ASSESSING THE INFLUENCE OF GENOTYPIC DIVERSITY ON SULFUR DYNAMICS IN THE SEAGRASS *HALODULE WRIGHTII* USING STABLE ISOTOPE ANALYSIS

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

Coastal development and other mounting anthropogenic pressures are threatening valuable seagrass habitats. The greatest risks posed to seagrasses are the effects of coastal eutrophication, which stimulates primary productivity and ultimately supplies abundant organic matter to marine sediments. The decomposition of this material is initially facilitated by aerobic microorganisms, depleting dissolved oxygen and generating anoxic conditions. Under these conditions, anaerobic microorganisms such as sulfate-reducing bacteria begin to dominate the degradation process, which reduce sulfate (SO_4^{2-}) to sulfide (H_2S) for energy production. The accumulation of H_2S in marine sediments is problematic for seagrasses, as this molecule can be highly toxic. Yet, seagrasses can withstand relatively high concentrations of H_2S in their environments. Stable isotope analyses have been used to investigate sulfide intrusion in seagrass meadows, as the unique isotopic signature of sediment-derived sulfur can be used to trace the uptake of H_2S and its distribution throughout the plant. This technique has allowed the study of factors that may influence sulfide production and intrusion, such as reduced light availability, organic matter enrichment, and high temperatures. However, few studies have examined the biological or biochemical features that enable seagrasses to withstand relatively high sedimentary sulfide levels. One biological feature that may help confer resistance is population genetic diversity, which has been identified as an important trait in the survival and performance of seagrass meadows under environmental stress. In general, genetic diversity is thought to play an important role in population resistance to environmental disturbance, as a wider assortment of functional traits encoded at the molecular level results in a variety of phenotypes likely to possess morphological and physiological differences that are complementary. This genotypic complementarity may extend to biochemical strategies associated with tolerance to, or

detoxification of, H_2S. The purpose of this study was to determine whether sulfide intrusion differs between genotypes of the seagrass *Halodule wrightii*, a prominent species in the Gulf of Mexico. Further, as the sulfur isotopic composition of marine sediments and seagrass vegetation is known to exhibit high spatial variability, this study also sought to assess sulfide intrusion between populations from distinct sites along the Texas Gulf Coast. Stable isotope data was used to infer the proportion of sulfur in *H. wrightii* tissues that was derived from sedimentary sulfide, while total sulfur (TS) data was also considered to understand the extent of sulfur accumulation within the plant. *H. wrightii* genotypes were determined by screening each sample at a series of microsatellite loci previously identified for this species. Although no difference in sulfide intrusion was observed between genotypes, sulfide uptake and distribution was significantly different between the three study sites. The results offer important insight to the effect of local conditions on sulfide intrusion in seagrass meadows and may guide future investigations concerned with the influence of genotypic diversity on H_2S metabolism in seagrasses.

DEDICATION

To the most loving and supportive parents a girl could ask for, Kerrie and Mark Girard. Mom and Dad, thank you for your unconditional belief in me and endless encouragement. Thank you for rooting for me from afar while always being just a phone call away. Thank you for everything! I love you more than you'll ever know.

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CHAPTER I: INTRODUCTION

Seagrasses, the only group of submerged, flowering plants known to inhabit the marine environment, are foundational to coastal ecosystems and perform a number of ecological services that support marine fauna and humans alike.[1] In addition to supporting a complex trophic food web, seagrasses also serve as a natural filter in coastal waters to improve water clarity and mitigate coastal erosion by stabilizing marine sediments.[2] Despite their demonstrated socioeconomic value and importance within the marine environment, seagrass habitats are declining worldwide.[3] While natural disturbances may induce reductions in seagrass coverage, this decline is largely driven by anthropogenic pressures, especially the eutrophication of coastal waters stimulated by nutrient runoff from increasingly developed coastal communities.[4]

Excessive nutrient input into coastal waters from urban, industrial, and agricultural runoff presents a steep challenge to the functioning of seagrass ecosystems. Coastal eutrophication rapidly enhances the biomass production of phytoplankton and macroalgae, increasing light attenuation in the water column that reduces photosynthetic activity. The organic material synthesized via eutrophication is eventually supplied to the underlying sediment, where it is readily oxidized by microbial respiration. Aerobic microbial respiration is the dominant oxidative process when oxygen is available; however, where organic matter deposition is high, the degradation process quickly consumes available oxygen in the sediment and subsequently shifts towards anaerobic mineralization pathways.[5] Among the various mechanisms of anaerobic microbial respiration, sulfate reduction is by far the most important of these strategies in marine sediments.

Sulfate (SO_4^{2-}) is the second most abundant anion dissolved in seawater, explaining the dominance of sulfate reduction as a pathway of organic matter mineralization despite being less

thermodynamically favorable compared to other electron acceptors such as nitrate, iron, and manganese.[6] Sulfate-reducing bacteria reduce SO_4^{2-} to hydrogen sulfide (H_2S, aka "sulfide") for energy production, and where anoxic conditions persist, H_2S is allowed to accumulate.[5] Sulfide is a demonstrated phytotoxin, interfering with metalloenzymes necessary for energy metabolism.[7] However, seagrasses have been widely demonstrated to tolerate H_2S in their environments to an extent, and are presumably adapted to these conditions given the likelihood of anoxia in marine sediments.[8] Still, resistance to sulfide toxicity is not fully understood.

To better understand the mechanisms allowing seagrasses to withstand high sulfide concentrations, stable sulfur isotope analyses using isotope ratio mass spectrometry (IRMS) can be implemented. This analytical technique is used to determine the isotopic composition of a sample based on the ratio of two stable isotopes within it. This value is then compared to the same ratio in a standard reference material, and the ratio of these two proportions is multiplied by 1000 to determine the sample's isotopic signature (denoted as $\delta^{34}S$). For sulfur analyses, the sulfur isotope ratio generally determined is $^{34}S/^{32}S$. The two sulfur sources available to seagrasses (SO_4^{2-} and H_2S) exhibit a large difference in $\delta^{34}s$ values. Due to the preferential uptake of SO_4^{2-} containing the lighter ^{32}S isotope during bacterial reduction, the H_2S that accumulates in the sediment has a distinctly negative $\delta^{34}S$ value between -15 to -25‰, as compared to the typical $\delta^{34}S$ value of SO_4^{2-} of approximately 20‰.[8] The level of fractionation observed in sedimentary H_2S is largely influenced by the rate of sulfate reduction, and these rates exhibit large spatial and temporal differences based on environmental factors such as temperature, organic matter contents, and sediment characteristics including oxygen and iron availability. By obtaining $\delta^{34}s$ values for seagrass tissues, the proportion of sulfur derived from each source can be estimated using a simple mixing model. This allows for the uptake and

distribution (intrusion) of sediment-derived sulfide to be traced in the plant. When paired with total sulfur (TS) data, it can provide some insight into the fate of H_2S following its intrusion into the plant.

Stable isotope analysis has been used to characterize the tissue sulfur isotopic composition of a number of seagrass species from locations around the world.[8] Further, this technique has allowed the study of factors that may influence sulfide production and intrusion, such as reduced light availability, organic matter enrichment, and high temperatures.[9-12] Yet, few studies have examined the biological or biochemical features that enable seagrasses to withstand relatively high sedimentary sulfide levels. One biological feature that may help confer resistance is population genetic diversity, which has been identified as an important trait in the survival and performance of seagrass meadows under environmental stress. In particular, populations with a large number of unique genotypes (high genotypic diversity), exhibit improved tolerance to grazing pressure and shading stress relative to less diverse ones.[13] Genotypically diverse seagrass meadows have also been shown to exhibit relatively high resilience following exposure to high temperatures, as demonstrated by lower loss of biomass and shorter periods of recovery compared to less diverse ones.[14]

In general, genetic diversity is thought to play an important role in population resistance to environmental disturbance, as a wider assortment of functional traits encoded at the molecular level results in a variety of phenotypes likely to possess morphological and physiological differences that are complementary. This genotypic complementarity may extend to biochemical strategies associated with tolerance to, or detoxification of, H_2S. The data presented in this study is one of the first to characterize genotypic differences in sulfide intrusion.

The purpose of this study was to determine whether sulfide intrusion differs between genotypes of the seagrass *H. wrightii*, or between populations from distinct sites along the Texas Coastal Bend. Clonal size (large vs. small genotypes) was also examined to determine whether this factor influenced intrusion. It was hypothesized that *H. wrightii* genotypes would differ in the proportion of sedimentary sulfide-derived sulfur in their tissues (root, rhizome, and leaf), and that intrusion in smaller genotypes would differ from larger ones. Further, it was expected that the unique characteristics of each study site would result in markedly different patterns of sulfide intrusion. The results could have implications for conservation and remediation efforts, where genetic variation is rarely taken into consideration. Additionally, they will help guide future studies investigating sulfide intrusion in seagrass meadows, while contributing to the growing body of knowledge concerned with the fate and metabolism of H_2S in seagrasses and the influence of genotypic diversity and clonal size on environmental stress resistance.

CHAPTER II: SEAGRASS BIOLOGY, ECOLOGY, AND DECLINE

2.1 Seagrass biology and physiology

Seagrasses are angiosperms (i.e., seed-producing plants), the only plant of this kind to inhabit marine environments. They are vascular plants, with specialized aerenchyma tissue interspersed with lacunae that facilitate the internal and external diffusion of gases such as CO_2 and O_2.[15] "Above-ground" seagrass tissues (i.e., leaves) are specialized for photosynthesis, with high concentrations of chloroplasts in leaf epidermal cells.[16] "Below-ground" tissues (i.e., root and rhizome) allow the plants to remain anchored in marine sediments and provide mechanical support. Additionally, roots perform nutrient uptake to support plant growth and survival and allow oxygen diffusion into the sediment to promote favorable growing conditions in the rhizosphere. Rhizomes (horizontal stems) offer a mechanism for vegetative growth by extending through sediments and supporting the production of new *ramets* (i.e., individual rhizome segments bearing leaf and root tissue, connected by nodes). A ramet represents the fundamental unit of a clonal plant, capable of surviving independently if separated from the rest of the clone.

The production of new ramets via rhizome extension describes vegetative (asexual) reproduction. This method is the dominant reproductive strategy of seagrasses, and the resulting ramets are genetically identical.[16] Seagrasses also reproduce sexually through pollination and seed production, which can generate genetic diversity within populations.[17] As this group of plants originated on land and eventually colonized the marine environment, their reproductive organs resemble those of terrestrial plants, with certain adaptations allowing for pollination and fertilization whilst submerged in the water (i.e., hydrophilous pollination). Seed dispersal is accomplished through biotic means, such as the ingestion and subsequent release of seeds by

grazing fauna, or abiotic means, such as wind, currents, and human interactions with seagrass habitats.[18]

2.2 Seagrass taxonomy and distribution

Seagrass meadows are globally distributed and form the foundation of coastal ecosystems in many parts of the world. Existing literature indicates that seagrasses occur in at least 191 countries with tropical or temperate coastlines.[19] Seagrass habitats are confined to shallow coastal environments, as they must be adequately exposed to light to perform photosynthesis. Light availability is generally the most important factor regulating seagrass distribution. Besides increasing depth of the seafloor, light attenuation may also be increased by high concentrations of dissolved organic matter, the presence of phytoplankton and leaf surface epiphytes, and sediment resuspension following intense storms or high energy wave activity.[20] Estimates of global seagrass coverage vary considerably due to inconsistent mapping efforts among the regions where they occur. However, it has been proposed that an area of approximately 4,320,000 km^2 could theoretically be colonized by seagrasses, while a recent conservative estimate based on published data suggested an area of 160,000 to 270,000 km^2 is currently occupied.[19]

In regards to taxonomy, seagrasses belong to four distinct families including Zosteraceae, Cymodoceaceae, Posidoniaceae, and Hydrocharitaceae. These four families encompass 12 genera, and approximately 60 species within these families are widely accepted as true seagrasses.[21] However, the inclusion of some species from the families Ruppiaceae and Zannichelliaceae as seagrasses would incorporate an additional two genera into this ecological group, bringing the total number to 72.[1]

Seagrass meadows are typically dominated by a single species as a result of the specific conditions each species has adapted to, which may relate to light availability, temperature, sediment conditions, nutrient availability, salinity, or biological interactions. While some seagrass genera are endemic to certain regions of the world, others such as *Zostera* and *Halophila* are widely distributed, with species occurring in both temperate and tropical seas across the globe.[22] The distribution of seagrass species is dependent on an interplay between anatomical and physiological features and environmental conditions. For example, seagrasses belonging to the genus *Phyllospadix* have evolved thicker and more fibrous below-ground tissues which allow them to inhabit relatively rocky substrates.[23] Similarly, adaptations in *Halophila decipiens* allow it to colonize the seafloor to depths over 40 m, and thrive in environments with levels of light attenuation, bioturbation, and herbivory that would exclude most other species.[24]

2.3 Ecological services

Seagrasses perform a multitude of ecological services from which other marine fauna benefit. Seagrass meadows host a diverse assemblage of benthic macrofauna that render them ideal foraging grounds for a wide variety of fish species and larger organisms such as sea turtles, dolphins, sharks, rays, otters and seals. Seagrass leaves are also a direct source of nutrition for sea turtles, manatees and dugongs.[2] Additionally, seagrass vegetation behaves as a filter for suspended particles and nutrients in the water. Organic and inorganic suspended solids may be physically trapped by seagrass leaves and deposited in the sediment, and concentrations of nutrients such as nitrate and ammonium in the water column are lower in seagrass beds relative to unvegetated coastal areas.[25] The sufficient food availability, high water quality, and physical sheltering characterizing seagrass meadows makes them suitable nursery grounds and permanent habitats for many marine organisms.

Besides playing a critical role in ecosystem functioning within the marine environment, seagrasses are valued for the numerous ecological services they perform that benefit humans. Seagrass meadows support commercial fisheries that create employment opportunities and perform nutrient cycling that has been valued at $1.9 trillion per year.[3] Further, these habitats are known to prevent coastal erosion, as the expansion of below-ground seagrasses tissues contributes to the stabilization of sediments, while the morphology of above-ground tissues imparts a reduction in wave energy impacting coastal shorelines. These features also help regulate the level of marine sediment surfaces, which is becoming increasingly important with rising sea levels associated with global warming.[26]

Seagrasses are important mediators of carbon cycling and sequestration within the oceans. Seagrass meadows serve as a 'blue carbon' sink. Seagrasses can utilize carbon dioxide that diffuses into marine waters during photosynthesis, which is then effectively sequestered in below-ground tissues in marine sediments. Whereas terrestrial carbon sinks that perform this same function may release carbon back into the atmosphere within decades, blue carbon sinks can retain buried carbon for thousands of years.[27] Seagrasses also demonstrate carbon sequestration through the export of their detritus to the deep sea. Below water column depths of 1000 m, seagrass carbon is effectively withheld from atmospheric exchange processes, and may be incorporated into the deep sea refractory dissolved organic carbon (DOC) pool. This implies that the effective area of carbon burial is not limited to the area physically colonized by seagrasses, but is integrated within the biogeochemical processes of the world's oceans beyond the constraints of their established habitats.

2.4 Seagrass decline

Despite their known value, seagrass populations are in decline. Facing extreme pressure in their environments, the global coverage of seagrasses is being diminished at a rate that has been accelerating over the past several decades.[3] Further, the risk of extinction and levels of endangerment faced by various species of seagrasses were established in an assessment based on criteria set by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species. Of the 63 species for which data was sufficient to be distinguished by the IUCN criteria, 10 were determined to be Endangered or Vulnerable, while an additional 5 were assigned the status of Near Threatened.[28]

Seagrass decline may be attributed to both natural and anthropogenic causes. Seagrasses in the dynamic marine environment are naturally subjected to geological and meteorological events, as well as disruptive biological interactions.[29] Examples of geological events that can hinder seagrass colonization include earthquakes, which can result in the elevation of shorelines and subsequent exposure of seagrass meadows, or light attenuation by smoke and ash resulting from volcanic activity. Meteorological events, such as tropical storms delivering heavy rainfall and winds to coastal environments, can increase turbidity in the water column and cause physical damage to seagrass meadows. Harmful biological interactions with seagrasses include the displacement of sediment through high levels of bioturbation by benthic organisms, grazing on seagrass vegetation in excess of productivity rates, and the introduction of diseases.

The natural stressors to which seagrass meadows are subjected can generally be tolerated by these resilient species, whereas human-induced disturbances are disproportionately accelerating their decline.[3] The anthropogenic threats most pertinent to seagrass decline include activities resulting in mechanical damage of seagrass vegetation or those resulting in decreased

water quality. For example, boat propellor scarring in seagrass beds not only results in immediate physical loss of vegetation, but also renders the affected areas more susceptible to damage by high energy currents.[30] Maritime dredging is another activity that can directly damage seagrass habitats, as coastal areas cleared by excavation of seafloor materials frequently possess beds.[31] Both propellor scarring and dredging may also increase turbidity of the water column, diminishing light availability for photosynthesis.

2.5 Coastal eutrophication

The most significant threat, however, is that posed by excessive nutrient loading into coastal waters. "Point" sources of nutrient pollution such as wastewater treatment and industrial discharges directly increase nutrient abundance in coastal waters, while "non-point" sources such as urban and agricultural runoff, as well as atmospheric deposition of nitrogen oxides generated by fossil fuel combustion, are also significant contributors.[32] High concentrations of nitrogen and phosphorus from these sources have been directly implicated in coastal eutrophication.[33]

Coastal eutrophication is especially harmful to seagrasses, as it presents several challenges to seagrass physiology. As coastal waters become more eutrophic, algal blooms characterized by the rapid proliferation of phytoplankton and macroalgae occur.[34] A bloom decreases light availability in the water column, exerting direct effects on seagrass productivity. This has been demonstrated by the group of Collier et al. who noted leaf shedding, decreases in shoot density, and reduced measurements of leaf tissue dimensions and production rates in four seagrasses subjected to experimental shading stress.[35] Further, the decomposition of micro- and macroscopic algae supplies labile organic matter to sediments, stimulating microbial activity. The decomposition of this material is initially facilitated by aerobic microorganisms, and anoxic conditions are subsequently generated as the available dissolved oxygen is consumed and other

mineralization pathways – such as sulfate reduction – begin to dominate the degradation process.[36]

2.6 Sediment sulfide (H_2S) production and toxicity to seagrasses

Sulfate-reducing organisms utilize sulfate (SO_4^{2-}) as a terminal electron acceptor in their energy metabolism, reducing it to hydrogen sulfide through a series of electron transfers.[37] In general, when anoxic conditions persist in the marine sediment, this reduced product of microbial anaerobic respiration is allowed to accumulate. This presents yet another threat to seagrass health, considering the toxic effects of H_2S for living organisms that have been well-documented.[7] Early studies of H_2S as a phytotoxin demonstrated how it could limit energy production by disrupting cytochrome c oxidase activity.[38] H_2S can also bind to trace metals such as iron, calcium and magnesium within the plant and limit their availability for metalloenzymes.[39] Further, reduced productivity of root tissue as a result of H_2S toxicity can lead to decreased nutrient uptake that is insufficient to support rhizome extension and the production of new leaf tissue.[40]

H_2S toxicity has been directly implicated in several instances of seagrass decline, such as meadows growing near fish farms and other areas impacted by excessive nutrient loading.[8] Fraser and Kendrick examined whether shoot density declines in the seagrass *Posidonia sinuosa* could be correlated with the high sedimentary sulfide concentrations observed in Cockburn Sound, Western Australia, which experienced a 75% loss in seagrass cover 40-60 years before the study.[41] They concluded that, despite water quality improvements since the initial die-off, shoot density continued to be limited in the sampled seagrass meadows, presumably from high levels of sedimentary H_2S that accumulated before any improvements to water quality were made. Chronic loss of *Thalassia testinidum* beds in Florida Bay has also been associated with

high levels of sulfide accumulation in sediment porewaters.[42] Heavily affected patches marked by active die-off exhibited the highest levels of sedimentary H_2S in the Florida Bay study, suggesting that sulfide intrusion may have been related to the decline as a secondary stressor, compounding the effects of the high temperatures and prolonged drought that were also observed. The die-off itself likely contributed to the increased production of sedimentary sulfide, stimulating a cyclic process of seagrass degradation and subsequent sulfide accumulation.

Still, seagrasses demonstrate relatively high resistance to sulfide toxicity, which seems reasonable considering the near certainty of diurnal anoxic periods in marine sediments, which are exaggerated by slow diffusion rates of O_2 through the water column.[43] There is, however, a high level of variability between seagrass species regarding resistance to sulfide exposure, and ambiguity regarding what mechanisms are responsible for its detoxification.[8] Additional information is needed to understand how particular species deal with environmental H_2S, and the conditions that stimulate its production.

CHAPTER III: SEDIMENT H_2S PRODUCTION IN SEAGRASS MEADOWS

3.1 Sulfur as a plant nutrient

Sulfur is among the 17 elements considered plant nutrients, which are distinguished as elements that are essential for the completion of a plant's life cycle.[44] It is incorporated into plant tissues in relatively small proportions, comprising around 0.1% of dry plant matter as opposed to nitrogen and carbon which constitute 1.5% and 45%, respectively.[45] Sulfur is a component of the amino acids methionine and cysteine, which are among the 20 amino acids necessary for protein synthesis. Methionine is critical to the initiation of protein translation in eukaryotic cells, while cysteine contributes to tertiary and quaternary protein structure through the formation of disulfide bonds with other cysteine residues.[46] Sulfur is present in a number of coenzymes and other relevant biomolecules due to the reactivity of the sulfhydryl group (i.e., functional groups in the form R-SH) and other sulfur moieties, which facilitate important catalytic and electrochemical processes.[45]

Plants primarily source their sulfur from sulfate (SO_4^{2-}), the predominant form of sulfur on Earth. Sulfate uptake in terrestrial plants mainly occurs via root cells, and is facilitated by a diverse series of transporters, the expression of which depends on the electrochemical gradient of sulfate across cell membranes.[47] Marine aquatic plants, including seagrasses, may also obtain sulfate from the water column via leaf tissue, as this anion is abundant in seawater where leaves are submerged.[48] Sulfur metabolism in plants involves assimilatory reductive pathways which produce methionine and cysteine through a series of enzyme-catalyzed electron transfers that reduce sulfate to S^{2-}.[47]

Seagrasses and other marine macrophytes (i.e., mangroves, salt marsh plants) additionally obtain sulfur from sulfides (H_2S, HS^-, S^{2-}) trapped in anoxic sediments.[49] While the contribution

of sulfides to sulfur nutrition in terrestrial and freshwater plants is generally negligible, reduced sulfur species are significant to the sulfur metabolism of marine macrophytes due to relatively high concentrations of sulfate in the seawater and the slow diffusion of O_2 into marine sediments, which limits aerobic oxidation of organic matter in these environments.

3.2 Sulfur cycling in marine sediments

Sulfur cycling in marine sediments has been extensively reviewed[5, 50-52]; however, the sulfur cycle is highly complex, and not completely understood. It is directly coupled with the cycling of other elements such as carbon, iron, and manganese.[50] The majority of sulfur cycling in the oceans occurs in coastal regions, as the shallow waters are characterized by sufficient light availability to support marine primary production, and are subsequently dominated by organic matter degradation and sedimentation processes relative to the deep sea. These properties largely regulate the marine sulfur cycle.

Dissimilatory sulfate reduction (DSR) is the driving force of sulfur cycling in marine sediments, and ultimately assumes a major role in the global sulfur cycle, as the oceans are collectively one of the largest and most dynamic sulfur pools on Earth. This mechanism of sulfate reduction differs from the process of assimilatory sulfate reduction (ASR) described above, which yields cysteine for the biosynthesis of proteins and has a minimal role in global sulfur cycling. Dissimilatory sulfate reduction is a process limited to a relatively small proportion of microorganisms compared to ASR which is widespread in plants, prokaryotes, and fungi. Both processes are marked by a series of enzyme-catalyzed electron transfers which reduces sulfate to sulfide; however, in dissimilatory sulfate reduction, sulfate is simply being used as a terminal electron acceptor to extract energy from the oxidation of organic matter.[53] Rather than undergoing subsequent reactions for the synthesis of cysteine, the resulting H_2S produced by the

DSR pathway is released from bacterial cells and allowed to accumulate. As such, the dissimilatory process has broad implications for the sulfur cycle of marine sediments.

Marine sediments represent a major sulfur sink; as DSR pathways result in the production of hydrogen sulfide, these reduced sulfur species can be mineralized through reactions with iron and manganese, giving way to metal sulfide complexes which may be buried deep within marine sediments. Reduced sulfur in these environments is said to belong to either the acid volatile sulfide (AVS) pool or chromium reducible sulfide (CRS) pool based on common extraction methods. The AVS pool contains the sulfur species H_2S and FeS, while the CRS pool contains S^0 and FeS_2. The entirety of reduced sulfides within the sediments are collectively known as the total reduced inorganic sulfur (TRIS) pool.[5]

3.3 Isotope ratio mass spectrometry

Isotope ratio mass spectrometry (IRMS) is a widely implemented analytical technique in the field of biogeochemistry. Due to the differences in atomic mass between isotopes, fractionation occurs in natural systems. Many chemical, physical, and biological processes utilize certain isotopes preferentially, resulting in variation in isotope abundance for particular elements. Fractionation processes may be either thermodynamic or kinetic in nature. Thermodynamic fractionation is the result of different binding energies among isotopes, whereas kinetic fractionation occurs as a consequence of varying reaction speeds for different isotopes that is dependent on their atomic mass. Fractionation events can be monitored using isotope ratio mass spectrometry (IRMS), and elemental sources can be defined by isotopic signatures that reflect the level of fractionation.[54]

Isotopic signatures are obtained by comparing the ratio of two isotopes in a sample (R_{sample}) against the same ratio in a standard with an internationally accepted isotopic

composition ($R_{standard}$). Reference materials are typically chosen to be characteristic of stable materials with relatively high abundances of minor isotopes.[55] Thus, the difference between R_{sample} and $R_{standard}$ in a source can provide insight to its relative enrichment or depletion of heavy isotopes, denoted respectively by positive or negative δ-values. These values are unitless, as they represent a relative relationship and are thus compared against each other to determine differences in isotopic composition between sources. Values in the δ-notation are most typically reported in parts per thousand (per mil, ‰), as the deviations in isotopic composition are incredibly slight.[56]

IRMS has proven useful for investigating seagrass sulfur metabolism, as the dissimilatory reduction of sulfate (SO_4^{2-}) to sulfide (H_2S) is a kinetic process that results in fractionation of sulfur isotopes, and produces a prominent distinction between sulfur sources. Sulfate-reducing microbes preferentially utilize sulfate containing ^{32}S as opposed to the heavier ^{34}S isotope, resulting in relatively low $\delta^{34}S$ values for sedimentary sulfide. These values are distinctly negative compared to the $\delta^{34}S$ values for seawater sulfate.[5, 37] This fractionation of isotopes is not thought to occur to a significant extent during the uptake of sulfur-containing molecules by plants and subsequent tissue assimilation.[57, 58] These processes are therefore conducive to sulfur stable isotope studies using isotope ratio mass spectrometry (IRMS). IRMS has been widely implemented to elucidate processes by which seagrass meadows respond to gaseous H_2S as it accumulates in marine sediments.[8]

3.4 Applications of stable isotope analysis in seagrass studies

Stable isotope analysis has revealed that sediment sulfides are taken up by seagrass roots and partitioned among root, rhizome, and leaf tissue; in some instances, little to no sediment-derived sulfur is translocated to the leaves, which primarily obtain sulfur from seawater sulfate.[48]

This has been demonstrated, for example, by the group of Fredericksen et al., who showed that below-ground tissues from the seagrasses *Z. marina* and *P. oceanica* had $\delta^{34}S$ values closer to that of sediment sulfides.[59] In contrast, leaf tissues were characterized by values more similar to seawater sulfate. The same study also determined a negative correlation between $\delta^{34}S$ values and total sulfur concentrations (TS), suggesting that increased sulfide intrusion was responsible for accumulating sulfur within the plant tissues. Similar patterns of sulfide intrusion, characterized by an increasing gradient of $\delta^{34}S$ values from root to rhizome to leaf tissues have been observed in a number of species, including *Halodule wrightii*.[9, 60-62] These studies all support the hypothesis that the total sulfur content of seagrass tissues increases as reduced sulfur species accumulate in anoxic sediments.

The resistance mechanisms used by seagrass meadows under sulfide stress have also been studied using IRMS. For example, Hasler-Sheetal and Holmer utilized stable isotope analysis to elucidate possible sulfide detoxification strategies in *Zostera marina*.[63] They found that tissue accumulation of S-containing compounds such as sulfate, thiols (i.e., cysteine and glutathione), and organosulfur compounds, as well as elemental sulfur (S^0) was positively correlated with concentrations of sediment sulfide. Based on these findings, the authors identified two categories of sulfide detoxification strategies: *avoidance* and *tolerance*. Using *avoidance* strategies, seagrasses can avoid sulfide intrusion by supplying oxygen to the plant's reduced rhizosphere. This is accomplished as oxygen, produced via photosynthesis or obtained from the water column, diffuses through aerenchyma from leaves to roots and leaks into the sediment, oxidizing reduced sulfides to nontoxic sulfates. This has also been demonstrated in a study by Broderson et al. that observed this mechanism in the seagrass species *Zostera muelleri*.[64] Under internal oxygen stress, such as when photosynthesis is disrupted or completely inactive (e.g., at night)

and water column oxygen concentrations are insufficient, this protection cannot be maintained and sulfide intrusion will occur.[65] Still, seagrasses may have the capacity to withstand sulfide intrusion through *tolerance* mechanisms. These involve the internal oxidation of sulfides to sulfate and the enzymatic assimilation of sulfides into thiol-containing compounds within the plant.[63] An additional *tolerance* strategy involves the oxidation of sulfide to S^0, which precipitates within aerenchyma tissue and prevents sulfide from affecting more sensitive tissues (i.e., leaf meristem).

CHAPTER IV: SEAGRASS GENOTYPIC DIVERSITY AND STRESS RESISTANCE

4.1 Relevance and assessment of genetic diversity

Biological diversity (biodiversity) broadly describes variability in life forms across any and all levels of biological organization. The main elements of biodiversity include ecological diversity, organismal diversity, and genetic diversity.[66] Ecological diversity can be understood at the level of habitats and ecosystems, and at larger scales such as bioregions and biomes. Organismal diversity may be concerned with measurements at certain levels of the taxonomic hierarchy such as species, genera, and families. Within populations of species or subspecies, genetic diversity may be discussed in terms of the genetic composition of individuals – this can be evaluated at the level chromosomes, genes, and even nucleotides. Variation within these elements is thought to shape and support a vast array of processes upon which living organisms depend. For example, species biodiversity has been demonstrated to influence ecosystem stability, as an increased number of species offers a greater number of functional traits that can contribute to the response of the ecosystem to environmental stress.[67] This concept may also be translated to the level of genetic diversity within ecosystems. In ecosystems where species diversity is poor, genetic diversity may serve as a functional substitute.[68] This idea has gained traction in seagrass meadow studies, as these habitats are typically dominated by a single seagrass species and face tremendous pressures in their environments.[69]

A number of features may be attributed to the overall genetic diversity of a population, at both the allelic and genotypic levels. Allelic diversity is measured by the average number of alleles per locus (i.e., site within a chromosome). This figure is dependent on the number of loci under examination, as the number of alleles is ultimately averaged across the number of different loci. Diversity at the allelic level may also be imparted by heterozygosity, which describes the

presence of two different alleles at a given locus. This is in contrast to homozygosity, which is defined as the presence of two identical alleles at a given locus. Genotypic diversity is concerned with the combinations of alleles that define an individual's genotype. Genotypes are most often inferred in population studies through the use of microsatellite markers. Microsatellites can be described as short nucleotide sequences (1-6 base pairs) which are repeated multiple times at a single locus.[70] Microsatellites loci do not code for functional products, yet have characteristics which make them ideal genetic markers for assigning a genotype to an individual. Particularly, microsatellite loci demonstrate high polymorphism (i.e., presence of two or more alleles per locus) and are widely distributed throughout the genome of eukaryotic organisms.[71] These markers are also known to exhibit codominance, allowing for levels of heterozygosity to be assessed. Microsatellite markers must be developed for each new species to be studied, and primers are created that target the DNA at these loci. These primers are then used along with DNA extracted from the subject of interest in the polymerase chain reaction (PCR), allowing for amplification of the microsatellite DNA. The genotypic identity of a given individual is ultimately defined by the combination of alleles identified at a series of microsatellite loci, typically 6-10 markers.[72]

4.2 Factors influencing population genetic diversity

The genetic diversity of clonal plant populations, such as seagrasses, is influenced by relative rates of sexual vs. clonal reproduction.[73] The latter strategy appears to dominate most seagrass populations, due to the inherent difficulties associated with successful hydrophilous pollination necessary for seagrass sexual reproduction.[74] Population genetic variation is also sensitive to a number of other factors which impart changes to allele frequencies over multiple generations, including mutation and migration.[67] Mutation describes a change within the DNA

sequence of an allele. These changes are ultimately responsible for all genetic diversity. The establishment of new alleles in a species through mutation is an incredibly slow process, contributing to the genetic diversity of a population over thousands of generations. Genetic diversity can increase within populations on a much faster scale through the process of migration. Migration can be defined as the mixing of alleles from genetically distinct populations as individuals from one population move into or out of another. Migration and sexual reproduction are the main factors controlling population genetic diversity in the near term, given the prominent changes they can introduce to allelic frequencies over relatively short periods of time.

Genetic diversity has been associated with a number of measurable population variables, such as productivity and response to disturbance. This has been demonstrated by increased levels of primary production and nutrient uptake in marine phytoplankton populations, which also demonstrated high resistance to salinity stress.[75] Population genetic diversity in a keystone species may also support species diversity at higher trophic levels within an ecosystem. For example, in a field study conducted to evaluate plant-arthropod interactions, arthropod abundance was markedly higher in diverse patches of evening primrose compared to monocultures.[76] Increased arthropod species diversity has also been demonstrated in genotypically diverse plots of tall goldenrod, where plots containing 12 genotypes of the perennial plant had levels of arthropod diversity that were 27% higher than those observed within single genotype plots.[77] Tall goldenrod net primary productivity was also higher in the diverse plots.

4.3 Seagrass stress resistance mediated by genotypic diversity and clonal size

Because seagrass reproduction is primarily achieved through clonal (vegetative) growth, and individual clones are often isolated from others in their habitats, seagrass meadows are susceptible to low genetic diversity. One particular aspect of genetic diversity that is often considered in seagrass studies is genotypic *richness*, which describes the number of unique genotypes within a population. Comparing the performance of populations with varying levels of genotypic richness can provide insight into the relationship between genotypic diversity and seagrass meadow stress resistance.

Several studies have been performed to investigate the relationship between seagrass population diversity and environmental stress. For example, Hughes and Stachowicz conducted a study in which four experimental *Zostera marina* plots were established with varying genotypic diversity and grazing pressure levels.[78] It was determined that the most genotypically diverse plots demonstrated the highest resistance to grazing, as explained by a faster recovery time to pre-disturbance shoot density levels. The more diverse plots also had the highest resistance to physiological stress associated with the transplantation of ramets during the experiment, noted by decreased shoot loss in the first two weeks following the transplantation when compared to less diverse plots. Another study demonstrating the importance of genotypic diversity was conducted by Reusch et al. to assess the response and recovery of the *Zostera marina* subjected to heat stress.[79] *Zostera* plots with varying levels of genotypic diversity were established at a site exhibiting water temperatures known to inhibit the growth of this species, and even induce heat-related mortality. The highest temperatures recorded during the study resulted in decreased shoot density and other consequences of heat-related stress. When temperatures were restored to those adequate for seagrass growth, the more genotypically diverse plots recovered faster (i.e., shoot

densities returned to pre-disturbance levels more quickly), produced higher biomass, and supported greater faunal abundance.

Clonal size, defined as the number of genetically-identical ramets joined by a continuous rhizome (underground stem) connection, may also play a role in resisting environmental stress. A substantial number of studies have noted the benefits of clonal integration in plants, as reviewed by Liu et al.[80] Clonal integration allows for the translocation of water and nutrients between clones, which is an important mechanism for portions of the clone that may be subjected to local nutrient deficiencies. Further, large clones can better combat environmental stressors such as high salinity and grazing relative to individual ramets, and are marked by higher productivity than smaller portions of the clone upon disintegration. These properties have been observed, for example, in the seagrass *Cymodocea nodosa* by the group of Tuya et al.[81] In that study, two patches of *C. nodosa* were subjected to simulated nutrient stress, and one of the patches was manipulated by severing integrated clones at the rhizome tissue. They found that integrated clones exhibited higher shoot density, above-ground biomass, and tissue carbohydrate levels relative to fragmented ones. This cooperativity between integrated ramets may also influence the uptake and distribution of sulfides in seagrass meadows, and contribute similarly to sulfide detoxification.

The benefits of genotypic diversity may also extend to biochemical processes. A study by Salo et al. investigated the influence of genotypic diversity on carbohydrate metabolism in the *Zostera marina*.[82] Though their results were statistically insignificant, due to a low number of genotypes, they found that some genotypes appeared to deplete and replenish stored carbohydrate reserves to a greater extent than others. If carbohydrate mobilization can vary

between genotypes, it could follow that other biochemical processes, such as response to sulfide stress, may vary as well.

CHAPTER V: METHODOLOGY

5.1 Objectives

There is a great need to better understand how seagrasses mitigate environmental stress, such as that induced by sedimentary sulfides. Population growth is projected to increase coastal eutrophication, leading to the accumulation of toxic sulfides in seagrass habitats. Seagrasses can withstand H_2S stress to some extent using various strategies, including avoidance of sulfide intrusion by re-oxidation to sulfate in the rhizosphere, or tolerance within the plant through re-oxidation and enzymatic assimilation. However, the mechanisms underlying these strategies have not been well resolved. Previous studies have correlated genotypic diversity with improved meadow resistance to, and recovery from, environmental stress. As such, it is reasonable to hypothesize that such diversity may also influence meadow response to sedimentary H_2S.

The purpose of this study was to assess whether the accumulation of H_2S-derived sulfur and its relative distribution amongst leaf, root, and rhizome tissues varies between seagrass (*Halodule wrightii*) genotypes and determine how H_2S-derived sulfur levels in *H. wrightii* vegetation differs across locations from the Texas Coastal Bend.

A number of objectives were established to investigate sulfide intrusion in *H. wrightii* meadows along the Texas coast with regard to spatial and genotypic effects, including:

1) To characterize the sulfur isotopic *composition* of *H. wrightii* tissues from various locations on the Texas coast, as well as that of the total reduced inorganic sulfur (TRIS) pool at each respective site
2) To determine whether sulfide *intrusion* differs among genotypes of *H. wrightii*
3) To determine whether sulfide *intrusion* differs between multi-ramet genotypes compared to single-ramet genotypes

4) To determine whether sulfide *intrusion* differs between *H. wrightii* meadows along the Texas Coastal Bend.

These objectives were based on hypotheses regarding sulfide intrusion in seagrass meadows and the effects of genetic diversity on environmental stress response. In particular, we hypothesized that (i) sulfide intrusion and sulfur accumulation are traits that will differ across genotypes, as demonstrated for other environmental pressures such as heat, shading, and grazing stress, that (ii) multi-ramet genotypes will demonstrate differences in sulfide uptake and distribution compared to single-ramet ones, as clonal plants have been shown to translocate resources between ramets in response to environmental stress, and that (iii) sulfide intrusion will differ among *H. wrightii* meadows from the various sites given their spatial differences in temperature, salinity, and anthropogenic impact. These effects are likely to influence the sulfur isotopic composition of sedimentary H_2S, and consequently that of the *H. wrightii* meadows that reside within them.

5.2 Study sites

H. wrightii samples were collected from two sites within the Upper Laguna Madre (ULM-1: 27.15363 N, -97.44308 W, ULM-2: 27.59654 N, -97.29709 W), approximately 50 km apart (Figure 1). Genotypic and isotopic data from a third site within Oso Bay (OB: 27.71053 N, -97.31845 W), collected as part of a previous project, was also incorporated to investigate genotypic and location-specific differences in sulfide-derived sulfur levels.[60]

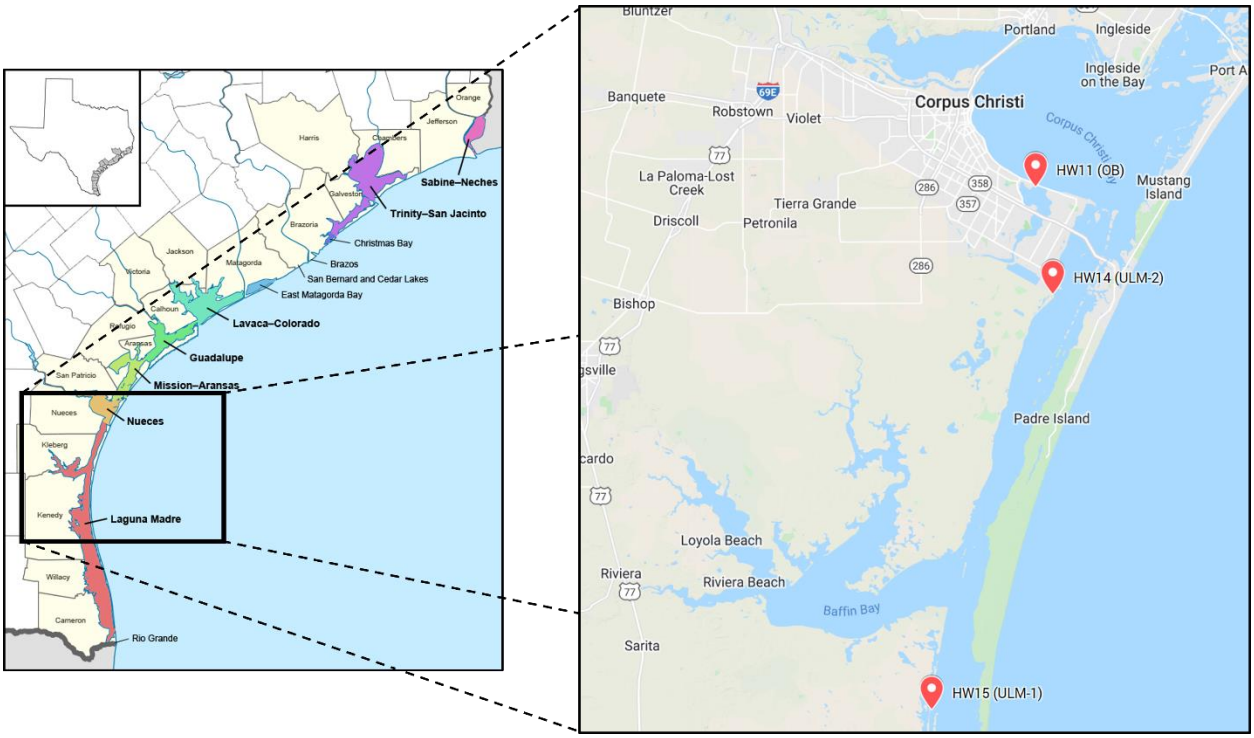

Figure 1. *H. wrightii* sampling sites (ULM-1, ULM-2, OB) from the Texas Coastal Bend.

5.3 Field sampling

5.3.1 H. wrightii tissue samples

OB samples were collected in July 2018, ULM-1 samples were collected in June 2021, and ULM-2 samples were collected in September 2019. *H. wrightii* samples (root, rhizome, and leaf tissue) were collected from a 6 x 22 m sampling grid, consisting of four parallel transects spaced 2 m apart, established in a meadow at each site. Twelve samples were collected along each transect, at intervals of 2 m, for a total of 48 samples from each site. A 10 cm x 10 cm (diameter x depth) coring device was used to extract vegetation from each sampling position. Vegetation was sieved free of sediment using a 0.5 mm mesh nylon bag and placed in a labeled Whirl-Pak® bag filled with seawater. Samples were stored on ice until returned to the laboratory for processing.

5.3.2 Sediment and seawater samples

Using a modified 30 ml syringe as a coring device, sediment samples were collected at set points (sample positions 3, 10, 18, 26, and 34) within each plot (10 cm depth) and transferred to a 50 ml centrifuge tube containing 10 ml of 1M zinc acetate to avoid oxidation of sedimentary sulfide. Five sediment samples each were collected from ULM sites and ten sediment samples were collected from OB. Approximately 500 ml of seawater was collected from above the seagrass canopy at each site using a sterile media bottle. All samples were stored on ice in the field and transported to the laboratory for further processing.

5.4 Sample processing for genetic/isotopic analyses

Vegetation from each core sample was further rinsed in the laboratory to remove any remaining sedimentary matter or detritus. For isotopic and genotypic analysis, a single rhizome fragment measuring 5-10 cm in length with sufficient intact root and leaf tissue was selected from each sample, while the remaining tissue was discarded. From the selected tissue bundle, leaf, root, and rhizome tissues were carefully separated, cleaned, and placed into separate, pre-labeled MPBio FastPrep® tubes. Tissue samples were flash-frozen in liquid nitrogen and lyophilized overnight. Dried tissues were ground to a fine powder using a MPBio FastPrep 24® ball mill and stored in a desiccator until genetic and isotopic analyses were performed. Seawater samples were filtered through a 0.2 mM ZapCap bottle top filter configured with a vacuum to remove particulate matter. The filtered seawater was then separated into five aliquots of 50 mL stored in sterile centrifuge tubes. The seawater and sediment samples were stored in a freezer until further processing for isotopic analysis.

5.5 Genetic (genotypic) analysis

DNA was extracted from ground rhizome tissue (ca. 10 mg) using the Plant DNeasy® kit (Qiagen) per the manufacturer's instructions. DNA was quantified using a Nanodrop® spectrometer. Each sample was genotyped at eight microsatellite DNA loci using a Multiplex Polymerase Chain Reaction (MPX-PCR) based assay. Each MPX-PCR assay (25 µL) consisted of 10-50 ng of DNA (5 µL of DNA extraction product), 12.5 µL of Type-it® Microsatellite Multiplex Master Mix (Qiagen), 2.5 µL of primer mix (2 µM) containing forward and reverse primers for each of the eight loci, 5 µL of RNase-free water, and 2.5 µL of Type-it® Q-Solution. PCR cycling was performed on a BioRad S1000® thermal cycler using the following program: an initial activation step of 5 minutes at 95ºC followed by 28 cycles of (i) denaturation (30 sec at 95ºC), (ii) annealing (90 sec at 54ºC) and (iii) extension (30 sec at 72ºC), with a final extension at 60ºC for 30 min. Amplification products were analyzed on an ABI 3730 XL genetic analyzer at the TAMUCC genomics facility. Microsatellite loci alleles were sized and scored using the Geneious Prime® software package and sample genotypes assigned using GenClone® (v. 2.0), a population genetic analysis software program.

5.6 Sulfur stable isotope analysis

5.6.1 Recovery of seawater sulfate

Sulfate for isotopic analyses was precipitated from seawater as barium sulfate ($BaSO_4$) according to the procedure established by Grasshoff et al.[83] To begin, seawater samples were thawed, diluted with 235 ml of deionized water, and treated with 10 ml of 1.3% picric acid solution and 5 ml of 12 M hydrochloric acid. The solution was heated to 90ºC, after which 10 ml of warm 10% barium chloride ($BaCl_2$) solution was added. The solution was kept warm and stirred for 10 minutes, allowing sulfate to precipitate as $BaSO_4$. The $BaSO_4$ precipitate was

collected by vacuum filtration using Whatman™ 42 filter paper and rinsed thoroughly with warm Milli-Q water by centrifugation to remove residual chloride. After a few rinses, the supernatant was transferred to a clean beaker and treated with $AgNO_3$ to assess whether chloride residues were present. If present, chloride ions would precipitate as AgCl upon the addition of $AgNO_3$ to the supernatant, as indicated by a shift from a clear to opalescent solution. Once sufficiently rinsed, the precipitate was transferred to a porcelain crucible and dried in an oven for 1 hour at 110°C. The precipitate was then combusted in a muffle furnace for 4 hours at 800°C to remove any residual organic matter prior to isotopic analysis. This procedure was performed on four replicate seawater samples from OB, and two samples from each of the ULM sites.

5.6.2 Recovery of sedimentary sulfides

Sediment sulfides (H_2S, HS^-, S^{2-}) were isolated as Ag_2S according to a distillation procedure adapted from work by Backlund et. al[84] and Fossing and Jorgensen.[85] In general, the distillation apparatus was comprised of four main compartments: 1) a sample chamber configured with a reflux coil condenser, situated within a fabric hemispherical mantle resting on a stir plate, 2) a buffering chamber containing 200 ml of 0.05 M potassium hydrogen phthalate (KHP) solution at pH 4.0 to neutralize free chloride ions in the system, 3) a trapping chamber containing 15 ml of 0.1 M $AgNO_3$ for collecting Ag_2S precipitate, and 4) an additional $AgNO_3$ trap in continuity with a flask containing NaOH for trapping gaseous waste. These components were joined by glass connection adapters and metal clamps. Glassware connections were further sealed using PTFE tape to prevent loss of gaseous sulfur species to the atmosphere, which could affect the isotopic composition of the distillation product.

After the distillation apparatus was configured, the four-neck round bottom flask (sample chamber) was removed and placed in a glove bag along with a sediment sample, a digital

balance, a spatula, a magnetic stir bar, and rubber septa for sealing the flask. The glove bag was sealed and filled with nitrogen gas such that the sediment could be transferred to the flask without risk of sulfide oxidation by atmospheric O_2. Approximately 10 g of sediment and the magnetic stir bar were added to the flask which was subsequently sealed. The round bottom flask containing the sample was then re-attached to the apparatus, while a flow of N_2 gas was simultaneously introduced. A temperate control unit was used to regulate the temperature of the sample chamber through the hemispherical mantle.

The sediment was treated with 10 ml of 50% ethanol, 50 ml of 6 M hydrochloric acid, and 50 ml of 1 M reduced chromium (Cr^{2+}) solution by injecting the solutions through a rubber septum into the sample chamber using a needle syringe. The solution was heated to 105°C and stirred, and the distillation was allowed to proceed for 1 hour under a continuous flow of N_2 gas. The volatile sulfides released reacted with $AgNO_3$ in the first trap described, where they were precipitated as Ag_2S. When the procedure was complete, the Ag_2S precipitate was collected via vacuum filtration using a disc of Whatman^{TM} 42 filter paper. The filter and precipitate were transferred to a porcelain crucible and lyophilized for 2 hours. This procedure was performed on ten sediment samples from OB, and five samples from each of the ULM sites.

5.6.3 Preparations for Isotope Ratio Mass Spectrometry (IRMS)

$BaSO_4$ precipitates were used to obtain average $\delta^{34}S$ value for seawater sulfate. Ag_2S precipitates were used to obtain average $\delta^{34}S$ for sedimentary H_2S. Ground and dried leaf, root, and rhizome tissue was analyzed for $\delta^{34}S$ and total sulfur (TS) values. All samples were encapsulated in 6x4 mm tin capsules at the Isotope Core Laboratory at Texas A&M University – Corpus Christi. The packaged samples were sent to the Stable Isotope Core Laboratory at Washington State University, where all isotopic and TS analyses were performed. TS and $^{34}S/^{32}S$

ratios were ultimately determined by elemental analyzer isotope ratio mass spectrometry (EA-IRMS) using an ECS 4010 CHNSO analyzer coupled to a Thermo Finnagan Delta PlusXP mass spectrometer.

5.7 Statistical analyses

Because the $\delta^{34}S$ values of the sulfur sources are likely to vary among locations, it would not be appropriate to compare tissue $\delta^{34}S$ across sites as a measure of sulfide intrusion. Rather, a simple mixing model was used to determine the proportion of sulfur within the tissues that was presumably derived from sedimentary sulfide. This parameter ($F_{sulfide}$) was calculated as follows[9]:

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

Where $\delta^{34}S_{tissue}$ represents the isotopic ratio for the particular tissue sample (root, rhizome, or leaf), $\delta^{34}S_{sulfate}$ represents the average value for the seawater from each location, and $\delta^{34}S_{sulfide}$ represents the average sedimentary sulfide value from each location. $F_{sulfide}$ values were calculated for all root, rhizome, and leaf tissue samples from all locations. Statistical analyses were performed using Rstudio. The average (mean) $F_{sulfide}$, δ^{34}s, and total sulfur (TS) values were determined for each level of tissue from each location. Spearman's rho correlation coefficients were determined to evaluate the relationship between $F_{sulfide}$ and TS for each level of tissue from each location.

Variations in mean $F_{sulfide}$ and TS among unique *H. wrightii* genotypes, and across locations, were evaluated for significance (α=0.05) using two-way analysis of variance (ANOVA) at each level of tissue. Tukey's HSD test was implemented for *post-hoc* evaluation of

significant ANOVA results, using the Westfall method for adjusting p-values to control familywise Type I error. Genotype was treated as a random factor, while location was treated as a fixed factor to determine whether sulfide intrusion in *H. wrightii* tissues differed between study sites – and if so, to what extent – after taking into account differences due to the genotypic identity of individuals. After building the mixed effects model, the variance was partitioned to determine the proportion of changes in $F_{sulfide}$ that could be explained by genotype as a random factor. Further, the effect of genotype on mean $F_{sulfide}$ was assessed for significance by creating a one-way ANOVA model using only location as a factor, then performing ANOVA between the mixed and simple models. Results from the ANOVA comparing the two models were evaluated to determine whether there was a difference in explanatory power between the mixed and simple models; that is, whether genotype could be excluded from the model as a random factor, while changes in $F_{sulfide}$ could still be sufficiently explained by location.

Certain assumptions must be met for the results of two-way ANOVA to be considered valid: the residuals (i.e., differences between observed values and those predicted by the model) for each level of any fixed factor must be normally distributed, the sample means for each level of any random factor must be normally distributed, and the data must exhibit homoscedasticity (i.e., equivalent variance in groups to be compared). For each analysis, data was assessed for normality and homoscedasticity by the Shapiro-Wilk test and Browne-Forsythe-Levene test, respectively (α=0.01).

CHAPTER VI: RESULTS

It should be noted that the genetic and isotopic data from the Oso Bay site (OB) were collected as part of a previous Master's thesis written by a graduate of our laboratory.[60] It was included with the goal of having sufficient site and genotypic data to determine whether significant differences in $\delta^{34}S$ or $F_{sulfide}$ values could be identified among locations and genotypes. The raw data was further analyzed to determine Spearman correlation coefficients and perform analysis of variance (ANOVA) in correspondence to the objectives of this particular study.

6.1 Estimates of genetic diversity

Each plant sample was assigned a multi-locus genotype (MLG) based on the collection of alleles amplified from the eight microsatellite loci. Some plant samples, however, differed slightly at one or two allelic scores. These differences are most likely the product of somatic mutations that occur within individual cells of the same vegetative clone, as compared to germ-line changes resulting from sexual reproduction that would produce distinct genetic individuals.[86] If samples varied slightly for two or less alleles compared to a similar genotype, they were assigned as members of the more common, similar genotype, and the collection of samples with slightly varying genotypes were designated as members of a common, multi-locus lineage (MLL). The common genetic identity of the members of a lineage was verified statistically by testing the hypothesis that slightly different MLGs were derived from distinct reproductive events. Significant evidence to reject this possibility ($P < 0.01$) resulted in the assignment of these given individuals to the same MLL, and the more common genotype was assigned as each member's genotypic identity.

At ULM-1 and OB, a total of 48 individual *H. wrightii* samples were assigned genotypes. However, in the processing of samples from ULM-2, the tissues of one individual were lost, and only 47 samples were assigned genotypes from this site. At ULM-1, no probable somatic mutations were identified, and the total number of MLLs was 40. At ULM-2, 10 MLGs were identified, with one MLG demonstrating a likely somatic mutation. This reduced the total number of unique genotypes to 9 based on MLL assignments. At OB, 14 MLGs were identified, and four of these demonstrated probable somatic mutations. The assignment of these MLGs to their respective MLLs resulted in 10 unique genotypes at this site.

The proportion of unique genotypes found at each site was estimated as (G-1)/(N-1), where G equals the number of unique genotypes and N equals the number of samples from a given site. This estimate is termed the genotypic *richness* (R) value. The R value was highest for ULM-1 at 0.85, while at ULM-2 and OB, the R values were 0.17 and 0.19 respectively (Table 1). At ULM-1, the mean clone size, indicating the average number of ramets assigned to one genotypic identity, was 1.2. These averages were 5.3 and 4.8 at ULM-2 and OB, respectively. Clone sizes ranged from 1 to 3 at ULM-1, 1 to 23 at ULM-2, and 1 to 16 at OB.

6.2 Sulfur isotopic composition of seawater and sediment

Silver sulfide (Ag_2S) was precipitated following the distillation of sediment samples from each site. Ag_2S represents the total reduced inorganic sulfur (TRIS) pool within the sediment, which is comprised of the various sulfide species that may be dissolved in the porewater (H_2S, HS^- and S^{2-}). At the Oso Bay site (OB), the average $\delta^{34}S$ of sedimentary sulfides was -27.38 ± 1.41‰ (n=10) (Table 1). At the Upper Laguna Madre sites, the average $\delta^{34}S$ value was -23.60 ± 0.16‰ for ULM-1 and -23.69 ± 0.21‰ for ULM-2 (n=5). Total sulfur (TS, % dry weight) values

for Ag_2S precipitates from OB, ULM-1, and ULM-2 sediments were 13.26 ± 0.78%, 12.98 ± 0.57%, and 12.92 ± 0.38%, respectively.

Barium sulfate ($BaSO_4$) was precipitated from seawater samples from each site. $BaSO_4$ represents seawater as a sulfur source, where most of the sulfur is present as dissolved sulfate. The average $\delta^{34}S$ of seawater sulfate from Oso Bay was 21.11 ± 0.76‰ (n=4). At the Upper Laguna Madre sites, average $\delta^{34}S$ values were 21.88 ± 0.08‰ for ULM-1, and 21.66 ± 0.24‰ for ULM-2 (n=2). These results were all very similar to the standard value of ca. 21‰ typically reported for seawater [9]. TS values for $BaSO_4$ precipitates from OB, ULM-1, and ULM-2 seawater were 13.92 ± 0.36%, 14.05 ± 0.12%, and 13.73 ± 1.84%, respectively.

Table 1. Genotypic characteristics, isotopic composition of sedimentary sulfides, and $F_{sulfide}$ values for tissues of *H. wrightii* from meadows along the Texas coast. With the exception of mean clone size, averages are given as sample mean (± SD).

Location	R	Mean clone size	$\delta^{34}S$ sedimentary sulfide (‰)	Root $F_{sulfide}$ (%)	Rhizome $F_{sulfide}$ (%)	Leaf $F_{sulfide}$ (%)
ULM-1	0.85	1.2	-23.60 ± 0.16	95.45 ± 6.72	63.17 ± 19.15	40.01 ± 6.64
ULM-2	0.17	5.3	-23.71 ± 0.20	88.93 ± 3.07	72.48 ± 5.08	54.45 ± 6.84
OB	0.19	4.8	-27.38 ± 1.41	55.03 ± 7.68	31.73 ± 6.95	16.23 ± 6.44

6.3 Sulfur composition of *H. wrightii* tissues

6.3.1 Tissue sulfur isotopic composition and within-site differences between tissues

The average $\delta^{34}S$ of root tissue from *H. wrightii* samples obtained from ULM-1 was -21.53 ± 3.04‰ (n=40), with values ranging from -25.25 to -12.48‰ (Figure 2). For rhizome tissue, the average $\delta^{34}S$ was -6.85 ± 8.71‰ (n=40), with values ranging from -18.46 to 10.95‰. For leaf tissue from ULM-1, the average $\delta^{34}S$ was 3.68 ± 3.02‰, with values ranging from -5.63

to 9.81‰ (n=40). The mean $\delta^{34}S$ values were significantly different between each level of tissue ($P < 0.001$ for all comparisons).

The average $\delta^{34}S$ of root tissue from *H. wrightii* samples obtained at ULM-2 was -18.69 ± 1.39‰ (n=40), with values ranging from -21.96 to -15.70‰ (Figure 2). For rhizome tissue the average $\delta^{34}S$ was -11.22 ± 2.30‰ (n=40), with values ranging from -15.10 to -4.76‰. For leaf tissue from ULM-2, the average $\delta^{34}S$ was -3.04 ± 3.10‰, with values ranging from -8.74 to 2.48‰ (n=40). The mean $\delta^{34}S$ values were significantly different between each level of tissue ($P < 0.001$ for all comparisons).

The average $\delta^{34}S$ of root tissue from *H. wrightii* samples obtained at OB was -5.56 ± 3.73‰ (n=48), with values ranging from -15.80 to 1.87‰ (Figure 2). For rhizome tissue the average $\delta^{34}S$ was 5.72 ± 3.37‰ (n=48), with values ranging from -0.22 to 11.66‰. For leaf tissue from OB, the average $\delta^{34}S$ was 13.24 ± 3.12‰, with values ranging from -2.91 to 16.35‰ (n=48). The mean $\delta^{34}S$ values were significantly different between each level of tissue ($P < 0.001$ for all comparisons).

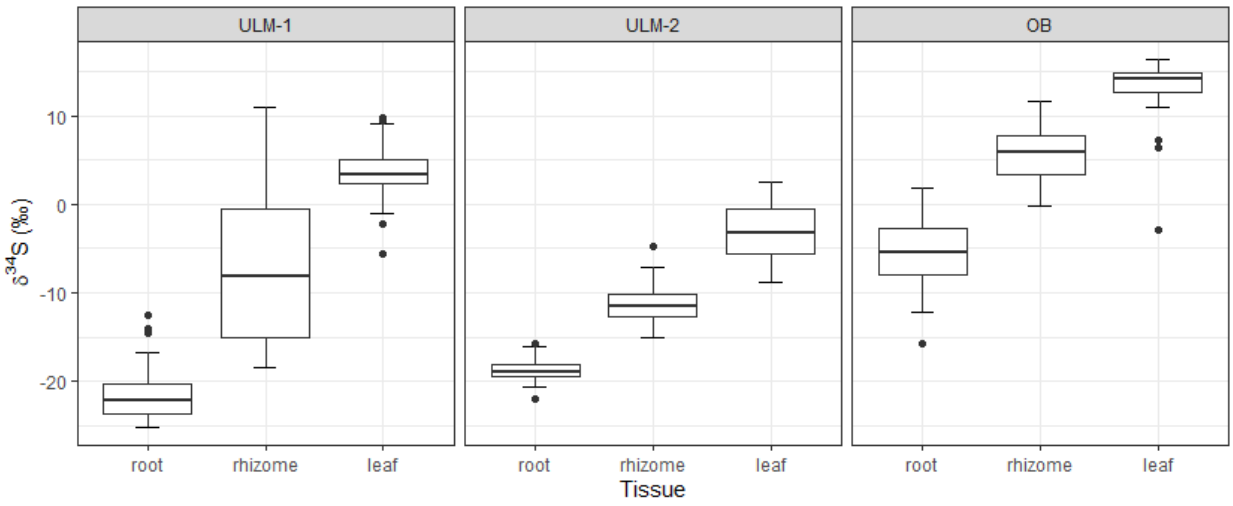

Figure 2. Distribution of $\delta^{34}S$ values for root, rhizome, and leaf tissues at each study site. Within each location, significant differences between all levels of tissue were observed. Statistical outliers are denoted by individual dots.

6.3.2 Total sulfur (TS) values and within-site differences between tissues

The root tissue samples from ULM-1 had an average TS value of 0.98 ± 0.78%, ranging from 0.18 to 4.11% (Table 2, Figure 3). The rhizome tissue samples had an average TS value of 0.80 ± 0.42%, ranging from 0.19 to 1.73%. The leaf tissue samples from ULM-1 had an average TS value of 0.40 ± 0.15%, ranging from 0.17 to 0.72%. Leaf tissues had significantly lower mean TS values than both root and rhizome tissues ($P < 0.001$), while root and rhizome tissues were not significantly different from one another ($P=0.13$) (Figure 3).

The root tissue samples from ULM-2 had an average TS value of 2.00 ± 0.74%, ranging from 0.63 to 3.54% (Table 2, Figure 3). The rhizome tissue samples had an average TS value of 0.80 ± 0.27%, ranging from 0.46 to 1.76%. The leaf tissue samples from ULM-2 had an average TS value of 0.65 ± 0.14%, ranging from 0.43 to 1.02%. Root tissues had significantly higher mean TS values as compared to rhizome and leaf tissues ($P < 0.001$), while no significant difference was observed between rhizome and leaf tissues ($P=0.14$).

The root tissue samples from OB had an average TS value of 0.55 ± 0.23%, ranging from 0.07 to 1.03% (Table 2, Figure 3). The rhizome tissue samples from OB had an average TS value of 0.49 ± 0.18%, ranging from 0.14 to 0.99%. The leaf tissue samples from OB had an average TS value of 0.55 ± 0.13%, ranging from 0.18 to 0.96%. Mean TS was not significantly different between any of the tissues at OB.

Table 2. Genotypic characteristics, isotopic composition of sedimentary sulfides, and TS values for tissues of *H. wrightii* from meadows along the Texas coast. With the exception of mean clone size, averages are given as sample mean (± SD).

Location	R	Mean clone size	$\delta^{34}S$ sedimentary sulfide (‰)	Root TS (% dw)	Rhizome TS (% dw)	Leaf TS (% dw)
ULM-1	0.85	1.2	-23.60 ± 0.16	0.98 ± 0.78	0.80 ± 0.42	0.40 ± 0.15
ULM-2	0.17	5.3	-23.71 ± 0.20	2.00 ± 0.74	0.80 ± 0.27	0.65 ± 0.14
OB	0.19	4.8	-27.38 ± 1.41	0.55 ± 0.23	0.49 ± 0.18	0.54 ± 0.12

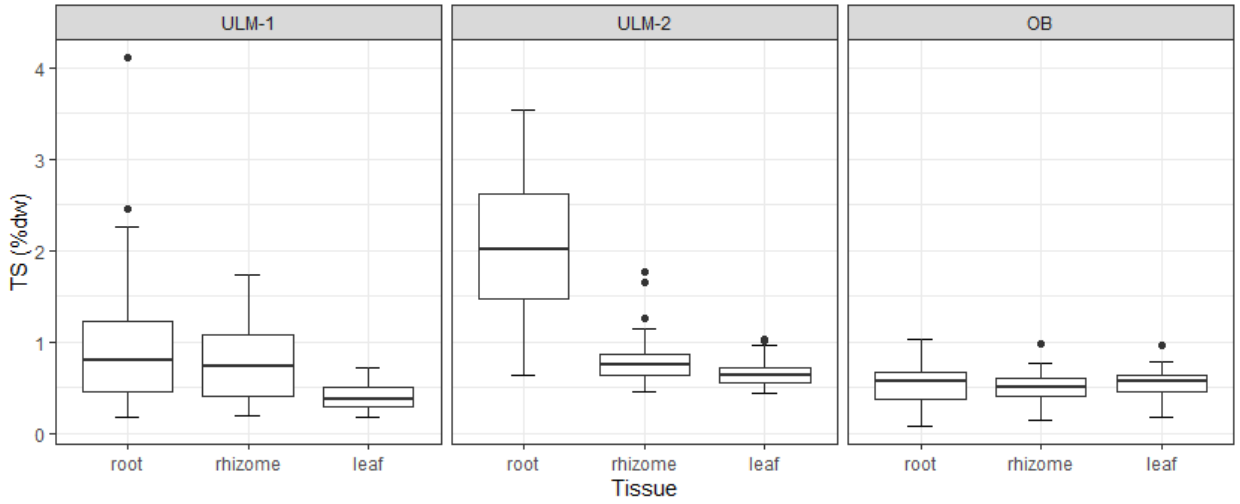

Figure 3. Distribution of TS values for root, rhizome, and leaf tissues at each study site. At ULM-1, leaf tissue TS was significantly lower than root and rhizome; at ULM-2, root tissue TS was significantly higher than rhizome and leaf tissue; at OB, no significant differences were found in TS between tissues. Statistical outliers are denoted by individual dots.

6.4 $F_{sulfide}$ estimates

The proportion of sulfur in plant tissues derived from sediment sulfide, denoted as $F_{sulfide}$, was estimated so that sulfide intrusion could be compared across tissues and study sites (Table 1, Figure 4). The highest levels of intrusion were observed in root tissues from the Upper Laguna Madre sites, with $F_{sulfide}$ values of 95.45 ± 6.72% and 88.93 ± 3.07% at ULM-1 and ULM-2, respectively. This trend was also observed for rhizome and leaf tissues; however, for these

tissues, values from ULM-2 were higher than those for ULM-1. Mean $F_{sulfide}$ values for rhizome tissues from ULM-1 and ULM-2 were 63.17 ± 19.15% and 72.48 ± 5.08%, respectively. For leaf tissue, the average $F_{sulfide}$ value was 40.01 ± 6.64% for ULM-1, 54.45 ± 6.84% for ULM-2, and 16.23 ± 6.43% for OB.

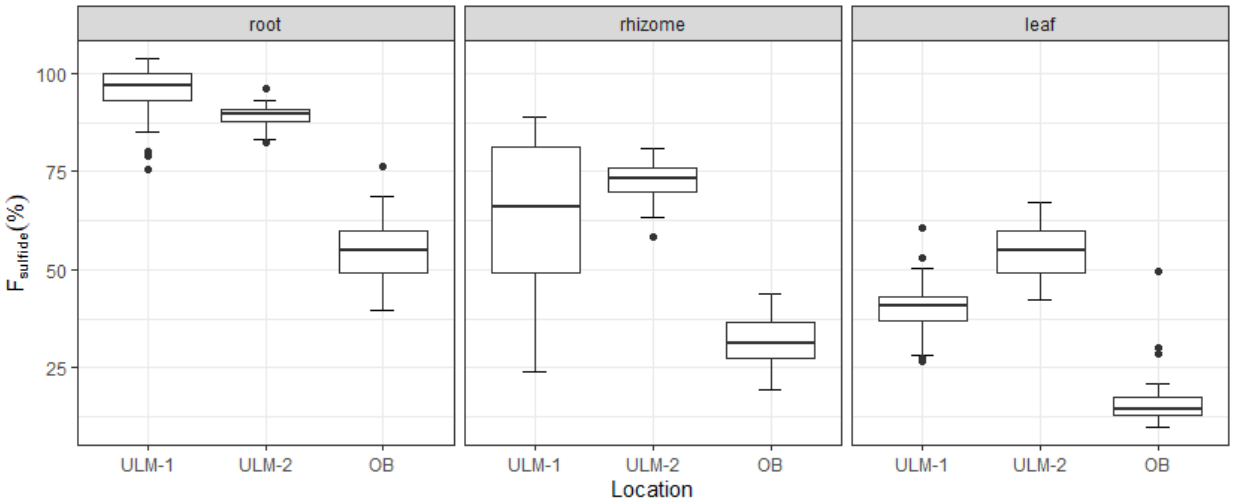

Figure 4. Distribution of $F_{sulfide}$ values grouped by tissue level for comparison across each site. Statistical outliers are denoted by individual dots.

6.5 Correlation between TS and $F_{sulfide}$

To assess the relationship between TS and $F_{sulfide}$ in each level of tissue, Spearman's rank correlation coefficients (ρ) were determined for each tissue type at each location (Figure 5). The Spearman correlation is useful for assessing whether two variable are monotonically related; that is, whether the value of one variable increases (or decreases) while that of the other variable also increases (or decreases), regardless of whether or not this relationship is linear. This rank-based test is particularly ideal in instances where statistical outliers are present in the data (as was the case of the $F_{sulfide}$ values obtained in this study), which render linear regression analyses less powerful or difficult to interpret.

The relationship between sulfur accumulation and the proportion of sulfur derived from sediment sulfide was not consistent across locations or tissue types. At ULM-1, for example, a

strong correlation was observed between TS and $F_{sulfide}$ in both root (ρ=0.78, $P < 0.001$) and rhizome tissue (ρ=0.79, $P < 0.001$), but not for leaves. At ULM-2, there was no evidence of a significant relationship between TS and $F_{sulfide}$ in either root or rhizome tissue, but these variables were moderately correlated in leaf tissue (ρ=0.37, P=0.017). At the Oso Bay site, a moderate correlation between TS and $F_{sulfide}$ was observed in root (ρ=0.32, P=0.025) and rhizome tissue (ρ=0.44, P=0.001), but these variables were not significantly related in leaf tissue.

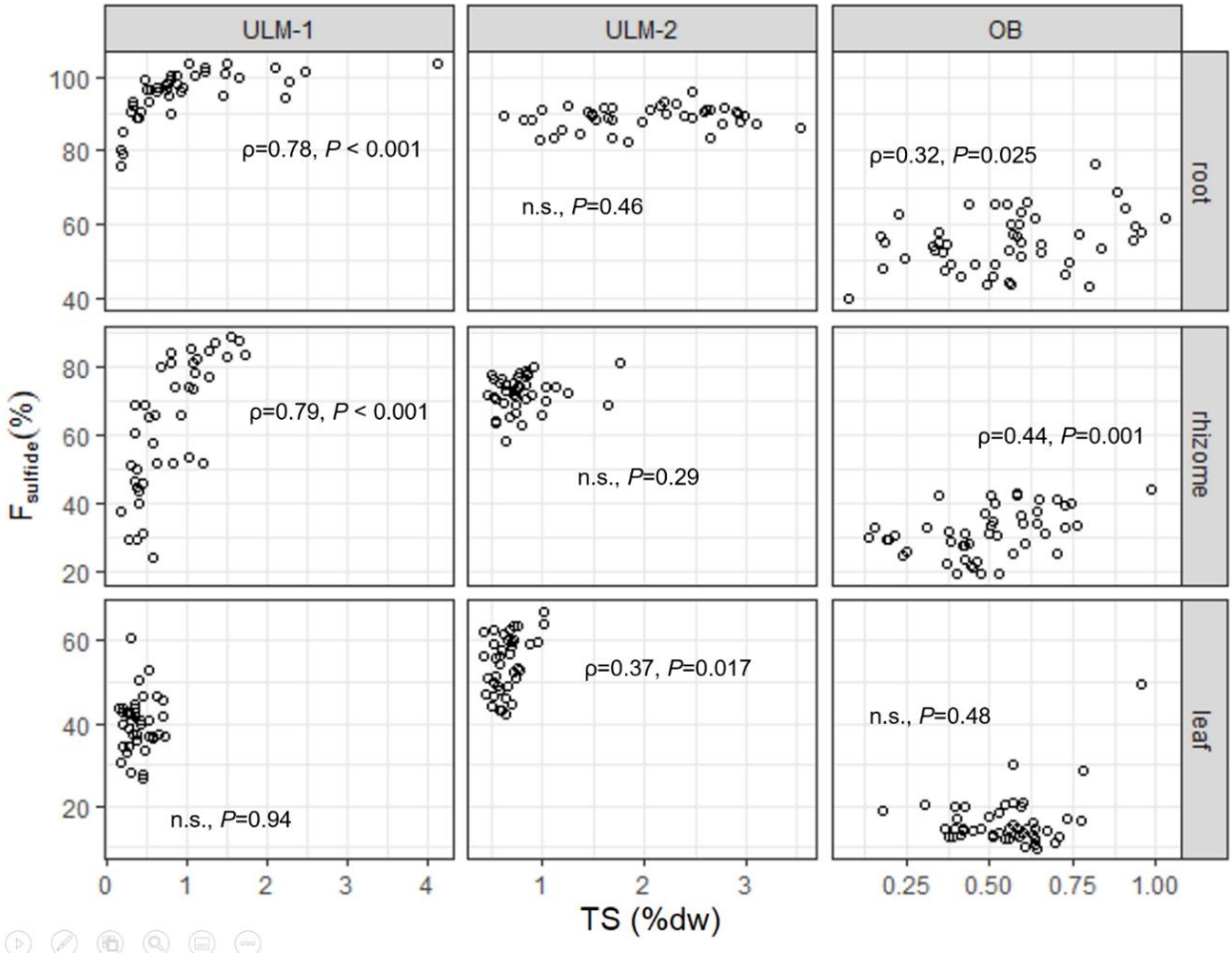

Figure 5. Correlation between $F_{sulfide}$ and TS for each tissue type across sites, with Spearman's correlation coefficients and associated p-values shown.

6.6 Genotypic differences for $F_{sulfide}$ and TS

6.6.1 Differences between MLLs

While differences across tissue types and sites were apparent, genotypic values for both $F_{sulfide}$ and TS generally tended to cluster together (Figures 6 and 7). Results of a two-way ANOVA, using location and genotype as factors to explain variation in $F_{sulfide}$ and TS, revealed no significant difference in mean $F_{sulfide}$ or TS values that could be explained by the genotypic identity of *H. wrightii* for leaf, root, or rhizome tissues.

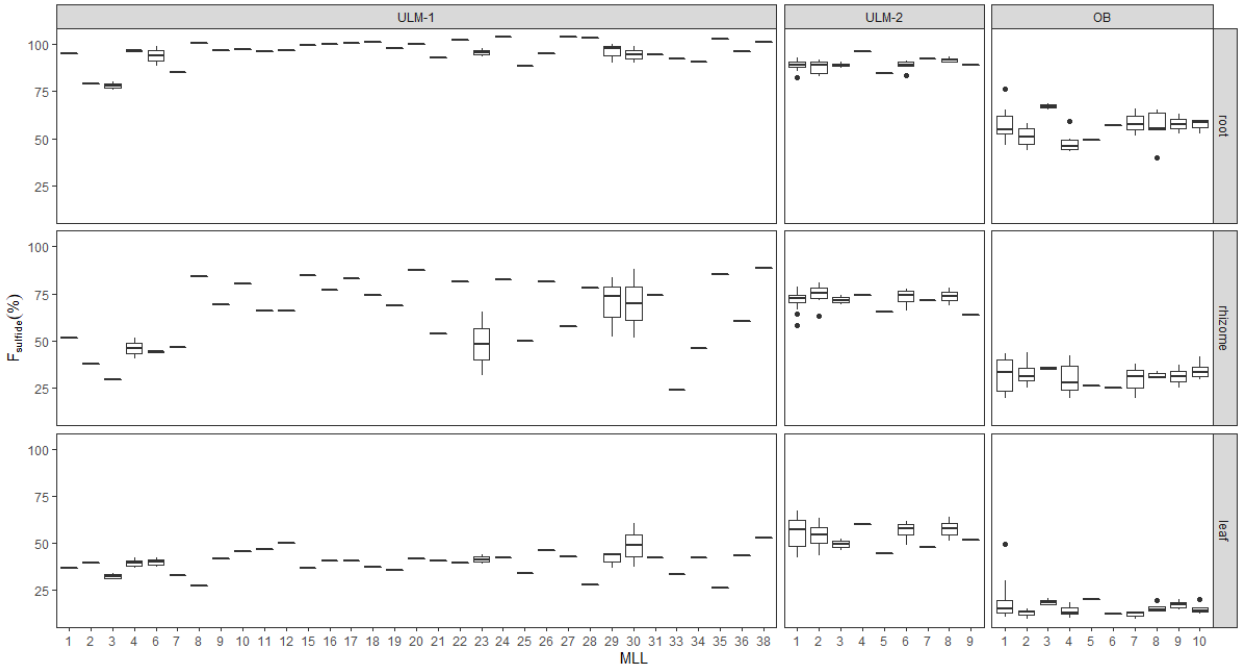

Figure 6. Variation in $F_{sulfide}$ values (root, rhizome, leaf) across genotypes for each location. Single-ramet genotypes are presented as straight lines representing their associated $F_{sulfide}$ value, while the mean and range of $F_{sulfide}$ values for multi-ramet genotypes are displayed with box-and-whisker plots. Statistical outliers are denoted by dots.

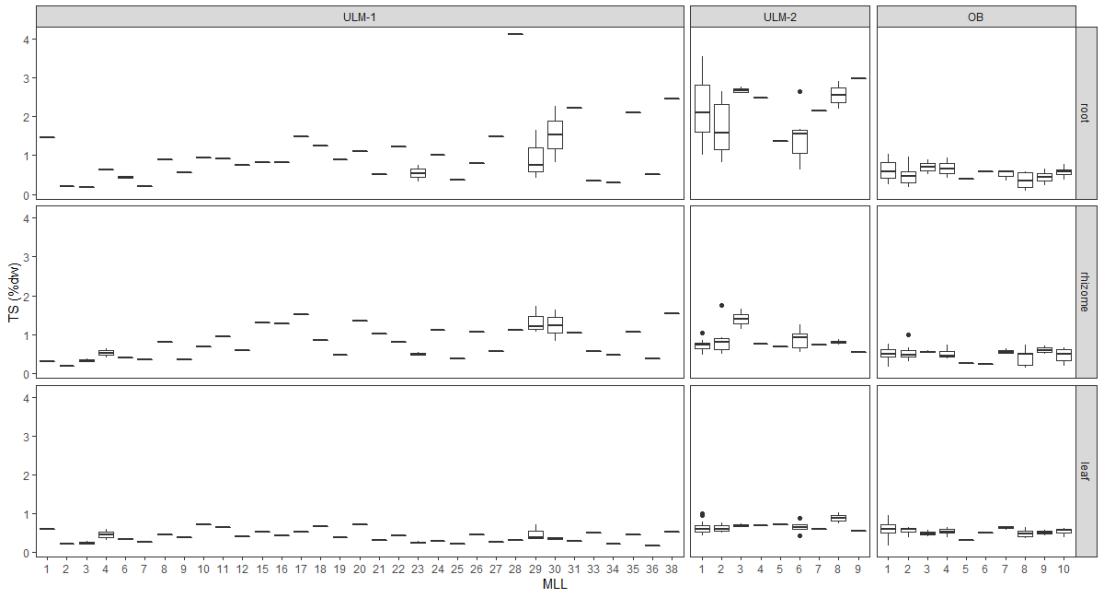

Figure 7. Variation in TS values (root, rhizome, leaf) across genotypes for each location. Single-ramet genotypes are presented as straight lines representing their associated TS value, while the mean and range of TS values for multi-ramet genotypes are displayed with box-and-whisker plots. Statistical outliers are denoted by dots.

6.6.2 Differences between single-ramet and multi-ramet genotypes

To assess the effect of clone size on $F_{sulfide}$ and TS, an additional two-way ANOVA was performed using location and clone size (single- vs. multi-ramet) as factors. Location was included due to differences identified between sites (see below). Unless otherwise noted, there is assumed to be no significant interaction between the two factors.

There was no significant difference in mean $F_{sulfide}$ values for either root or leaf tissues between single- and multi-ramet genotypes at any site (Figure 8). However, for rhizomes, significant evidence of an interaction between location and clone size was found. In this instance, the effect of clone size on $F_{sulfide}$ was dependent on the location in question, and therefore this effect had to be investigated separately for each location. Mean $F_{sulfide}$ values for rhizome tissues at ULM-1 were significantly lower in multi-ramet genotypes compared to single-ramet genotypes (P=0.015). The average $F_{sulfide}$ value for rhizome tissues from multi-ramet clones at

ULM-1 was 52.76 ± 19.26%, while that for single-ramet genotypes was 68.18 ± 17.12. The effect of clone size at other sites was not significant for rhizome tissues.

There was no significant evidence of a difference in mean TS values between single- and multi-ramet genotypes observed in any level of tissue, from any site.

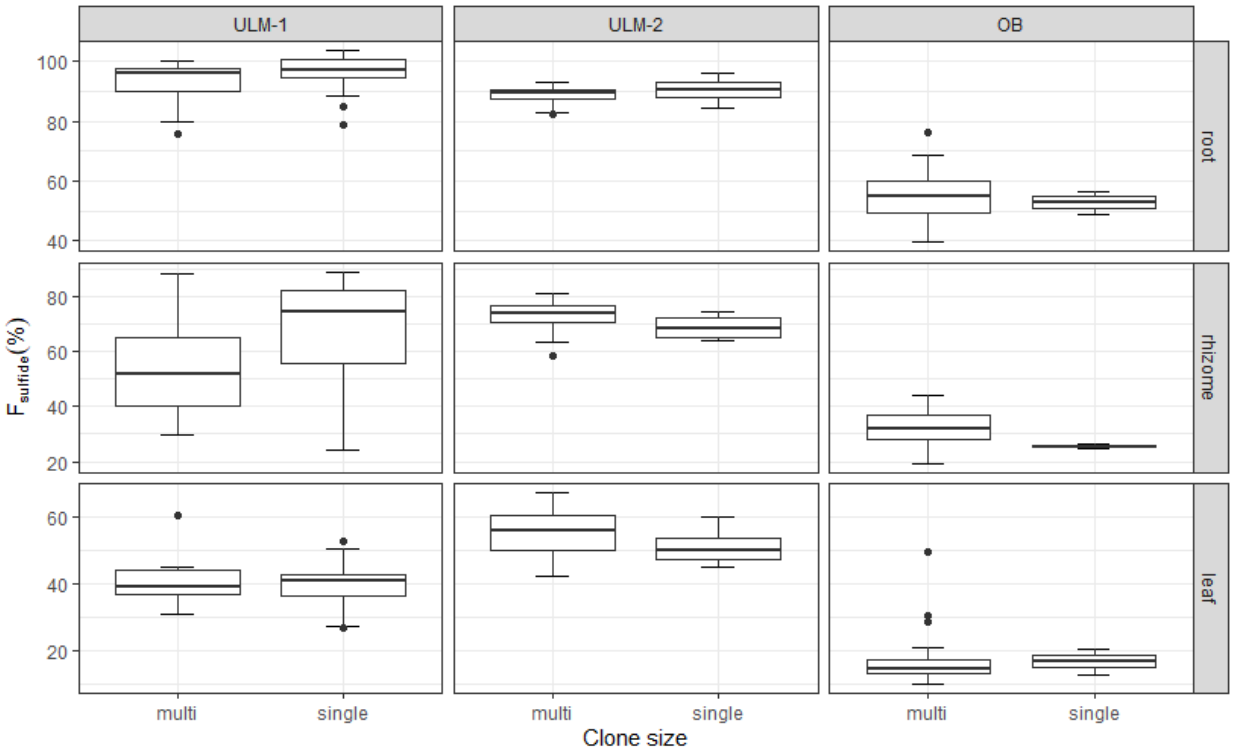

Figure 8. Variation in $F_{sulfide}$ values (root, rhizome, leaf) across groupings of single- and multi-ramet genotypes for each site. The only significant difference observed in group means was between those for rhizome tissues from ULM-1.

6.7 Variation in $F_{sulfide}$ and TS as explained by location

Despite the inability of genotype to explain differences in $F_{sulfide}$ and TS, it was retained in the model as a blocking factor to account for the fact that individual samples with the same genotype are members of the same clone. This was to ensure that p-values associated with comparisons between locations would not be influenced by slight differences existing between unique clones, even if these differences did not yield significant results for the factor as a whole. There were significant differences in sulfide intrusion and sulfur accumulation, as noted by mean

$F_{sulfide}$ and TS values, between the three study sites. These differences were noted at almost every level of tissue, as presented below.

6.7.1 Root $F_{sulfide}$ and TS

Mean $F_{sulfide}$ values for root tissue differed significantly between each of the study sites ($P < 0.001$) (Figure 9). Mean values were lowest at OB, and highest at ULM-1. Mean TS values for root tissue also differed significantly between each of the study sites ($P < 0.001$) (Figure 10). Mean root TS values were lowest at OB, and highest at ULM-2.

6.7.2 Rhizome $F_{sulfide}$ and TS

Mean $F_{sulfide}$ values for rhizome tissue also differed significantly between each of the study sites ($P < 0.001$ for differences between ULM sites and OB, $P=0.003$ for difference between ULM-1 and ULM-2) (Figure 9). Mean rhizome $F_{sulfide}$ values were lowest at OB, while values were highest at ULM-2. Mean TS values for rhizome tissue differed significantly between OB and the ULM sites ($P=0.008$ for difference between OB and ULM-1, $P=0.010$ for difference between OB and ULM-2), while no significant difference in TS values was observed between the ULM sites (Figure 10). Mean rhizome TS values were lowest at OB, while values were higher at the ULM sites (at each of which mean TS values were nearly identical).

6.7.3 Leaf $F_{sulfide}$ and TS

Mean $F_{sulfide}$ values for leaf tissue differed significantly as well between each of the study sites ($P < 0.001$) (Figure 9). Mean leaf $F_{sulfide}$ values were lowest at OB, while values were highest at ULM-2. Mean TS values for leaf tissue were significantly different between each of the study sites. Mean leaf TS values were lowest at ULM-1, and highest at ULM-2 (Figure 10).

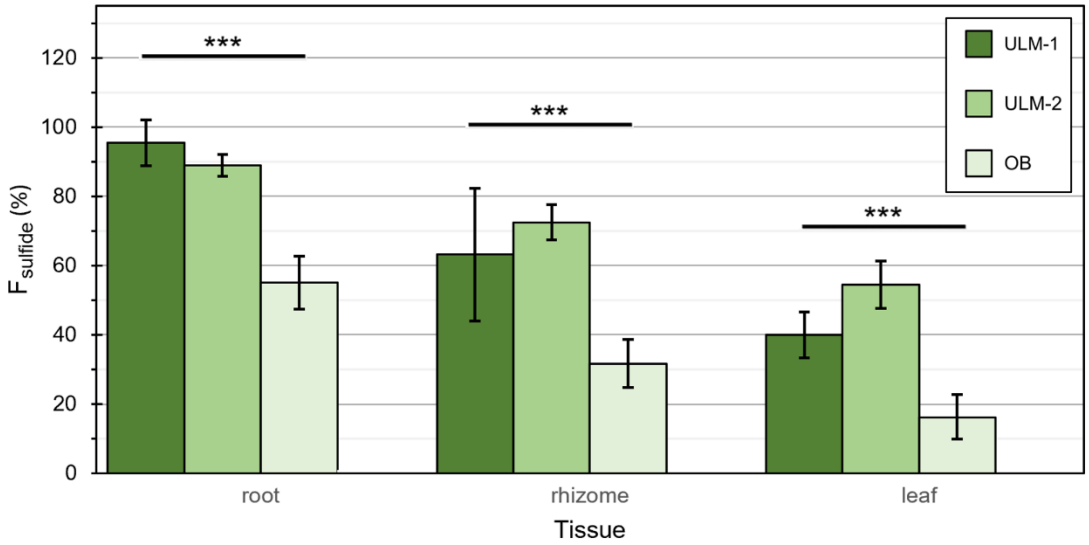

Figure 9. Comparison of mean $F_{sulfide}$ values across the various study sites, grouped by tissue level, where the height of each block represents the mean and the overlaying error bars represent ± 1 SD for each respective grouping. '***' denotes comparisons significant at α=0.01.

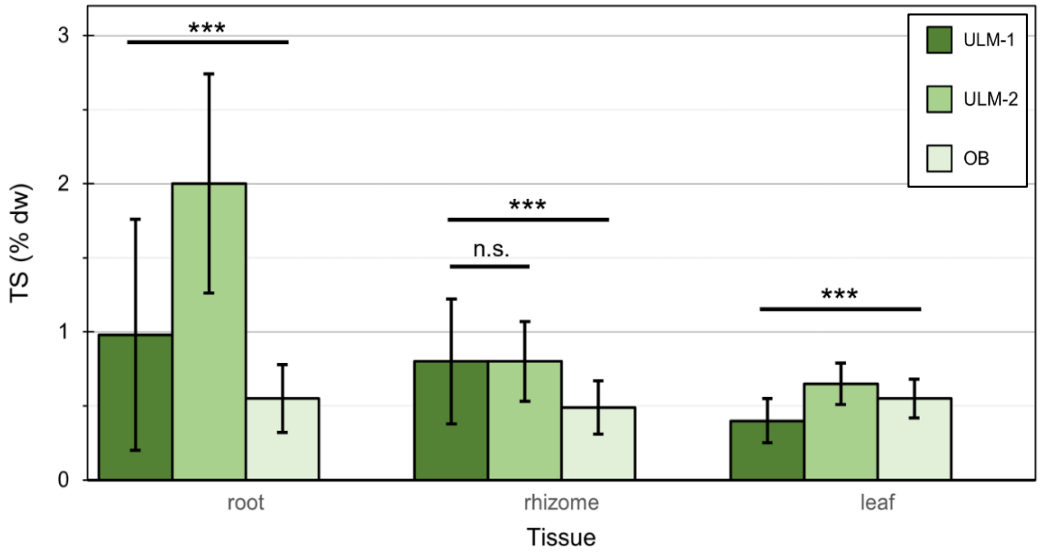

Figure 10. Comparison of mean TS values across the various study sites, grouped by tissue level, where the height of each block represents the mean and the overlaying error bars represent ± 1 SD for each respective grouping. '***' denotes comparisons significant at α=0.01. 'n.s.' denotes comparisons that were not significantly different ($P > 0.1$).

CHAPTER VII: DISCUSSION

Seagrass habitats across the globe are declining in the wake of mounting anthropogenic pressures, primarily those associated with coastal eutrophication. As coastal waters become increasingly eutrophic the threat of toxic sulfide accumulation within seagrass habitats grows stronger. There is a need to characterize the effects of this phenomenon and investigate strategies by which seagrasses may cope with environmental stress. Population genetic diversity has been shown to be correlated with resistance to, and recovery from, a number of environmental stressors. While data for sulfide intrusion in seagrasses within the Gulf of Mexico is lacking, this study (which includes data obtained from a previous project in our laboratory)[60] is believed to be the first to investigate whether sulfide intrusion in seagrasses, from multiple populations, is regulated at the genotypic level.

Our original objectives were to determine whether sulfide intrusion differs among different genotypes of *H. wrightii* or between single- and multi-ramet genotypes. Additionally, we sought to determine whether sulfide intrusion differs between *H. wrightii* meadows from two Texas bay systems. We hypothesized that sulfide intrusion and sulfide accumulation would be variable between genotypes based on a large body of evidence implicating the genotypic regulation of several traits involved in seagrass stress resistance, and that intrusion and accumulation would also vary between locations given the spatial characteristics distinguishing each site. While differences in sulfide intrusion could not be explained by the genotypic identity of *H. wrightii*, it was determined that there were distinct differences between the three study sites.

7.1 $\delta^{34}S$, $F_{sulfide}$, and TS values

H. wrightii across each study site exhibited a gradient in $\delta^{34}S$ and $F_{sulfide}$ values, from root to rhizome to leaf tissues, as reported widely for other seagrass species. This suggests that H_2S is taken up by the roots and translocated throughout the plant. The accumulation of sulfide is highest in the below-ground tissues, and while some sulfur in the leaves is derived from sulfide, it is apparent that leaves obtain much of their sulfur from sulfate in the water column. However, significant differences in average $F_{sulfide}$ and TS values indicates that sulfide intrusion and sulfur accumulation varied across sites. Surprisingly, although $\delta^{34}S$ values for sedimentary sulfides were notably lower at OB compared to ULM sites, tissue $\delta^{34}S$ levels at OB were much higher. This implies that intrusion was lower at OB compared to the ULM sites.

Relatively low mean $F_{sulfide}$ values from OB suggest that this population avoided sulfide intrusion through the reoxidation of sedimentary sulfide via root tip leakage of photosynthetically-derived oxygen. If tolerance mechanisms (i.e., biochemical assimilation of intruding sulfide) were responsible, this should have resulted in higher $F_{sulfide}$ values, as the isotopic composition of the metabolized sulfide would be retained. Still, at least some intrusion occurred at OB, and the accumulation of sulfide-derived sulfur is supported by a positive correlation between $F_{sulfide}$ and TS in root and rhizome tissues. Excessive sulfide may have been oxidized to S^0 or SO_4^{2-} and stored within cellular components of these tissues. No correlation was observed between $F_{sulfide}$ and TS in the leaf tissues, suggesting that any sulfur originating from sedimentary sulfide was likely used to meet metabolic demands. SO_4^{2-} is found in all seagrass tissues and is generally presumed to be the primary transport molecule of sulfur in plants.[87] It plays an important role in regulating sulfur metabolism, signaling the up- or downregulation of sulfate transporters.[88] Sulfide taken up by root tissues that becomes

chemically oxidized and translocated to leaf tissues may signal a downregulation of sulfate transporters in these tissues to decrease the uptake of sulfate from the seawater.

Evidence of sulfide avoidance in the OB population is contrasted by high intrusion in the ULM populations. In root tissues from ULM-1, for example, approximately 95% of the sulfur was derived from sedimentary sulfide, while those from ULM-2 derived 88% from sedimentary sulfide. Rhizome and leaf tissues from both ULM sites also exhibited significantly higher proportions of sediment-derived sulfur compared to those from OB. In these populations, tolerance mechanisms must have been more prominent. This assertion is supported not only by $F_{sulfide}$ values, but also by trends in TS data. At ULM-1, a strong positive correlation between $F_{sulfide}$ and TS was observed in root and rhizome tissues, suggesting sulfide detoxification products accumulated in these tissues. Significantly higher TS values for these tissues compared to those at OB demonstrate that sulfur accumulated to a greater extent in ULM-1 due to higher sulfide intrusion. At ULM-2, TS values for all tissues were also significantly higher than those from OB, although no correlation between $F_{sulfide}$ and TS was observed.

Interestingly, although $F_{sulfide}$ and TS were not correlated in the root and rhizome tissues at ULM-2, the ranges and maximum values of TS were much larger in these tissues compared to those from OB. This suggests that sulfur did accumulate to a greater extent in ULM-2. However, particularly in root tissues, $F_{sulfide}$ and TS are not correlated due to minimal variation in $F_{sulfide}$ values. This may indicate that in some instances, intruding sulfide meets the nutritional demands of the plant, while in others, sulfide is present in excess and is subsequently allowed to accumulate as S^0 or sulfate. Terrestrial plants have been shown to reduce the uptake of sulfate via leaf cells when exposed to increasing concentrations of H_2S. Under these conditions, the assimilatory reduction of sulfate is also decreased, evidenced by downregulation of a key

enzyme involved in the pathway.[87] Instead, cysteine is seemingly produced directly from intruding sulfide. This suggests that sulfide exposure may be tolerated through a decreased uptake of sulfate to meet nutritional needs, and this strategy could similarly be used by seagrasses. The assimilation of sulfide into organic molecules has been noted in the seagrass *Zostera marina*; however, this mechanism has not been demonstrated to entirely replace assimilatory sulfate reduction.[63] The role of H_2S as a nutritional source of sulfur in seagrasses appears worthy of investigation, especially in seagrasses facing high sulfide exposure.

The ability of seagrasses to avoid sulfide intrusion through radial oxygen loss from the root tips depends on a number of environmental factors. In general, conditions that may be attributed to both increased respiration rates and sulfide accumulation in the sediment – such as high temperatures, high organic matter content, and low oxygen availability in the water column – increase the threat of sulfide intrusion. The conditions of the sites investigated in this study are known to be considerably different. Oso Bay is an enclosed secondary bay within the Nueces Estuary, receiving freshwater inflow from Oso Creek, which is heavily dominated by industrial and wastewater pollution.[89] A large wastewater treatment plan serving the Corpus Christi area also deposits operational waste directly into Oso Bay. As a result, organic matter contents within Oso Bay are typically very high. The Laguna Madre is one of only 6 hypersaline lagoons in the world, where the only freshwater input is generally from rainwater, which rapidly evaporates in the South Texas climate.[90] The Upper Laguna Madre is relatively pristine, but can be polluted by agricultural runoff from adjacent ranches. ULM-1 and ULM-2 are both located within the Upper Laguna Madre, yet the two sites are distinct regarding their proximity to developed shorelines and dominant sources of nutrient inputs. ULM-1 is relatively isolated from urban areas, with essentially no developed coastline within at least 45 km. The site is within Kenedy County,

which, according to the 2020 U.S. Census, has a population of approximately 350 and is thereby the fourth least populous county in the United States. On the other hand, ULM-2 is within Nueces County, which is densely populated with a census estimate of 353,079 residents. In particular, the ULM-2 site is within a few kilometers of the highly developed coastline of Corpus Christi.

In a recent report to the Texas General Land Office, Oso Bay and the Upper Laguna Madre were characterized in terms of a number parameters known to influence sulfide production and accumulation.[91] It stated that Oso Bay receives higher levels of nutrient pollution than the Upper Laguna Madre, with average NO_3^- porewater concentrations up to 3600 times that observed in the ULM. Dissolved oxygen concentrations, however, were substantially higher in Oso Bay, especially during the winter months when average values exceeded 3.0 mg/L, compared to 0.5 mg/L for the ULM. Conversely, the salinity in Oso Bay was far lower, with a yearly average of 26.3 compared to 40.5 for the ULM.

Low oxygen availability in the water column has been shown to induce sulfide intrusion in the seagrass *Zostera marina.*[12] In a mesocosm study conducted by Pedersen et al., high internal oxygen partial pressures (e.g., above 7.4 kPa) prevented the intrusion of sulfide into *Z. marina* tissues, suggesting these levels of internal oxygen were sufficient for oxygen diffusion into the rhizosphere. However, when water column oxygen levels were experimentally depleted, the internal oxygen partial pressure was quickly reduced and sulfide soon began to diffuse into below-ground tissues. Water column hypoxia could have been responsible for the high sulfide intrusion observed at both ULM sites, and although dissolved oxygen was not measured in this study, existing data suggests that oxygen availability in the Upper Laguna Madre is relatively low. This is especially important considering that even under pristine conditions, such as

minimal nutrient pollution and turbidity, respiration will still occur at night when photosynthesis is inactive and internal plant oxygen will be consumed. Without diffusion of oxygen from the water column into the sediment, or the ability of the plant to obtain oxygen and supply it to the rhizosphere, sulfide intrusion is bound to occur.[12, 65]

High salinities are also expected to have an effect on sulfide intrusion. A mesocosm study was performed by the group of Koch and Erskine to investigate the interplay between high sulfide concentrations, high salinity, and high temperature, and the effect of these combined characteristics on sulfide intrusion in the seagrass *Thalassia testudinum*.[92] When exposed to high concentrations of sulfide at ambient temperature and salinity, no indication of mortality was observed; however, under high temperature and salinity conditions, traditional signs of seagrass "die-back" were observed such as tissue rotting and necrotic lesions. An additional study demonstrated that *T. testudinum* in high salinity conditions experienced higher levels of intrusion in terms of tissue H_2S concentration and faced longer periods of exposure compared to control groups.[93] These results implicate high salinity as an additional stressor driving sulfide intrusion. This may have influenced sulfide intrusion at the ULM sites, as the $F_{sulfide}$ and TS data suggests that high proportions of sulfur in *H. wrightii* from these sites were derived from sedimentary sulfide, and the Upper Laguna Madre is known to demonstrate consistently high salinity.[90, 91] This also seems reasonable given salinity and dissolved oxygen are directly related, with increased salinity reducing oxygen saturation in the water column.[94]

7.2 Remarks for $F_{sulfide}$ values that are over 100%

At ULM-1, some individual root $F_{sulfide}$ values exceeded 100%; that is, the mixing model suggests that over 100% of the sulfur in these root tissue samples was derived from sedimentary sulfide. Clearly, this is not possible, and there must be some explanation as to how these

observations may occur. Algebraically, values of $F_{sulfide}$ that exceed 100% are the result of a tissue $\delta^{34}S$ value which is lower than that of sediment sulfide. It is unlikely that there is significant error in the $\delta^{34}S$ value for sediment sulfide, as the values obtained for each of the Ag_2S replicates were within ± 0.16‰ of one another. One potential explanation may be that at the time the sediment-derived sulfur was assimilated into the tissues, the $\delta^{34}S$ of sediment sulfide was lower. Because the plants take some time to fully develop, their tissues are not necessarily an immediate reflection of the sediment characteristics at the time of sampling. This phenomenon may also be the result of an unidentified fractionation effect occurring with the uptake of sulfur compounds. For example, gaseous sulfide diffusing into root tissues may be an equilibrium fractionation process, and the isotopic composition of belowground tissues may therefore be temperature dependent. If large concentrations of sulfide are available in the sediment, and temperatures are low enough to slow the rate of H_2S diffusion into root tissues, then there may be a preferential uptake of the lighter ^{32}S-H_2S, and the tissues would be depleted of the heavier sulfur isotope.

7.3 Genotypic diversity and sulfide intrusion

Our hypothesis that sulfide intrusion would exhibit genotypic differences was not supported. In addition, although multi-ramet genotypes did exhibit lower $F_{sulfide}$ values, this was only significant for one tissue type (rhizomes) at one location (ULM-1). This phenomenon, however, may be worth further investigation. Besides the sediment and water column characteristics influencing sulfide intrusion, there are several possible strategies utilized by seagrasses to mitigate this stress. For example, O_2 derived from photosynthesis may be transported from leaf to root tissues and supplied to the sediment to re-oxidize sulfides. Certain genotypes may have a relatively higher photosynthetic capacity and produce larger amounts of

oxygen that can be supplied to the sediment, or demonstrate more efficient radial oxygen loss to oxidize the rhizosphere more readily. Further, plants with a greater internal availability of oxygen may tolerate greater amounts of intruding sulfide through reoxidation to sulfate or S^0. The direct utilization of sulfide as a nutritional source is another aspect of sulfide tolerance, where the downregulation of enzymes involved in sulfate assimilation may be an important mechanism to avoid excessive sulfur accumulation. All of these traits could have the potential to be regulated at a genotypic level, similar to other traits involved in stress resistance that appear to be enhanced in populations with high genotypic diversity. In this study, however, a number of the unique genotypes were only observed once (one ramet). Many more multi-ramet genotypes, from multiple locations, may need to be assessed before a genotypic influence can be confidently dismissed. Ideally, these genotypes should be well-represented by several replicates to understand how sulfide intrusion realistically scales within a given clone. This could allow for a more powerful examination of the relationship between genotypic diversity and sulfide invasion in *H. wrightii* meadows.

7.4 Future studies

Given the significant differences in sulfide intrusion and sulfur accumulation among study sites, future studies could work to identify which features are most responsible. Porewater sulfide, sedimentary organic matter and iron content are all factors that can influence sulfide accumulation and intrusion.[10] Organic matter stimulates sulfide production, while iron reacts with reduced sulfur, decreasing the likelihood of seagrass intrusion. Spatial and temporal variation in dissolved oxygen and salinity may also be important, as they have also been directly linked to intrusion and play a critical role in the reoxidation of reduced sulfur.[12, 92] Assessing the abundance and diversity of benthic macrofauna could provide additional insight, as these

organisms facilitate mixing of water column oxygen with marine sediments through bioturbation.[95]

In terms of experimental design, future studies could benefit from a larger sample collection, in the hope of obtaining more multi-ramet replicates to better test the influence of genotypic identity and size on intrusion. Such a sampling design would also allow a robust analysis of other population genetic features, such as genotypic *evenness* and *architecture*. Sampling periods should also be consistent (e.g. same month across sites), to avoid temporal effects in temperature and organic matter deposition that may influence sulfide production and uptake. Better yet, collecting samples across seasons (e.g., one year) could allow greater insight into the dynamics regulating sulfide production and intrusion is seagrass meadows.

Potential fractionation effects should also be investigated, such as those that may occur during sulfide uptake and distribution in the plant, or during its isolation (i.e. sediment distillation procedure). It has been assumed in previous studies regarding sulfide intrusion in seagrasses, as well as studies focusing on sulfur metabolism in terrestrial plants, that little to no fractionation is associated with the uptake of sulfate or sulfide. In terrestrial plants, tissue sulfur $\delta^{34}S$ has been reported to vary from source values by as little as 1 to 2 per mil.[58] Still, fractionation effects in terms of the uptake and distribution of sulfur compounds have not been investigated in seagrasses, where abundant sulfur availability from numerous sources may influence isotope selectivity. Considering the large fractionation associated with bacterial enzymes facilitating the dissimilatory reduction of sulfate, it seems unlikely that similar effects would not be observed in sulfur assimilation in plants. Studies focusing on the sulfur isotope composition of terrestrial plants have also demonstrated that the $\delta^{34}S$ values of sulfur metabolites, while providing valuable insight to their origins, are not a direct reflection of their

sources. Rather, there appears to be a number of fractionation effects associated with the various fates of sulfate as it undergoes assimilatory reduction.[96] While the present study offers a glimpse into the extent of sulfide intrusion in the species *H. wrightii* within a particular region and insight to the effects of local conditions on sulfide invasion, the results may be confounded by unknown fractionation effects associated with sulfur metabolism. Compound-specific $\delta^{34}S$ values may give way to a deeper knowledge of the mechanisms regulating sulfide detoxification in seagrasses, such that the fate of sulfide-derived sulfur may be better elucidated. Quantifying fractionation effects at the levels of sulfur uptake from each source, the translocation of sulfur species between different plant organs, and the various sulfur metabolites could allow for a more precise mixing model to determine the proportion of sulfide-derived sulfur that is actually tolerated within seagrass tissues.

REFERENCES

1. Short, F.; Short, C.; Novak, A., Seagrasses. 2016.
2. Potouroglou, M.; Grimsditch, G.; Weatherdon, L.; Lutz, S. *Out of the Blue: The value of seagrasses to the environment and to people*; 978-92-807-3780-6; 2020.
3. Waycott, M.; Duarte, C. M.; Carruthers, T. J. B.; Orth, R. J.; Dennison, W. C.; Olyarnik, S.; Calladine, A.; Fourqurean, J. W.; Heck, K. L.; Hughes, A. R.; Kendrick, G. A.; Kenworthy, W. J.; Short, F. T.; Williams, S. L., Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *Proceedings of the National Academy of Sciences* **2009,** *106* (30), 12377-12381.
4. Burkholder, J. M.; Tomasko, D. A.; Touchette, B. W., Seagrasses and eutrophication. *Journal of Experimental Marine Biology and Ecology* **2007,** *350* (1), 46-72.
5. Jørgensen, B. B.; Findlay, A. J.; Pellerin, A., The Biogeochemical Sulfur Cycle of Marine Sediments. *Frontiers in Microbiology* **2019,** *10* (849).
6. Schink, B., Microbially driven redox reactions in anoxic environments: pathways, energetics, and biochemical consequences. *Engineering in Life Sciences* **2006,** *6* (3), 228-233.
7. Lamers, L.; Govers, L.; Janssen, I.; Geurts, J.; Van der Welle, M.; Van Katwijk, M.; Van der Heide, T.; Roelofs, J.; Smolders, A., Sulfide as a soil phytotoxin—a review. *Frontiers in Plant Science* **2013,** *4* (268).
8. Holmer, M.; Hasler-Sheetal, H., Sulfide intrusion in seagrasses assessed by stable sulfur isotopes—a synthesis of current results. *Frontiers in Marine Science* **2014,** *1* (64).
9. Frederiksen, M.; Holmer, M.; Diaz-Almela, E.; Marba, N.; Duarte, C., Sulfide invasion in the seagrass Posidonia oceanica at Mediterranean fish farms: Assessment using stable

sulfur isotopes. *Marine Ecology-progress Series - Mar Ecol-Progr Ser* **2007,** *345*, 93-104.

10. Holmer, M.; Kendrick, G. A., High Sulfide Intrusion in Five Temperate Seagrasses Growing Under Contrasting Sediment Conditions. *Estuaries and Coasts* **2013,** *36* (1), 116-126.

11. Holmer, M.; Wirachwong, P.; Thomsen, M. S., Negative effects of stress-resistant drift algae and high temperature on a small ephemeral seagrass species. *Marine Biology* **2011,** *158* (2), 297-309.

12. Pedersen, O.; Binzer, T.; Borum, J., Sulphide intrusion in eelgrass (Zostera marina L.). *Plant, Cell & Environment* **2004,** *27* (5), 595-602.

13. Evans, S. M.; Vergés, A.; Poore, A. G. B., Genotypic Diversity and Short-term Response to Shading Stress in a Threatened Seagrass: Does Low Diversity Mean Low Resilience? *Frontiers in Plant Science* **2017,** *8* (1417).

14. Reusch Thorsten, B. H.; Ehlers, A.; Hämmerli, A.; Worm, B., Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *Proceedings of the National Academy of Sciences* **2005,** *102* (8), 2826-2831.

15. Papenbrock, J., Highlights in Seagrasses' Phylogeny, Physiology, and Metabolism: What Makes Them Special? *ISRN Botany* **2012,** *2012*, 103892.

16. Kuo, J.; Hartog, C. d., Seagrass Morphology, Anatomy, and Ultrastructure. In *Seagrasses: Biology, Ecologyand Conservation*, Larkum, A. W. D.; Orth, R. J.; Duarte, C. M., Eds. Springer Netherlands: Dordrecht, 2006; pp 51-87.

17. Ackerman, J. D., Sexual Reproduction of Seagrasses: Pollination in the Marine Context. In *Seagrasses: Biology, Ecologyand Conservation*, Larkum, A. W. D.; Orth, R. J.; Duarte, C. M., Eds. Springer Netherlands: Dordrecht, 2006; pp 89-109.

18. Orth, R. J.; Harwell, M. C.; Inglis, G. J., Ecology of Seagrass Seeds and Seagrass Dispersal Processes. In *Seagrasses: Biology, Ecologyand Conservation*, Larkum, A. W. D.; Orth, R. J.; Duarte, C. M., Eds. Springer Netherlands: Dordrecht, 2006; pp 111-133.

19. McKenzie, L. J.; Nordlund, L. M.; Jones, B. L.; Cullen-Unsworth, L. C.; Roelfsema, C.; Unsworth, R. K., The global distribution of seagrass meadows. *Environmental Research Letters* **2020,** *15* (7), 074041.

20. Ralph, P.; Durako, M.; Enríquez, S.; Collier, C.; Doblin, M., Impact of light limitation on seagrasses. *Journal of Experimental Marine Biology and Ecology* **2007,** *350*, 176-193.

21. Hartog, C.; Kuo, J.; Larkum, A.; Orth, R.; Duarte, C., Taxonomy and Biogeography of Seagrasses. 2007; pp 1-23.

22. Short, F.; Carruthers, T.; Dennison, W.; Waycott, M., Global seagrass distribution and diversity: A bioregional model. *Journal of Experimental Marine Biology and Ecology* **2007,** *350* (1), 3-20.

23. Cooper, L. W.; McRoy, C. P., Anatomical adaptations to rocky substrates and surf exposure by the seagrass genus Phyllospadix. *Aquatic Botany* **1988,** *32* (4), 365-381.

24. Hammerstrom, K. K.; Kenworthy, W. J.; Fonseca, M. S.; Whitfield, P. E., Seed bank, biomass, and productivity of Halophila decipiens, a deep water seagrass on the west Florida continental shelf. *Aquatic Botany* **2006,** *84* (2), 110-120.

25. Moore, K. A., Influence of Seagrasses on Water Quality in Shallow Regions of the Lower Chesapeake Bay. *Journal of Coastal Research* **2004,** (10045), 162-178.

26. Potouroglou, M.; Bull, J. C.; Krauss, K. W.; Kennedy, H. A.; Fusi, M.; Daffonchio, D.; Mangora, M. M.; Githaiga, M. N.; Diele, K.; Huxham, M., Measuring the role of seagrasses in regulating sediment surface elevation. *Sci Rep* **2017,** *7* (1), 11917-11917.

27. Röhr, M. E.; Boström, C.; Canal-Vergés, P.; Holmer, M., Blue carbon stocks in Baltic Sea eelgrass (Zostera marina) meadows. *Biogeosciences* **2016,** *13* (22), 6139-6153.

28. Short, F.; Polidoro, B.; Livingstone, S.; Carpenter, K.; Bandeira, S.; Bujang, J. S.; Calumpong, H.; Carruthers, T.; Coles, R.; Dennison, W.; Erftemeijer, P.; Fortes, M.; Freeman, A.; Jagtap, T. G.; Kamal, A. H.; Kendrick, G.; Kenworthy, W.; La Nafie, Y.; Nasution, I.; Zieman, J., Extinction Risk Assessment of the World's Seagrass Species. *Biological Conservation* **2011,** *144*, 1961–1971.

29. Short, F.; Wyllie-Echeverria, S., Natural and human-induced disturbance of seagrasses. *Environmental Conservation* **1996,** *23*, 17-27.

30. Dunton, K. H.; Schonberg, S. V., Assessment of Propeller Scarring in Seagrass Beds of the South Texas Coast. *Journal of Coastal Research* **2002**, 100-110.

31. Erftemeijer, P. L. A.; Robin Lewis, R. R., Environmental impacts of dredging on seagrasses: A review. *Marine Pollution Bulletin* **2006,** *52* (12), 1553-1572.

32. Tong, Y.; Zhao, Y.; Zhen, G.; Chi, J.; Liu, X.; Lu, Y.; Wang, X.; Yao, R.; Chen, J.; Zhang, W., Nutrient Loads Flowing into Coastal Waters from the Main Rivers of China (2006–2012). *Sci Rep* **2015,** *5* (1), 16678.

33. Howarth, R. W.; Sharpley, A.; Walker, D., Sources of Nutrient Pollution to Coastal Waters in the United States: Implications for Achieving Coastal Water Quality Goals. *Estuaries* **2002,** *25* (4), 656-676.

34. Smith, V. H.; Tilman, G. D.; Nekola, J. C., Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution* **1999,** *100* (1), 179-196.

35. Collier, C. J.; Waycott, M.; Ospina, A. G., Responses of four Indo-West Pacific seagrass species to shading. *Marine Pollution Bulletin* **2012,** *65* (4), 342-354.

36. Holmer, M.; Bondgaard, E., Photosynthetic and growth response of eelgrass to low oxygen and high sulfide concentrations during hypoxic events. *Aquatic Botany - AQUAT BOT* **2001,** *70*, 29-38.

37. Leavitt, W. D.; Bradley, A. S.; Santos, A. A.; Pereira, I. A. C.; Johnston, D. T., Sulfur Isotope Effects of Dissimilatory Sulfite Reductase. *Frontiers in Microbiology* **2015,** *6* (1392).

38. Allam, A., Sulfide Inhibition of Oxidases in Rice Roots. *Phytopathology* **1972,** *62*, 634.

39. Moore Jr., P. A.; Patrick Jr., W. H., Calcium and Magnesium Availability and Uptake by Rice in Acid Sulfate Soils. *Soil Science Society of America Journal* **1989,** *53* (3), 816-822.

40. Zhang, P.; Luo, Q.; Wang, R.; Xu, J., Hydrogen sulfide toxicity inhibits primary root growth through the ROS-NO pathway. *Sci Rep* **2017,** *7* (1), 868.

41. Fraser, M. W.; Kendrick, G. A., Belowground stressors and long-term seagrass declines in a historically degraded seagrass ecosystem after improved water quality. *Sci Rep* **2017,** *7* (1), 14469.

42. Carlson, P.; Yarbro, L.; Barber, T., Relationship of Sediment Sulfide to Mortality of Thalassia Testudinum in Florida Bay. *Bulletin of Marine Science* **1994,** *54*, 733-746.

43. Brodersen, K. E.; Kühl, M.; Nielsen, D. A.; Pedersen, O.; Larkum, A. W. D., Rhizome, Root/Sediment Interactions, Aerenchyma and Internal Pressure Changes in Seagrasses. In *Seagrasses of Australia: Structure, Ecology and Conservation*, Larkum, A. W. D.; Kendrick, G. A.; Ralph, P. J., Eds. Springer International Publishing: Cham, 2018; pp 393-418.

44. Barker, A. V.; Pilbeam, D., *Handbook of plant nutrition*. 2016; p 1-773.

45. Leustek, T.; Martin, M. N.; Bick, J. A.; Davies, J. P., Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. *Annu Rev Plant Physiol Plant Mol Biol* **2000,** *51*, 141-165.

46. Brosnan, J. T.; Brosnan, M. E., The Sulfur-Containing Amino Acids: An Overview. *The Journal of Nutrition* **2006,** *136* (6), 1636S-1640S.

47. Leustek, T., Sulfate Metabolism. *The Arabidopsis Book* **2002,** *2002* (1).

48. Fry, B.; Scalan, R. S.; Winters, J. K.; Parker, P. L., Sulphur uptake by salt grasses, mangroves, and seagrasses in anaerobic sediments. *Geochimica et Cosmochimica Acta* **1982,** *46* (6), 1121-1124.

49. Raven, J. A.; Scrimgeour, C. M., The Influence of Anoxia on Plants of Saline Habitats with Special Reference to the Sulphur Cycle. *Annals of Botany* **1997,** *79* (suppl_1), 79-86.

50. Van Cappellen, P.; Wang, Y., Cycling of iron and manganese in surface sediments; a general theory for the coupled transport and reaction of carbon, oxygen, nitrogen, sulfur, iron, and manganese. *American Journal of Science* **1996,** *296* (3), 197-243.

51. Habicht, K. S.; Canfield, D. E., Isotope fractionation by sulfate-reducing natural populations and the isotopic composition of sulfide in marine sediments. *Geology* **2001,** *29* (6), 555-558.

52. Schippers, A.; Jørgensen, B. B., Biogeochemistry of pyrite and iron sulfide oxidation in marine sediments. *Geochimica et Cosmochimica Acta* **2002,** *66*, 85-92.

53. Kushkevych, I.; Cejnar, J.; Treml, J.; Dordević, D.; Kollar, P.; Vítězová, M., Recent Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria. *Cells* **2020,** *9* (3), 698.

54. Berto, D.; Calace, N.; Rampazzo, F.; Saccomandi, F., *Isotopes: from theory to practice*. 2019.

55. Muccio, Z.; Jackson, G., Isotope Ratio Mass Spectrometry. *The Analyst* **2009,** *134*, 213-22.

56. Prohaska, T.; Irrgeher, J.; Zitek, A.; Jakubowski, N., *Sector Field Mass Spectrometry for Elemental and Isotopic Analysis*. 2014; p 666.

57. Winner, W. E.; Smith, C. L.; Koch, G. W.; Mooney, H. A.; Bewley, J. D.; Krouse, H. R., Rates of emission of H2S from plants and patterns of stable sulphur isotope fractionation. *Nature* **1981,** *289* (5799), 672-673.

58. Trust, B. A.; Fry, B., Stable sulphur isotopes in plants: a review. *Plant, Cell & Environment* **1992,** *15* (9), 1105-1110.

59. Frederiksen, M.; Holmer, M.; Pérez, M.; Invers, O.; Ruiz Fernandez, J. M.; Knudsen, B., Effect of increased sediment sulfide concentrations on the composition of stable sulfur isotopes ($\delta^{34}S$) and sulfur accumulation in the seagrasses Zostera marina and

Posidonia oceanica. *Journal of Experimental Marine Biology and Ecology* **2008,** *358,* 98-109.

60. Rubiano-Rincon, S. Assessment of sulfide intrusion and genotypic diversity in the seagrass Halodule wrightii from the Texas Gulf Coast. Texas A&M University-Corpus Christi, 2019.

61. Kilminster, K.; Forbes, V.; Holmer, M., Development of a 'sediment-stress' functional-level indicator for the seagrass Halophila ovalis. *Ecological Indicators* **2014,** *36,* 280-289.

62. Holmer, M.; Frederiksen, M. S.; Møllegaard, H., Sulfur accumulation in eelgrass (Zostera marina) and effect of sulfur on eelgrass growth. *Aquatic Botany* **2005,** *81* (4), 367-379.

63. Hasler-Sheetal, H.; Holmer, M., Sulfide Intrusion and Detoxification in the Seagrass Zostera marina. *PLoS ONE* **2015,** *10,* e0129136.

64. Brodersen, K. E.; Nielsen, D. A.; Ralph, P. J.; Kühl, M., Oxic microshield and local pH enhancement protects Zostera muelleri from sediment derived hydrogen sulphide. *New Phytologist* **2015,** *205* (3), 1264-1276.

65. Borum, J.; Pedersen, O.; Greve, T. M.; Frankovich, T. A.; Zieman, J. C.; Fourqurean, J. W.; Madden, C. J., The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, Thalassia testudinum. *Journal of Ecology* **2005,** *93* (1), 148-158.

66. Sodhi, N. S.; Ehrlich, P. R., *Conservation biology for all*. Oxford University Press: 2010.

67. Frankham, R.; Ballou, J. D.; Briscoe, D. A., *A Primer of Conservation Genetics*. Cambridge University Press: Cambridge, 2004.

68. Thorsten, B. H. R.; Hughes, A. R., The Emerging Role of Genetic Diversity for Ecosystem Functioning: Estuarine Macrophytes as Models. *Estuaries and Coasts* **2006,** *29* (1), 159-164.

69. Massa, S. I.; Paulino, C. M.; Serrão, E. A.; Duarte, C. M.; Arnaud-Haond, S., Entangled effects of allelic and clonal (genotypic) richness in the resistance and resilience of experimental populations of the seagrass Zostera noltii to diatom invasion. *BMC Ecology* **2013,** *13* (1), 39.

70. Abdul-Muneer, P. M., Application of microsatellite markers in conservation genetics and fisheries management: recent advances in population structure analysis and conservation strategies. *Genet Res Int* **2014,** *2014*, 691759-691759.

71. Genetic diversity. In *A Primer of Conservation Genetics*, Briscoe, D. A.; Ballou, J. D.; Frankham, R., Eds. Cambridge University Press: Cambridge, 2004; pp 12-30.

72. Conservation Genetics. In *Molecular Ecology*, 2011; pp 319-366.

73. Ellstrand, N. C.; Roose, M. L., Patterns of Genotypic Diversity in Clonal Plant Species. *American Journal of Botany* **1987,** *74* (1), 123-131.

74. Pazzaglia, J.; Nguyen, H. M.; Santillán-Sarmiento, A.; Ruocco, M.; Dattolo, E.; Marín-Guirao, L.; Procaccini, G., The Genetic Component of Seagrass Restoration: What We Know and the Way Forwards. *Water* **2021,** *13* (6), 829.

75. Sjöqvist, C. O.; Kremp, A., Genetic diversity affects ecological performance and stress response of marine diatom populations. *ISME J* **2016,** *10* (11), 2755-2766.

76. Johnson, M. T. J.; Lajeunesse, M. J.; Agrawal, A. A., Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. *Ecology Letters* **2006,** *9* (1), 24-34.

77. Crutsinger Gregory, M.; Collins Michael, D.; Fordyce James, A.; Gompert, Z.; Nice Chris, C.; Sanders Nathan, J., Plant Genotypic Diversity Predicts Community Structure and Governs an Ecosystem Process. *Science* **2006,** *313* (5789), 966-968.

78. Hughes, A. R.; Stachowicz, J. J., Genetic diversity enhances the resistance of a seagrass ecosystem to disturbance. *Proceedings of the National Academy of Sciences* **2004,** *101* (24), 8998-9002.

79. Reusch, T. B. H.; Ehlers, A.; Hämmerli, A.; Worm, B., Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (8), 2826-2831.

80. Liu, F.; Liu, J.; Dong, M., Ecological Consequences of Clonal Integration in Plants. *Frontiers in Plant Science* **2016,** *7* (770).

81. Tuya, F.; Viera-Rodríguez, M. A.; Guedes, R.; Espino, F.; Haroun, R.; Terrados, J., Seagrass responses to nutrient enrichment depend on clonal integration, but not flow-on effects on associated biota. *Marine Ecology Progress Series* **2013,** *490,* 23-35.

82. Salo, T.; Reusch, T.; Boström, C., Genotype-specific responses to light stress in eelgrass Zostera marina, a marine foundation plant. *Marine Ecology Progress Series* **2015,** *519*.

83. Kremling, K., Determination of the major constituents. In *Methods of Seawater Analysis*, 1999; pp 229-251.

84. Backlund, K.; Boman, A.; Fröjdö, S.; Åström, M., An analytical procedure for determination of sulphur species and isotopes in boreal acid sulphate soils and sediments. *Agricultural and Food Science* **2005,** *14* (1), 70-82.

85. Fossing, H.; Jørgensen, B. B., Measurement of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. *Biogeochemistry* **1989,** *8* (3), 205-222.

86. Arnaud-Haond, S.; Duarte, C. M.; Alberto, F.; Serrão, E. A., Standardizing methods to address clonality in population studies. *Molecular Ecology* **2007,** *16* (24), 5115-5139.

87. Rennenberg, H., The Fate of Excess Sulfur in Higher Plants. *Annual Review of Plant Physiology* **1984,** *35* (1), 121-153.

88. Takahashi, H., Sulfate transport systems in plants: functional diversity and molecular mechanisms underlying regulatory coordination. *Journal of Experimental Botany* **2019,** *70* (16), 4075-4087.

89. Nicolau, B. A. *Water quality and biological characterization of Oso Creek & Oso Bay, Corpus Christi, Texas*; 2001.

90. Bugica, K.; Sterba-Boatwright, B.; Wetz, M., Water quality trends in Texas estuaries. *Marine pollution bulletin* **2020,** *152*, 110903.

91. Dorina Murgulet, A. D., Cody Lopez, Bimal Gyawali, Valeriu Murgulet *Impacts of Temporal and Spatial Variation of Submarine Groundwater Discharge on Nutrient Fluxes to Texas Coastal Embayments*; 2019.

92. Koch, M. S.; Erskine, J. M., Sulfide as a phytotoxin to the tropical seagrass Thalassia testudinum: interactions with light, salinity and temperature. *Journal of Experimental Marine Biology and Ecology* **2001,** *266* (1), 81-95.

93. Johnson, C. R.; Koch, M. S.; Pedersen, O.; Madden, C. J., Hypersalinity as a trigger of seagrass (Thalassia testudinum) die-off events in Florida Bay: Evidence based on shoot

meristem O2 and H2S dynamics. *Journal of Experimental Marine Biology and Ecology* **2018,** *504*, 47-52.

94. Song, H.; Wignall, P. B.; Song, H.; Dai, X.; Chu, D., Seawater Temperature and Dissolved Oxygen over the Past 500 Million Years. *Journal of Earth Science* **2019,** *30* (2), 236-243.

95. Glud, R. N., Oxygen dynamics of marine sediments. *Marine Biology Research* **2008,** *4* (4), 243-289.

96. Tcherkez, G.; Tea, I., 32S/34S isotope fractionation in plant sulphur metabolism. *New Phytologist* **2013,** *200*.

APPENDIX A

$\delta^{34}S$, $F_{SULFIDE}$, AND TS VALUES FOR *H. WRIGHTII* FROM UPPER LAGUNA MADRE (ULM-1)

Sample ID	Tissue	$\delta^{34}S$ (‰)	$F_{sulfide}$ (%)	TS (% dw)	Genotype
HW.15.01	Root	-21.27	94.88	1.46	MLL-1a
HW.15.02	Root	-14.06	79.03	0.20	MLL-2a
HW.15.03	Root	-12.48	75.55	0.18	MLL-3a
HW.15.04	Root	-21.8	96.05	0.62	MLL-4a
HW.15.06	Root	-18.46	88.70	0.41	MLL-6a
HW.15.07	Root	-23.21	99.15	0.47	MLL-6a
HW.15.08	Root	-16.84	85.14	0.21	MLL-7a
HW.15.09	Root	-23.84	100.54	0.89	MLL-8a
HW.15.10	Root	-22.09	96.69	0.56	MLL-9a
HW.15.11	Root	-22.35	97.26	0.95	MLL-10a
HW.15.12	Root	-21.86	96.18	0.92	MLL-11a
HW.15.13	Root	-22.06	96.62	0.76	MLL-12a
HW.15.15	Root	-14.52	80.04	0.19	MLL-3a
HW.15.17	Root	-22.34	97.24	0.62	MLL-4a
HW.15.18	Root	-23.31	99.37	0.81	MLL-15a
HW.15.19	Root	-23.73	100.29	0.81	MLL-16a
HW.15.20	Root	-23.89	100.65	1.48	MLL-17a
HW.15.21	Root	-24.16	101.24	1.24	MLL-18a
HW.15.22	Root	-22.65	97.92	0.89	MLL-19a
HW.15.23	Root	-23.62	100.05	1.11	MLL-20a
HW.15.24	Root	-20.47	93.13	0.52	MLL-21a
HW.15.25	Root	-24.74	102.52	1.23	MLL-22a
HW.15.26	Root	-22.65	97.92	0.75	MLL-23a
HW.15.27	Root	-25.25	103.64	1.02	MLL-24a
HW.15.28	Root	-18.46	88.70	0.38	MLL-25a
HW.15.29	Root	-21.33	95.02	0.79	MLL-26a
HW.15.30	Root	-25.25	103.64	1.49	MLL-27a
HW.15.31	Root	-25.21	103.55	4.11	MLL-28a
HW.15.32	Root	-22.53	97.66	0.74	MLL-29a
HW.15.33	Root	-23.59	99.99	1.66	MLL-29a
HW.15.34	Root	-22.99	98.67	2.26	MLL-30a
HW.15.35	Root	-19.03	89.96	0.81	MLL-30a
HW.15.36	Root	-21.1	94.51	2.23	MLL-31a
HW.15.39	Root	-20.48	93.15	0.33	MLL-23a
HW.15.40	Root	-20.05	92.20	0.34	MLL-33a
HW.15.41	Root	-19.28	90.51	0.30	MLL-34a
HW.15.42	Root	-24.84	102.74	2.10	MLL-35a
HW.15.43	Root	-21.92	96.31	0.50	MLL-36a
HW.15.45	Root	-19.22	90.38	0.42	MLL-29a
HW.15.46	Root	-24.23	101.39	2.46	MLL-38a
HW.15.01	Rhizome	-1.53	51.47	0.31	MLL-1a
HW.15.02	Rhizome	4.72	37.73	0.19	MLL-2a
HW.15.03	Rhizome	8.36	29.72	0.29	MLL-3a
HW.15.04	Rhizome	3.53	40.34	0.40	MLL-4a
HW.15.06	Rhizome	1.42	44.98	0.38	MLL-6a

HW.15.07	Rhizome	2.06	43.58	0.41	MLL-6a
HW.15.08	Rhizome	0.65	46.68	0.35	MLL-7a
HW.15.09	Rhizome	-16.46	84.31	0.80	MLL-8a
HW.15.10	Rhizome	-9.59	69.20	0.36	MLL-9a
HW.15.11	Rhizome	-14.68	80.39	0.68	MLL-10a
HW.15.12	Rhizome	-8.24	66.23	0.94	MLL-11a
HW.15.13	Rhizome	-8.03	65.77	0.60	MLL-12a
HW.15.15	Rhizome	8.35	29.74	0.38	MLL-3a
HW.15.17	Rhizome	-1.67	51.78	0.64	MLL-4a
HW.15.18	Rhizome	-16.6	84.61	1.29	MLL-15a
HW.15.19	Rhizome	-13.23	77.20	1.27	MLL-16a
HW.15.20	Rhizome	-15.8	82.86	1.51	MLL-17a
HW.15.21	Rhizome	-11.88	74.23	0.86	MLL-18a
HW.15.22	Rhizome	-9.35	68.67	0.47	MLL-19a
HW.15.23	Rhizome	-17.9	87.47	1.35	MLL-20a
HW.15.24	Rhizome	-2.61	53.85	1.02	MLL-21a
HW.15.25	Rhizome	-15.1	81.32	0.81	MLL-22a
HW.15.26	Rhizome	-7.78	65.22	0.54	MLL-23a
HW.15.27	Rhizome	-15.73	82.70	1.12	MLL-24a
HW.15.28	Rhizome	-0.83	49.93	0.38	MLL-25a
HW.15.29	Rhizome	-15.11	81.34	1.07	MLL-26a
HW.15.30	Rhizome	-4.32	57.61	0.57	MLL-27a
HW.15.31	Rhizome	-13.7	78.24	1.11	MLL-28a
HW.15.32	Rhizome	-11.58	73.57	1.07	MLL-29a
HW.15.33	Rhizome	-16.21	83.76	1.73	MLL-29a
HW.15.34	Rhizome	-18.06	87.83	1.64	MLL-30a
HW.15.35	Rhizome	-1.67	51.78	0.83	MLL-30a
HW.15.36	Rhizome	-11.86	74.19	1.03	MLL-31a
HW.15.39	Rhizome	7.54	31.53	0.45	MLL-23a
HW.15.40	Rhizome	10.95	24.03	0.57	MLL-33a
HW.15.41	Rhizome	0.86	46.22	0.46	MLL-34a
HW.15.42	Rhizome	-16.99	85.47	1.06	MLL-35a
HW.15.43	Rhizome	-5.66	60.56	0.37	MLL-36a
HW.15.45	Rhizome	-1.77	52.00	1.21	MLL-29a
HW.15.46	Rhizome	-18.46	88.70	1.54	MLL-38a
HW.15.01	Leaf	5.23	36.61	0.59	MLL-1a
HW.15.02	Leaf	3.84	39.66	0.22	MLL-2a
HW.15.03	Leaf	6.3	34.25	0.29	MLL-3a
HW.15.04	Leaf	5.09	36.91	0.59	MLL-4a
HW.15.06	Leaf	4.87	37.40	0.34	MLL-6a
HW.15.07	Leaf	2.56	42.48	0.35	MLL-6a
HW.15.08	Leaf	6.83	33.09	0.26	MLL-7a
HW.15.09	Leaf	9.41	27.41	0.46	MLL-8a
HW.15.10	Leaf	2.86	41.82	0.37	MLL-9a
HW.15.11	Leaf	1.09	45.71	0.71	MLL-10a
HW.15.12	Leaf	0.68	46.61	0.64	MLL-11a
HW.15.13	Leaf	-1.02	50.35	0.40	MLL-12a
HW.15.15	Leaf	7.91	30.71	0.19	MLL-3a
HW.15.17	Leaf	2.73	42.10	0.30	MLL-4a
HW.15.18	Leaf	5.05	37.00	0.52	MLL-15a
HW.15.19	Leaf	3.27	40.92	0.42	MLL-16a
HW.15.20	Leaf	3.4	40.63	0.52	MLL-17a
HW.15.21	Leaf	4.88	37.38	0.66	MLL-18a
HW.15.22	Leaf	5.55	35.90	0.38	MLL-19a
HW.15.23	Leaf	2.81	41.93	0.71	MLL-20a

HW.15.24	Leaf	3.4	40.63	0.31	MLL-21a
HW.15.25	Leaf	3.78	39.79	0.43	MLL-22a
HW.15.26	Leaf	4.13	39.02	0.29	MLL-23a
HW.15.27	Leaf	2.53	42.54	0.28	MLL-24a
HW.15.28	Leaf	6.32	34.21	0.22	MLL-25a
HW.15.29	Leaf	0.77	46.41	0.45	MLL-26a
HW.15.30	Leaf	2.45	42.72	0.27	MLL-27a
HW.15.31	Leaf	9.05	28.20	0.31	MLL-28a
HW.15.32	Leaf	5.08	36.94	0.72	MLL-29a
HW.15.33	Leaf	1.5	44.81	0.36	MLL-29a
HW.15.34	Leaf	-5.63	60.49	0.32	MLL-30a
HW.15.35	Leaf	4.91	37.31	0.39	MLL-30a
HW.15.36	Leaf	2.7	42.17	0.29	MLL-31a
HW.15.39	Leaf	1.93	43.86	0.21	MLL-23a
HW.15.40	Leaf	6.72	33.33	0.49	MLL-33a
HW.15.41	Leaf	2.53	42.54	0.21	MLL-34a
HW.15.42	Leaf	9.81	26.53	0.46	MLL-35a
HW.15.43	Leaf	2.09	43.51	0.17	MLL-36a
HW.15.45	Leaf	1.95	43.82	0.37	MLL-29a
HW.15.46	Leaf	-2.15	52.84	0.53	MLL-38a

APPENDIX B

$\delta^{34}S$, $F_{SULFIDE}$, AND TS VALUES FOR *H. WRIGHTII* FROM UPPER LAGUNA MADRE (ULM-2)

Sample ID	Tissue	$\delta^{34}S$ (‰)	$F_{sulfide}$ (%)	TS (% dw)	Genotype
HW.14.01	Root	-20.48	92.89	2.31	MLL-1b
HW.14.02	Root	-19.53	90.79	2.61	MLL-2b
HW.14.03	Root	-19.39	90.49	2.59	MLL-3b
HW.14.05	Root	-19.23	90.13	2.22	MLL-1b
HW.14.06	Root	-19.77	91.32	1.68	MLL-1b
HW.14.07	Root	-21.96	96.15	2.48	MLL-4b
HW.14.08	Root	-20.08	92.01	1.26	MLL-2b
HW.14.09	Root	-16.62	84.38	1.37	MLL-5b
HW.14.11	Root	-18.16	87.77	1.97	MLL-1b
HW.14.12	Root	-19.58	90.91	2.65	MLL-6b
HW.14.13	Root	-18.58	88.70	1.65	MLL-1b
HW.14.14	Root	-19.87	91.54	2.79	MLL-1b
HW.14.15	Root	-18.60	88.74	2.48	MLL-1b
HW.14.16	Root	-20.14	92.14	2.16	MLL-7b
HW.14.17	Root	-18.52	88.57	1.68	MLL-2b
HW.14.18	Root	-19.78	91.35	1.60	MLL-6b
HW.14.19	Root	-20.64	93.24	2.19	MLL-8b
HW.14.20	Root	-19.15	89.96	2.92	MLL-8b
HW.14.21	Root	-19.54	90.82	2.05	MLL-2b
HW.14.22	Root	-18.01	87.44	2.76	MLL-3b
HW.14.23	Root	-16.16	83.37	1.68	MLL-6b
HW.14.24	Root	-18.48	88.48	1.52	MLL-6b
HW.14.27	Root	-19.61	90.97	1.00	MLL-1b
HW.14.28	Root	-19.51	90.75	2.90	MLL-1b
HW.14.29	Root	-18.46	88.44	0.90	MLL-6b
HW.14.30	Root	-18.99	89.60	0.63	MLL-6b
HW.14.31	Root	-19.00	89.63	1.48	MLL-1b
HW.14.32	Root	-17.27	85.81	1.20	MLL-1b
HW.14.33	Root	-18.84	89.27	2.99	MLL-9b
HW.14.34	Root	-15.70	82.35	1.84	MLL-1b
HW.14.35	Root	-17.98	87.38	3.10	MLL-1b
HW.14.37	Root	-18.31	88.11	0.82	MLL-2b
HW.14.39	Root	-19.42	90.55	1.45	MLL-1b
HW.14.42	Root	-18.83	89.25	2.40	MLL-2b
HW.14.43	Root	-16.17	83.39	2.64	MLL-2b
HW.14.44	Root	-16.24	83.54	1.11	MLL-2b
HW.14.45	Root	-18.23	87.93	2.94	MLL-1b
HW.14.46	Root	-16.02	83.06	0.97	MLL-2b
HW.14.47	Root	-17.39	86.08	3.54	MLL-1b
HW.14.48	Root	-19.19	90.05	1.49	MLL-2b
HW.14.01	Rhizome	-7.60	64.50	0.54	MLL-1b
HW.14.02	Rhizome	-15.10	81.03	1.76	MLL-2b
HW.14.03	Rhizome	-11.97	74.13	1.14	MLL-3b
HW.14.05	Rhizome	-9.87	69.50	0.62	MLL-1b
HW.14.06	Rhizome	-8.47	66.42	0.74	MLL-1b
HW.14.07	Rhizome	-11.96	74.11	0.76	MLL-4b

HW.14.08	Rhizome	-7.07	63.33	0.81	MLL-2b
HW.14.09	Rhizome	-7.91	65.18	0.69	MLL-5b
HW.14.11	Rhizome	-10.53	70.96	0.55	MLL-1b
HW.14.12	Rhizome	-10.15	70.12	1.03	MLL-6b
HW.14.13	Rhizome	-4.76	58.24	0.65	MLL-1b
HW.14.14	Rhizome	-12.56	75.43	0.73	MLL-1b
HW.14.15	Rhizome	-10.98	71.95	0.73	MLL-1b
HW.14.16	Rhizome	-10.72	71.38	0.74	MLL-7b
HW.14.17	Rhizome	-12.4	75.08	0.85	MLL-2b
HW.14.18	Rhizome	-13.55	77.61	0.87	MLL-6b
HW.14.19	Rhizome	-9.62	68.95	0.74	MLL-8b
HW.14.20	Rhizome	-13.82	78.21	0.87	MLL-8b
HW.14.21	Rhizome	-14.70	80.15	0.92	MLL-2b
HW.14.22	Rhizome	-9.72	69.17	1.65	MLL-3b
HW.14.23	Rhizome	-11.13	72.28	1.25	MLL-6b
HW.14.24	Rhizome	-8.29	66.02	0.99	MLL-6b
HW.14.27	Rhizome	-10.89	71.75	0.46	MLL-1b
HW.14.28	Rhizome	-10.50	70.89	0.84	MLL-1b
HW.14.29	Rhizome	-13.00	76.40	0.60	MLL-6b
HW.14.30	Rhizome	-13.12	76.67	0.53	MLL-6b
HW.14.31	Rhizome	-13.4	77.28	0.77	MLL-1b
HW.14.32	Rhizome	-12.00	74.20	1.03	MLL-1b
HW.14.33	Rhizome	-7.29	63.81	0.54	MLL-9b
HW.14.34	Rhizome	-11.55	73.20	0.64	MLL-1b
HW.14.35	Rhizome	-12.08	74.37	0.78	MLL-1b
HW.14.37	Rhizome	-12.62	75.56	0.58	MLL-2b
HW.14.39	Rhizome	-11.54	73.18	0.75	MLL-1b
HW.14.42	Rhizome	-13.74	78.03	0.50	MLL-2b
HW.14.43	Rhizome	-10.90	71.77	0.90	MLL-2b
HW.14.44	Rhizome	-10.72	71.38	0.52	MLL-2b
HW.14.45	Rhizome	-12.33	74.92	0.64	MLL-1b
HW.14.46	Rhizome	-13.77	78.10	0.79	MLL-2b
HW.14.47	Rhizome	-14.06	78.74	0.84	MLL-1b
HW.14.48	Rhizome	-12.55	75.41	0.73	MLL-2b
HW.14.01	Leaf	-0.61	49.09	0.56	MLL-1b
HW.14.02	Leaf	-1.00	49.95	0.52	MLL-2b
HW.14.03	Leaf	0.76	46.07	0.65	MLL-3b
HW.14.05	Leaf	0.56	46.51	0.53	MLL-1b
HW.14.06	Leaf	2.16	42.98	0.58	MLL-1b
HW.14.07	Leaf	-5.53	59.93	0.70	MLL-4b
HW.14.08	Leaf	1.57	44.28	0.50	MLL-2b
HW.14.09	Leaf	1.38	44.70	0.71	MLL-5b
HW.14.11	Leaf	-6.67	62.45	0.68	MLL-1b
HW.14.12	Leaf	-2.95	54.25	0.59	MLL-6b
HW.14.13	Leaf	2.48	42.28	0.64	MLL-1b
HW.14.14	Leaf	-5.52	59.91	0.96	MLL-1b
HW.14.15	Leaf	-2.27	52.75	0.78	MLL-1b
HW.14.16	Leaf	-0.09	47.94	0.59	MLL-7b
HW.14.17	Leaf	2.05	43.23	0.61	MLL-2b
HW.14.18	Leaf	-5.67	60.24	0.72	MLL-6b
HW.14.19	Leaf	-1.51	51.07	0.74	MLL-8b
HW.14.20	Leaf	-7.40	64.06	1.02	MLL-8b
HW.14.21	Leaf	-5.03	58.83	0.70	MLL-2b
HW.14.22	Leaf	-2.19	52.57	0.73	MLL-3b
HW.14.23	Leaf	-5.33	59.49	0.88	MLL-6b

HW.14.24	Leaf	-0.49	48.83	0.66	MLL-6b
HW.14.27	Leaf	0.19	47.33	0.45	MLL-1b
HW.14.28	Leaf	-1.47	50.99	0.46	MLL-1b
HW.14.29	Leaf	-3.87	56.28	0.43	MLL-6b
HW.14.30	Leaf	-6.28	61.59	0.63	MLL-6b
HW.14.31	Leaf	-5.12	59.03	0.52	MLL-1b
HW.14.32	Leaf	-4.07	56.72	0.68	MLL-1b
HW.14.33	Leaf	-1.76	51.62	0.55	MLL-9b
HW.14.34	Leaf	-6.67	62.45	0.53	MLL-1b
HW.14.35	Leaf	-6.50	62.07	0.43	MLL-1b
HW.14.37	Leaf	-3.60	55.68	0.54	MLL-2b
HW.14.39	Leaf	-4.47	57.60	0.61	MLL-1b
HW.14.42	Leaf	-1.63	51.34	0.55	MLL-2b
HW.14.43	Leaf	-3.83	56.19	0.58	MLL-2b
HW.14.44	Leaf	-5.71	60.33	0.66	MLL-2b
HW.14.45	Leaf	-7.18	63.57	0.76	MLL-1b
HW.14.46	Leaf	-7.11	63.42	0.72	MLL-2b
HW.14.47	Leaf	-8.74	67.01	1.01	MLL-1b
HW.14.48	Leaf	-2.62	53.52	0.76	MLL-2b

APPENDIX C

$\delta^{34}S$, $F_{SULFIDE}$, AND TS VALUES FOR *H. WRIGHTII* FROM OSO BAY

Sample ID	Tissue	$\delta^{34}S$ (‰)	$F_{sulfide}$ (%)	TS (% dw)	Genotype
HW.11B.01	Root	1.87	39.68	0.07	MLL-8c
HW.11B.02	Root	-0.04	43.62	0.56	MLL-4c
HW.11B.03	Root	-1.38	46.38	0.73	MLL-1c
HW.11B.04	Root	-5.41	54.69	0.66	MLL-2c
HW.11B.05	Root	-3.89	51.56	0.59	MLL-7c
HW.11B.06	Root	-3.64	51.04	0.24	MLL-1c
HW.11B.07	Root	-5.69	55.27	0.19	MLL-8c
HW.11B.08	Root	-2.31	48.30	0.18	MLL-2c
HW.11B.09	Root	-6.36	56.65	0.17	MLL-2c
HW.11B.10	Root	-10.55	65.29	0.52	MLL-3c
HW.11B.11	Root	-6.39	56.71	0.58	MLL-6c
HW.11B.12	Root	-5.12	54.09	0.33	MLL-1c
HW.11B.13	Root	-5.54	54.96	0.35	MLL-8c
HW.11B.14	Root	-10.65	65.50	0.44	MLL-1c
HW.11B.15	Root	-7.91	59.85	0.59	MLL-10c
HW.11B.16	Root	-8.92	61.93	1.03	MLL-1c
HW.11B.17	Root	-4.28	52.36	0.36	MLL-10c
HW.11B.18	Root	-10.62	65.44	0.55	MLL-8c
HW.11B.19	Root	-15.8	76.12	0.82	MLL-1c
HW.11B.20	Root	-1.86	47.37	0.36	MLL-1c
HW.11B.21	Root	-6.86	57.68	0.34	MLL-7c
HW.11B.22	Root	-9.35	62.82	0.23	MLL-9c
HW.11B.23	Root	-0.09	43.72	0.49	MLL-2c
HW.11B.24	Root	-6.81	57.58	0.57	MLL-1c
HW.11B.25	Root	0.30	42.92	0.80	MLL-4c
HW.11B.26	Root	-5.84	55.58	0.93	MLL-1c
HW.11B.27	Root	-9.65	63.44	0.59	MLL-8c
HW.11B.28	Root	-6.98	57.93	0.96	MLL-2c
HW.11B.29	Root	-6.65	57.25	0.77	MLL-10c
HW.11B.30	Root	-2.78	49.27	0.46	MLL-2c
HW.11B.31	Root	-5.30	54.46	0.37	MLL-1c
HW.11B.32	Root	-1.25	46.11	0.51	MLL-4c
HW.11B.33	Root	-10.22	64.61	0.91	MLL-1c
HW.11B.34	Root	-8.91	61.91	0.64	MLL-1c
HW.11B.35	Root	-10.79	65.79	0.61	MLL-7c
HW.11B.36	Root	-3.11	49.95	0.74	MLL-4c
HW.11B.37	Root	-4.93	53.70	0.83	MLL-1c
HW.11B.38	Root	-4.58	52.98	0.56	MLL-1c
HW.11B.39	Root	-7.72	59.46	0.94	MLL-4c
HW.11B.40	Root	-7.99	60.01	0.57	MLL-10c
HW.11B.41	Root	-0.24	44.03	0.56	MLL-2c
HW.11B.42	Root	-12.26	68.82	0.88	MLL-3c
HW.11B.43	Root	-2.70	49.10	0.52	MLL-1c
HW.11B.44	Root	-2.66	49.02	0.39	MLL-5c
HW.11B.45	Root	-1.21	46.03	0.41	MLL-4c
HW.11B.46	Root	-5.51	54.90	0.60	MLL-1c
HW.11B.47	Root	-4.38	52.57	0.65	MLL-9c
HW.11B.48	Root	-4.64	53.10	0.33	MLL-2c
HW.11B.01	Rhizome	4.68	33.88	0.50	MLL-8c

HW.11B.02	Rhizome	10.21	22.48	0.37	MLL-4c
HW.11B.03	Rhizome	7.49	28.09	0.61	MLL-1c
HW.11B.04	Rhizome	5.87	31.43	0.67	MLL-2c
HW.11B.05	Rhizome	11.65	19.51	0.53	MLL-7c
HW.11B.06	Rhizome	10.59	21.70	0.44	MLL-1c
HW.11B.07	Rhizome	6.20	30.75	0.21	MLL-8c
HW.11B.08	Rhizome	6.09	30.98	0.42	MLL-2c
HW.11B.09	Rhizome	5.07	33.08	0.31	MLL-2c
HW.11B.10	Rhizome	4.18	34.91	0.51	MLL-3c
HW.11B.11	Rhizome	8.99	24.99	0.24	MLL-6c
HW.11B.12	Rhizome	6.93	29.24	0.20	MLL-1c
HW.11B.13	Rhizome	6.42	30.29	0.14	MLL-8c
HW.11B.14	Rhizome	5.00	33.22	0.16	MLL-1c
HW.11B.15	Rhizome	6.74	29.63	0.19	MLL-10c
HW.11B.16	Rhizome	0.46	42.59	0.35	MLL-1c
HW.11B.17	Rhizome	5.60	31.99	0.38	MLL-10c
HW.11B.18	Rhizome	6.35	30.44	0.52	MLL-8c
HW.11B.19	Rhizome	0.17	43.18	0.58	MLL-1c
HW.11B.20	Rhizome	9.67	23.59	0.43	MLL-1c
HW.11B.21	Rhizome	6.06	31.04	0.50	MLL-7c
HW.11B.22	Rhizome	3.09	37.16	0.49	MLL-9c
HW.11B.23	Rhizome	0.42	42.67	0.50	MLL-2c
HW.11B.24	Rhizome	10.85	21.16	0.45	MLL-1c
HW.11B.25	Rhizome	7.55	27.96	0.42	MLL-4c
HW.11B.26	Rhizome	1.73	39.97	0.74	MLL-1c
HW.11B.27	Rhizome	5.15	32.91	0.73	MLL-8c
HW.11B.28	Rhizome	-0.22	43.99	0.99	MLL-2c
HW.11B.29	Rhizome	4.44	34.38	0.60	MLL-10c
HW.11B.30	Rhizome	8.94	25.10	0.57	MLL-2c
HW.11B.31	Rhizome	11.62	19.57	0.40	MLL-1c
HW.11B.32	Rhizome	11.66	19.49	0.47	MLL-4c
HW.11B.33	Rhizome	4.51	34.23	0.64	MLL-1c
HW.11B.34	Rhizome	1.59	40.26	0.51	MLL-1c
HW.11B.35	Rhizome	2.68	38.01	0.64	MLL-7c
HW.11B.36	Rhizome	0.62	42.26	0.58	MLL-4c
HW.11B.37	Rhizome	4.83	33.57	0.77	MLL-1c
HW.11B.38	Rhizome	4.50	34.25	0.60	MLL-1c
HW.11B.39	Rhizome	1.92	39.58	0.73	MLL-4c
HW.11B.40	Rhizome	0.99	41.49	0.65	MLL-10c
HW.11B.41	Rhizome	7.24	28.60	0.44	MLL-2c
HW.11B.42	Rhizome	3.50	36.32	0.59	MLL-3c
HW.11B.43	Rhizome	9.85	23.22	0.46	MLL-1c
HW.11B.44	Rhizome	8.42	26.17	0.25	MLL-5c
HW.11B.45	Rhizome	7.54	27.99	0.42	MLL-4c
HW.11B.46	Rhizome	1.09	41.29	0.70	MLL-1c
HW.11B.47	Rhizome	8.88	25.22	0.70	MLL-9c
HW.11B.48	Rhizome	6.96	29.18	0.38	MLL-2c
HW.11B.01	Leaf	11.43	19.96	0.40	MLL-8c
HW.11B.02	Leaf	15.01	12.58	0.38	MLL-4c
HW.11B.03	Leaf	15.15	12.29	0.56	MLL-1c
HW.11B.04	Leaf	13.65	15.38	0.57	MLL-2c
HW.11B.05	Leaf	16.35	9.82	0.64	MLL-7c
HW.11B.06	Leaf	14.74	13.14	0.42	MLL-1c
HW.11B.07	Leaf	13.28	16.15	0.63	MLL-8c
HW.11B.08	Leaf	14.39	13.86	0.45	MLL-2c

HW.11B.09	Leaf	14.17	14.31	0.39	MLL-2c
HW.11B.10	Leaf	13.01	16.70	0.40	MLL-3c
HW.11B.11	Leaf	14.94	12.72	0.51	MLL-6c
HW.11B.12	Leaf	14.34	13.96	0.42	MLL-1c
HW.11B.13	Leaf	14.09	14.48	0.37	MLL-8c
HW.11B.14	Leaf	11.87	19.06	0.18	MLL-1c
HW.11B.15	Leaf	15.00	12.60	0.39	MLL-10c
HW.11B.16	Leaf	-2.91	49.54	0.96	MLL-1c
HW.11B.17	Leaf	14.10	14.46	0.58	MLL-10c
HW.11B.18	Leaf	14.10	14.46	0.47	MLL-8c
HW.11B.19	Leaf	11.10	20.64	0.60	MLL-1c
HW.11B.20	Leaf	14.53	13.57	0.53	MLL-1c
HW.11B.21	Leaf	14.83	12.95	0.58	MLL-7c
HW.11B.22	Leaf	11.39	20.05	0.43	MLL-9c
HW.11B.23	Leaf	14.52	13.59	0.60	MLL-2c
HW.11B.24	Leaf	14.19	14.27	0.42	MLL-1c
HW.11B.25	Leaf	16.03	10.48	0.64	MLL-4c
HW.11B.26	Leaf	13.16	16.40	0.77	MLL-1c
HW.11B.27	Leaf	14.02	14.62	0.56	MLL-8c
HW.11B.28	Leaf	14.14	14.37	0.64	MLL-2c
HW.11B.29	Leaf	11.27	20.29	0.55	MLL-10c
HW.11B.30	Leaf	16.32	9.88	0.61	MLL-2c
HW.11B.31	Leaf	14.92	12.77	0.59	MLL-1c
HW.11B.32	Leaf	12.78	17.18	0.50	MLL-4c
HW.11B.33	Leaf	6.44	30.25	0.57	MLL-1c
HW.11B.34	Leaf	15.04	12.52	0.71	MLL-1c
HW.11B.35	Leaf	14.36	13.92	0.68	MLL-7c
HW.11B.36	Leaf	14.79	13.03	0.51	MLL-4c
HW.11B.37	Leaf	11.53	19.76	0.59	MLL-1c
HW.11B.38	Leaf	7.28	28.52	0.78	MLL-1c
HW.11B.39	Leaf	12.10	18.58	0.53	MLL-4c
HW.11B.40	Leaf	14.20	14.25	0.61	MLL-10c
HW.11B.41	Leaf	15.18	12.23	0.54	MLL-2c
HW.11B.42	Leaf	10.92	21.01	0.57	MLL-3c
HW.11B.43	Leaf	15.71	11.14	0.70	MLL-1c
HW.11B.44	Leaf	11.14	20.56	0.30	MLL-5c
HW.11B.45	Leaf	14.84	12.93	0.63	MLL-4c
HW.11B.46	Leaf	12.83	17.08	0.74	MLL-1c
HW.11B.47	Leaf	14.04	14.58	0.58	MLL-9c
HW.11B.48	Leaf	15.22	12.15	0.64	MLL-2c