate that live cultures of *L. zosterae* can be cultured from seagrass beach wrack. This finding suggests a possible mechanism for *Labyrinthula* to spread to new beds and leaves. However, because leaf blades from *Z. marina* populations adjacent to the beach wrack were not sampled, the relationship between the levels of infection in beach wrack and frequency of disease in the nearshore population could not be quantified. Such information could be helpful in understanding how drift accumulations of seagrass could function as vectors, and might give researchers some assistance in selecting efficient methods for gathering samples for regional monitoring efforts. Also, because these samples were taken from the beach late in the season, density and viability of *Labyrinthula* might have been less than if the beach wrack had been sampled in the spring or summer. For this reason, comparisons between the two sampling methods could not be made at this time. Still, it is clear that *Labyrinthula* can be isolated and transferred from beach wrack, so the pathogen is clearly capable of surviving out of water in the natural environment, and the potential exists for floating mats of *Z. marina* to serve as a vector for infection. Future research could address the role of flotsam, including algae and seagrass, in the distribution of *Labyrinthula zosterae* and other marine pathogens.

Variation in *Labyrinthula* Strain Identification Through Colony Morphology Characterization

Using colony morphology as a method of rapid identification of strain variability in cultured samples could save biologists time and money by eliminating the need for genetic testing of isolations to confirm pathogenic strains. The genetic analyses needed to show whether there are distinct strains of *L. zosterae* is still forthcoming. However, the data presented here show it may be possible for morphology to change within one re-plating event. Observer bias is also likely, suggesting that, when possible, repeated observations should be made by a single individual or replicated by multiple observers.

Effects of Refrigeration and Freezing on the Ability to Culture *Labyrinthula*

The ability to sample *Z. marina* beds in the field and store subsequent samples prior to processing would allow for empirical studies of disease to be accomplished in more remote areas where sufficient laboratory space is unavailable or inconvenient. Currently, samples are gathered and processed the same day, resulting in very long days in the laboratory that can become burdensome to investigators. Areas where research laboratories are not available are not currently being researched with the same rigor as site located near laboratories. These findings may also provide insight into the ecology of this disease by showing the temperature range of survival is quite a bit wider than expected. If anything, it may be helpful for technicians to refrigerate samples prior to processing. These results suggest further testing of collection and storage techniques may be fruitful.

Effect of Light on *Labyrinthula* Culture Transfer Success

The data suggests scientists should not place newly transferred isolates in the dark following processing. When compared with transfer data from previous experiments, this is a reduction in the success of transfer by approximately 75%. Out of the 10 samples with positive growth, 100% were observed as thick varieties with continuous edge characteristics, suggesting that the pathogenic strain of *Labyrinthula,* may be able to infect organisms and survive in long periods of total darkness and could possibly live outside of the littoral zone of the ocean, expanding the known range of the organism. This information may be helpful for researchers investigating the ecology of this elusive organism.

Summary

This research paper provides a comprehensive analysis of the historical research on the 'wasting-disease' and sheds light on areas of research in the culture and ecology of the disease that are still emerging today. Pilot studies in this paper show some areas where research may benefit scientists interested in improving isolation, identification and culture of *Labyrinthula* and may help shed light on the ecology of this elusive disease.

In the laboratory, these pilot studies suggest freezing and refrigeration of samples prior to processing may be an adequate method of storage. Another paired sampling test would be wise to confirm these results, but this pilot study revealed a clear ability to culture *L. zosterae* equally between fresh, frozen and refrigerated samples. Further, the green and green/brown tissue provided the greatest number of positive isolates following processing. This suggests future isolation efforts may benefit from focusing on plating greener material vs. the browner necrotic tissues that are identifying features of the disease. Samples should not be stored in the dark following re-plating efforts as growth was slowed or halted in most cases. Under a microscope there is some error in defining colony morphologies between technicians or the cultures morphologies may be changing between plating events, therefore it may be a good idea to develop clear definitions of key morphological features to reduce this error. Genetic testing of the morphological differences would also help determine if the various morphological strains are genetically different from one another and if any morphology can be associated with the pathogenic strain.

Ecologically, this research shows that flotsam and beach wrack are a viable vector of *Labyrinthula,* which may suggest a method for the infection to spread to other beds over wider areas. The studies also show infection rates may be variable between sites and that proximity to the shoreline has no significant effect on the ability to culture the organism from eelgrass blades.

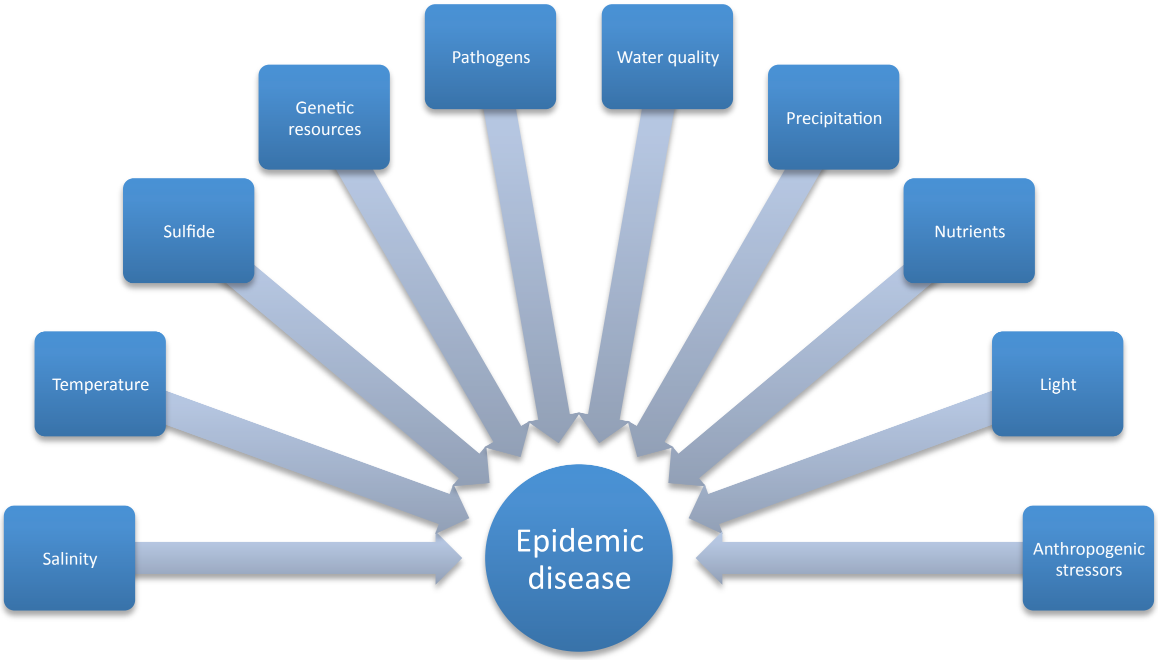


Figure 16: Conceptual diagram of causal agents in epidemic disease

The findings of this research clearly demonstrate a trend towards the existence of multiple players in the wasting disease of seagrass (Figure 16). Further analyses of these players, the relative role of those players and the relationships that exist between them should be assessed to pinpoint defining characteristics of ecosystems as risk of acute seagrass loss. The ecologies of *Labyrinthula* and seagrasses are still being discovered, and the geographic relationships of these organisms have not been adequately studied. What is clear is that more work is needed to address critical gaps in our understanding of the risks of extinction and acute loss of seagrass meadows, which serve as a critical habitat for many of our largest commercially viable fisheries and serves as a traditional hotspot of biological diversity in the ocean.

While these studies were undertaken with great care, limitations in time, funding and other resources, prevented detailed data collection and empirical analyses and testing of the multiple-players hypothesis.

Recommendations for future research

Despite a significant amount of published work on the causes of seagrass decline, losses to seagrass appear to be continuing all over the world. In the Wadden Sea eelgrass extinction was recorded as recent in 2004 (Katwijk et al. 2010). The connection between seagrass declines and disease, let alone other collaborating casual agents is still in its infancy. Since many commercial fisheries are dependent upon eelgrass as spawning and rearing habitat, it is unclear why a monitoring program has not been initiated in the Pacific Northwest to assess the role of disease in observed local seagrass declines, especially with existing seagrass monitoring occurring throughout the Puget Sound Georgia Basin.

With increased awareness of health and climate issues discussed in the Puget Sound Partnership action agenda, the role of disease in seagrass declines should be considered for future research. This research can serve as a guide for future work, which attempts to understand the role of disease in seagrass ecology and the implications of these findings for seagrass affected by global climate change. Further climate analyses, listed in Appendix A can serve as a starting point for more empirical research of the role of *L. zosterae* in seagrass decline. Monitoring efforts could also benefit from these studies, since disease monitoring has been sporadic and isolated if it has been conducted at all in most seagrass beds around the world.

Where some scientists have reported one pathogen, *L. zosterae*, as the etiological agent responsible for the mass wasting of seagrass species, this research suggests it may be critical to recognize there are many key players in the ‘wasting disease’ of seagrass. The ecological variables of *Labyrinthula* habitat should not be overlooked when addressing stressors and etiological agents in seagrass. Much like western medicine benefits from Eastern medicine’s holistic view of the physiology of the human body and recognition of the interrelated mechanisms needed to treat many common diseases, the epidemiologists who study decline in seagrass are wise to understand the range of environmental conditions that lead to degraded health of seagrass ecosystems and the interrelated mechanisms of infection and disease therein.

Identifying extinction hotspots: A framework for assessing stressors and risk factors through spatial analysis of environmental conditions

After reviewing the literature regarding the biology and stressors of both seagrasses and *Labyrinthula*, and reviewing existing literature about the known disease phenomenon and climate predictions, it seems logical to coalesce the known information about environmental conditions for successful growth of host and pathogen into a useable format for nearshore mangers and policy makers to assist in making key management decisions. It seems likely that a combination of ideal environmental conditions for the pathogen coupled with conditions that reduce vitality of the host could result in a range of effects, from minimal to catastrophic.

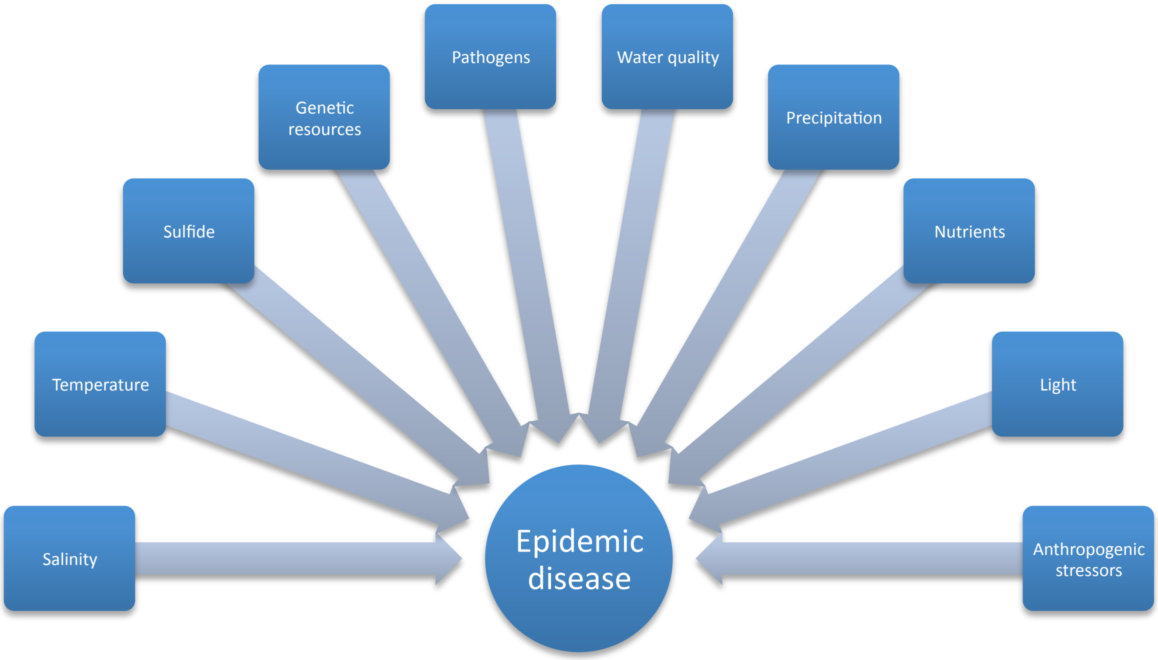


Figure 17: Conceptual diagram of key players in risk of disease outbreak

Under increasing pressure from climate change it is wise to review optimum conditions for both *Labyrinthula* and seagrass species. The science of climate change is becoming more precise all the time and theories about the ecology of disease in seagrass must respond to those changes. For instance, while CO_2 enrichment in nearshore ecology may benefit *Z. marina* to some degree (Palacios and Zimmerman 2007), given the tables below (Tables 11 and 12), assigning a net benefit to seagrass communities for all scenarios of global climate change is premature (MEA 2005). Harvell et al. (2002) found that under a variety of global warming scenarios there is a general increased risk of disease outbreaks due in part to milder winter temperatures, which may reduce winter die-back of pathogens. Further, research by Helmuth et al. (2002) suggests global warming may increase heat stress in interintertidal areas of Washington more than Oregon and California, suggesting a possibility that local seagrass may be at a higher risk of desiccation stress during summer low tides than in areas closer to the poles. That study suggests a simple model of heat increasing at the equator and a subsequent shift of existing populations towards the North or South Pole may not be an accurate way of modeling stress in nearshore communities.

In order to respond to new climate research much more information about the basic ecology of seagrasses and especially *Labyrinthula* must be developed.

Table 11: Draft conceptual framework for analyzing variation in optimum ecological conditions between eelgrass and *Labyrinthula*.

Key Ecological Measurement (KEM)	**Observed Ecological Event (OEE) in seagrass community**	***L. zosterae*** **(host) optimum**	***Z. marina*** **(pathogen) optimum**
Salinity	Reduced ocean mixing	24-42 $^{00}/_{000}$, 20 best (Pokorny 1967)	
Temperature	Eutrophication, interintertidal heat stress	14-25º C, not > 30ºC (Pokorny 1967)	
pH	Ocean acidification	Okay 4-9, 8 suggested (Pokorny 1967)	
Turbidity	Reduce photosynthesis, encourage epiphytic growth, sediment deposits		
Light	Reduced photosynthesis		Min 10-11% insolation (Pokorny 1967)
Hydrogen sufide	Sediment hypoxia,		
Oxygen	anoxia		
Precipitation	Fresh events, drought		
Nutrients	Plankton blooms, eutrophication	Thiamine and Calcium required (Pokorny 1967)	
Wind	Sediment deposits, nutrient rich dust deposits, burial, turbidity, algal blooms, bathymetry shifts		
UV-B Radiation			
Pathogens	Significant biological loss		

Table 12: Draft stressor-response geographic analysis for seagrass and *Labyrinthula*. Responses are either positive, negative or neutral. Known published responses are cited, unpublished responses are noted with a (?) and a response hypothesis is given.

Potential stressors	Predictable geography	*Labyrinthula* response	*Z. marina* response
Increased temperature	Surface and shallow water, interintertidal (summer lows), aspect, low mixing	?positive	Negative (Short and Neckles 1999)
Lowering pH	Water column	? negative	Neutral (Beer et al. 2006)
Increased salinity	Away from river outlets, deeper water	positive	?negative
Increased plankton and algal blooms	Nutrient rich waters (wind blown dust, septic failures, sewer outfalls, upwelling, heavy fertilizer use)	?positive	Negative (Katwijk, Bos, de Vries 2010)
Increased hydrogen sulfide	Hypoxic and anoxic sediments	?	Negative (Holmer and Bondgaard 2001)
Reduced taxonomic diversity	Ecosystem level of organisms	Positive	?Neutral
Increased turbidity	Nearshore, wind driven waves, soft sediments	?	Negative (Short and Neckles 1999)
Precipitation extremes	Near river outlets and stormwater outfalls,	?Negative	?
Loss of genetic diversity	Individual organisms	?	Negative (Wyllie-Echeverria 2010)
Increased storms and winds	Coasts and wind exposed inlets and bays	?	Negative (Short and Neckles 1999)
Increased sediment loading	Soft sediment beaches, river outlets,	?	Negative (Short and Neckles 1999)
Reduced light	Die-back and senescence, discoloration of leaf blades	?negative	Negative (Short and Neckles 1999)
Reduced water movement	Minimum tides, shallow beaches	?	Negative (Short and Neckles

			1999)
UV-B radiation	?	?	Negative
Increased atmospheric CO_2	?	?	Positive (Palacios and Zimmerman 2007)

Based on this preliminary assessment, it is clear that more research is needed to understand the ecology of the *Labyrinthula* pathogen is needed. Once tables such as these can be developed to describe disease phenomenon and ecological conditions, more detailed analyses of risk based on coupling and modeling of key factors which contribute to increased stress in seagrass ecosystems and elevated disease can be analyzed for regional and possibly even more site specific populations.

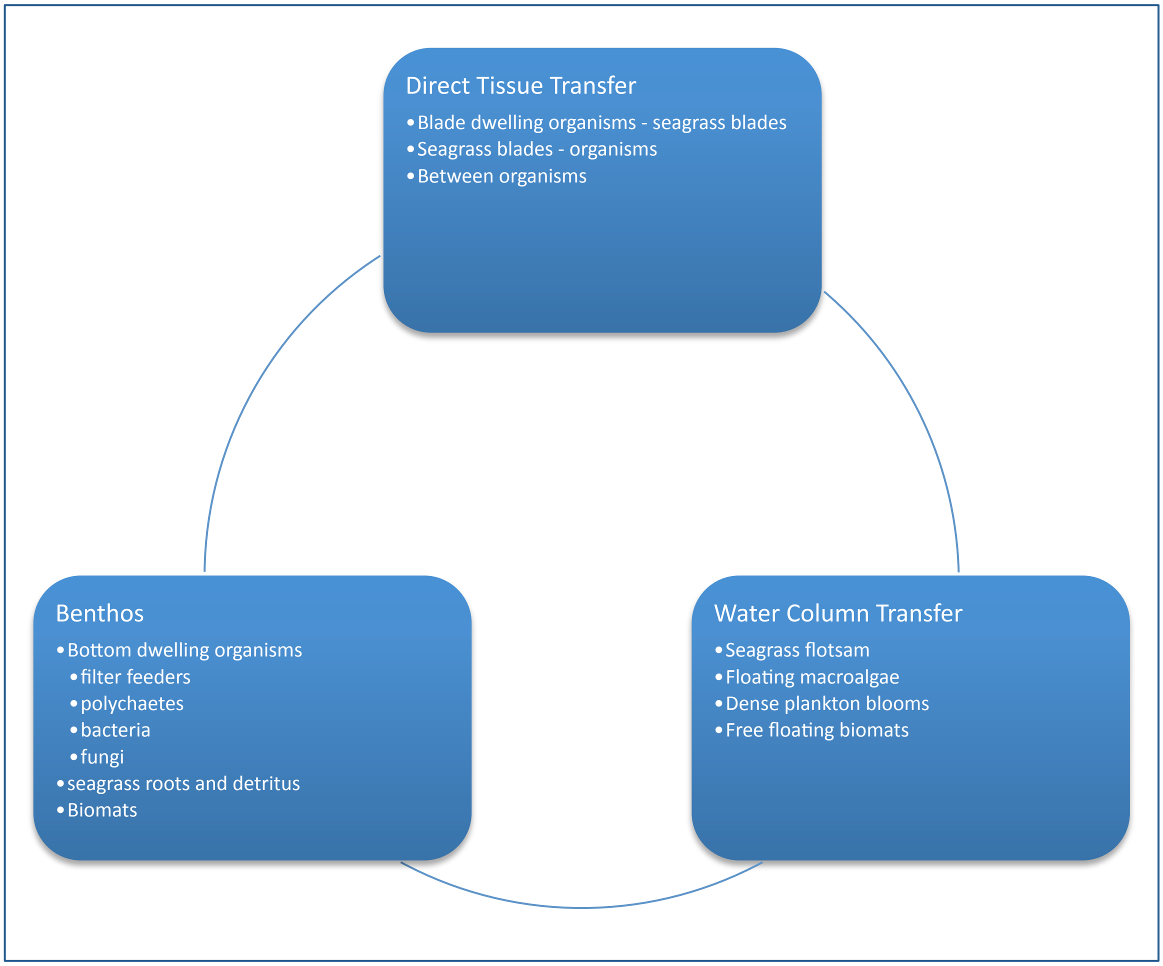


Figure 13: Conceptual model of wasting-disease movement in an eelgrass ecosystem. Effective spread of pathogen in the ecosystem may be density dependent.

A final area of research that warrants consideration in the future is the discovery of alternate host-pathogen interactions and the location of *Labyrinthula* in the ecosystem (Figure 13). Much of the research in the past 100 years or so has focused on the relationship between *Labyrinthula* and seagrasses, mostly *Zostera marina*. Attempting isolation from other organisms, especially organisms that inhabit seagrasses, may enhance our understanding about organisms that contribute to the transfer and spread of wasting disease in seagrasses. There is some information in the literature about strains of *Labyrinthula* in algae and as predators of diatoms, however the link between pathogenicity and these and other organisms is largely unstudied.

Making the case for including disease monitoring into existing monitoring efforts

Seagrass beds are being monitored all over the world. While it may not be possible to monitor all beds for disease symptoms, a system of risk analysis and collaboration with existing monitoring efforts may prove effective in advancing the science of seagrass disease ecology while providing insight to beds experiencing loss. Below is an experimental design that is tailored to piggy-back the existing monitoring structure, resulting in reduced planning, implementation, personnel and overall cost to monitoring agencies.

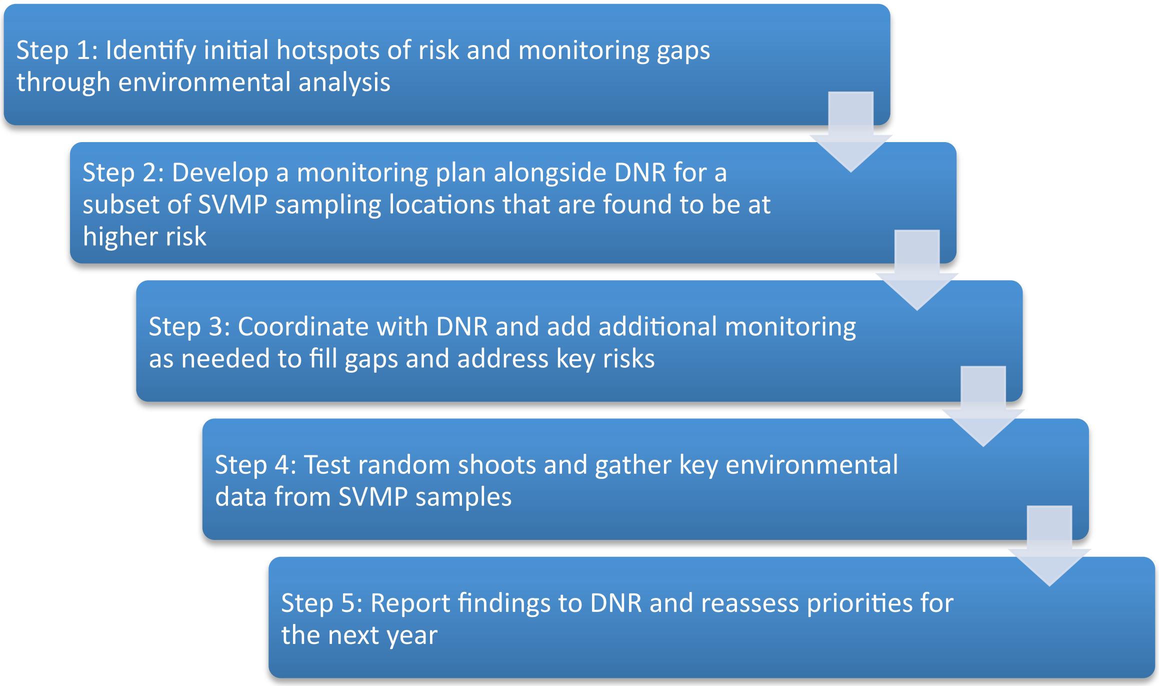


Figure 18: Framework for incorporating disease monitoring in existing monitoring efforts

The Puget Sound Submerged Vegetation Monitoring Project (SVMP) at the Washington State Department of Natural Resources (DNR) has been monitoring eelgrass coverage and density for over 10 years now. Currently, the project is focused on sampling for coverage and density of seagrasses (predominantly eelgrass, *Z. marina*) in Puget Sound, a marine inlet located in the Northwestern part of Washington State. The project has proceeded to collected monitoring data for over 10 years now, publishing frequent detailed reports on research findings. Monitoring results are summarized by assigning a status of increasing, decreasing and stable in coverage and density. These findings have greatly contributed to accessible data for oceanographers and fisheries scientists who are concerned about seagrass ecosystem health, and also for managers who understand the critical role eelgrass habitat plays in providing critical ecosystem services for the region.

The SVMP was originally designed to provide statistically powerful analysis of data with a goal of being able to detect a 20 percent loss or gain in coverage during any given year. After the first few years of monitoring the experimental design was tested to ensure accuracy and dependability in results. Since the framework is already in place to support additional monitoring services, consideration of how disease monitoring may fit into the goals of the project are shown here in a qualitative matter to demonstrate the possibility of enhancing monitoring efforts sound-wide.

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