ASSESSMENT OF SULFIDE INTRUSION AND GENOTYPIC DIVERSITY IN THE SEAGRASS *HALODULE WRIGHTII* FROM THE TEXAS GULF COAST

A Thesis

by

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Seagrasses are marine angiosperms that provide key ecological services to coastal areas. Unfortunately, seagrasses are experiencing a progressive decline, driven by natural and anthropogenic stressors, including sulfide (H_2S) intrusion from high sediment sulfide concentrations. Seagrasses cope with sulfides through avoidance (*reoxidation in the sediment*) or tolerance (*assimilation by tissues*). Previous studies also suggested that seagrass response to environmental stress can be influenced and benefit from having genotypic (clonal) diversity. Although these mechanisms have been studied in some species, few have examined sulfide intrusion and its relation to genotypic diversity in seagrasses from the Texas Gulf Coast. In this study, we used stable sulfur isotopes and a microsatellite-based DNA marker assay to assess sulfide intrusion in the seagrass *Halodule wrightii* and investigate whether genotypic diversity plays a role in its response to sulfide stress. We found a gradient in $\delta^{34}S$ values (-5.58 ± 0.54‰ to 13.58 ± 0.30‰), from roots to leaves, suggesting that H_2S enters through underground tissues and is then distributed throughout the plant. The presence of sulfide-derived sulfur in varying proportions (15% to 76%) among the leaf, rhizome and root tissues indicates they are able to assimilate it into non-toxic, metabolic forms. Although sulfide intrusion did not significantly vary among the different genotypes ($P > 0.05$), this seagrass population had signs of being genetically diverse, indicating that it has the necessary genetic material to face and resist environmental stress. We hope that this study serves as the basis for further exploration of the genetics of sulfur assimilation and metabolism in seagrasses.

DEDICATION

To my parents, Ernesto and Marcela, whose sacrifice, devotion, and unconditional love encouraged me to overcome adversity and find happiness in every journey I embark on far from home. I love you with all my heart.

Para mis padres, Ernesto y Marcela, cuyo sacrificio, entrega, y amor incondicional me motivaron a superar la adversidad y hallar felicidad en cada aventura que vivo lejos de casa. Los amo con todo mi corazón.

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CHAPTER I: INTRODUCTION

Seagrasses are marine angiosperms, usually found in shallow-waters from tropical and temperate coastal areas around the world.[1] They provide key ecological services to marine ecosystems, including provision of food and support to a variety of marine species, regulation of nutrient cycles, stabilization of marine bottom sediments, and improvement of water conditions.[1,2]

Unfortunately, seagrasses are experiencing a global crisis because their habitats are declining. In the Gulf of Mexico, such decline is marked by reports of reductions in seagrass populations in estuaries in the northern parts over the last half century.[3,4] This is a serious issue given that more than half of the total US, and up to 5% of global seagrass habitats are located in this region.[4] In Texas, seagrass beds are also experiencing a gradual decline, characterized by large bed fragmentation and loss of seagrass cover.[5] These negative changes have been attributed to diverse stressors, including natural disturbances from storms, ecological competition with invasive species, and anthropogenic impacts, from which increased nutrient input from sewage waste and agricultural run-off is a major concern.[1,3,5] Excessive release of nutrients triggers eutrophication events which are usually accompanied by high deposition of organic matter in sediments. Its degradation, in turn, may promote anaerobic conditions resulting in accumulation of reduced toxic substances such as sulfides (in the form of hydrogen sulfide, H_2S).[1,2,6,7]

High sulfide concentrations in coastal sediments can be a potential problem given that sulfide is known to be toxic to eukaryotic cells, including seagrass tissues.[1,2,7] In anoxic sediments, sulfides can diffuse into roots and rhizomes (intrusion), potentially inducing seagrass mortality. However, and surprisingly, certain seagrass species have adapted to grow in sulfidic sediments. Although this is still a relatively unexplored field, it has been found that seagrasses can cope with sulfide intrusion via two major detoxification strategies.[2,7] Seagrasses can either avoid sulfide intrusion

by promoting sulfide re-oxidation in the sediment or can develop sulfide tolerance inside the tissues by enzymatic assimilation and further oxidation of sulfide into organic compounds that can be used for coping with nutritional needs.[2,6,7] If such detoxification capacities are exceeded and sulfide manages to accumulate inside the tissues, the plants may suffer the toxic effects resulting in seagrass mortality.[2,7]

Sulfide intrusion is only one of many environmental stressors that can affect seagrass ecosystems. Other features, such as population genetic diversity can also affect population survival. For example, if the size of a seagrass population decreases, the number of individuals that genetically contribute to the next generation also decreases, leading to *genetic drift* (loss of some alleles, fixation of others) that will result in reduced population *genetic diversity*. This can limit the ability of a population to respond and adapt to environmental change, resulting in a reduced probability of long-term survival.[8]

A number of studies over the past two decades have noted the impact that one aspect of population genetic diversity, *genotypic* (clonal) diversity, has had on resistance to environmental stress. Populations with a greater genotypic diversity have been shown to be more resistant to, and recover more quickly from, environmental disturbance related to heat stress, grazing, algal blooms, and shading.[9–15] While exact mechanism(s) are unknown, it appears that genotypic "complementarity" is an important factor. That is, more diverse collection of genotypes exhibit a variety of morphological, physiological, and perhaps even biochemical differences that complement one another, enabling the population to counteract disturbances.[9–15] As some of these disturbances (e.g. shading, heat stress, eutrophication) are directly linked to sulfide production and intrusion, it may well be that genotypic diversity within a seagrass bed contributes to a combination of sulfide detoxification strategies.

Thus far, literature on sulfide intrusion in seagrasses has focused on just a few species, particularly *Z. marina, T. testudinum,* and *P. oceanica.*[2,6,7,16–22] Few studies have examined sulfide uptake in seagrasses from the Gulf of Mexico, a major center of seagrass habitats that is frequently dominated by species such as *Halodule wrightii* and *Thalassia testudinum.*[5,23–26] In addition, to the best of our knowledge, no studies have examined the genetic effects on sulfide intrusion in seagrasses, such as whether this phenomenon is influenced by genotypic diversity.

The objective of this study was to develop a robust method for distilling reduced sulfur from marine sediment, investigate sulfur uptake in a previously uncharacterized species of seagrass, and examine whether its source (seawater sulfate or sediment sulfide), distribution (root, rhizome, leaf) and total amount retained differ among genotypes found in a single population. We expect that such results will lay the groundwork for further investigations into the genetics of sulfur metabolism in seagrasses and their resistance to environmental stressors. We also hope these findings will have important implications for seagrass conservation and restoration, especially in *Halodule*-rich areas such as the Gulf of Mexico and Caribbean Sea.

CHAPTER II: SEAGRASS BIOLOGY, ECOLOGY AND DISTRIBUTION

2.1. General Remarks on Seagrasses

Seagrasses are marine angiosperms found in coastal areas of tropical and/or temperate latitudes. They also extend into high latitude coastal waters in both hemispheres, including the temperate North Atlantic, tropical Atlantic, Mediterranean, temperate North Pacific, tropical Indo-Pacific, and temperate south Atlantic and Pacific oceans.[27] Seagrasses in the United States are found along the Pacific, Atlantic and Gulf of Mexico coastlines, for which the Texas coast encompass around 90,000 ha of seagrass beds.[5,25] Texas is home to five species: *Halodule wrightii* (Ascherson) (shoal grass), *Syringodium filiforme* (manatee grass), *Thalassia testudinum* (turtle grass), *Halophila engelmanni* (star grass), and *Ruppia maritima* (widgeon grass).[25,28]

Taxonomically, seagrasses are organized into four families, Zosteraceae, Cymodoceaceae, Posidoniaceae, Hydrocharitaceae, which include 12 genera containing about 60 species.[1,27,29,30] Altogether, they can be referred as the "eurysaline" ecological group of aquatic plants, i.e. species that can grow in waters with an unstable salinity.[30] Seagrasses can also be grouped according to morphological differences based on leaf shape and aggregation (e.g. strap-shaped leaves vs. round-tubular leaves) and rhizome growth direction (e.g. horizontal vs. vertical extension).[2,27,29]

Seagrass distribution is often limited by the amount of light that reaches the canopy, salinity, and sediment composition. By being rooted phototrophs, they require adequate substrates (muddy to sandy sediments), sufficient immersion in seawater, and illumination to maintain growth.[1,31] While they can also tolerate a wide range of salinity, from full-strength seawater to either brackish or hypersaline waters, they do have limits. Different species have different response to these factors, so seagrass meadows are usually dominated by one or few species, resulting in typically low and skewed species diversity estimates.[1]

Seagrasses can reproduce both sexually and asexually, with most species exhibiting vegetative (clonal) reproduction.[32] Clonal growth is achieved through rhizome extension, which provides unique genetic individuals (*genets*) the opportunity to survive and expand in both space and time.[30] Such expansion often results in large beds or meadows dominated by a single seagrass species.[27] Rhizome extension is not restricted to a linear growth. Seagrass rhizomes are able to branch in different directions, leading to different spatial distributions of the genets within meadows. In addition, clonal expansion, fast growth, and branching facilitate the rapid recovery of seagrass meadows from disturbance, mainly because they allow the distribution and sharing of resources between individuals growing under stress or with low nutrients.[1,33,34]

Most (9 out 12 genera) species of seagrass are dioecious, meaning that individual plants are either female or male.[1] Sexual reproduction occurs through hydrophilous pollination. Specialized pollen grains from the male flowers are released into the water column to fertilize females.[1,27,33] The resulting seeds are dispersed by both biotic (e.g. waterfowl, manatees, dugongs and fish that ingest the seeds) and abiotic (e.g. wind, currents, and human activities, such as seeds attached to ocean vessels or boat trailers) agents.[27,35]

Similar to terrestrial and freshwater plants, the basic unit of seagrass structure is called a *ramet*, which consists of a section of rhizome, associated roots, and a leaf bundle (Figure 1). Rhizomes are the plant stems that extend either horizontally or vertically near the sediment surface and are responsible for the extension of the ramet in space. They connect and integrate neighboring units and include air spaces (*lacunae*) that serve as storage organs and allow for gas exchange.[1,29,33]

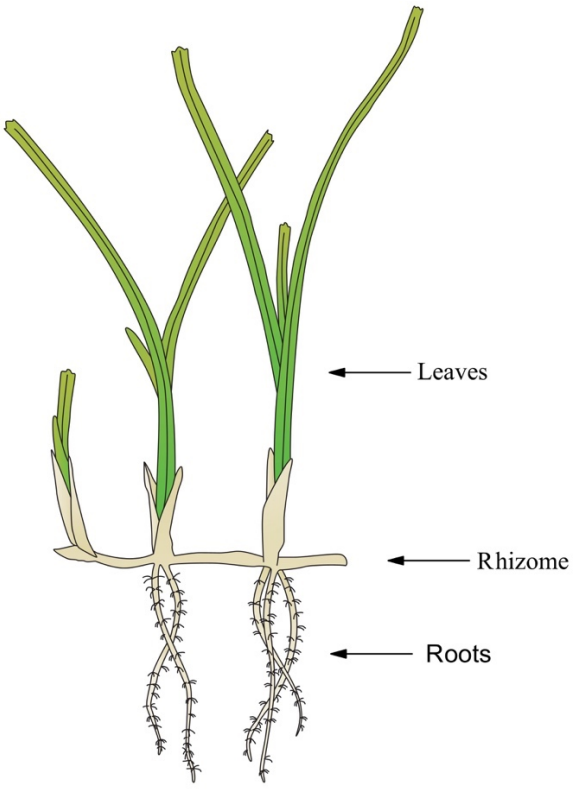

Figure 1. Illustration of *Halodule wrightii* ramets (root/rhizome/leaf bundles) connected by rhizome structures. In this case all ramets have arisen from the same parental tissue and would belong to the same *genet*. *Source*: Tracey Saxby, Integration and Application Network (IAN), University of Maryland, Center for Environmental Science (http://ian.umces.edu/imagelibrary/)

Most seagrass species have strap-like leaves, which are generally long and relatively narrow, and are often grouped in bundles attached to the rhizome.[1] Seagrass leaves lack stomata, and their surfaces are covered by a thin porous cuticle, across which gas and mineral nutrients exchange can occur. Seagrass leaves are rich in chloroplasts making them photosynthetically active.[1,27,29]

Seagrass roots are adventitious, generally arising from the lower surface of the rhizomes. Besides anchoring the plant to the sediment and participating in nutrient uptake, seagrass roots support a diversity of microorganisms on their surface, especially bacteria.[29,30] Under anaerobic conditions, these microorganisms use seawater sulfate (SO_4^{-2}) as an electron acceptor, converting it to H_2S in energy-yielding metabolic pathways.[36,37] This results in an increase in the concentration

of sulfide in the surrounding sediment. Seagrass roots can reduce the concentration of H_2S in the rhizosphere by releasing photosynthetic oxygen, which creates an oxic zone that re-oxidizes the sulfide to sulfate.[1,29]

2.2. Ecological Services of Seagrasses

Despite their low taxonomic diversity (less than 0.02% of all angiosperm species)[1] seagrasses provide key ecological services that profoundly influence the physical, chemical, and biological environments of coastal ecosystems.[3,31] In terms of provision and support, seagrasses are important food sources for marine fauna. While some animals can graze on seagrasses blades, rhizomes and roots, others consume the epiphytes growing on their leaves or search for prey in seagrass meadows. In total, seagrass beds serve as nursery and foraging areas for approximately 250 faunal organisms, including migratory waterfowl, manatees, dugongs, sea turtles, and numerous fish and shellfish species.[1,3]

Seagrass beds also have important roles in nutrient cycling and maintenance of the aquatic environment. First, they are primary producers that contribute significant amounts of photosynthetically-derived oxygen and organic carbon to the detrital pool.[1] Particularly, they are estimated to be responsible for 20% of the global organic carbon buried in marine sediments ("Blue Carbon"),[2] making seagrass beds important carbon sinks. Second, they reduce fine particle loads in the water column and absorb dissolved nutrients, improving water clarity and quality of aquatic systems.[1,3] Such nutrients trapping also prevents and reduces rapid growth and turnover of light shading phytoplankton.[2]

Thirdly, seagrasses rhizome and root systems hold and stabilize marine sediments, preventing coastline erosion due to storms and hurricanes. Their leaves help to deflect currents and

dissipate wave energy, favoring sediment deposition and retention.[1,2] Indirectly, seagrasses act as biological sentinels, or "coastal canaries". Changes in seagrass distribution and bed morphology ("patchy" vs. "contiguous") can signal changes in the quality of the marine environment, warning of potential losses in ecosystem services.[3] In this sense, their distribution allows assessment of environmental trends in coastal regions and can be used as an indicator of water quality.[5]

Seagrasses also have direct economic uses and applications. For example, *P. oceanica* and *Z. marina* leaves that accumulate on beaches (beach wrack) are used as fertilizers and raw material for roof thatching in certain parts of the world.[2] Several studies have also reported the use of compounds extracted from seagrasses as pharmaceuticals. For example, the seagrass *Zostera asiatica* produces a bioactive pectin, *zosterin*, which decreases toxicity of anti-tumor drugs and purges heavy metals from human organisms.[31] Likewise, *thalassiolins*, isolated from the seagrass *Thalassia testudinum,* are considered promising anti-HIV agents thanks to their inhibition of HIV integrase activity.[38]

2.3. Seagrass Cover Reduction and Decline

Given their ecological services and uses, seagrass habitats should be preserved and protected. Unfortunately, seagrasses are experiencing a global crisis, as their habitats are declining at a rate of approximately 1.5% per year, with a total loss of 29% (3,370 km^2) of seagrass beds between 1879 and 2006 (i.e. 27 km^2 per year).[39] Ten seagrass species (14% of total) have an elevated risk of extinction, with three species qualifying as endangered.[31] The Gulf of Mexico (GOM) is not exempt from this trend, as seagrass losses have been reported at more than 500,000 ha since 1950.[5] This includes Texas, home to the second-largest concentration of seagrasses on the US Gulf coast, where beds covering approximately 90,000 ha have also experience a progressive decline.[4]

Natural causes of seagrass declines include geological and meteorological events and specific biological interactions with other species.[1] For example, earthquakes can cause a rise of shorelines, exposing seagrass vegetation to air and damaging the beds. Storms producing strong waves and currents also disrupt seagrass vegetation and cause erosion of bottom sediments. This physically removes vegetation and creates additional stress by increasing water turbidity that can hinder primary production.[1,2] In some instances, excessive grazing by animals such as sea cucumbers can result in meadow deterioration and destruction.[1]

Human activities, however, are primarily responsible for seagrass declines, especially those related to industrial and urban development, or agricultural activities in proximity of coastal areas.[40] For example, channel dredging in support of maritime transport directly destroys seagrass habitats. Dredging also results in sediment resuspension, which increases water turbidity and reduces photosynthesis by decreasing light penetration.[27] Propeller scarring from recreational boats and intensive fishing practices can also disturb bottom sediments, resulting in reductions of seagrass cover.[1]

The greatest impact probably occurs from increased nutrient input from sewage waste as well as municipal and agricultural runoff. Excessive nutrients trigger eutrophication events, characterized by rapid growth ("blooms") of micro- and macroalgae (e.g., phytoplankton), which in turn reduce the amount of light reaching seagrass leaves. Blooms also absorb nutrients and subsequent degradation consumes dissolved oxygen, limiting their availability to other species.[1,27] Increased nutrient inputs also causes a decrease in the redox potential of sediments, favoring the establishment of anoxic conditions. Under this stress, microbial respiration shifts towards the use of alternative electron acceptors, such as sulfate, resulting in production of gaseous hydrogen sulfide (H_2S) that is toxic to seagrasses.[1,41] Gaseous H_2S can pass through seagrass membranes

and poison photosynthetic enzymes, resulting in increased stress and mortality.[40,41] Therefore, accumulation of sulfides in sediments, in combination with poor water quality (e.g., light reduction, water column hypoxia), represent environmental stressors that significantly contribute to seagrass decline.

CHAPTER III: SULFIDE INTRUSION IN SEAGRASSES

3.1. Sulfur Sources for Seagrasses

Sulfur is an essential component of living cells, found in molecules with key roles in cellular metabolism, structure, and defense. As such, sulfur it is part of the amino acids cysteine and methionine, which are key building blocks for proteins. Sulfur is also found in coenzymes, vitamins, and electron carriers, and as constituent of cell walls, photosynthetic membranes, and connective tissues in plants.[42,43] Seagrasses can acquire sulfur from up to three potential sources: seawater sulfate (SO_4^{2-}), porewater sulfate, or sediment-derived sulfide (H_2S). Sulfate can enter the plants through active uptake from the water column by leaves or from sediment pore-water through roots, while dissolved H_2S from sediment can enter plants through roots by passive diffusion across cellular membranes.[2,6,7]

Sulfate from seawater and porewater is the major inorganic form of sulfur and it is found at a high concentration in the oceans (ca. 29 mM or 2.7 g L^{-1}, at a salinity of 35%).[44] Sulfate-uptake systems include: i) sulfate permeases, which cotransport $3H^+$ into tissues when taking up SO_4^{2-} or, ii) facilitated transport systems, which rely on ATP-mediated uptake.[43] Sulfate serves as an important electron acceptor for oxidation of organic matter via microbial respiration under anaerobic conditions, such as those resulting from eutrophication events.[45] Organoclastic or dissimilatory sulfate reduction is considered to be among the most important anaerobic mineralization pathways in marine sediments.[36] It is carried out by sulfate-reducing bacteria, according to the following equation:[44]

$$2CH_2O_{(s)} + SO_4^{2-}{}_{(aq)} \rightarrow 2HCO_3^-{}_{(aq)} + H_2S_{(g)}$$

This reaction produces sulfide (as H_2S), which can be re-oxidized to sulfate by oxygen present in pore water sediment[44] or trapped in sediment by precipitation with dissolved metal ions (Fe and Mn).[46] Approximately 90% of the resulting sulfides are re-oxidized with oxygen, which can also be accelerated by enzymatic catalysis of microorganisms in the root tips of seagrasses.[46] If precipitated, the remaining sulfides react, for example, with iron in the sediment to form insoluble iron-sulfides (e.g. FeS or FeS_2),[44,47] preventing the accumulation of free sulfide in pore water.[36,48]

Sulfide trapping by iron, in the form of iron oxyhydroxides (FeOOH),[36] separates sulfur into two pools. The first sedimentary sulfur pool consists of acid-volatile sulfides (AVS), which are iron-trapped, dissolved sulfides that can be liberated as gaseous H_2S following addition of a strong acid.[49,50] The most important species in the AVS pool are dissolved sulfides (i.e. HS^- and H_2S) and aqueous iron sulfide clusters, commonly referred to as FeS_{aq} (e.g. iron(II) monosulfide (amorphous FeS, mackinawite (crystalline FeS), and greigite (Fe_3S_4)). The other sulfur pool mainly consists of pyrite (Fe_2S) and elemental sulfur (S^0), known as the chromium reducible sulfides (CRS).[49,51] Pyrite is the major reservoir of reduced S in sediments and it is not usually considered to be a component of the AVS because sulfide from pyrite is not liberated with simple addition of the strong acid.[49,52]

Both the AVS and CRS pools form the Total Inorganic Reduced Sulfur (TRIS) pool in sediments. In rapidly or newly deposited sediments sulfur from the AVS (AVS-S) pool tends to occur in greatest abundance relative to pyrite in the TRS pool, whereas in slowly accumulating sediments AVS-S is a minor TRIS component.[49] Therefore, sulfides intruding into seagrass tissues may originate from different iron-sulfide pools, depending on the sediment conditions where the plants grow.

3.2. Strategies for Limiting Sulfide Intrusion

Sulfide intrusion into seagrass tissues has been found to be driven by pools of dissolved sediment sulfides created when reoxidation of sulfide in sediment does not keep up with microbial sulfate reduction rates.[2,7] This is problematic given that sulfide is a potentially harmful, phytotoxic substance that interferes with cytochromes crucial to the electron transport chain in plants. This results in decreased ATP production that affects their cellular and metabolic energy balance.[1,45]

Seagrasses cope with high sulfide intrusion through *avoidance* (re-oxidation) or *tolerance* (assimilation into non-toxic forms).[6,16] In *avoidance*, seagrasses transport photosynthetic oxygen produced in leaves to roots, via a system of internal gas spaces (lacunae) that extend virtually throughout the ramet.[18,46] Oxygen then leaks from the roots into surrounding sediment due to differences in partial pressure of the gas, where it creates an "oxygen microshield" in the rhizosphere that promotes oxidation of sulfide to non-toxic sulfur species such as sulfate, elemental sulfur (S^0), or other intermediate sulfur compounds.[6,46] Re-oxidation can also occur in the lacunae of seagrass tissues, where resulting sulfate and S^o can be stored in cell vacuoles or precipitate on cell walls. *Toleration* occurs when intrusive sulfide is converted to organic forms that become enzymatically assimilated into metabolites throughout the plant.[6,18,45]

It has been assumed that the amount of oxygen released from roots is sufficient to detoxify the high amounts of sulfide in sediment. However, sulfide-oxidizing bacteria can also participate in detoxification by internally reoxidizing sulfide to sulfate while fixing organic carbon from CO_2.[31,42,46,53] Moreover, iron pools in non-carbonate sediments can also act as a buffer against sulfide toxicity, because they can precipitate sulfides as pyrite (FeS_2) and iron mono-sulfides (FeS).[42,45]

Sulfide detoxification by oxygen is, however, light-dependent, as oxygen supply is the result of seagrass photosynthesis. At night, when light conditions deteriorate, oxygen concentration is depleted inside the plant and oxygen flow to roots and rhizomes diminishes.[1,6] This situation re-establishes anoxic conditions that facilitates sulfide intrusion into the plant. Such conditions are further enhanced by oxygen depletion in the water column due to high respiratory activity or during calm wind conditions with low air-water gas exchange and flushing.[45] Nonetheless, oxygen depletion in the rhizosphere at night does not occur instantaneously, as some oxygen transport out of the roots occurs even during dark conditions. Oxygen from the water column or stored within seagrass tissues can be translocated to roots after cessation of photosynthesis, where it will diffuse out due to the negative concentration gradient ($[O_2]_{plant} > [O_2]_{sediment}$). This allows seagrasses to withstand short periods of sulfide exposure at night.[2]

In the case of tolerance, assimilation can take a number of pathways. Sulfide is probably enzymatically incorporated directly into the amino acid cysteine and subsequently metabolized to other organic sulfur compounds useful in metabolic pathways, as observed in terrestrial plants.[6] Sulfide-derived sulfate can be reduced back to sulfide and incorporated into cysteine, or activated to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) for assimilation into organic molecules.[43] Activated sulfate can be linked to an oxygen atom to form a sulfate ester, to a nitrogen atom to form a sulfamate, or to a carbon atom to form a sulfonic acid, resulting in a variety of compounds such as additional sulfur-containing amino acids used in protein synthesis (e.g. methionine); group-transfer coenzymes and vitamins (e.g. coenzyme A, S-adenosyl-l-methionine (SAM), thiamine, biotin, lipoic acid); chloroplast membrane components (e.g. sulfolipids such as sulfoquinovosyldiacylglycerol); signaling molecules (e.g. sulfated lipooligosaccharides, turgorin); and peptide hormones (e.g. phytosulfokines).[43] Glutathione and its derivatives are also important

metabolites resulting from reductive sulfate assimilation, as they play key roles in storage and transport of reduced sulfur, in signal transduction pathways, in detoxifying xenobiotics, in activating and conjugating phenylpropanoids and hormones, and as cellular antioxidants. Glutathione also serves as starting material for synthesis of phytochelatins, which are small cysteine-rich oligopeptides that bind and detoxify plants from heavy metals such as cadmium (Cd) and copper (Cu).[43]

3.3. Characterization of Sulfide Intrusion

Sulfide intrusion and uptake in seagrasses was recently studied using stable sulfur isotopes. Isotopic analysis can be used to distinguish the source and fate of sulfur assimilated into seagrass tissues, given that the isotopic signature of sulfates from the water column is markedly different from sediment-derived sulfides.[7,17] The distinction between the sulfur sources is possible because sulfate and sulfide isotope values remain fairly unchanged in plant uptake and assimilation, meaning that the isotopic composition of seagrass tissues reflects the sulfur source.[2,7,42]

3.3.1. Sulfur Stable Isotopes

Naturally-occurring sulfur contains four stable isotopes, with the following abundances: ^{32}S (94.93%), ^{33}S (0.76%), ^{34}S (4.29%), and ^{36}S (0.02%).[54,55] Accordingly, there are a number of sulfur isotope ratios that can be measured, but the $^{34}S/^{32}S$ is generally used for measuring isotope fractionation. The accepted sulfur isotope ratio measurement is the delta value $\delta^{34}S$ (given in per mill (‰) units), which expresses the deviation from an international standard, according to the following equation:[54–56]

$$\delta^{34}S_{sample} = \left(\frac{(R)_{Sample}}{(R)_{CDT}} - 1 \right) \times 1000$$

where R represents the $^{34}S/^{32}S$ ratio and CDT corresponds to the Cañon Diablo standard. This standard contains sulfur from the troilite of an iron meteorite found at Meteor Crater, Arizona, USA. For many years, CDT has been used as the reference standard for sulfur isotope analysis. However, it has been found that CDT is not as homogeneous as originally reported and may display variations in ^{34}S up to 0.4‰. Therefore a new reference scale, Vienna-CDT or V-CDT, has been introduced to account for instrument-caused deviation during isotope analyses.[54,55]

Sulfur isotope ratios are measured by Isotope Ratio Mass Spectrometry (IRMS), in which charged isotopes are separated on the basis of their masses and motions in electromagnetic fields. Relative intensities of the beams of different masses can be used to calculate the isotope masses or ratios. Before IRMS analysis, sulfur samples are combusted to SO_2 gas by oxidation at high temperatures with either O_2 gas or an oxidized species such as copper oxide or vanadium(V) oxide. Evolving gas can either be chemically trapped or separated on Gas Chromatography (GC) columns, and then directed to the MS, where it is ionized, focused into a coherent beam and accelerated down the flight tube. Ion beams are deflected in a magnetic field in relation to the charge/mass ratio of the ion. Charged particles enter the various collectors (usually, Faraday cups) where the current produced is sent through resistors that generate voltages that are amplified and recorded. Intensities of voltages produced are proportional to abundance of isotopes, and their ratios and delta values are automatically calculated by the software that operates the machine. Standardization is completed through the use of an added internal standard, whose isotopic composition has been previously determined by conventional techniques.[54,55]

Sulfur isotope ratios analysis by IRMS is challenging. First, although SO_2 is easily produced and easily analyzed on most mass spectrometers, it is a highly polar molecule that fouls the ion source of the mass spectrometer, potentially affecting the results of other samples. This can

be worsened when trace amounts of water are present, leading to the formation of corrosive sulfuric acid. Fortunately, this issue can be significantly reduced with use of continuous flow systems, in which gas is directly introduced into the source of the mass spectrometer. With such technique, only very small amounts of SO_2 are necessary for analysis, which means that the source is kept clean.[54,55]

Another challenge with SO_2 measurements is that there is an unavoidable mass spectrometer uncertainty, arising from the possibility of having the same mass ratio from SO_2 isotopologues with different sulfur (^{34}S or ^{32}S) and oxygen (^{18}O or ^{16}O) isotopes. This situation is alleviated by comparing results from sulfate standards with sulfate samples and sulfide standards with sulfide samples, although this assumes that the ^{18}O value of sulfate samples and standards are similar.[54] Finally, SF_6 has been used an alternative gas for sulfur isotopes measurements because it is cleaner and allows for the measurement of rare isotope ratios (e.g. $^{33}S/^{32}S$, $^{36}S/^{32}S$). However, using this compound requires a large-radius mass spectrometer specifically configured for sulfur isotope analysis.[54]

3.3.2. Isotopic Signatures of Sulfur Sources for Seagrasses

As mentioned, sulfide intrusion can be assessed by sulfur stable isotopes measurements thanks to the difference in the $\delta^{34}S$ signal between seawater sulfate and sediment sulfide.[2,7] This isotopic difference is established by a kinetic isotope effect, associated with dissimilatory sulfate reduction by bacteria. Bacteria discriminate against the heavier ^{34}S isotope, preferring lighter ^{32}S, as it has a lower dissociation energy that allows the bonds to be more easily broken. This yields sulfides with an isotopic signal enriched for the lighter isotope.[2,7,54,55,57] While the $\delta^{34}S$ value for sulfate in modern seawater has a near constant value of 21.0‰ ± 0.25‰[18,54], the $\delta^{34}S$ value of

sediment-derived sulfides varies considerably, ranging from –10‰ to –20‰, with individual values as low as –50‰.[18,54,56] Accordingly, seagrasses that have been exposed to sulfide intrusion tend to have lower $\delta^{34}S$ signals in their tissues compared to plants that have not.[18]

Fractionation of 40 ± 10‰ observed between sulfate and sulfide $\delta^{34}S$ signals is explained by different enzyme-catalyzed steps involved in sulfate reduction by bacteria. These include sulfate uptake, reduction to sulfite, and reduction of sulfite to sulfide, each of which occur at a different reaction rate.[54,58] However, the rate-limiting step that contributes the most to fractionation is breaking of the first S-O bond in reduction of sulfate to sulfite by the sulfite-reductase enzyme.[55,58]

Sulfide oxidation in the rhizosphere also results in fractionation, but to a lesser extent than sulfate reduction. Fractionation values range from nearly nil to -5.2‰ for oxidations carried out by chemolithotrophic aerobic bacteria, and from -4.2‰ to 2‰ for anaerobic oxidizing bacteria.[57] These activities yield porewater sulfate depleted in the ^{34}S isotope compared to seawater. If rhizosphere-associated bacteria use the depleted sulfate as an electron acceptor, the resulting sulfides have even lower ^{34}S isotope values,[55] further increasing the contrast in $\delta^{34}S$ values of the sulfur pools.

3.4. Previous Works on Sulfide Intrusion in Seagrasses

While work with stable sulfur isotopes in plants extends back to the early 1980's, it was Frederiksen et al.[17] who performed a key study on sulfide intrusion in the seagrass *Zostera marina*, where they examined differences in ^{34}S uptake and distribution, taking into consideration spatial and temporal variations. The authors found that the mechanisms for sulfide intrusion are complex, and subject to factors beside the free sulfide concentrations of sediment. The authors concluded

that plant morphology and environmental factors need to be considered when assessing the potential for sulfide intrusion in seagrasses. Regardless, this study demonstrated the promising role of stable sulfur isotopes as indicators of environmental stress in seagrass communities. The authors also suggested that further experimental work is required to obtain more robust knowledge on the relationship among sediment sulfide concentrations, plant oxygen status, and sulfide intrusion.[17]

In a later study, Frederiksen et al.[19] evaluated the effect of increased sediment sulfide on the sulfur isotope composition, total sulfur content (TS), and elemental sulfur (S^0) concentrations in leaves, rhizomes and roots of two seagrass species: *Zostera marina* and *Posidonia oceanica*. They concluded that TS and S^0 differences among tissues are correlated with $\delta^{34}S$ from sediment sulfide and seawater sulfate, implying different pathways of sulfur assimilation. The authors also addressed the effect of sulfide on growth and survival of the two species. In particular, they found that, while both species were tolerant to sulfide exposure, *P. oceanica* was the more sensitive and showed a clear trend of reduced growth and reduced survival with higher sulfide concentrations. This study demonstrated that both intrusion and toxicity can vary among seagrass species, and established the need to examine these mechanisms in additional seagrasses, including those from tropical and sub-tropical environments.[19]

Holmer et al.[22] examined sulfide intrusion in two seagrass species. Their research examined oxygen dynamics and sulfide intrusion in the seagrasses *Thalassia testudinum* and *Syringodium filiforme* from the US Virgin Islands. They found that sulfide intrusion was influenced by a combination of plant parameters such as shoot density, leaf morphology, nutrient content, and sediment biogeochemistry (e.g., Fe concentration), as presence of iron pools in sediments can modulate sulfide invasion by precipitating sulfides as iron-sulfides.[22] More recently, Hasler-Sheetal and Holmer[6] characterized the identity and origin of specific sulfur compounds in *Z.*

marina subject to sulfide intrusion. They found that sediment-derived sulfide was mostly reoxidized to S^0 and sulfate, or metabolized into organic thiols in the underground tissues (roots and rhizomes), where sulfide intrusion was greatest. S_o precipitated onto cell walls while sulfate could be stored throughout the plant. Thiols were used in sulfur metabolism as well as stored in vacuoles. They concluded that avoidance of sulfide toxicity through re-oxidation or incorporation into organic forms are likely major survival strategies for seagrasses in sulfidic sediments.[6]

Reviews on sulfide intrusion[2,7] show that the $\delta^{34}S$ values are now known for approximately half of all seagrass species, but most of the work on sulfide intrusion has focused on seagrass species from the Mediterranean, North Atlantic and the South Pacific.[16] The only information available from the Texas coast includes a few observations from the 1980s on the stable sulfur isotope composition of *H. wrightii* leaves and roots.[2,7,37] There are no available data on the $\delta^{34}S$ values of rhizome tissue or the sulfur content (TS) from sediment-derived sulfides in the tissues. Therefore, there is a clear need for documenting $\delta^{34}S$ of seagrass species in the Gulf of Mexico and using the findings to assess sulfide intrusion in them.

CHAPTER IV: GENOTYPIC DIVERSITY IN SEAGRASSES

4.1. The Importance of Genetic Variation

When exposed to environmental stressors, seagrasses can tolerate moderate disturbance through morphological and physiological adaptations. However, strong disturbances (e.g. excessive sulfide intrusion) can result in seagrass loss, ranging from a thinning of the meadow to widespread die-off events.[59] The common feature of such declines is that population size is reduced, which is usually accompanied by the loss of genetic material in the form of fewer alleles and genotypes. Overall, this results in reduced genetic variation within the population.[23,26]

Reduced genetic variation affects the ability of a population to respond to environmental pressures.[8,23,60] When a population's size is large or genetic variation is high, it is statistically more likely that one or more individuals will have genotypes that are better able to withstand stressful conditions. Genotypes are expressed as different *phenotypes* that are able to combat stress through morphological or biochemical features that provide them with an advantage over the stressor.[15] For example, if one genotype results in the production of a phenotype with larger diameter lacunae, this could allow more oxygen to leak into the rhizosphere to oxidize sulfides. In contrast, when population size or genetic variation is low, the population may not have enough genetic resources to resist, adapt and/or recover from environmental disturbances.[8,60] Therefore, the assessment of genetic variation within a seagrass population can be used as proxy for the likehood of the presence of different phenotypes, and a means to evaluate their relative fitness.

4.2. Genetic Variation Measurements

Genetic variation can be measured using a variety of DNA-based, molecular markers, of which *microsatellites* are widely used in seagrass research.[8,61] Microsatellites, also known as

simple sequence repeats (SSRs) or *short tandem repeats* (STRs), are short DNA sequences consisting of tandemly repeated motifs 1 to 6 base pairs (bp) in length.[60] They are advantageous as markers because they show very high levels of polymorphism compared to regular DNA sequences. They serve as proxies for the amount of genetic variation present at loci that actually do code for proteins responsible for an individual's phenotype. Microsatellite markers are also codominant in diploid or polyploid species,[8] meaning that they may be used to distinguish individuals that are *homozygous* (contain the same allele) at a particular microsatellite locus from those that are *heterozygous* (different alleles). The high level of polymorphism also means that a population may harbor multiple alleles at any given microsatellite locus. By examining microsatellite alleles at multiple loci, an individual's genotype can be assigned and compared to others in the population to find which samples share the same genotype, and are thus members of the same clone.[8,60]

Genetic variation can be assessed among individuals within a population or among populations of the same species.[8,32,60] In both cases it involves the analysis of the variety of alleles and genotypes present in a population, or populations. Genetic variation is assessed through estimates of allelic and/or genotypic frequencies. Within a population, allelic diversity can be used to estimate several aspects of genetic variation, including *allelic richness* (average number of alleles per locus), *heterozygosity* (proportion of individuals or loci with heterozygous genotypes), the *inbreeding coefficient* (degree of inbreeding within a population), and *clonal richness* (proportion of individuals with unique genotypes).[8,60,62] The idea of clonal diversity can also be extended to take into account other aspects of genotypic variation such as *clonal evenness,* the equitability with which clonal (gene) membership is distributed among individuals (ramets) within

a population, and clonal *aggregation* or *architecture*, the extent to which different genotypes are segregated or intermingled within a seagrass bed.[63]

Microsatellite variation is detected by DNA amplification using the Polymerase Chain Reaction (PCR) and synthesized oligonucleotide "primers" that target the desired, microsatellite-containing DNA segment. The amplified segments are separated according to size using electrophoretic techniques combined with different, spectroscopy-based detection mechanisms. Multiple (6-10) microsatellite loci are typically assayed in each individual to assign genotypes.[8,60]

In Texas, research on seagrass genetic variation has involved two species, *T. testudinum* and *H. wrightii*.[25,32] Larkin et al.[26,64] carried out some of the first studies to assess genetic variation in these species from the western Gulf of Mexico, and found moderate levels of genetic variation similar to those found for other species with comparable life histories and breeding systems. Additional studies by the same authors examined the effect of disturbance events (e.g. propeller scarring) on genetic variation in *H. wrightii* from Redfish Bay, Texas, USA,[23] and developed a successful microsatellite assay with 8 polymorphic markers for genetic studies in the same species.[24]

In a more recent study, Larkin et. al.[25] further verified and extended previous work by assessing genetic diversity, structure, and connectivity in *H. wrightii* from ten locations along the Texas Gulf Coast. They found that *H. wrightii* from this region exhibits variable clonal diversity, moderate allelic diversity, and relatively high heterozygosity, compared to other species. In addition, they showed that genetic diversity and structure was not strongly affected by geographic barriers along the Texas Gulf Coast. Their results suggest that seagrass expansion and colonization is mediated via drifting vegetative fragments instead of dispersal by seeds.[25]

Besides genetic variation *per se*, several studies have begun to examine the relationship between genetic diversity, ecological traits, and resistance to environmental stress. For instance, multiple studies showed that populations with higher genotypic *richness* have increased resistance to grazing[9] and enhanced resistance to invasion.[13] Such populations also influence grazer biomass,[10] produce more flowering shoots with higher leaf shoot density, and thus, have higher seed germination success.[65] In another study, Reynolds et al.[66] evaluated the effect of microsatellite allelic diversity on ecological traits of *Z. marina* beds from restored plots. The authors found that more genetically diverse plots survived longer, increased shoot density faster, and possessed greater invertebrate density compared to less diverse plots.[21] However, perhaps the most interesting finding was that enhancement of these ecosystem services occurred without obvious signs of ecological stress or disturbance to the plots.[66]

On the other hand, Reusch et al.[12] studied the effect of genotypic (*clonal*) diversity on the short-term ecological response of *Zostera marina* beds to high water temperature stress. They found that when exposed to near-lethal water temperatures, beds with greater genotypic diversity showed greater rates of seagrass recovery once the stress ceased, exhibiting enhanced biomass production, plant density, and faunal abundance. They also found that the response could be better explained by genotypic *complementarity* (different genotypes exhibiting different traits) rather than through the performance of particularly robust individual genotypes. They concluded that genetic and genotypic diversity should be consider in biodiversity conservation and environmental management strategies, as such factors may enhance seagrass recovery in the face of global climate change and increasing perturbations.[12]

Finally, in one of the few investigations to examine the relationship between genetic diversity and seagrass metabolism, Tomas et al.[67] showed that genotypes differed in biochemical

characteristics associated with nutritional quality (percentage of nitrogen, carbon to nitrogen ratios, concentrations of feeding deterrents) and biomass productions, which affected grazing pressure by isopods. The differences in key traits among genotypes had important consequences for the communities and ecosystems that depend on the seagrass beds. Furthermore, these genetic effects were not overwhelmed by known environmental stressors, such as excessive nutrient input.[67]

From these and other studies, it is clear that genetic variation, in the form of allelic or genotypic diversity, can influence seagrass bed ecology, production, and metabolism. It is also clear, however, that the mechanisms behind are still not well understood, and that there is a growing need for research that examines the role of genetic variation in the response of seagrasses to environmental stressors such as sulfide intrusion.

CHAPTER V: METHODOLOGY

5.1. Objectives

The purpose of this study was to assess levels of genetic variation and sulfide intrusion in a population of the seagrass *Halodule wrightii* from the Texas Gulf Coast. Specifically:

1. To estimate the degree of genetic variation, including genotypic diversity, in a *H. wrightii* population using a DNA-based microsatellite marker assay.
2. To assess and quantify sulfide intrusion in the same *H. wrightii* population.
3. To evaluate the distribution of sulfur derived from sediment sulfide among primary tissues (root, rhizome, and leaf) of *H. wrightii*.
4. To compare accumulation of sulfur derived from sediment sulfide in *H. wrightii* to other temperate and tropical seagrass species.
5. To investigate whether sulfur source, distribution, and accumulation in seagrass tissues varies among genotypes found in the *H. wrightii* population.

5.2. Study Site

Halodule wrightii samples were collected from a single location in Oso Bay (27°42'38.2"N 97°19'03.9"W), a secondary bay near Texas A&M University-Corpus Christi (Ward Island) on the Texas Gulf Coast (Figure 2). This site was selected based on previous similar studies[23,25,26,68] with the same seagrass species and for its proximity to campus.

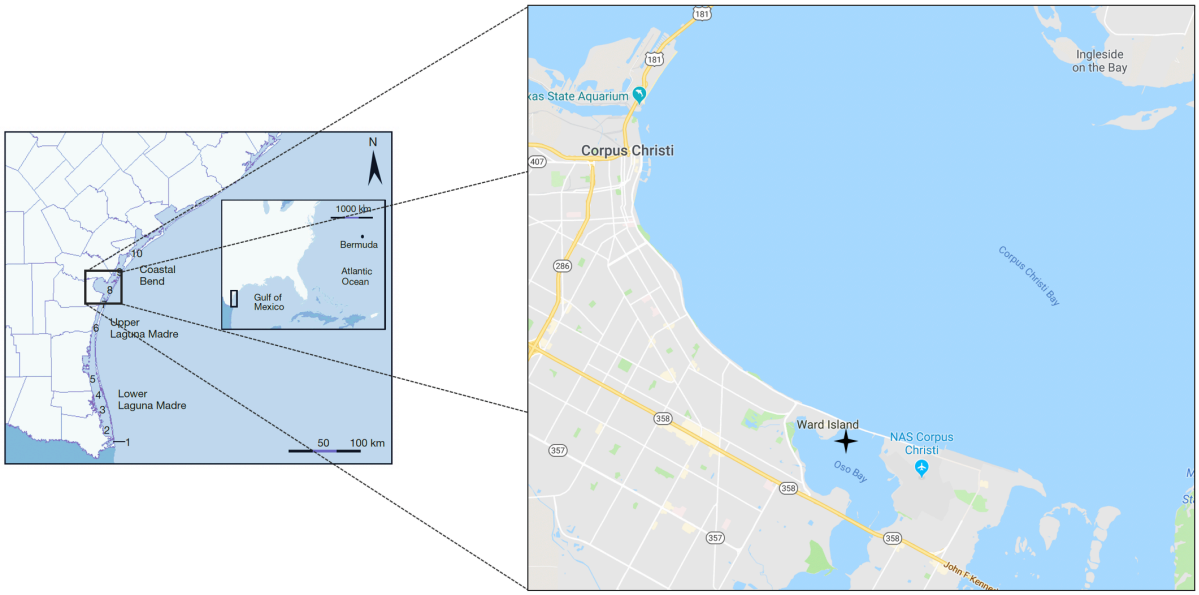

Figure 2. Sampling site for *H. wrightii* in Oso Bay, Texas. Map on the left shows sampling sites on Texas Gulf Coast from previous studies. Map on the right shows sampling location marked with a star. *Source:* Adapted from Larkin, P. D.; Maloney, T. J.; Rubiano-Rincon, S.; Barrett, M. M. *Mar. Ecol. Prog. Ser.* **2017**, 567, 95–107 and Google Maps, 2019.

Oso Bay is an enclosed bay located on the southern shore of Corpus Christi Bay, classified as a soft sediment estuarine area with an average depth of <1.0 m. It receives freshwater from Oso Creek, a small, low gradient stream, whose stream is dominated by treated effluents from wastewater treatment plants input (e.g., from Robstown, Corpus Christi Greenwood, and Oso Wastewater Treatment Plants). Oso Creek exchanges water only with the Corpus Christi Bay through a pass located on the east side of Ward Island. Oso Bay sediments consists of clays, silt, and sands and include some sites with high organic material deposition, such as those close to the City of Corpus Christi Oso Wastewater Treatment Plant (OWWTP).[69] Oso Bay has numerous areas of seagrass beds, mostly comprised of *Halodule wrightii*,[25] and a well-defined wetland area located adjacent to the OWWTP outfall.[69]

5.3. Field Sampling

5.3.1. Seagrass Samples

H. wrightii samples were collected from a seagrass meadow in Oso Bay in July 2018, using a 6 x 22 m rectangular plot (grid) placed roughly parallel to the nearest shore. The grid comprised four parallel, 22 m long transects spaced at 2 m intervals. Samples were collected regularly at 2 m intervals along each transect, resulting in 48 samples total (Figure 3). A 10 cm x 10 cm (diameter x depth) coring device was used to collect sediment/vegetation cores from each position on the grid. Core contents were sieved free of sediment using a 0.5 mm mesh nylon bag and a 5−10 cm section of a single rhizome, containing multiple roots, rhizome and leaf bundles, was collected. Total vegetation samples were collected from 3 grid points for biomass and shoot density determinations. All samples were stored in seawater and kept on ice until return to the laboratory for processing.

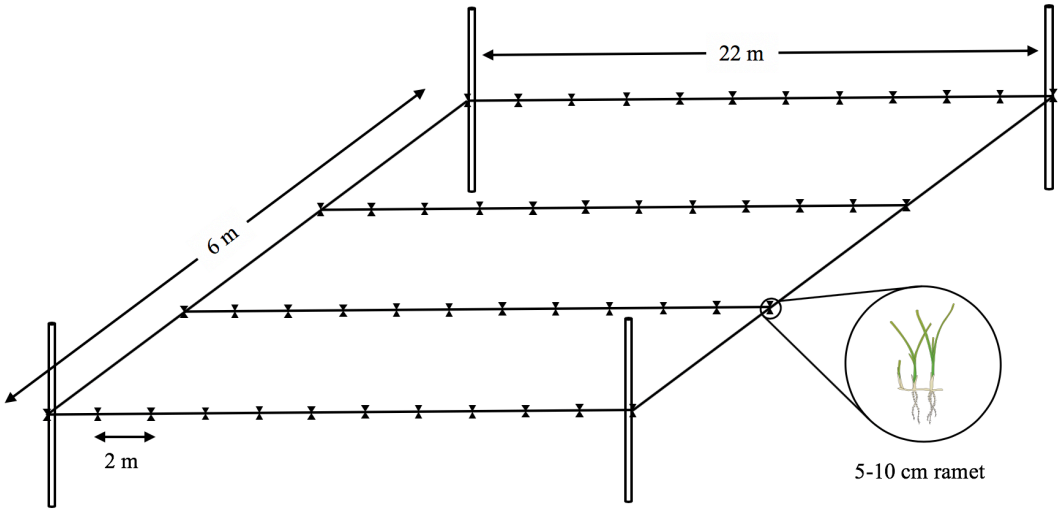

Figure 3. Sampling grid diagram used for collecting H. wrightii samples (ramets) from a seagrass bed in Oso Bay, Texas.

In the laboratory, seagrass samples were thoroughly rinsed with deionized (DI) water to remove sand, salts, and organic materials. Epiphytes were gently removed from leaf blades with a moist paper towel. For genetic variation analysis, 1-2 cm sections of rhizome tissue (10-20 mg)

were transferred into pre-labeled MPBio FastPrep tubes, flash-frozen with liquid nitrogen and freeze-dried for DNA extraction at a later time. For sulfur isotopic analysis, root, leaf, and remaining rhizome tissues were separated and placed into small paper envelopes. Samples were oven-dried at 60°C overnight, transferred to pre-labeled MPBio FastPrep tubes and stored in a desiccator cabinet. For biomass determination, above-ground tissue (leaves, sheath material, and floral parts) were separated from below-ground tissues (rhizomes and roots) using a small razor blade. Epiphytes were also removed and the number of leaves was recorded. Above- and below-ground tissues were placed into aluminum envelopes and oven-dried at 60°C until a constant weight was obtained.

5.3.2. Sediment and Seawater Samples

Ten sediment samples were collected at random from within the 6 x 22 m grid using an open-ended 50 mL syringe. The deeper 5 cm (approximately 25 mL) of each sediment from each sampling position was quickly transferred into a pre-labeled 50 mL centrifuging tube containing 10 mL of 1M zinc acetate, capped and shaken vigorously to fix free sulfides. The sediment-zinc acetate mixture was transferred to the laboratory on ice and kept frozen until needed for sulfide distillation. One (~500 L) seawater sample was collected above the seagrass canopy with a glass bottle and stored on ice. In the laboratory, particulate matter was removed by filtering through a 0.2 mM ZapCap bottle top filter using vacuum. The filtered solution was aliquoted into pre-labeled 50 mL centrifuge tubes and frozen until needed for sulfate precipitation.

5.4. Genetic Diversity Analysis

5.4.1. DNA Extraction

Approximately 20 mg of freeze-dried rhizome tissue from each sample was pulverized using the Lysing Matrix A Kit (MP Biomedicals) in a FastPrep® 24 instrument. DNA was extracted from the homogenized sample using the Plant DNeasy® kit (Qiagen) following the manufacturer's protocols. DNA was quantitated using a QuanIT® dsDNA BR fluorometric assay and a Qubit ® fluorometer (Invitrogen) according to manufacturer's protocols. DNA samples were then stored at 4ºC until use.

5.4.2. Microsatellite Amplification

Each sample was screened at 8, previously described microsatellite loci[24,25] using a Multiplex Polymerase Chain Reaction (MPX-PCR)-based assay. Amplification reactions were performed with the Type-it® Microsatellite PCR kit (Qiagen), and consisted of 12.5 µL of Type-it Multiplex PCR Master Mix, 2.5 µL of Primer Mix containing forward and reverse WellRED D_2, D_3 and D_4 Fluorescent dye-labeled primers, 2.5 µL of Q-Solution, 10-50 ng of genomic DNA (1-7.5 µL) and sufficient RNase-free Water (0-6.5 µL) to obtain a total of 25 µL volume per PCR reaction. PCR cycling was performed on a BioRad S1000 thermal cycle. Cycling conditions included a 5 min initial activation step at 95ºC followed by 28 cycles of (i) denaturation (30 sec at 95ºC), (ii) annealing (90 sec at 60ºC) and (iii) extension (30 sec at 72ºC). A final extension at 60ºC for 30 min ended the program.

Microsatellite amplification products were separated and sized on a CEQ 8000® Genetic Analyzer (Beckman-Coulter). Amplification products were diluted 1:10 with DI water. Two µL of each diluted product were loaded into a 96-well plate together with 0.5 µL of 400 bp size standards previously diluted in 35 µL of Sample Loading Solution (Beckman-Coulter). Wells were topped off with a drop of mineral oil to prevent evaporation. Separately, a buffer plate was filled (ca. 100 µL per well) with Separation Buffer (Beckman-Coulter). Allele scoring was performed using the

Beckman–Coulter CEQ 8000 Fragment Analysis Software, v (9.0). Ten percent of the samples were run in duplicate to confirm the reproducibility of results.

5.5. Sulfur Stable Isotopes Analysis

5.5.1. Sulfate Precipitation from Seawater Samples

Sulfate (SO_4^{2-}) from seawater was precipitated as barium sulfate ($BaSO_4$) following the protocol of Grasshoff et al.[70] Briefly, 50 mL of a seawater aliquot (5 aliquots total) were thawed and heated to 90°C with 235 mL of DI water, 10 mL of 1.3% picric acid solution, and 5 mL of 12 M hydrochloric acid (HCl). $BaSO_4$ was precipitated by adding warm 10% barium chloride ($BaCl_2$) solution while stirring. The $BaSO_4$ precipitate was filtered by vacuum using a Whatman™ 42 filter paper and washed with warm DI water until all chloride residues were removed. The filtrate water was tested for chloride with drops of 0.1M silver nitrate ($AgNO_3$). If the solution turned opalescent, the $BaSO_4$ precipitate still contained some chloride. The precipitate was rinsed with warm DI water until the filtrate gave no opalescence with addition of $AgNO_3$. The precipitate was then transferred to a porcelain crucible, oven-dried at 110°C for 1 hour, and then ignited at 800°C in a muffle furnace for 2 hours to eliminate impurities. Dried $BaSO_4$ was transferred to glass vials and stored in a desiccator until sample encapsulation for isotope analysis.

5.5.2. Sulfide Distillation from Sediment Samples

Sulfides (from the Total Reduced Inorganic Sulfur (TRIS) pool) were extracted from sediment samples and precipitated as silver sulfide (Ag_2S) following a distillation protocol based on Backlund et al.[71] and Fossing & Jorgensen.[52] The distillation apparatus (Figure 4) consists of a hemispherical mantle supported on a hot plate, a four-neck round bottom flask, a reflux coil condenser, a buffer bottle (containing 200 mL of 0.05 M potassium hydrogen phthalate (KHP)

buffer solution, pH 4), two trap bottles (containing 15 mL of 0.1 M $AgNO_3$), a N_2 gas dispersion tube, and a NaOH trap flask. The glassware is connected by glass connection adapters, pinch-type clamps, joint clips, and rubber tubing. A temperature control with sensor was included to regulate temperature during distillation.

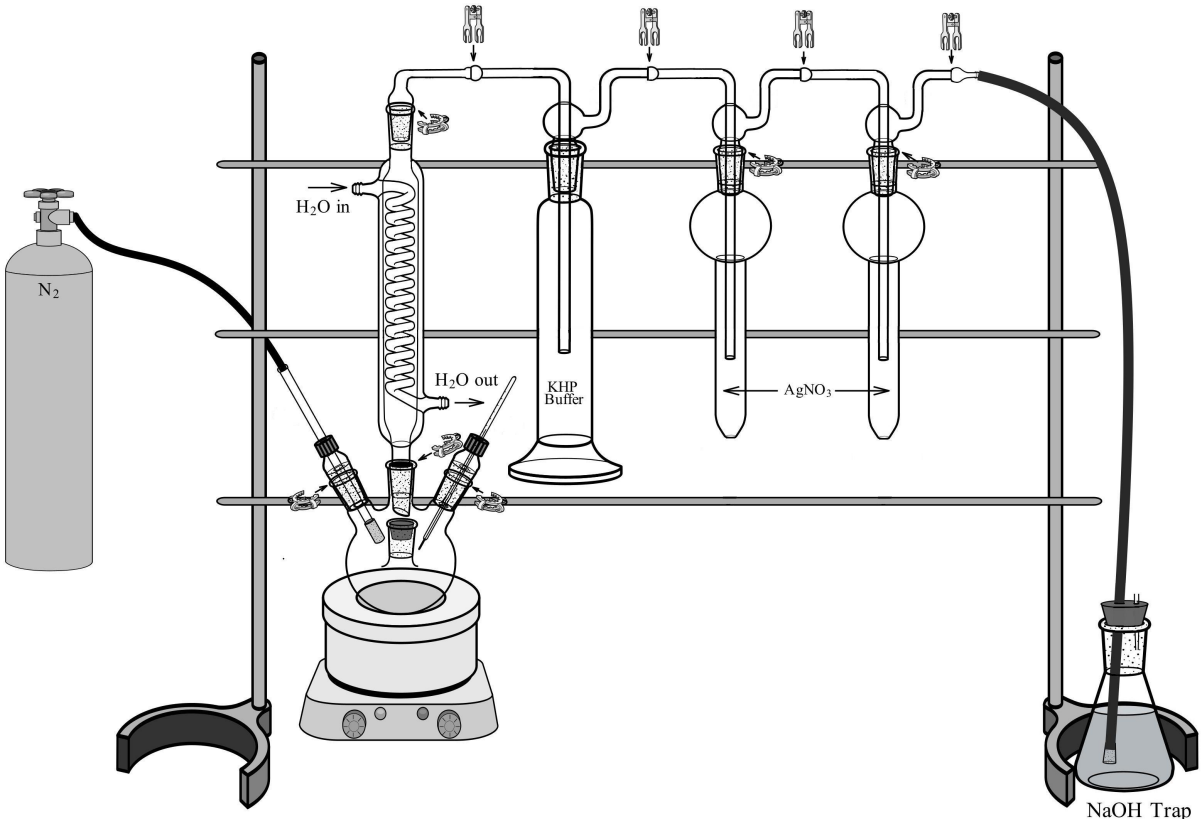

Figure 4. General scheme for sulfide distillation apparatus.

Ten grams of zinc-acetate fixed sediment was thawed and transferred to a four-neck, round bottom flask in a nitrogen-filled glove bag to prevent oxidation of sulfides. A triangular stir bar was added and the sediment-containing flask was connected to the distillation apparatus and 10 mL of 50% EtOH was added while stirring. The distillation reagents were deoxygenated with nitrogen gas before the procedure to minimize oxidation of liberated H_2S.

The apparatus was degassed with N_2 for 10 minutes at a flow rate of approximately 5 to 10 bubbles per second. Sediment distillation proceeded with the injection of 50 mL of 6M HCl and 50 mL of a 1M reduced chromium (Cr^{2+}) solution in 0.5 M HCl while boiling for 1 hour with a constant N_2 flow. The Cr^{2+} solution was previously prepared by percolating 1M chromium(III) chloride hexahydrate ($CrCl_3*6H_2O$) in 0.5 N HCl through a Jones Reductor (Figure 5). The reductor was set up using a glass-column with an integral sinter at the bottom and filled with Hg-amalgamated granular zinc. An efficient reduction of the chromium ions from the $CrCl_3*6H_2O$ solution was verified by a color change from dark green (Cr^{3+}) to bright blue (Cr^{2+}). The solution was collected in 50 mL plastic syringes and capped with rubber tip caps and parafilm to prevent re-oxidation.

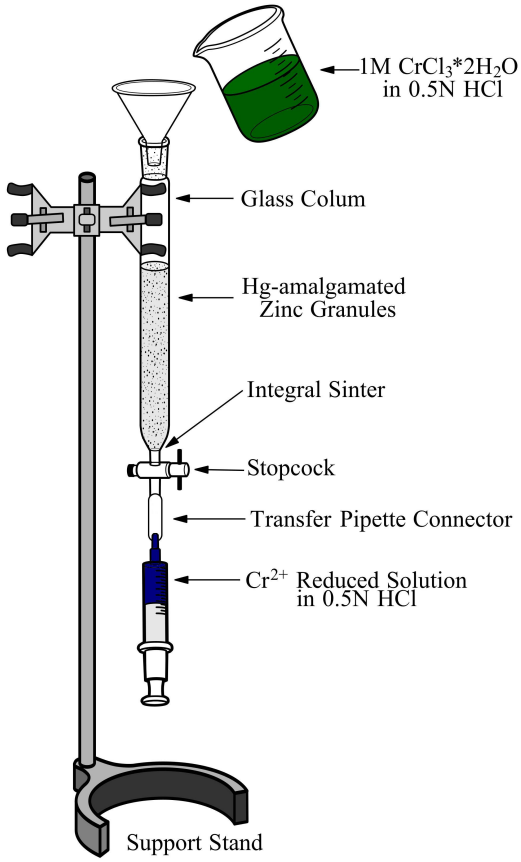

Figure 5. General scheme for Jones Reductor apparatus for preparation of Cr^{2+} solution.

During distillation, H_2S liberated from the TRIS pool in the sediment was first transported to the KHP buffer bottle, where any free chlorine ions in the system were neutralized to prevent precipitation of silver chloride (AgCl). Hydrogen sulfide was then transported to the sulfide traps, where it precipitated as Ag_2S. Ag_2S was collected by vacuum filtration using Whatman™ 42 filter paper. The precipitate was transferred to a glass vial and placed in a lyophilizer for vacuum-drying overnight. Vials were stored in a desiccator until sample encapsulation for isotope analysis.

After each distillation was complete, the apparatus was disassembled and the glassware was cleaned with base (1M NaOH in 2-propanol) and acid (1M HCl) baths to ensure removal of residues and contaminants.

5.5.3. IRMS Sample Analyses

Seagrass tissues (root, rhizome, and leaves), and precipitates (Ag_2S and $BaSO_4$) of sediment sulfide and seawater sulfate were analyzed for total sulfur (TS; %dw) and stable sulfur isotope ratio ($^{34}S/^{32}S$) at the Stable Isotopes for Biosphere Science (SIBS) laboratory at Texas A&M University by elemental analyzer combustion continuous flow isotope ratio mass spectroscopy (Thermo EA IsoLink CNSOH coupled to a Thermo Scientific Delta V Advantage Isotope Ratio Mass Spectrometer with Universal Triple Collector and HD Collector).

For encapsulation, seagrass tissue samples were finely ground and homogenized using the FastPrep® 24 instrument. Samples were encapsulated in 6x4 mm tin capsules (Elemental Microanalysis) using the facilities of the Isotope Core Laboratory (ICL) at Texas A&M University-Corpus Christi. Sample size in each capsule was either 0.75 +/- 0.05 mg of seagrass tissue or 0.05 +/- 0.01 mg of Ag_2S or $BaSO_4$. No V_2O_5 was added to the capsules.

The $^{34}S/^{32}S$ ratios were reported in standard delta notation ($\delta^{34}S$, units per mill, ‰). Isotope and %TS normalization (quality assurance) was performed using four IAEA silver sulfide and barium sulfate standards (S2, S3, SO-6, and NBS127). Quality control was performed using IAEA standards (S1 and SO5) and two commercially available hair standards (USGS42 and USGS43 from USGS).

5.6. Statistical Analyses

5.6.1. Genetic Variation Estimates

Allele scores were exported to a Microsoft Excel® spreadsheet (Appendix I) for statistical analysis. Samples with questionable allele scores were reanalyzed. Unique multi-locus genotypes (MLGs) were identified from the allelic scores and used for obtaining estimates of genetic variation. MLGs that differed at only one locus were re-assayed using additional loci (including the variable locus) to determine if the differences were the result of scoring errors or somatic mutations.

Estimates of genetic diversity included: mean number of alleles (A); mean allelic richness (A_R), representing allele diversity standardized to a particular sample size; mean expected and observed heterozygosity (H_e and H_o, respectively), representing the proportion of heterozygous individuals statistically expected or actually observed in the population; the inbreeding coefficient (F_{IS}), calculated as $(H_e\text{-}H_o)/H_e$; and clonal richness (R), representing the proportion of samples within a population that are comprised of unique genotypes. R is calculated as $(G\text{-}1)/(N\text{-}1)$, where G represents the number of unique genotypes within the sample set and N is the sample size.[72]

Additional attributes of genotypic (clonal) diversity were also estimated, including clonal *evenness*, which describes the equitability with which clonal membership is distributed among the

samples within a population. It was estimated using the Simpson's evenness index (E_D) and the slope of the Pareto distribution (β). The Aggregation Index (A_c) was also used to quantify clonal *architecture*, i.e. the degree to which clones in a population exhibit a *guerilla* (intermingled) or *phalanx* (clumped) distribution.[63] All estimates were obtained using the GenClone (v. 2.0),[73] GenAlEx® (v. 6.5),[74] and FSTAT®[75] population genetic analysis software programs.

5.6.2. Sulfur Stable Isotopes

$\delta^{34}S$ values from seagrass tissues and sediments may involve a certain degree of discrepancy due to differences in the timing of seasonal variations in temperature, sulfate reduction rates, or organic matter availability.[7] For adjusting for the possible variation from the sulfur sources, Frederiksen et al.[17] proposed the parameter $F_{Sulfide}$, which represents the percentage of tissue sulfur that is derived from sedimentary sulfide. This parameter is defined as the contribution of sedimentary sulfide to the sulfur found in plants, and is calculated as:

$$F_{Sulfide} = \frac{\delta^{34}S_{tissue} - \delta^{34}S_{sulfate}}{\delta^{34}S_{sulfide} - \delta^{34}S_{sulfate}} \times 100$$

wherein $\delta^{34}S_{Tissue}$ represents the value measured in the seagrass tissue (leaf, rhizome or root), $\delta^{34}S_{Sulfate}$ corresponds to the seawater sulfate value, and $\delta^{34}S_{Sulfide}$ represents the sediment sulfide value.

All data ($\delta^{34}S$, %TS, $F_{Sulfide}$, and MLGs) for all samples were exported to a Microsoft Excel® spreadsheet (Appendix II). Statistical analyses were conducted in R (R Core Team[76]) and figures were produced using the package ggplot2 (Wickham[77]) and Microsoft Excel®. Data were tested for normality and homogeneity of variance. Linear regression analyses were used to compare and find significant correlations between %TS and $\delta^{34}S$ as well as $F_{Sulfide}$ and %TS. Mean $F_{Sulfide}$, $\delta^{34}S$, and %TS variations among seagrass tissue were evaluated using a One-way ANOVA

(factor: seagrass tissue). Variation in $F_{Sulfide}$ mean values among unique genotypes (MLGs) and seagrass tissues was evaluated using a Two-way ANOVA (factors: genotype and seagrass tissue). Significant ANOVA results were followed by *post-hoc* Tukey's Honest Significant Difference (HSD) adjusted Westfall test.[78] $F_{Sulfide}$ distribution among genotypes was also tested for genotypic evenness and aggregation using the GenClone (v. 2.0) software. Influential data points were determined by measuring Cook's Distance values.[79] Mean values were presented with standard deviation (mean ± SD) or standard error (mean ± SE) and the tests were performed at the $P < 0.05$ significance level.

CHAPTER VI: RESULTS

6.1. Genetic Variation Estimates

6.1.1. Multi-Locus Genotypes (MLGs) and Multi-Locus Lineages (MLLs)

Genetic variation estimates were based on the genotypes derived for each ramet (root/rhizome/leaf shoot bundle) sample using the suite of 8 microsatellite markers. Two terms are used to describe genotypes: *Multi-Locus Genotypes* (MLGs) and *Multi-Locus Lineages* (MLLs). A MLG is the combination of alleles that are present across multiple loci in a particular individual. Because seagrasses are capable of clonal reproduction, more than one sample (*ramet*) in a population can have the same MLG, and thus belong to the same clone (*genet*). Multi-Locus Lineages refer a collection of MLGs that differ at only 1 or 2 alleles. Because the mutation rate in microsatellite DNA is much higher than that of regular DNA,[8,60] these differences likely represent *somatic* (vs *germ-line*) mutations that occur in individual cells, vs. true genetic differences that result from sexual reproduction. Individual samples that differ in terms of somatic mutations are not considered to be distinct genotypes, but rather members of the same clone. They are classified as belonging to the same MLL, whose formal identity is that of the dominant (most numerous) genotype in the group. Slightly distinct MLGs that are suspected to belong to the same MLL can be analyzed using specific statistical analyses. MLGs are considered to belong to the same MLL when the probability that their genotypes were derived from distinct reproductive events (P_{sex}) is lower than 0.01.[63]

Amplification and allele scoring (Appendix I) of the 48 samples of *H. wrightii* from Oso Bay resulted in 10 MLLs from 14 MLGs (Table 1). That is, 14 genotypes (MLGs) were found, but 4 of those differed at only one or two alleles ($P_{sex} < 0.01$). There were consolidated to produce 10 unique genotypes (MLLs) from the population. All loci were polymorphic, meaning that at least

two alleles per locus were found in the population. Genetic diversity estimates for the Oso Bay population are shown in Table 2.

Table 1. Unique genotypes (MLLs with corresponding MLGs) for *H. wrightii* samples from Oso Bay, Texas. Alleles that differed among genotypes from the same MLL are in bold and underlined.

Genotype		No. of Samples	Microsatellite Marker per Locus							
MLL	*MLG*		*180*	*190*	*196*	*212*	*214*	*222*	*228*	*232*
1	1	11	232 235	131 139	178 187	295 298	214 224	219 219	269 278	265 265
	2	5	232 235	131 139	178 187	295 298	214 224	219 **223**	269 278	265 265
2	3	2	232 244	125 131	178 **178**	280 289	212 230	223 235	272 278	265 269
	4	6	232 244	125 131	178 187	280 289	212 230	223 235	272 278	265 269
3	5	2	232 244	131 133	178 187	295 298	212 212	215 235	269 269	263 265
4	6	1	232 244	131 135	178 187	280 298	212 224	179 **219**	272 278	269 269
	7	5	232 244	131 135	178 187	280 298	212 224	179 223	272 278	269 269
5	8	1	232 244	131 137	178 187	283 295	212 218	235 243	269 278	269 269
6	9	1	232 244	131 137	178 187	295 298	212 218	231 263	269 278	269 269
7	10	3	232 247	131 135	178 187	280 298	210 218	231 239	269 278	263 269
8	11	5	235 244	135 137	178 178	298 298	210 214	219 219	278 278	265 269
9	12	1	244 244	135 135	178 178	289 298	214 222	**219** 227	278 278	269 269
	13	1	244 244	135 135	178 178	289 298	214 222	223 227	278 278	269 269
10	14	4	247 250	135 137	178 178	298 298	210 224	211 287	278 278	269 269

Table 2. Genetic diversity estimates for *H. wrightii* from Oso Bay, Texas. *N*: number of samples; *G*: number of genotypes (MLLs); *R*: genotypic (clonal) richness; *A*: average number of different alleles; A_R: mean allelic richness; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient.

Site	Basin	*N*	*G*	*R*	*A*	A_R	H_o	H_e	F_{IS}
Oso Bay	Coastal Bend	48	10	0.19	5.38	4.74	0.75	0.66	-0.14

Compared to other populations of *H. wrightii* from the Texas Gulf Coast,[25] we found the Oso Bay population to be quite genetically diverse. The *Mean Number of Alleles* (*A*) was the third highest among the Texas populations and above the average (4.84 ± 0.36). Similarly, *Allelic Richness* (A_R) was the second highest but was closer to the average value for all the Texas populations (4.27 ± 0.18). Heterozygosity estimates were also substantially higher than (mean: H_o

= 0.62 ± 0.03; H_e = 0.55 ± 0.03) while F_{IS} were low compared to other populations (-0.12 ± 0.02), confirming the higher observed than expected heterozygosity estimate for Oso Bay.

6.1.2. Clonal Diversity

Clonal diversity encompasses several attributes such as clonal *richness*, *evenness* and *architecture.* Clonal richness (*R*) estimates the proportion of unique genotypes (MLLs) within a population and provides an idea of the number of individuals contributing to the pool of genetic variation. *R* varies from 0 when individual samples possess the same genotype (i.e., are all members of the same clone), to 1.0 when all individuals possess a different genotype.[72] Therefore, it is used as an indicator of the extent of clonal growth (*vegetative reproduction through rhizome extension*) vs. sexual reproduction. *H. wrightii* from the Oso Bay site had an R value of 0.19 (10 genotypes from 48 individuals), suggesting the population consists of a relatively small number of clones. Clonal diversity in Oso bay was below the average for all populations from the Texas Gulf Coast (0.32 ± 0.07) (Figure 6).

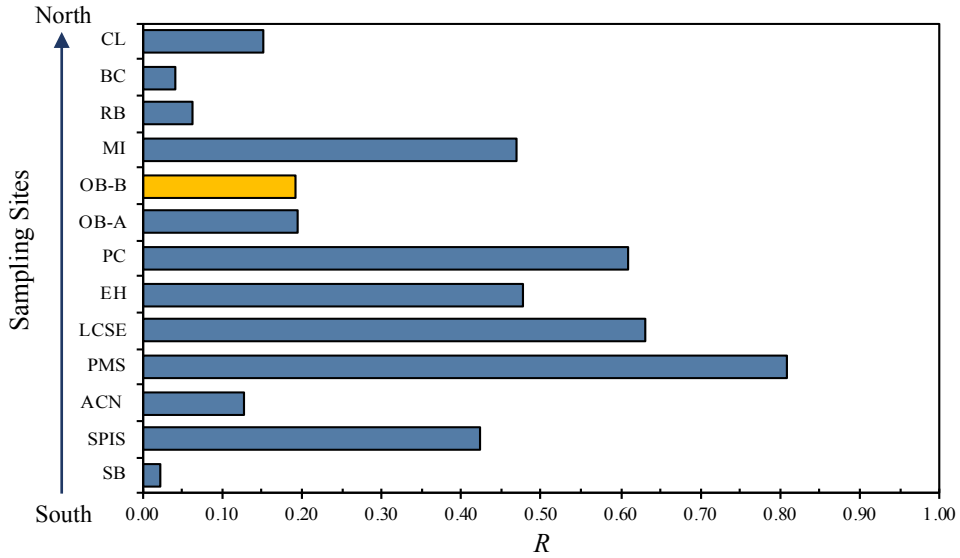

Figure 6. Clonal Diversity (R) estimates for *H. wrightii* populations from the Texas Gulf Coast. Values are organized according to population's location from south to north. This study's population (Site 11B at Oso Bay, OB-B) is highlighted in yellow.

Clonal diversity was be further assessed by examining the distribution of clonal membership within the population (*Evenness*) and their spatial relationship to one another (*Architecture*, Figure 7). *Evenness* was estimated using the Simpson's evenness index (E_D) and the slope of the Pareto distribution (β). The Simpson's evenness index ranges from 0 to 1, where higher values represent a more equitable distribution of clonal membership and lower values represent a more skewed one, indicating the majority of samples belonged to only one or a few genotypes. In a similar fashion, the slope of the Pareto distribution increases as the distribution of individuals among genotypes becomes more even.[68] For our population, the E_D value was 0.901 and β was 1.621, suggesting a high degree of evenness.

Figure 7. Multi-Locus Lineage (MLL) distribution in Oso Bay, Texas population. Colors and numbers represent positions of unique genotypes (MLL). Genotypic (clonal) richness (R); Simpson's evenness index (E_D); slope of the Pareto distribution of clonal membership (β); and Aggregation index (A_c) values are included at right. *A_c value was not significant ($P > 0.05$).

Clonal architecture, which describes the spatial relationship of clones in a population, was quantified using the *Aggregation index* (A_c). The A_c value measures the probability that nearest neighbors are identical clones, and thus whether genotypes reside in a segregated (*phalanx*) or intermingled (*guerilla*) spatial distribution.[63] A_c values vary from 0 to 1, with higher values reflecting a greater probability of residing next to the same MLL (*phalanx*) and lower values a lesser probability (*guerilla*). The A_c value for the Oso Bay site was very low (0.0064), suggesting an intermingled spatial distribution, but not significant ($P > 0.05$) probably due to the low number

of MLLs (10) in the sample. Visual inspection of the results, however, supports this conclusion (Figure 7). While dominated by only a few clones, they are broadly distributed in space.

6.2. Sulfur Stable Isotopes Analyses

6.2.1. Sulfur Isotope Composition of Seawater and Sediments

Sulfides (e.g., H_2S, HS^-) from the Total Reduced Inorganic Sulfur (TRIS) pool were distilled from 10 sediment samples. On average, 9.91 ± 5.11 mg of Ag_2S was recovered and 0.05 ± 0.01 mg of each sample was used for isotope analysis. The mean $\delta^{34}S$ value of the sediment samples was -27.38 ± 1.41‰ (N=10), with values ranging from -29.49‰ to -25.21‰.

Sulfate (SO_4^{2-}) was precipitated from 5 replicate seawater samples and, on average, 202.20 ± 13.55 mg was recovered. As for sediment, 0.05 ± 0.01 mg of each sample was used for isotope analysis. The mean $\delta^{34}S$ value was calculated from 4 of the 5 samples, as the combustion of one was incomplete. The mean $\delta^{34}S$ value of the seawater samples was 21.11 ± 0.76‰, with values ranging from 20.28‰ to 22.05‰.

$\delta^{34}S$ values for sulfur sources are summarized in Table 3 and Appendix II. Total Sulfur (TS, % dry weight, dw) of sediment sulfide and seawater sulfate samples was 13.26 ± 0.78% and 13.92 ± 0.36%, respectively. Precision of $\delta^{34}S$ analyses was estimated to approximately ± 0.08‰, and measurements on IAEA standards S2 ($\delta^{34}S$ 22.62‰ VCDT), S3 ($\delta^{34}S$ -32.49‰ VCDT), SO-6 ($\delta^{34}S$ -34.05‰ VCDT), and NBS127 ($\delta^{34}S$ 21.12‰ VCDT) gave $\delta^{34}S$ values of 22.56‰, -32.40‰, -34.22‰, and 21.11‰, respectively. Quality control was performed using IAEA standards S1 ($\delta^{34}S$ -0.30‰ VCDT) and SO5 ($\delta^{34}S$ 0.49‰ VCDT) and USGS hair standards USGS42 ($\delta^{34}S$ 7.84‰ VCDT) and USGS43 ($\delta^{34}S$ 10.46‰ VCDT). These standards gave the $\delta^{34}S$ values of -0.19‰, 0.69‰, 7.43‰, and 9.95‰, respectively.

Table 3. $\delta^{34}S$ of sulfur sources (sediment sulfide-TRIS or seawater sulfate) from *H. wrightii* meadow at Oso Bay, Texas. Values are given as sample mean (±SD). *N* = number of observations.

Species	**Sediment (TRIS)**		**Seawater**	
Location	***N***	**$\delta^{34}S$ (‰)**	**N**	**$\delta^{34}S$ (‰)**
Halodule wrightii TX, USA	10	-27.38 ± 1.41	4	21.11 ± 0.76

6.2.2. Sulfur Isotope Composition of Seagrass Tissues

Root, rhizome, and leaf tissues were obtained from 48 samples of the *H. wrightii* population from Oso Bay. On average, 40.11 ± 16.27 mg of leaf tissue, 36.60 ± 19.28 mg of rhizome tissue, and 14.26 ± 7.38 mg of root tissue were collected and ground to fine powder. For isotope analysis, 0.75 ± 0.05 mg of each sample was used. The mean $\delta^{34}S$ and TS values for the three tissues are summarized in Table 4 and Appendix II.

Table 4. TS and $\delta^{34}S$ of *H. wrightii* leaves, rhizomes, and roots from Oso Bay, Texas. Values are given as sample mean (±SD). *N* = number of observations.

Species **Location**	**Leaf**			**Rhizome**			**Root**		
	N	**TS (%dw)**	**$\delta^{34}S$ (‰)**	***N***	**TS (%dw)**	**$\delta^{34}S$ (‰)**	***N***	**TS (%dw)**	**$\delta^{34}S$ (‰)**
H. wrightii TX, USA	47	0.54 ± 0.12	13.58 ± 2.04	48	0.49 ± 0.18	5.72 ± 3.37	48	0.55 ± 0.23	-5.58 ± 3.73

The $\delta^{34}S$ values of leaf tissues originally ranged from -2.91‰ to 16.35‰. However, the -2.91‰ value, found in one sample, was considered to be an outlier as it was 1.5 IQRs (Interquartile Range, 2.25) below the Q1 (First Quartile, 12.61). Furthermore, this value was considered to be an *influential point*, according to the Cook's distance test,[79] which states that a point is influential if removing it from the dataset substantially changes any possible relationship (e.g., regression). Any point with a Cook's distance greater than 1 should be considered as influential. The -2.91‰ value had a Cook's distance of 3.298, meaning that it could negatively influence any possible

linear trends within the data. For this reason, it was decided to exclude it from the statistical analyses.

Thus, the amended $\delta^{34}S$ values for leaf tissues ranged from 6.44‰ to 16.35‰, with a mean value of 13.58 ± 2.04‰ (N = 47). The $\delta^{34}S$ values of rhizome tissues ranged from -0.22‰ to 11.66‰, with a mean value of 5.72 ± 3.37‰ (N = 48). The $\delta^{34}S$ values of root tissues ranged from -15.80‰ to 1.87‰, with a mean value of -5.58 ± 3.73‰ (N = 48). Neither rhizome or root tissues and exhibited influential points. Variation in the $\delta^{34}S$ signal was higher in roots and rhizomes (16‰ and 12‰, respectively) than in leaves (only 9‰). Differences in $\delta^{34}S$ values among seagrass tissues are shown in Figure 8.

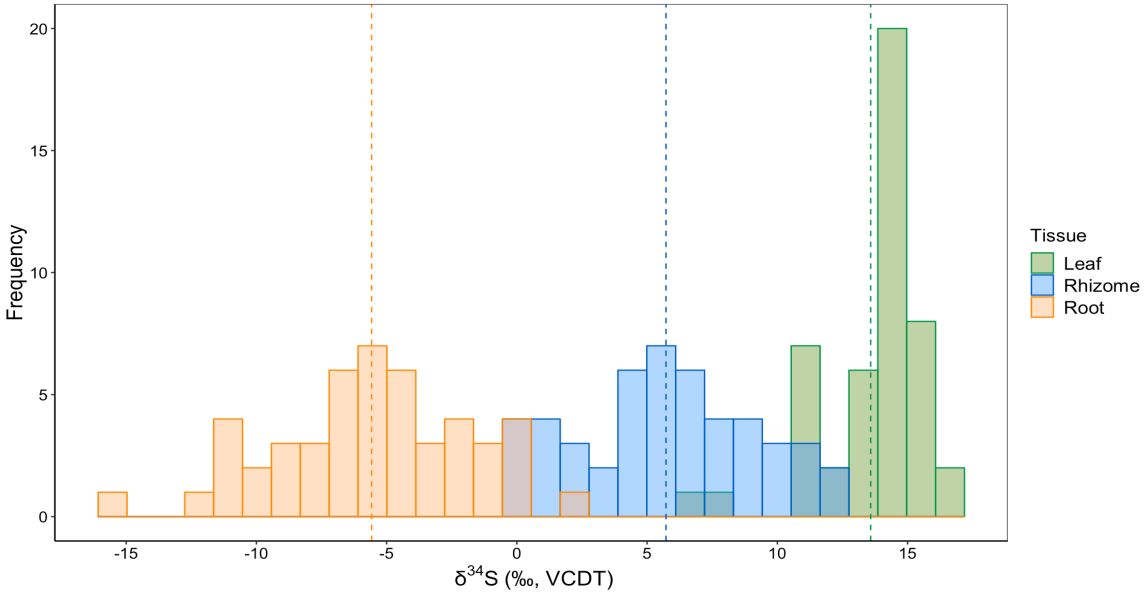

Figure 8. Histogram of $\delta^{34}S$ (‰) values for *H. wrightii* leaves, rhizomes, and roots from Oso Bay, Texas. Dashed lines represent averages.

Calculations of the $F_{sulfide}$ parameter showed that, in roots and rhizomes, 55.02 ± 7.68% and 31.72 ± 6.95%, respectively, of the sulfur content was derived from sediment sulfide. Values in roots ranged from 39.67% to 76.11% and those in rhizomes from 19.48% to 43.98%. On the other

hand, only 15.51 ± 4.2% of the sulfur in leaves was derived from sediment sulfide, with values ranging from 9.81% to 30.24%.

While the proportion of sulfur derived from sediment sulfide varied, the total sulfur content (%TS) across tissues was similar. TS values in leaves ranged from 0.18% to 0.78% (mean = 0.54 ± 0.12%), 0.13% to 0.98% in rhizomes (mean 0.49 ± 0.18%), and from 0.07% to 1.02%, in roots (mean = 0.55 ± 0.23%).

6.2.3. Relationships Between TS, $\delta^{34}S$ and $F_{sulfide}$ Among Seagrass Tissues

Correlation and linear regression analyses were used to explore relationships between sulfur content and isotope composition in H. wrightii tissues. Correlations (Pearson's *r*) between $\delta^{34}S$ and TS were computed for roots, rhizomes and leaves. While a negative correlation was shown between $\delta^{34}S$ and TS for rhizomes ($r(48) = -0.41$, $P = 0.003$) and roots ($r(48) = -0.34$, $P = 0.018$), there was no significant correlation for leaves ($r(47) = 0.04$, $P = 0.796$). Regression models showed that $\delta^{34}S$ in roots and rhizomes decreased with increasing TS ($R^2 = 0.1153$, $P = 0.018$ for roots and $R^2 = 0.1718$, $P = 0.003$) while there was no significant linear relationship for leaves (Figure 9).

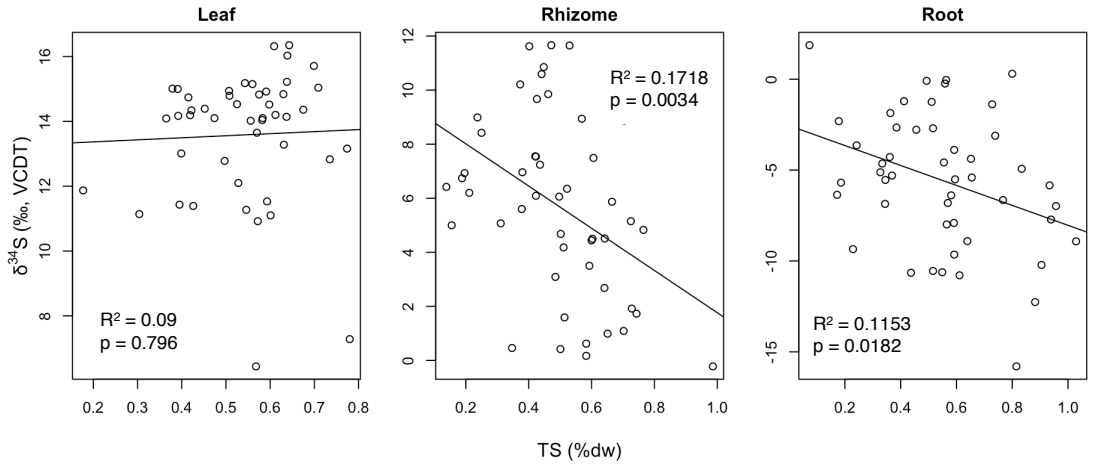

Figure 9. Regressions between total sulfur content (TS, %dw) and $\delta^{34}S$ (‰) from different *H. wrightii* tissues. Correlation coefficients (R^2) and *p*-values are shown.

Pearson's *r* correlations between $F_{sulfide}$ and TS were computed to evaluate the contribution of sediment-derived sulfide to total sulfur in seagrass tissues. There was a positive correlation between $F_{sulfide}$ and TS in rhizomes ($r = 0.41$, $N = 48$, $P = 0.003$) and roots ($r = 0.34$, $N = 48$, $P = 0.018$) but no correlation with leaves ($r = -0.04$, $N = 47$, $P = 0.797$). Likewise, regression models showed that $F_{sulfide}$ increases in roots and rhizomes with increasing TS ($R^2 = 0.1153$, $P = 0.018$ for roots and $R^2 = 0.1718$, $P = 0.003$) but not in leaves (Figure 10).

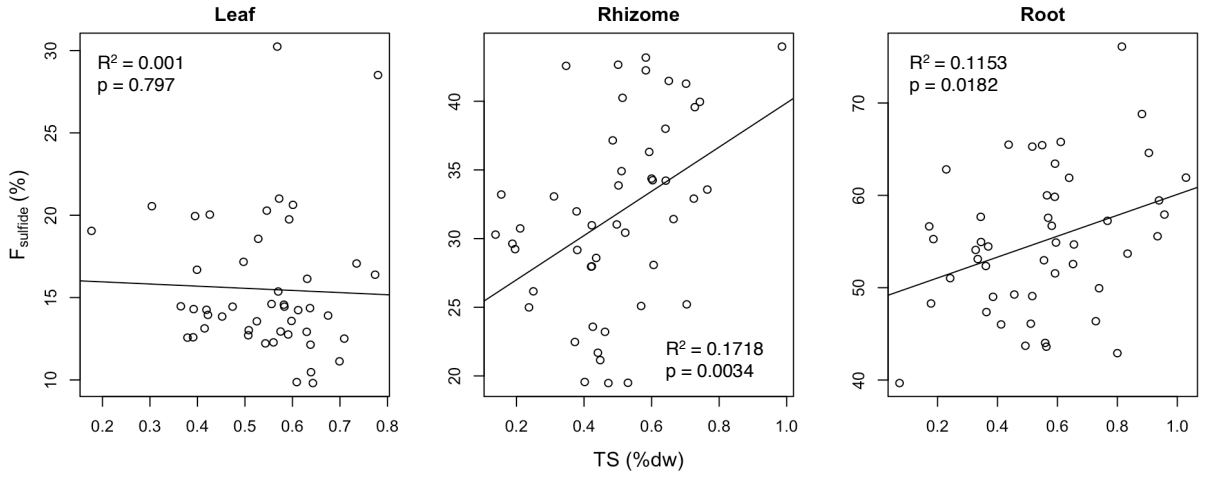

Figure 10. Regressions between total sulfur content (TS, %dw) and $F_{sulfide}$ (%, fraction of tissue sulfur derived from sediment sulfide) for different *H. wrightii* tissues. Correlation coefficients (R^2) and *p*-values are shown.

6.2.4. Variation in $F_{sulfide}$, $\delta^{34}S$ and TS as Explained by Seagrass Tissue and MLLs

Variation in $F_{sulfide}$, $\delta^{34}S$ and TS content among tissues was assessed with a One-way ANOVA test. Results showed significant differences between mean $F_{sulfide}$ values ($F(2,140) = 448.13$, $P < 2.2e\text{-}16$) (Figure 11a) and $\delta^{34}S$ ($F(2,140) = 448.15$, $P < 2.2e\text{-}16$) (Figure 11b) for the seagrass tissues. There were no statistically significant differences among TS means ($F(2,140) = 1.3601$, $P = 0.26$) (Figure 11c). *Post hoc* analyses using the Tukey's Honest Significant Difference (HSD) adjusted Westfall test indicated that the mean $\delta^{34}S$ value was significantly lower ($P < 2.2e\text{-}16$) in roots than in other tissues and that mean $\delta^{34}S$ value for rhizomes was significantly lower

($P < 2.2e-16$) than in leaves. A similar trend was found with the $F_{sulfide}$ parameter. The mean sulfur percentage derived from sediment sulfide in roots was significantly higher ($P < 2.2e-16$) than in rhizomes or leaves, while the mean percentage in rhizomes was significantly higher ($P < 2.2e-16$) than in leaves.

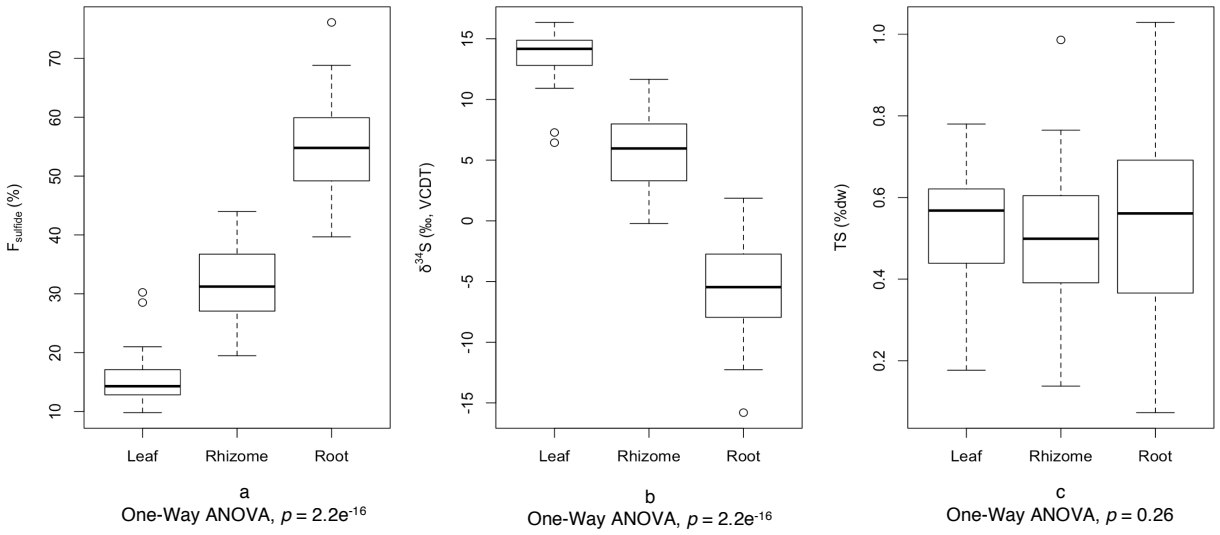

Figure 11. Boxplots representing variation in mean $F_{sulfide}$ (%), $\delta^{34}S$ (‰), and TS (%dw) among different *H. wrightii* tissues. *P*-values from One-Way ANOVAs are included below each graph. Individual dots represent outliers, as defined by any number larger than 3rd Quantile (Q_3) + 1.5*IQR* or smaller than 1st Quantile (Q_1) - 1.5*IQR*. Lines outside the box (whiskers) extend to the smallest and largest non-outliers.

A Two-way ANOVA was used to assess the relationship between $F_{sulfide}$ and genotype (MLL) to determine if there is a genetic basis to the proportion of tissue sulfur attributable to sulfide intrusion (Figure 12). Factors included 3 levels of seagrass tissue and 6 levels of unique genotypes. Only genotypes (MLLs) with 3 or more replicates were analyzed. While there were differences among individual genotypes, the overall effect of genotype was not statistically significant ($F(5,107) = 1.7202$, $P = 0.14$). The interaction effect (variation explained by both tissue and genotype) was also non-significant ($F(1, 10) = 0.8413$, $P = 0.5901$).

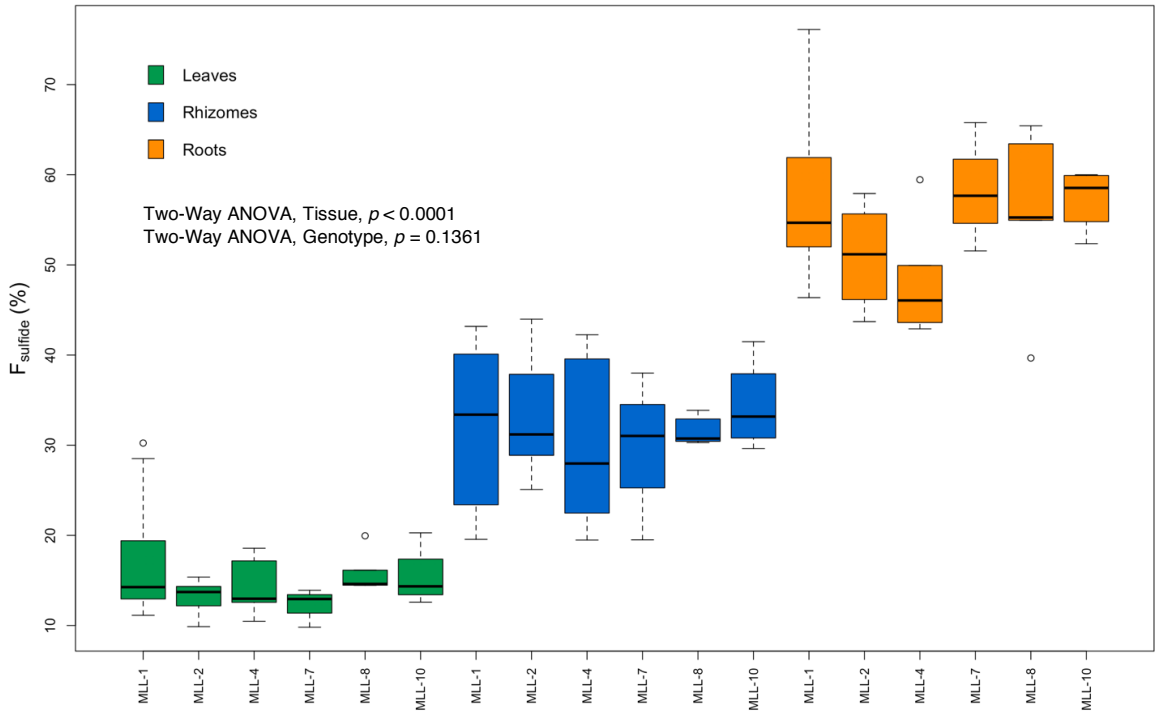

Figure 12. Boxplot representing the variation in mean $F_{sulfide}$ among unique genotypes (MLLs) for each *H. wrightii* tissue. Individual dots represent outliers, as defined by any number larger than 3rd Quantile (Q_3) + 1.5*IQR* or smaller than 1st Quantile (Q_1) - 1.5*IQR*. Lines outside the box (whiskers) extend to the smallest and largest non-outliers. Only genotypes (MLLs) with more than 3 replicates were used in the analysis.

6.2.5. Spatial Distribution of Sulfide Intrusion

The spatial distribution of sulfide in the Oso Bay meadow was estimated using the position and $F_{sulfide}$ value for each sample. This analysis was done to determine whether any region of the bed had unusually high or low values that may have skewed the results. We assigned each sample tissue to a different level of sulfide intrusion (*low, moderate, high*) based on its $F_{sulfide}$ value (Figure 13). These levels were established by using the mean $F_{sulfide}$ value for each tissue and its corresponding standard deviation (SD). Low values were those greater than 1 SD *below* the mean, moderate values were less than 1 SD above *or* below the mean, and high values were greater than 1 SD *above* the mean. The evenness with which these levels were distributed throughout the meadow were estimated using the same parameters (*ED,* β) as those used to assess clonal evenness. Results showed that sulfide levels did not appear to be especially high or low in any particular

region and are thus not likely to have skewed the results. The majority of levels fell within the *moderate* range and were fairly evenly distributed. The only exception may have been in leaves, where there were many more *high* values than *low*.

Unique Genotypes (*as reference*)

8	4	1	2	7	1	8	2	2	3	6	1
8	1	10	1	10	8	1	1	7	9	2	1
4	1	8	2	10	2	1	4	1	1	7	4
1	1	4	10	2	3	1	5	4	1	9	2

$F_{sulfide}$ in Leaves

Low (L): $F_{sulfide} < 11.31\%$; **Moderate (M):** $11.31\% \leq F_{sulfide} \leq 19.72\%$; **High (H):** $F_{sulfide} > 19.72\%$

H	M	M	M	L	M	M	M	M	M	M	M
M	M	M		M	M	H	M	M	H	M	M
L	M	M	M	H	L	M	M	H	M	M	M
H	H	M	M	M	H	L	H	M	M	M	M

E_D = 0.600

β = 1.123

$F_{sulfide}$ in Rhizomes

Low (L): $F_{sulfide} < 24.77\%$; **Moderate (M):** $24.77\% \leq F_{sulfide} \leq 38.67\%$; **High (H):** $F_{sulfide} > 38.67\%$

M	L	M	M	L	L	M	M	M	M	M	M
M	M	M	H	M	M	H	L	M	M	H	L
M	H	M	H	M	M	L	L	M	H	M	H
M	M	H	H	M	M	L	M	M	H	M	M

E_D = 0.809

β = 1.329

$F_{sulfide}$ in Roots

Low (L): $F_{sulfide} < 47.34\%$; **Moderate (M):** $47.34\% \leq F_{sulfide} \leq 62.71\%$; **High (H):** $F_{sulfide} > 62.71\%$

L	L	L	M	M	M	M	M	M	H	M	M
M	H	M	M	M	H	H	M	M	H	L	M
L	M	H	M	M	M	M	L	H	M	H	M
M	M	M	M	L	H	M	M	L	M	M	M

E_D = 0.750

β = 1.249

Figure 13. Sulfide intrusion distribution for *H. wrightii* population from Oso Bay, Texas, using $F_{sulfide}$ values. Colors and letters represent sulfide intrusion levels. Simpson's evenness index (E_D) and the slope of the Pareto distribution (β) are indicated next to each chart.

CHAPTER VI: DISCUSSION

Seagrasses from the Texas Coastal Bend (region in the Texas northern coast comprising the Nueces and Mission Aransas estuary systems[80] and approximately 15% of Texas seagrass cover) are experiencing seagrass bed fragmentation[5], resulting from natural and anthropogenic stressors, including H_2S intrusion from high sediment sulfide concentrations. Although seagrasses have different mechanisms for coping with such pressure (e.g., *avoidance* and *tolerance*), their response is also influenced from their level of genotypic (clonal) diversity. We investigated the extent of sulfur uptake in a *H. wrightii* seagrass population from this region and examined whether its genotypic diversity played a role in its response to sulfide stress.

7.1. Genetic Variation in *H. wrightii* from Oso Bay

First, it was found that this population is genetically diverse. When compared to other seagrasses in Texas, this population had a large number of alleles, high heterozygosity, and low inbreeding. These findings suggest that *H. wrightii* from Oso Bay has the necessary genetic material, and is thus relatively fit to face environmental change and stress. However, compared to other seagrass populations in the Coastal Bend, clonal *richness* (*R*) in Oso Bay was relatively low. This may be the result of geographical isolation. Oso Bay has only one connection (Corpus Christi Bay) with other water bodies in this region.[69] This may have reduced gene transfer with other populations, and favored clonal growth or sexual reproduction (seeds) among a limited number of genotypes as the dominant means of bed expansion. This could have implications on long-term genetic diversity if alleles become fixed due to limited parentage.

H. wrightii samples seem to be evenly distributed among the few (10) genotypes present, as suggested by high values for the Simpson's evenness index and the Pareto distribution slope. However, when looking at actual number of samples for each genotype (MLL), these findings may

seem contradictory. For example, the dominant genotype was found in 16 of the 48 samples. Another was present in 8, while two more were found in 6 and 5 samples, respectively. The remaining genotypes were found in three or fewer samples, and three were found only once. Thus, while clonal distribution was skewed towards the dominant genotype, remaining samples were more evenly distributed among the remaining nine, resulting in a fairly *even* overall clonal distribution in the population. Genotypic mapping (Figure 7) and a low Aggregation Index (A_c) suggest this seagrass population has an intermingled architecture. That is, different clones have expanded among and across each other (*guerrilla* growth)[68] without segregating or clustering in particular locations. This type of spatial distribution suggests that the clones have fully occupied the meadow for some time, with relatively weak competitive interactions.[63]

Genotypically rich populations are more resistant and resilient to, and recover more quickly from environmental change and stressors. Hughes and Stachowicz,[65] for example, showed that clonal *richness* (R) in *Z. marina,* enhances resilience to intense biomass removal and resistance to macroalgal blooms. Similarly, Massa et al.[13] found that allelic and genotypic richness combined to have a positive influence on the ability of *Z. noltii* populations to resist and recover from algal blooms. Populations with *low* clonal richness (such the *H. wrightii* population from this study) can also face environmental disturbances. However, Arnaud-Haond et al.[81] showed that mortality was lower in less genotypically-rich beds of *P. oceanica* exposed to stressors from nearby fish farms. They suggested that larger clones do better at environmentally impacted sites because of their broader distribution in space. This might allow at least a portion of the clone to occupy microsites less negatively affected by stressors, enhancing the probability of survival.

Intermingled and evenly distributed architecture of the *H. wrightii* population may also be advantageous when it comes to population survival. For example, intermingled (*guerrilla*) patterns

of clonal expansion seem to improve the opportunities for *outcrossing* (sexual reproduction between non-related individuals).[82] Outcrossing, through seed production, results in individuals with novel genotypes and can lead to a higher than expected heterozygosity, increasing the level of genetic variation of the individuals. In turn, this higher variation can help the population to recover from and reverse the effects of inbreeding depression, ensuring its long-term survival.[8] The *guerrilla* strategy also results in clonal growth that enable plants to spread quickly in horizontal space and escape adverse environmental conditions (e.g. when resources level are low or competitive stress is high) and find suitable sites for expansion.[83–85] This growth pattern might be advantageous for seagrasses exposed to environmental toxins, such as H_2S, as their detoxification capacities (particularly, *avoidance*) can be enhanced. For example, by occupying a larger area through the intermingled pattern, different clones could find and colonize areas with lower sulfide concentrations, and thus, less exposure to this environmental stressor. Also, with the intermingled expansion, a genotype that results in a phenotype with advantageous features (e.g., more or larger lacunae) could colonize larger areas where it could neutralize higher amounts of sulfides in sediments. This could potentially limit and combat the stress caused by sulfide intrusion into the genets.

7.2. Sulfide Intrusion and Sulfur Distribution in *H. wrightii* from Oso Bay

Seagrasses take up sulfur from two primary sources: sulfate (SO_4^{2-}) from seawater and/or porewater and sulfides (e.g., gaseous H_2S) from the sediment. A good portion of the laboratory work for this study focused on developing a distillation protocol that would yield enough material for isotope analysis. We developed a modified, one-step extraction of the Total Reduced Inorganic Sulfur (TRIS pool), that included both the acid-volatile (porewater H_2S, FeS) and chromium-reducible (S^o, FeS_2) sulfur. The $\delta^{34}S$ signal for sulfate from the *H. wrightii* population (21.11 ±

0.76‰) was consistent with the global oceanic value of 21.0 ± 0.25‰.[18,54] Those for sediment sulfides were also very close to the range reported in the literature.[2,7] $\delta^{34}S$ values from root, rhizome, and leaf tissue matched those reported for other seagrass species, including *H. wrightii,* confirming that our extraction, distillation, and isotope preparation protocols worked as well as those reported in the literature.

We found a gradient in $\delta^{34}S$ values from roots to leaves, suggesting that sulfide enters the roots and then passes up into the rhizome and leaf tissue. This was quantified as $F_{sulfide}$, which estimated that approximately 55%, 32%, and 15% of the sulfur in roots, rhizomes, and leaves respectively, came from sediment-derived sulfides. The range of values suggests a mixing of the sulfur pools (seawater sulfate and sedimentary sulfide) in the various tissues. Higher $F_{sulfide}$ values have been reported for other species such as *Z. marina* with 86% in roots and 68% in leaves d, *T. testudinum* with 96% in roots and 21% in leaves, and *H. ovalis* with 100% in roots and 11% in leaves. For *H. wrightii*, it seems that mixing with seawater sulfate and/or re-oxidation (*avoidance)* may prevent higher accumulations of sulfide in the different tissues. The lower $F_{sulfide}$ values for the *H. wrightii* population could be explained by the following: (1) less sulfide is accumulating in this top section of the sediment, compared to other areas. This needs to be further verified by determining the AVS and CRS pools concentrations; (2) the sulfide in the sediment is re-oxidized or precipitated to a greater extent before intruding the plants. This also needs to take into consideration the spatial and temporal variation of sulfide oxidation by abiotic (e.g., temperature) and biotic (e.g., microorganisms) agents in sediments; (3) less sulfide is intruding into *H. wrightii* compared to other species. These last two explanations depend directly on the plant's abilities to produce and leak out more oxygen; finally, (4) sulfide-derived sulfur inside the plant is mixed to a greater degree with sulfate-derived sulfur compared to other species. This is also dependent on

the plant's ability to uptake sulfate from the seawater (or porewater) and its assimilation mechanisms.

The significant linear relationships between $\delta^{34}S$ and $F_{sulfide}$ values with total sulfur content (TS) in roots and rhizomes showed that the amount of sulfur in below-ground seagrass tissues is directly dependent on sediment sulfide. The relationship was much weaker for leaves, indicating that sulfur accumulation is independent of sulfur source for this tissue. There was no significant difference in TS among tissues, suggesting that their metabolic needs, or assimilatory capacity, is similar. Overall, findings suggest that sulfides enter plants through roots, and are then assimilated into non-toxic forms (e.g. sulfate or organic thiols) that can travel to rhizomes and leaves, where they will accumulate and mix with sulfur-containing compounds derived from seawater sulfate.

Sulfur assimilation and distribution inside plants has been discussed by Hasler-Sheetal and Holmer[6] for the seagrass *Z. marina.* They found that intruding sulfides were assimilated into different sulfur compounds such as elemental sulfur (S^0), sulfate, organic sulfur, and thiols in below-ground tissues. S^o appears to precipitate on the cell walls of below-ground tissues, while much of the sulfate is either transported to other tissues or stored in the vacuoles. Thiols (e.g., cysteine and glutathione) appear to have been further metabolized into organic sulfur or oxidized to sulfate, and then translocated to the different tissues to satisfy the metabolic needs of the plants. Further research is required to identify the specific fate of the intruding sulfides in *H. wrightii*. Even though these findings established that sulfide intrusion occurs through below-ground tissues and that there is even a possible sulfate (from seawater) gradient from leaves to roots, the particular distribution of assimilation products needs to be elucidated (i.e. what are the various forms of sulfur that can be taken up and/or stored by the tissues).

7.3. Genotypic Variation and Sulfide Intrusion

Another goal of this project was to determine whether there were genetic differences in sulfide uptake and distribution. Particularly, whether lower or higher $F_{sulfide}$ values were associated with particular genotypes. In this study, however, no significant difference in mean $F_{sulfide}$ values was found among genotypes, provided that the sample size was quite small (only 6 genotypes contained enough samples for statistical analyses). Variation in $F_{sulfide}$ among root tissue genotypes, however, appeared possible. MLL No. 4, in particular, had a very low $F_{sulfide}$ value compared to the others. Statistically, this difference is not significant but the probability of this difference being unusual is marginally significant at the alpha level = 0.1 ($P = 0.1361$). Given that variation in sulfur content (TS) among tissues was non-significant, there may be additional factors or mechanisms that lowered accumulation of H_2S in this genotype. For example, this genotype may do a better job of releasing oxygen from the roots to the sediment, favoring reoxidation of sulfides and reducing their intrusion into the plant.

7.4. Research Improvement and Expansion

Additional research should address the accuracy of the sediment distillation protocol and include additional genotypes to explore the relationship between genetic variation and sulfide accumulation. Despite having $\delta^{34}S$ values for sediment sulfides that match the reported ranges, it is still necessary to investigate whether isotopic fractionation occurs during the distillation.

There is no specific information on possible isotope fractionation during sediment distillation. The available protocols only discuss replication of results and general recoveries. For example, Fossing and Jørgensen[52] reported that the single-step distillation procedure (on which the protocol of this study was based) is simpler and also more accurate. They found that more than 95% of the H_2S released is recovered after the first 20 minutes of the distillation.[52] Backlund et al.[71] suggested that H_2S is not lost from individual distillation steps,[71] making the procedure

reliable and efficient. However, there is no discussion of fractionation. Determination of sulfur concentrations in sediment (AVS and CRS pools) and seawater (SO_4^{2-}) samples could also determine whether all of the sulfur is being distilled and recovered as Ag_2S. It is important to also consider the net turnover of sulfide in terms of *H. Wrightii* oxidation, abiotic oxidation and biotic oxidation. To fully understand sulfide stress on seagrasses it is necessary to address spatial and temporal variation in the relative contribution of these oxidation factors. This should include as well the role of porewater sulfate and the extent of reoxidation of sulfates in the rhizosphere.

Investigation of additional *H. wrightii* populations, from Oso Bay or similar locations, should help to clarify whether unusual samples in this population (for example, very low $\delta^{34}S$ values or high TS content in leaves) are valid or due to sampling errors. Also, a greater number of MLLs with multiple replicates could provide greater statistical power to test the relationship between sulfide uptake/distribution and genotype. Additional research should also be performed to explore the mechanisms of sulfur assimilation and metabolism in this seagrass species.

In summary, this study found that a population of *H. wrightii* population from Oso Bay, Texas is dominated by few clones with a high level of genetic variation. Stable sulfur isotope indicators showed a gradient in sediment-derived sulfur from roots to leaves, demonstrating that *H. wrightii* has the capacity to obtain sulfur from both sedimentary H_2S and seawater sulfate. Sedimentary sulfide is likely assimilated into non-toxic compounds, which are then distributed throughout the plant. While there were no significant differences in sulfide-derived sulfur among genotypes, the sample size may have been too small to detect an effect. Information gathered in this thesis shall serve as the basis for further investigation into the genetics of sulfur metabolism in seagrasses, and their resistance to environmental stressors.

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LIST OF APPENDICES

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APPENDIX I – Allele scores for *Halodule wrightii* samples from Oso Bay, Texas.

Sample No.	Microsatellite Loci															
	180		190		196		212		214		222		228		232	
HW.11B.01	235	244	135	137	178	178	298	298	210	214	219	219	278	278	265	269
HW.11B.02	232	244	131	135	178	187	280	298	212	224	179	223	272	278	269	269
HW.11B.03	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.04	232	244	125	131	178	187	280	289	212	230	223	235	272	278	265	269
HW.11B.05	232	247	131	135	178	187	280	298	210	218	231	239	269	278	263	269
HW.11B.06	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.07	235	244	135	137	178	178	298	298	210	214	219	219	278	278	265	269
HW.11B.08	232	244	125	131	178	187	280	289	212	230	223	235	272	278	265	269
HW.11B.09	232	244	125	131	178	187	280	289	212	230	223	235	272	278	265	269
HW.11B.10	232	244	131	133	178	187	295	298	212	212	215	235	269	269	263	265
HW.11B.11	232	244	131	137	178	187	295	298	212	218	231	263	269	278	269	269
HW.11B.12	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.13	235	244	135	137	178	178	298	298	210	214	219	219	278	278	265	269
HW.11B.14	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.15	247	250	135	137	178	178	298	298	210	224	211	287	278	278	269	269
HW.11B.16	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.17	247	250	135	137	178	178	298	298	210	224	211	287	278	278	269	269
HW.11B.18	235	244	135	137	178	178	298	298	210	214	219	219	278	278	265	269
HW.11B.19	232	235	131	139	178	187	295	298	214	224	219	223	269	278	265	265
HW.11B.20	232	235	131	139	178	187	295	298	214	224	219	223	269	278	265	265
HW.11B.21	232	247	131	135	178	187	280	298	210	218	231	239	269	278	263	269
HW.11B.22	244	244	135	135	178	178	289	298	214	222	223	227	278	278	269	269
HW.11B.23	232	244	125	131	178	187	280	289	212	230	223	235	272	278	265	269
HW.11B.24	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.25	232	244	131	135	178	187	280	298	212	224	179	223	272	278	269	269
HW.11B.26	232	235	131	139	178	187	295	298	214	224	219	223	269	278	265	265
HW.11B.27	235	244	135	137	178	178	298	298	210	214	219	219	278	278	265	269
HW.11B.28	232	244	125	131	178	178	280	289	212	230	223	235	272	278	265	269
HW.11B.29	247	250	135	137	178	178	298	298	210	224	211	287	278	278	269	269
HW.11B.30	232	244	125	131	178	187	280	289	212	230	223	235	272	278	265	269

HW.11B.31	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.32	232	244	131	135	178	187	280	298	212	224	179	219	272	278	269	269
HW.11B.33	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.34	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.35	232	247	131	135	178	187	280	298	210	218	231	239	269	278	263	269
HW.11B.36	232	244	131	135	178	187	280	298	212	224	179	223	272	278	269	269
HW.11B.37	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.38	232	235	131	139	178	187	295	298	214	224	219	223	269	278	265	265
HW.11B.39	232	244	131	135	178	187	280	298	212	224	179	223	272	278	269	269
HW.11B.40	247	250	135	137	178	178	298	298	210	224	211	287	278	278	269	269
HW.11B.41	232	244	125	131	178	187	280	289	212	230	223	235	272	278	265	269
HW.11B.42	232	244	131	133	178	187	295	298	212	212	215	235	269	269	263	265
HW.11B.43	232	235	131	139	178	187	295	298	214	224	219	223	269	278	265	265
HW.11B.44	232	244	131	137	178	187	283	295	212	218	235	243	269	278	269	269
HW.11B.45	232	244	131	135	178	187	280	298	212	224	179	223	272	278	269	269
HW.11B.46	232	235	131	139	178	187	295	298	214	224	219	219	269	278	265	265
HW.11B.47	244	244	135	135	178	178	289	298	214	222	219	227	278	278	269	269
HW.11B.48	232	244	125	131	178	178	280	289	212	230	223	235	272	278	265	269

APPENDIX II – $\delta^{34}S$, $F_{Sulfide}$, and TS values for *Halodule wrightii* samples from Oso Bay, Texas.

Sample No.	Tissue	$\delta^{34}S$ (‰, VCDT)	TS (%dw)	$F_{Sulfide}$ (%)	Genotype
HW.11B.01	Leaf	11.43	0.395	19.95	MLL-8
HW.11B.02	Leaf	15.01	0.379	12.57	MLL-4
HW.11B.03	Leaf	15.15	0.560	12.28	MLL-1
HW.11B.04	Leaf	13.65	0.570	15.37	MLL-2
HW.11B.05	Leaf	16.35	0.643	9.81	MLL-7
HW.11B.06	Leaf	14.74	0.415	13.13	MLL-1
HW.11B.07	Leaf	13.28	0.631	16.14	MLL-8
HW.11B.08	Leaf	14.39	0.452	13.85	MLL-2
HW.11B.09	Leaf	14.17	0.392	14.30	MLL-2
HW.11B.10	Leaf	13.01	0.399	16.69	MLL-3
HW.11B.11	Leaf	14.94	0.507	12.71	MLL-6
HW.11B.12	Leaf	14.34	0.422	13.95	MLL-1
HW.11B.13	Leaf	14.09	0.365	14.47	MLL-8
HW.11B.14	Leaf	11.87	0.177	19.05	MLL-1
HW.11B.15	Leaf	15.00	0.391	12.59	MLL-10
HW.11B.16	Leaf	-2.91	0.959	49.53	MLL-1
HW.11B.17	Leaf	14.10	0.583	14.45	MLL-10
HW.11B.18	Leaf	14.10	0.474	14.45	MLL-8
HW.11B.19	Leaf	11.10	0.601	20.63	MLL-1
HW.11B.20	Leaf	14.53	0.525	13.56	MLL-1
HW.11B.21	Leaf	14.83	0.575	12.94	MLL-7
HW.11B.22	Leaf	11.39	0.426	20.04	MLL-9
HW.11B.23	Leaf	14.52	0.598	13.58	MLL-2
HW.11B.24	Leaf	14.19	0.419	14.26	MLL-1
HW.11B.25	Leaf	16.03	0.639	10.47	MLL-4
HW.11B.26	Leaf	13.16	0.774	16.39	MLL-1
HW.11B.27	Leaf	14.02	0.556	14.61	MLL-8
HW.11B.28	Leaf	14.14	0.637	14.36	MLL-2
HW.11B.29	Leaf	11.27	0.546	20.28	MLL-10
HW.11B.30	Leaf	16.32	0.609	9.87	MLL-2

HW.11B.31	Leaf	14.92	0.591	12.76	MLL-1
HW.11B.32	Leaf	12.78	0.497	17.17	MLL-4
HW.11B.33	Leaf	6.44	0.568	30.24	MLL-1
HW.11B.34	Leaf	15.04	0.709	12.51	MLL-1
HW.11B.35	Leaf	14.36	0.675	13.91	MLL-7
HW.11B.36	Leaf	14.79	0.508	13.02	MLL-4
HW.11B.37	Leaf	11.53	0.593	19.75	MLL-1
HW.11B.38	Leaf	7.28	0.780	28.51	MLL-1
HW.11B.39	Leaf	12.10	0.528	18.57	MLL-4
HW.11B.40	Leaf	14.20	0.612	14.24	MLL-10
HW.11B.41	Leaf	15.18	0.543	12.22	MLL-2
HW.11B.42	Leaf	10.92	0.572	21.01	MLL-3
HW.11B.43	Leaf	15.71	0.699	11.13	MLL-1
HW.11B.44	Leaf	11.14	0.304	20.55	MLL-5
HW.11B.45	Leaf	14.84	0.630	12.92	MLL-4
HW.11B.46	Leaf	12.83	0.735	17.07	MLL-1
HW.11B.47	Leaf	14.04	0.582	14.57	MLL-9
HW.11B.48	Leaf	15.22	0.638	12.14	MLL-2
HW.11B.01	Rhizome	4.68	0.502	33.87	MLL-8
HW.11B.02	Rhizome	10.21	0.373	22.47	MLL-4
HW.11B.03	Rhizome	7.49	0.606	28.08	MLL-1
HW.11B.04	Rhizome	5.87	0.665	31.42	MLL-2
HW.11B.05	Rhizome	11.65	0.530	19.50	MLL-7
HW.11B.06	Rhizome	10.59	0.441	21.69	MLL-1
HW.11B.07	Rhizome	6.20	0.211	30.74	MLL-8
HW.11B.08	Rhizome	6.09	0.423	30.97	MLL-2
HW.11B.09	Rhizome	5.07	0.311	33.07	MLL-2
HW.11B.10	Rhizome	4.18	0.511	34.91	MLL-3
HW.11B.11	Rhizome	8.99	0.237	24.99	MLL-6
HW.11B.12	Rhizome	6.93	0.196	29.23	MLL-1
HW.11B.13	Rhizome	6.42	0.138	30.29	MLL-8
HW.11B.14	Rhizome	5.00	0.155	33.21	MLL-1
HW.11B.15	Rhizome	6.74	0.188	29.63	MLL-10
HW.11B.16	Rhizome	0.46	0.347	42.58	MLL-1

HW.11B.17	Rhizome	5.60	0.378	31.98	MLL-10
HW.11B.18	Rhizome	6.35	0.522	30.43	MLL-8
HW.11B.19	Rhizome	0.17	0.583	43.18	MLL-1
HW.11B.20	Rhizome	9.67	0.426	23.58	MLL-1
HW.11B.21	Rhizome	6.06	0.497	31.03	MLL-7
HW.11B.22	Rhizome	3.09	0.485	37.15	MLL-9
HW.11B.23	Rhizome	0.42	0.501	42.66	MLL-2
HW.11B.24	Rhizome	10.85	0.448	21.15	MLL-1
HW.11B.25	Rhizome	7.55	0.421	27.96	MLL-4
HW.11B.26	Rhizome	1.73	0.743	39.96	MLL-1
HW.11B.27	Rhizome	5.15	0.725	32.91	MLL-8
HW.11B.28	Rhizome	-0.22	0.986	43.98	MLL-2
HW.11B.29	Rhizome	4.44	0.600	34.37	MLL-10
HW.11B.30	Rhizome	8.94	0.569	25.09	MLL-2
HW.11B.31	Rhizome	11.62	0.402	19.56	MLL-1
HW.11B.32	Rhizome	11.66	0.472	19.48	MLL-4
HW.11B.33	Rhizome	4.51	0.642	34.22	MLL-1
HW.11B.34	Rhizome	1.59	0.514	40.25	MLL-1
HW.11B.35	Rhizome	2.68	0.641	38.00	MLL-7
HW.11B.36	Rhizome	0.62	0.583	42.25	MLL-4
HW.11B.37	Rhizome	4.83	0.765	33.57	MLL-1
HW.11B.38	Rhizome	4.50	0.603	34.25	MLL-1
HW.11B.39	Rhizome	1.92	0.728	39.57	MLL-4
HW.11B.40	Rhizome	0.99	0.651	41.48	MLL-10
HW.11B.41	Rhizome	7.24	0.436	28.59	MLL-2
HW.11B.42	Rhizome	3.50	0.593	36.31	MLL-3
HW.11B.43	Rhizome	9.85	0.462	23.21	MLL-1
HW.11B.44	Rhizome	8.42	0.250	26.16	MLL-5
HW.11B.45	Rhizome	7.54	0.423	27.98	MLL-4
HW.11B.46	Rhizome	1.09	0.702	41.28	MLL-1
HW.11B.47	Rhizome	8.88	0.704	25.21	MLL-9
HW.11B.48	Rhizome	6.96	0.380	29.17	MLL-2
HW.11B.01	Root	1.87	0.073	39.67	MLL-8
HW.11B.02	Root	-0.04	0.563	43.61	MLL-4

HW.11B.03	Root	-1.38	0.728	46.37	MLL-1
HW.11B.04	Root	-5.41	0.655	54.68	MLL-2
HW.11B.05	Root	-3.89	0.592	51.55	MLL-7
HW.11B.06	Root	-3.64	0.242	51.03	MLL-1
HW.11B.07	Root	-5.69	0.186	55.26	MLL-8
HW.11B.08	Root	-2.31	0.178	48.29	MLL-2
HW.11B.09	Root	-6.36	0.172	56.64	MLL-2
HW.11B.10	Root	-10.55	0.516	65.28	MLL-3
HW.11B.11	Root	-6.39	0.581	56.70	MLL-6
HW.11B.12	Root	-5.12	0.327	54.09	MLL-1
HW.11B.13	Root	-5.54	0.345	54.95	MLL-8
HW.11B.14	Root	-10.65	0.437	65.49	MLL-1
HW.11B.15	Root	-7.91	0.591	59.84	MLL-10
HW.11B.16	Root	-8.92	1.029	61.92	MLL-1
HW.11B.17	Root	-4.28	0.361	52.35	MLL-10
HW.11B.18	Root	-10.62	0.549	65.43	MLL-8
HW.11B.19	Root	-15.80	0.815	76.11	MLL-1
HW.11B.20	Root	-1.86	0.363	47.36	MLL-1
HW.11B.21	Root	-6.86	0.344	57.67	MLL-7
HW.11B.22	Root	-9.35	0.229	62.81	MLL-9
HW.11B.23	Root	-0.09	0.493	43.71	MLL-2
HW.11B.24	Root	-6.81	0.569	57.57	MLL-1
HW.11B.25	Root	0.30	0.800	42.91	MLL-4
HW.11B.26	Root	-5.84	0.934	55.57	MLL-1
HW.11B.27	Root	-9.65	0.592	63.43	MLL-8
HW.11B.28	Root	-6.98	0.957	57.92	MLL-2
HW.11B.29	Root	-6.65	0.767	57.24	MLL-10
HW.11B.30	Root	-2.78	0.456	49.26	MLL-2
HW.11B.31	Root	-5.30	0.369	54.46	MLL-1
HW.11B.32	Root	-1.25	0.511	46.10	MLL-4
HW.11B.33	Root	-10.22	0.905	64.60	MLL-1
HW.11B.34	Root	-8.91	0.639	61.90	MLL-1
HW.11B.35	Root	-10.79	0.611	65.78	MLL-7
HW.11B.36	Root	-3.11	0.739	49.94	MLL-4

HW.11B.37	Root	-4.93	0.834	53.69	MLL-1
HW.11B.38	Root	-4.58	0.555	52.97	MLL-1
HW.11B.39	Root	-7.72	0.939	59.45	MLL-4
HW.11B.40	Root	-7.99	0.565	60.00	MLL-10
HW.11B.41	Root	-0.24	0.559	44.02	MLL-2
HW.11B.42	Root	-12.26	0.882	68.81	MLL-3
HW.11B.43	Root	-2.70	0.516	49.09	MLL-1
HW.11B.44	Root	-2.66	0.385	49.01	MLL-5
HW.11B.45	Root	-1.21	0.412	46.02	MLL-4
HW.11B.46	Root	-5.51	0.595	54.89	MLL-1
HW.11B.47	Root	-4.38	0.652	52.56	MLL-9
HW.11B.48	Root	-4.64	0.334	53.10	MLL-2