Tolerances and responses of seagrasses to hydrogen sulfide and implications to ecology and restoration

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Abstract

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Predicting an individual or species response to climate change, ecosystem disruptions or any environmental change is highly problematic and often requires assumptions. Currently *Zostera marina* (eelgrass) and other seagrasses have undergone extensive changes within their ecosystems much of which has effected there population size and fecundity. In the Pacific Northwest (PNW) changes have occurred within the ecosystems since the industrial revolution, and correspondingly reductions in the meadows have been observed. Many of these changes are anthropogenic in nature. Bio-matter accumulation, increases in temperature and prevalence of disease are only a few of many factors influencing survival. In the late 1990's and early 2000's Westcott bay, a typical eelgrass habitat, abruptly went extinct. Many hypotheses were developed in hopes of explaining the disappearance, and restoration efforts tried yet failed to re-establish a viable population in the bay.

In 2007 I started evaluating *Z. marina* response to different environmental conditions and as I transitioned into my graduate career I used these and other experiments to explain in part the

reductions and extinctions observed. Here, I report on the tolerances of seagrasses found in the PNW to hydrogen sulfide (H_2S), its physiological interactions, and implication to restoration and seed banking. Additionally, I report on the concentrations of H_2S/sulfide found in embayments throughout the San Juan archipelago and surrounding areas within the Salish Sea (PNW).

Based on laboratory experiments I found that eelgrass is highly vulnerable to low concentrations of H_2S (LD_{50} = 334µM). More specifically it was determined that the H_2S decreased photosynthetic output and inhibited photosystem II. No reduction in respiration was detected until ~10mM. This is also the case with *Phyllospadix scouleri*, however based on its physiology and adaptions for a rockier habitat the lethal limit was significantly lower (LD_{50} = 86µM). Field observations indicate that concentrations vary dramatically based on site location, sediment type and biomatter accumulation, but is highly correlated with eelgrass presence (or absence). And in cases when there are no shoots found concentrations are higher than those observed to be lethal. Sites experiencing high concentrations of sulfide(s) may be a candidate for restoration: capping. In populations which are undergoing reductions seed storage may be necessary, however it is highly problematic, and I report that seed viability decreases with each year of storage and plantation may not be plausible after 2 years.

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DEDICATION

To my fellow 12's!

Chapter 1

Conceptual Framework:

Understanding ecological processes and predicting the response of an individual or species to climate change presents a major challenge in ecology (Long et al. 2004). Here I use physiological experiments and in the field measurements to elicit an understanding of how eelgrass (*Zostera marina*; figure 1.1), an ecological and financial important species, responds to changes within the ecosystem (Kenworthy et al., 2006), and more specifically how it and other seagrasses respond to hydrogen sulfide.

Currently, *Z. marina* grows in sub-arctic, temperate and sub-tropical environments in the northern hemisphere (Short and Coles, 2001; Wyllie-Echeverria and Ackerman, 2003), and the genus has been present since the Cretaceous (McCoy and Heck, 1976; Phillips and Menez, 1988). These sites are colonized by the combined strategy of creeping rhizomes and seed dispersal that result in large contiguous underwater "prairies" which harbor a large diversity of other organisms (Moore and Short, 2006). Seagrass beds are found in sediments which are often anoxic and rich in sulfide compounds (Pedersen et al. 2004; Frederiksen et al. 2006; Mascaro et al., 2009). *Z. marina*, like many other seagrasses, are important to a wide variety of animals as food or shelter during some part of their life cycle (Moore and Short, 2006; Dooley et al., 2013a). However, seagrass beds have experienced declines and local extinctions (Short and Wyllie-Echeverria 1996, Dooley et al. 2013a,b). Understanding why these seagrasses have undergone these reductions may be key to restoration efforts. Often this involves deciphering the specific kill mechanism within the individual and across the community.

While these kill mechanisms are almost never caused by a single factor, and in fact a variety of environmental changes can result in a similar physiological mechanism, just as any single environmental stimulus can elicit multiple physiological responses. Myriad observations from around the world have documented what appears to be substantial mortality in many different seagrass species (Short and Wyllie-Echeverria, 1996; Waycott et al., 2009), yet elucidation of the kill mechanisms themselves remain often ad hoc. Proposed kill mechanisms include: disease (Bull et al. 2012); temperature (Hoffle et al. 2011; Reusch et al. 2008); ocean acidification (Hendriks et al. 2013); changes in salinity (Philips 1972) and hydrogen sulfide (H_2S) (Dooley et al. 2013b,c). Here, I demonstrate through a series of experiments designed to test hypothesis in which I use to elude a potential kill mechanism (the physiologically disruptive process that causes death) in Puget Sound seagrass.

Early in my graduate carrier I attempted to identify plausible reasoning for the declines observed in one bay (Westcott, San Juan Island, WA, USA). Like other bays, Westcott seagrass went extinct rapidly at the turn of the 21st century. Plausible explanations involved changes in temperature; salinity; CO_2; metals; disease and sulfide(s) (figure 1.2). Changes in CO_2, metals and salinity were less supported based on the known literature and field testing. While wasting disease (*Labyrinthula spp*.) has been observed, more recently in other meadows throughout the San Juans, its role in extinction is unknown. Currently in Fisherman Bay (Lopez Island, WA, USA) wasting disease is of high prevalence and the meadow is experiencing a decline in density (as well as increases in sulfides; see chapter 3). The reduction of these variables allowed me to focus my study on two variables: temperature and H_2S (figure 1.3).

Using a hobo sensor we recorded the water temperature(s) in Picnic Cove (Shaw Island, WA, USA) over a year (figure 1.4; imbedded). We determined that temperature does fluctuate in

local bays over the course of a year and on can reach 30°C. To evaluate the effects of temperature on seeds, seedlings and mature plants, we collected seeds and performed a germination trial. Once germinated we grew seedlings and adult shoots under various temperatures. I determined that while high temperatures (figure 1.4), which do occur in the bays, can kill *Z. marina* it is unlikely to do so based on the short interval in which the individual plants would experience the high temperature. Lethality only occurs after a prolonged period of exposure. This experiment provided the frame work for all the following future experiments.

Based on the above observations and the results from my initial study I decided that the next plausible hypothesis which could be tested was on H_2S toxicity.

Increases in H_2S can be caused by several factors, biotic (decomposing biomass – algae blooms) to abiotic (hydrothermal vents). Human activities, such as increasing organic and nutrient loading, have provided conditions in which H_2S production is increased (Short and Burdick 1996; Kamp-Nielsen et al. 2001; Halun et al. 2002). Indications, based on field studies in Commencement Bay in Puget Sound (Elliott et al., 2006; and our sampling [ch. 3]), have suggested that hydrogen sulfide concentrations may control *Z. marina* expansion and re-colonization in these regions (Goodman et al. 1995; Holmer and Bondgaard, 2001; Plus et al. 2003; Pedersen et al., 2004). Additionally, new research into past mass extinctions coincide with increased sulfur loads in most marine systems (Berner and Ward, 2006; Ward, 2006).

The toxicity of hydrogen sulfide (H_2S) has been studied for over 200 years in animal systems (Lloyd, 2006) and recently toxicity in seagrasses is being studied. It is known that H_2S and sulfides causes an array of physiological responses, and this model organism (eelgrass) has morphological modifications to the plant to help prevent sulfide toxicity (Penhale and Wetzel, 1983). This plant forms aerenchyma (fig. 1.5), which transport oxygen to the roots when the

plant is photosynthesizing (Mascaro et al., 2009), which in turn reacts with H_2S to produce SO_4^{2-} and H_2O; thus diminishing the negative effects of H_2S in the rhizosphere (Pedersen et al., 2004; Koch et al., 2007). Still, seedlings still may be impacted. Seedling rhizomes are small when compared to that of mature plants, photosynthetic capacity may be limited, and seedlings may be less resilient, possibly making this a critical stage in re-establishment (e.g., Plus et al., 2003). **Prediction:** *Z. marina* will experience lethality and senescence at H_2S concentrations equal to or lower than those found in locations where seagrass is declining or locally extinct. Just as importantly one could assume that a species of seagrass that does not normally live in an environment which may experience these conditions will not have as strong of resilience to H_2S. *Phyllospadix scouleri* is another common species of seagrass in the PNW. *P. scouleri* is found in rockier intertidal embayments, therefore **Prediction**: *P.* scouleri is vulnerable to H_2S concentrations lower than that of *Z. marina*.

Understanding how H_2S causes senescence in eelgrass is still unknown. It is known that H_2S inhibits respiration in animals by binding to the cytochrome c complex in the electron transport chain held within the mitochondria. Lethal limits are known to be as low as 10µM. However, plants unlike animals have an additional component to its metabolism (photosynthesis). Photosynthesis in eelgrass is primarily conducted in the two photosystems. *Zostera marina* has both Photosystem I and II (Nakamura et al., 1976), with the relative concentration of these photosystems occurring at a ratio of 2.8, which is within the ratio range for higher plants/fresh water algae but not marine algae (Nakamura et al., 1976), but is typical of land-based monocots. The photosystems, distributed within the chloroplast, is made up of two basic units. Photosystem II (PSII), associated with the light harvesting complex P680 and the breaking of water, and Photosystem I (PSI), associated with P700 and cycles electrons. Between

the two systems there is an electron transport complex with proteins, very similar to that of respiration, that may be vulnerable to H_2S (figure 1.6) .

Research conducted by Holmer et al. (2001) suggest that respiration is inhibited in eelgrass with exposure to high levels of H_2S. Here I will explore the competing view that photosynthesis is more vulnerable, has a lower LD_{50}, then respiration. **Prediction**: H_2S is lethal to *Z. marina* because it inhibits photosystem II.

In setting up the experiments evaluation of many independent variables [media, exunicia, environmental conditions (temperature, light, and ph)] had to be considered due to the reactive nature of H_2S.

H_2S is known to react with many inorganic compounds and forms different ions under different pH, because of this a specific media, seawater with a select micronutrient formula, was selected and maintained at a pH close to the equilibrium coefficient especially during the manufacturing process. Specific details regarding this process and experimental methods are in the related chapters.

Here I report results in support of the above predictions, as well as other factors and conditions.

Figure 1.1

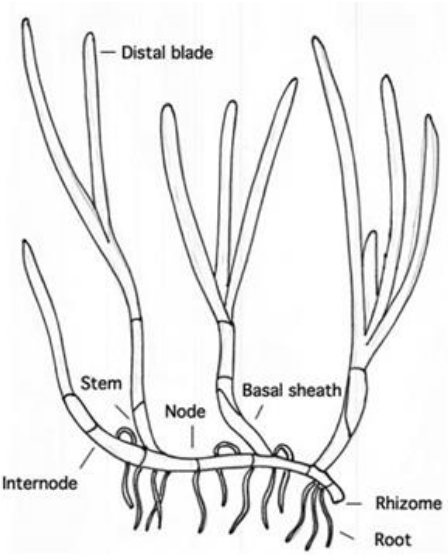


Figure 1.1: Diagram illustrating a typical eelgrass structure

Figure 1.2: seagrass interaction map

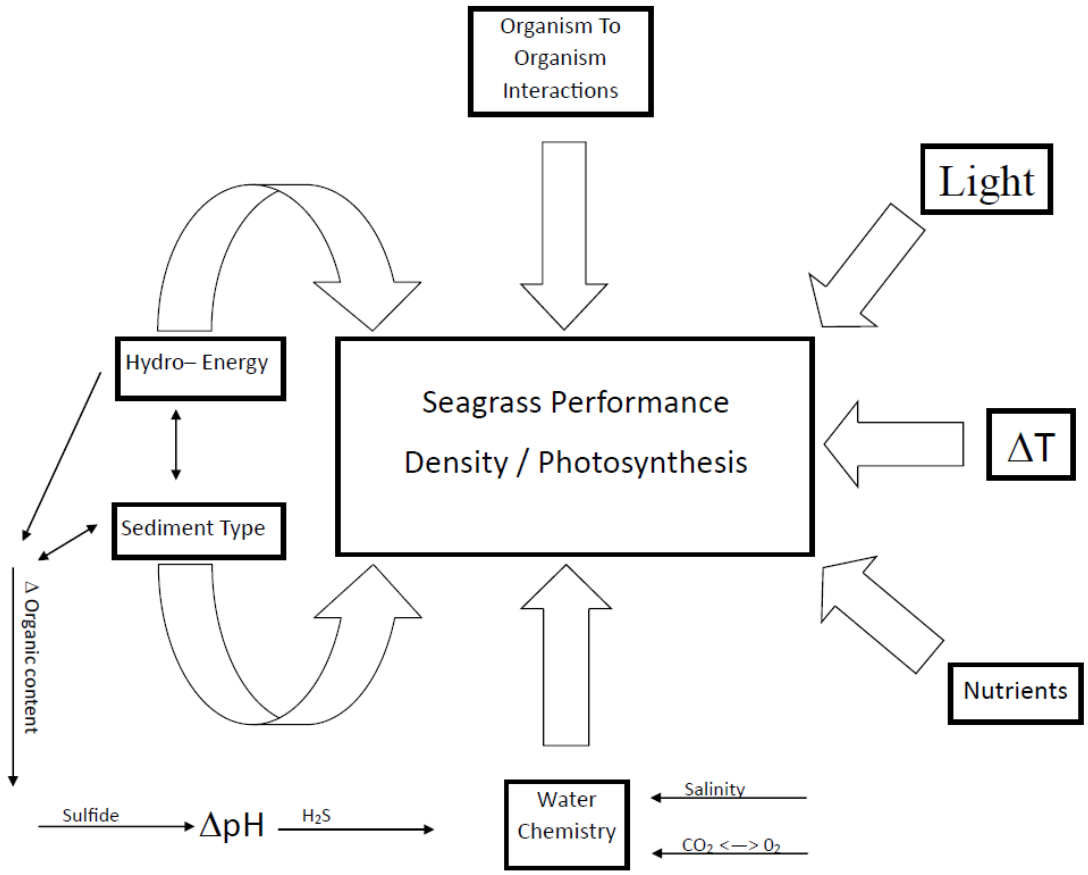


Figure1.2: this figure is a graphical representation of the many factors that affect seagrass performance as defined by density and/or photosynthesis. Each of these individual factors can and often interact with one another forming large complex interactive webs.

Figure 1.3: H_2S interaction diagram

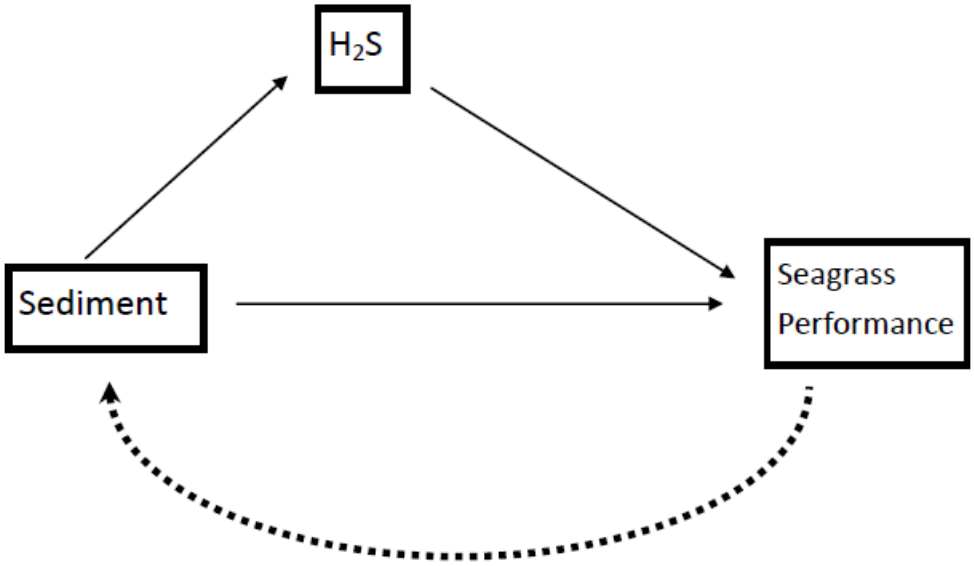


Figure 1.4: Percent survival of *Z. marina* at various temperatures, and temperature recordings at Picnic Cove

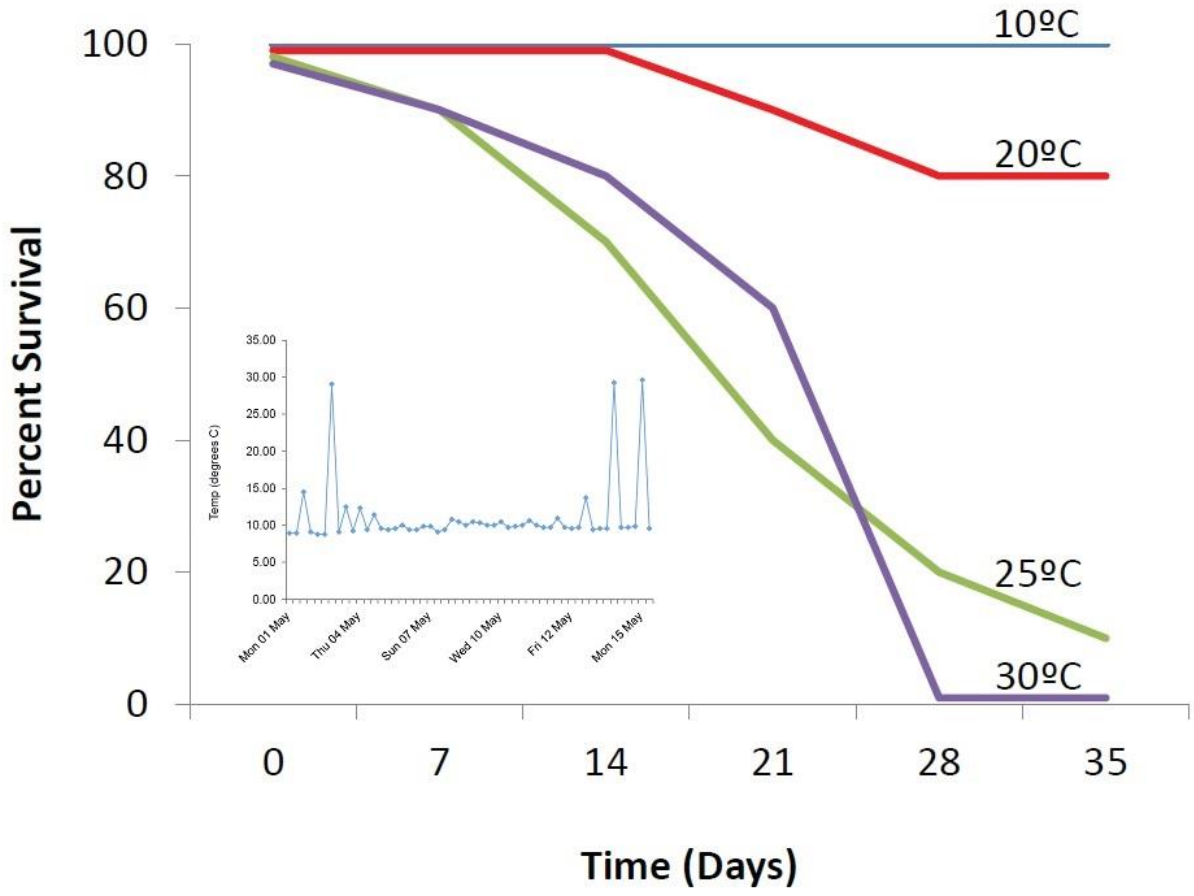


Figure 1.4: percent survival of Z. marina at various temperatures. Inside figure is temperature recordings at Picnic Cove, Washington, USA, throughout the year. Notice that 30°C is reached multiple times of the year, well beyond the normal 10°C average. Generally these high temperatures are recorded at low tide, and on hot days.

Figure 1.5: Cross section of *Z. marina*

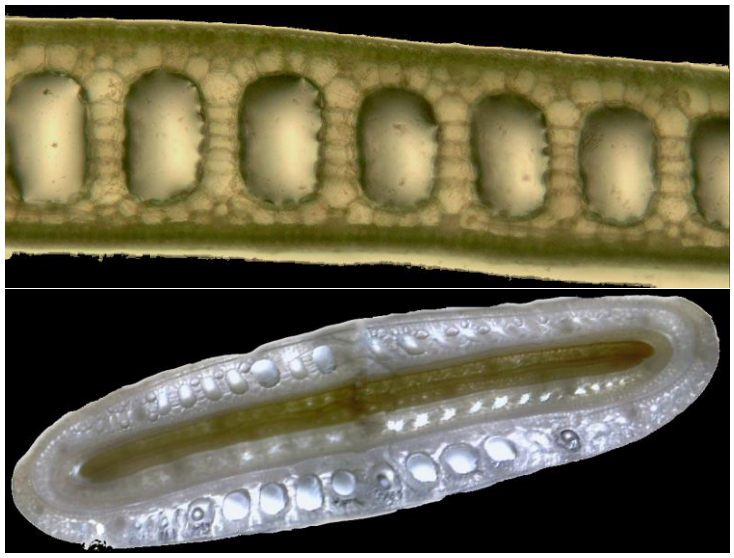
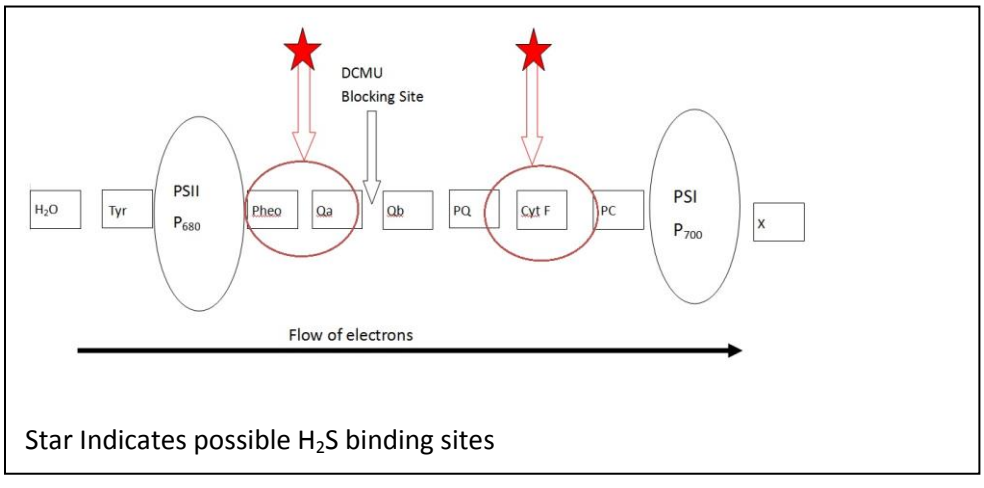


Fig. 1.5: *Z. marina* leaf with lacuna (top); stem, near meristem with aerenchyma (bottom)

Figure 1.6 Photosystems flow chart



Star Indicates possible H_2S binding sites

Chapter 2

Long-term Seed Storage and Viability of *Zostera marina*

Chapter 2 introduces seed and seedling populations of Zostera marina, there survival and possible implications to restoration. Portions of this chapter were originally published in the Journal of Aquatic Botany in collaboration of Sandy Wyllie-Echeverria and Elizabeth Van Volkenburgh. Dooley, F., Wyllie-Echeverria, S., Van Volkenburgh, E. 2013. Long-term seed storage and viability of Zostera marina. Aquatic Botany. 111: 130-134 © Elsevier and are reproduced with permission from Elsevier.

Abstract

Successful establishment of seedlings in populations of *Zostera marina* (eelgrass), especially for restoration efforts using stored seeds, depends in part on viability and germination of seeds. Seeds of *Z. marina* were collected from plants and stored in seawater at 5ºC for up to several years. Seed viability, assessed with the viability stain, tetrazolium chloride, decreased steadily over a four-year period. There was a strong correlation between age and viability; viability of fresh seeds was approximately 77% whereas for four-year old seeds was 32%. However, only 51 of 975 of fresh seeds that germinated (~5%) developed leaves. The physical structure of the seed was evaluated to understand the effects of aging. It was determined that as seeds age there is an increase in the number of fractures on the seed coat. These data combined with the recent awareness that global seagrass populations are declining present valuable information to help maintain viable seed repositories which may contribute to the conservation and restoration of these wild plants.

Key words

eelgrass, restoration, seagrass, seed storage, tetrazolium chloride

2.1. Introduction

Appropriate seed storage methods were, and still are, critical to providing an adequate supply of food for human beings and domestic animals (Biasutti owen, 1956; Hong and Ellis, 1996; Nabhan, 2009). Both short term storage from harvest to planting in the following year, and long term seed preservation are necessary to insure crop sustainability (Hong and Ellis, 1996). Those engaged in the restoration of important wild plant species also recognize the value of developing seed storage techniques (Young and Young, 1986; Guerrant et al., 2004). While there are general conditions such as temperature and moisture that must be considered to ensure seeds do not degrade, the response of seeds to a storage environment can vary widely among species (Hong and Ellis, 1996; Young and Young, 1986; Guerrant et al., 2004). The response of seeds to storage conditions must be evaluated on a species by species basis before a specific storage technique is recommended. Knowledge related to seed storage for wild plants becomes more important with the advance of seeding programs to restore the valuable habitat they provide (Guerrant et al., 2004).

The restoration of seagrasses or marine flowering plants into areas globally threatened by human and other activities (Short and Wyllie-Echeverria, 1996; Orth et al., 2006a; Waycott et al., 2009) now benefits from programs that deliver seed to the seafloor (reviewed in Orth et al., 2006b and Orth et al., 2012). Moreover, the collection, processing, short-term storage and

sowing of seeds from a single species is now an accepted restoration practice (Pickerell et al., 2005; Marion and Orth, 2010; Busch et al., 2010; Tanner et al., 2010). However while these techniques have clarified issues related to short-term storage (< 1 year) little is known about the viability of seeds over longer periods of time.

In earlier work, McMillan (1991) found, by measuring percent germination in the laboratory, that some portion of the seeds of *Halodule wrightii*, *H. univervis*, *Syringodium filiforme* and *Halophila engelmannii* were viable for between two and four years depending on the species. Additionally, Zipperle et al. (2009) found that *Zostera noltii* seeds persist in the wild up to three years, however this work has not been replicated and no other studies discuss the behavior of *Zostera marina* seeds in long-term storage treatments. This information may be a critical component of future restoration efforts (e.g. Walmsley and Davy, 1997; Liu and Spira, 2001).

The primary goal of our research was to determine the feasibility of long-term seed storage for *Zostera marina*. Objectives were first to determine seed viability, using both germination and viability staining, for seeds stored over several years, and second to evaluate seed coat behavior in long-term storage using SEM.

2.2. Materials and Methods

2.2.1 Seed Collection

Z. marina generative shoots were collected in the Fall of 2005 through 2010 from False Bay, San Juan Island, Washington State USA. Generative shoots were placed in outdoor tanks

serviced by flowing seawater at Friday Harbor Laboratories (FHL), University of Washington. Once released, seeds were collected by sieving tank water. Collected seeds were placed in 20 ml seawater in scintillation vials, in batches of 100, and stored in the dark at 5ºC until experiments were initiated. Seeds for experiments were randomly selected from stock and placed into treatments.

2.2.2 Seed Metrics

Each seed used in experiments described, plus 168 seeds haphazardly selected each year (2005-2011) from fresh seeds collected in the field were measured to determine seed size classes (Wyllie-Echeverria et al., 2003). To do this the length and width of each seed was measured to the nearest 0.05 cm. These measurements allowed us to determine seed weight using the model:

$Y = ax + b$

where Y = seed weight (mg), a = 2.01, x = cross-sectional area in mm^2 and b = -2.4 (Wyllie-Echeverria et al., 2003).

After seed weights were determined individual seeds were assigned to a small, medium or large size class (Wyllie-Echeverria et al., 2003).

2.2.3 Determination of Seed Viability by Staining

Seed viability was assessed by immersing seeds in a 1% (w : v) tetrazolium chloride (TTC) solution. The TTC test is an internationally accepted test to determine seed viability and

has been previously used with success in seagrasses (e.g. Conacher et al., 1994; Alexandre et al., 2006; Cabaço & Santos 2010). Using a sterile scalpel, a small incision was made in the seed coat to allow the TTC to enter. After absorbing TTC the embryo, if viable, undergoes a redox reaction changing color from white to reddish brown (Supplemental 1) during cellular respiration (Smith, 1951). When we initiated the study in 2008 fifty seeds from each year (2005-2008) were tested. This test was repeated in 2009, 2010 and 2011. Before immersion, length and width (to the nearest 0.05 mm) and weight (to the nearest 0.05 mg) of each seed were recorded (Wyllie-Echeverria et al., 2003). During TTC treatment, seeds were placed in the dark and held at 20ºC (Philips, 1972). Twenty-four and 48 hours later, the number of viable seeds (based on embryo color (Supplemental 1)) in each batch was recorded.

2.2.4 Determination of Seed Viability by Germination

On 14 February 2009 germination experiments were initiated with two replicate treatments (n=35 seeds in each treatment) for each year (2005-2008). Each seed was placed in a petri dish filled with 25ml, sterile seawater (10 PSU). Petri dishes were randomly distributed on a rack and kept in the dark in a climate-controlled room [10ºC, 1 ATM, ~ 50% relative humidity]. The development of each seed to seedling was monitored between 14 February and 1 June 2009. In addition, 975 fresh seeds were placed in uncapped, plastic test tubes serviced by fresh nutrient enriched seawater medium [$NaNO_3$ + Na_2HPO_4 + $MnCl_2.4H_2O$ + Ferric-Sodium EDTA + H_3BO_3 + HCl] (AC Churchill unpublished data) held at 15ºC. Seeds were allowed to germinate and grow to determine the relationship between seed viability and seedling development. For the purposes of this experiment, we defined germination as the stage when

hypocotyl extension occurs (see Churchill, 1992, and Supplemental 2). After the seed germinated it was monitored for the development of leaves.

2.2.5 Scanning Electron Microscope (SEM) Images of Stored Seeds

SEM images were taken of randomly selected seeds representing various years of storage; eleven seeds were scanned for 2011, ten for 2010, 2007 and 2006. Seeds from 2008 and 2009 were not available. The seeds were obtained from the same seed stock used in germination experiments. Each seed was individually prepared, mounted and scanned using the following standard procedure; (1) seeds were gently blotted with a Kimwipe until dry; (2) dry seeds were mounted with conductive tape to a specimen block; (3) seeds were coated with gold/palladium using a Sputter Coater; and (4) each seed was individually scanned using JCM-5000 NeoScope by JELO. Scanned seeds were compared to each other by counting the number of fractures in the seed coat, and by assigning a value using the classification chart (Table 3).

2.2.6 Statistical Analysis

Statistics were computed in R (R version 2.14.2). A chi-squared test was performed to investigate the different means. A GLM model was developed and one-way ANOVA was computed to analyze the seed fractures data and seed class differences.

2.3. Results

2.3.1 Seed size classes

Mean seed length, width and weight remained relatively constant from year to year (Table 2.1). Mean seed weight was more variable, especially when comparing 2005 weights to 2011 weights; however this difference was not statistically different. Except for 2005, seed size classes were also relatively constant (Table 2.2). In 2005 there were more large-sized seeds than the other two classes combined, which was significantly different from other years (χ^2 =213.29, df =12, P<0.001).

2.3.2 Determination of Seed Viability by Staining

Combined mean seed viability for 2005-2008 was 54.3% but with a large variation between years. Maximum viability was 77% for the year-old seeds. The viability of two-year-old seeds only dipped slightly to 71%. However after three years the viability decreased to 37%, and dropped to 31% in the fourth year. There was a strong and significant relationship between age and decrease in viability ((Fig. 2.1); χ^2=23.03; P<0.001). There was a positive correlation between seed size and viability. Medium and large seeds have a higher percent viability than the smaller seeds. This relationship holds true except in the case of year four when the large seeds may not, in fact, be large seeds but smaller ones that have bloated due to breakage in the seed coat (SEM images support this conjecture).

2.3.3 Determination of Seed Viability by Germination

Germination rates of *Z. marina* were consistent in trend but lower than viability rates. One-year-old seeds had the highest germination rate at 68% and dropped to 15 % after three years (χ^2=22.04, P<0.001) (Fig. 2.2). Hypocotyl extension followed the same trend, decreasing with each consecutive year (χ^2=25.3, P<0.001). These data demonstrate that although a seed may be viable, it still may not produce a seedling. There was a high frequency of embryonic abortion; of the 975 fresh seeds used in this study only 51, i.e. ~5% produced successful seedlings growing more than 10 cm. In older seeds, germination was often followed by fungal infection and embryonic failure.

2.3.4 Scanning Electron Microscope (SEM) Images of Stored Seeds

We determined that the *Z. marina* seed coats are formed from an epidermal layer of cells (Moèise et al. 2005) that forms multiple ridges and valleys around the seed (Fig. 2.3a). It appears as if seed-coat separation from the embryo during germination occurs along the ridges (Fig. 2.3a) (Anderson et al., pers. comm.). For the purposes of this study we examined the seed coat as a proxy for permeability of the seed coat structure over time. Results are listed in table 2.4. It was determined that young seeds had few if any fractures in the seed coat (Table 2.4; Fig. 3b). Fractures, when observed, occurred perpendicular to the ridges and occurred with more frequency as the seed aged (P=0.009; Table 2.4: Fig. 2.3c, d, e, f, g). Additionally, using the classification chart the difference between years, listed in Table 2.2, was highly significant (P <0.001).

2.4. Discussion

Although seagrasses take advantage of asexual growth to maintain extant meadows (Tomlinson, 1974), the seed rain carries propagules to suitable habitat adjacent to existing populations or more distant locations (Orth et al., 2006b). This reproductive strategy can contribute favorably to restoration programs. For example the collection, processing, short-term storage and sowing of *Z. marina* seed is now an accepted and successful restoration practice (Pickerell et al., 2005; Marion and Orth, 2010; Busch et al., 2010; Tanner et al., 2010). Techniques associated with this effort are now being used to develop protocols to restore other seagrass species using seeds (Dominiquez et al., 2010; Zarranz et al., 2010; Kishima et al., 2011). However, while this research describes procedures to store seeds effectively from collection to planting in the same year, except for early work by McMillan (1991), no information related to the behavior (expressed as percent viable) of seeds stored for a longer time period is available. Research related to seed behavior in long-term seed storage for terrestrial flowering plants describes the need to develop procedures to ensure long-term storage does not degrade seed condition (Hong and Ellis, 1996; Guerrant et al., 2004). Our work demonstrates that while *Z. marina* seeds appear to be viable during storage for at least three years, as observed for the natural seed-banks of *Z. noltii* (Zipperle et al. 2009), only 15% of these germinated. Further, viability is reduced after two years and only a fraction of those seeds will produce a seedling.

In this experiment we demonstrate that if each flowering shoot produces approximately 20 viable seeds (Wyllie-Echeverria et al., 2003), and only 5-10% of viable seeds produce a seedling (Harrison, 1993; Cabaco et al., 2010) then one-year old seeds will only produce ~1 seedling per flowering shoot [equation = 22(seeds per shoot)*0.78(viability) *0.075(average

viability to seedling ratio)], and by year three it will require multiple flowering shoots to produce one new seedling. We postulate that this viability reduction is linked to several different factors, and represent an important bottleneck in the species life-strategy. For example, it is known that seeds require a minimum amount of energy and hormones present to complete germination (Finch-Savage et al., 2006); however, if hormones or stored lipids, sugars and starches, are degraded or leached through fractures in the seed coat, as observed in SEM images, viability of older seeds could be compromised. In a pilot experiment we found that exposing older seeds to gibberellin (10^{-9} M) increased ($P<0.001$) the frequency of germination. SEM evaluation of seed coat integrity suggests that fractures in the coat as the seed ages result in embryonic death. While we lack information to describe the biochemical process forcing seed coat fracturing, one obvious line of inquiry would be to explore the relationship between various storage media and environments on seed viability over time.

The increase in projects to restore *Z. marina* using seeds was guided by a series of publications to assist practitioners with collection, short-term storage and sowing techniques (Pickerell et al., 2005; Orth et al., 2006c). These publications form the basis for an *operations manual* to continue *Z. marina* restoration projects and potentially direct program development for other seagrass species. Consequently we recommend the same process be considered here. That is, it seems wise to compile an operations manual that explains a step-wise procedure to prevent the degradation of seagrass seeds in long-term storage. Our recommendation is based in the awareness that the global seagrass decline is on the rise (Short and Wyllie-Echeverria, 1996; Orth et al., 2006a; Waycott et al., 2009) and viable seed repositories contribute to the conservation and restoration of wild plants (Nabhan, 1989).

Table 2.1: Mean Seed Size Metrics ± SE (n = 168 each year)

Year	Length (mm)	Width (mm)	Weight (mg)
2005	3.75 ± 0.18	1.71 ± 0.16	7.76 ± 1.28
2006	3.71 ± 0.18	1.63 ± 0.13	7.14 ± 0.96
2007	3.88 ± 0.23	1.62 ± 0.11	7.52 ± 1.02
2008	3.74 ± 0.15	1.65 ± 0.14	7.31 ± 0.94
2009	3.82 ± 0.19	1.61 ± 0.13	7.34 ± 0.86
2010	3.80 ± 0.18	1.59 ± 0.11	7.13 ± 0.90
2011	3.62 ± 0.23	1.61 ± 0.13	6.84 ± 1.14

Table 2.2: Seed Size Frequency Per Year (n = 168 each year)

Year	Small	Medium	Large
2005	5	78	85
2006	6	149	13
2007	4	137	27
2008	4	149	15
2009	3	152	13
2010	4	148	16
2011	18	134	16

Table 2.3: Seed Classification Chart Concerning the Integrity of the Seed Coat

1	2	3	4	5
Seed is intact, few to no fractures, ridges are continuous and sealed, germination is highly likely	Seed is mostly intact, few fractures, ridges are continuous and sealed, germination is likely	Seed is partially intact, few fractures, ridges are mostly continuous, germination is somewhat likely	Seed is highly fractured and not entirely intact, ridges are semi-continuous and germination is unlikely	Seed is not intact, multiple fractures, ridges are no longer continuous and germination is highly unlikely

Table 2.4: Summary of Seed classification, using Table 2.3, and number of fractures observed in seed coat (mean ± SD)

Year	Classification	Number of Fractures
2011	1.5 ± 0.7	1.8 ± 2.1
2010	1.3 ± 0.4	0.4 ± 0.9
2007	3.7 ± 1.2	2.6 ± 1.5
2006	3.2 ± 1.0	3.3 ± 2.8

Figure 2.1 Seed viabilty by year, determined by TTC-test (mean ± SD).

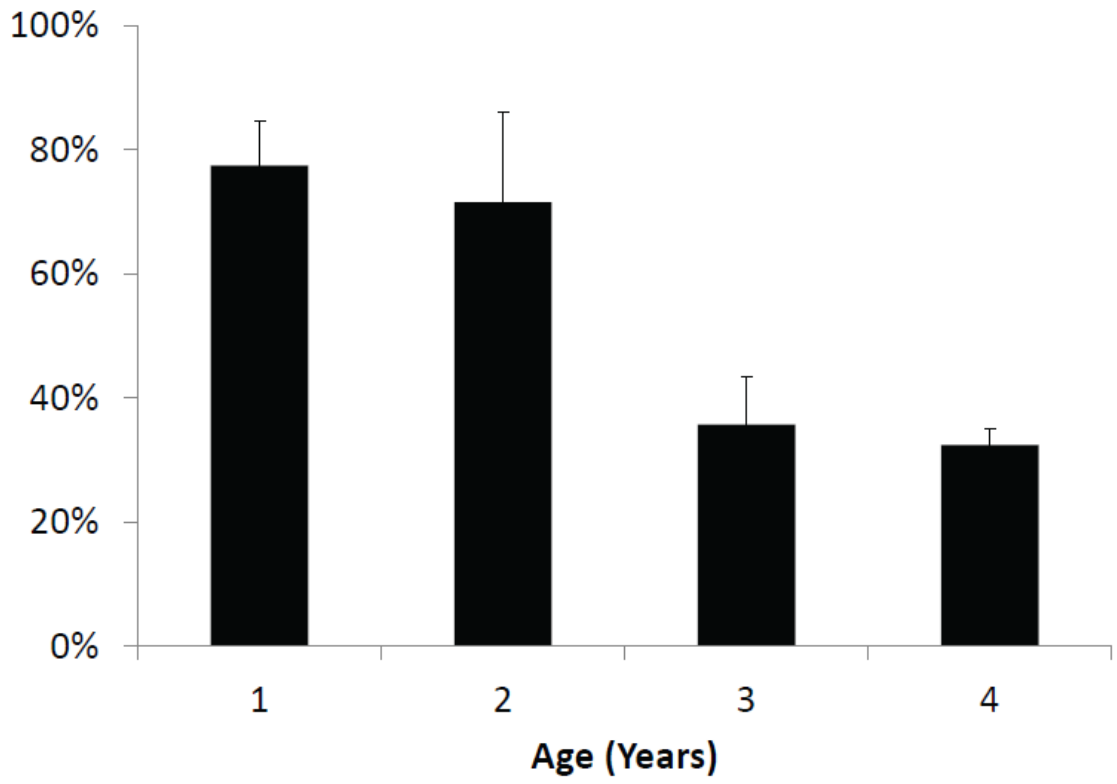


Figure 2.2 Data showing percent of sample showing each trait: germination, hypocotyl extension and fungal development over time.

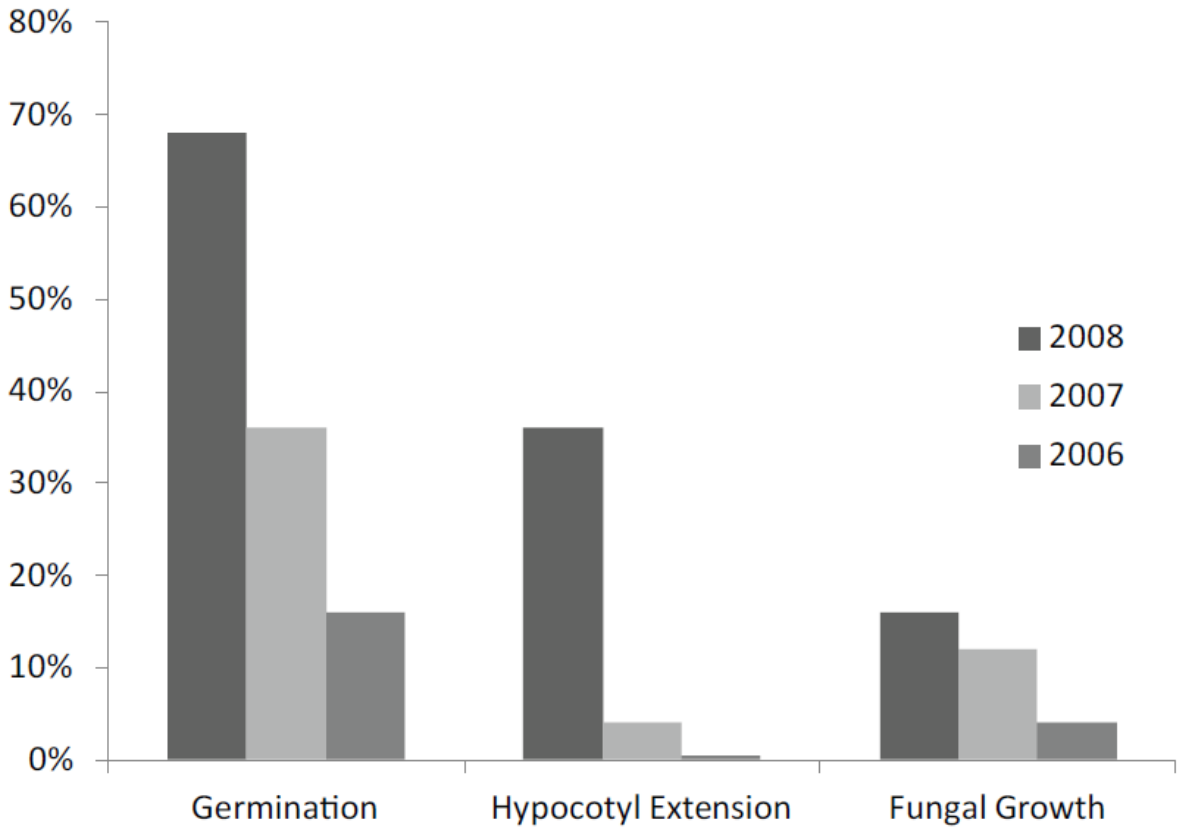


Figure 2.3a Separation of the seed coat from the embryo. Notice the separation occurs along the ridge.

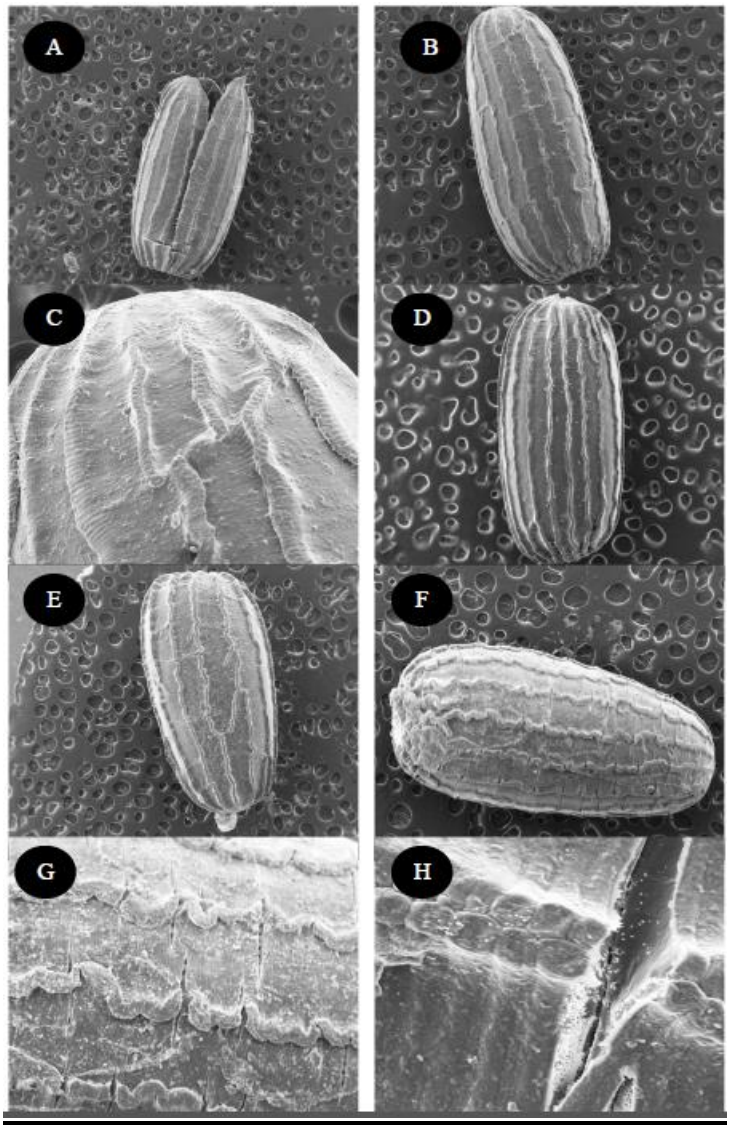
Figure 2.3b and c SEM image of a fresh seed (1 year old). Notice that the seed surface is made up of ridges and valleys. There are no observable cracks in the surface of the seed.

Figure 2.3d Two year-old seed. Notice that there are still few fractures within the seed coat however at the top of the seed the seed coat is opening.

Figure 2.3e Three year-old seed. Notice there are more fractures within the seed coat; and the embryo extends out through an opening at the end of the seed.

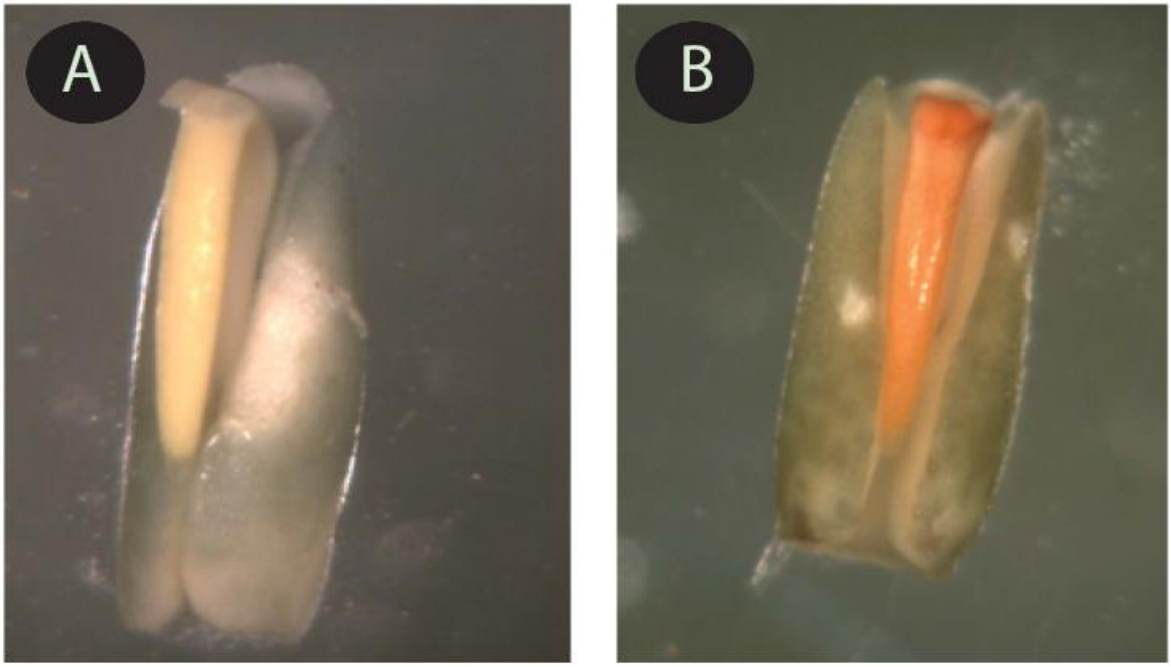
Figure 2.3f and g Four year-old seed. Notice that there are an abundant amount of fractures within the surface of the seed. The ridges are no longer continuous nor are they straight along the seed length.

Figure 2.3h SEM image of *Z. marina* seed at 1000x. This is a close up of a ridge along the seed with a fracture going through the ridge.

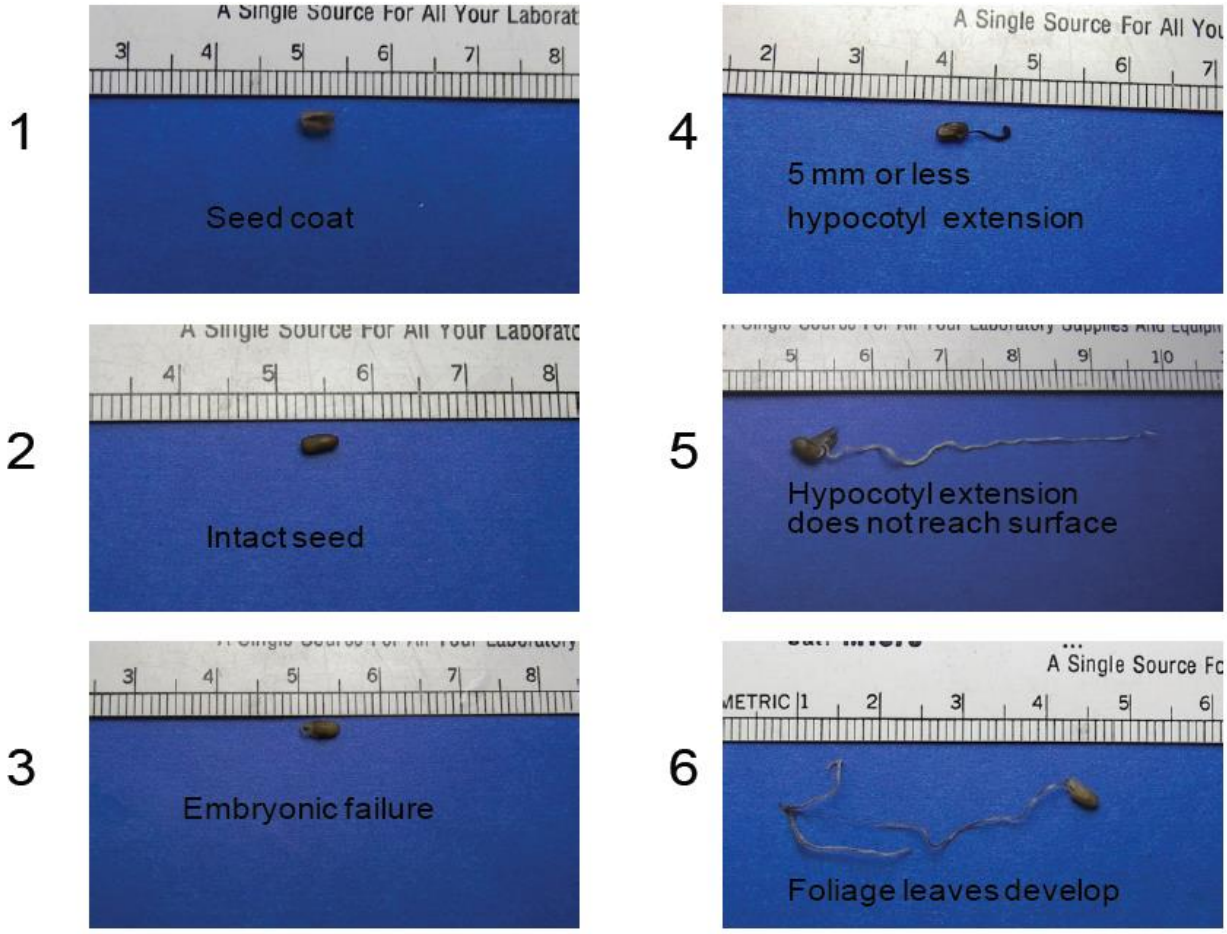

A
B
C
D
E
F
G
H

Supplemental Figures Legends

Supplemental 2.1 Indication of seed viability, the seed embryo white in color (A) is not viable while the seed embryo with the dyed pinkish-red parts (B) is viable.



Supplemental 2.2 Development Stages of *Z. marina* seeds and seedlings



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Chapter 3

The influence of hydrogen sulfide on the distribution of seagrass in the mid Salish Sea.

Chapter 3 introduces the distribution of seagrasses in the San Juans and portions of the Salish Sea, as well as concentrations of H_2S, pH, dissolved oxygen and other sediment and water column chemistry found in these field sites. This chapter, at the time of defense, was under review at Estuarine, Coastal and Shelf Science. This work was done in collaboration with Sandy Wyllie-Echeverria, Erin Licata, Sam Barr, Olga Vitruk, Eric Gupta, Jefferson Emm, and Marco Hatch.

Abstract

Seagrass meadows provide many ecosystem services [food, habitat, shoreline stabilization, carbon sequestration, etc.], however many of these meadows are under threat. Populations locally within the Pacific Northwest (PNW) of the United States and globally have undergone reductions and in extreme cases localized extinctions. Many hypotheses have been proposed as to the cause of these reductions, including some studies which have correlated increases in hydrogen sulfide (H_2S) to decreased health and mortality eelgrass seedlings. Here we report on local populations of *Z. marina* located within the Salish Sea, Washington USA, and the environmental conditions within the stands. We evaluated both sites with current meadows and those with extinct or declining populations, focusing on hydrogen sulfide (H_2S) concentrations found within sediments and directly above the sediment (~5 cm). Using extensive field surveys we determined that in locations with healthy *Z. marina* meadows, determined by

high density, low disease prevalence and high photosynthetic capacity, sediments had low (0 - 10µM) levels of H_2S and organic matter (%OM). However, in places experiencing reductions or extirpation, H_2S levels (100 µM to mM) and %OM are statistically higher than the sites with eelgrass. Additionally, we found two stations, out of 35, in Fidalgo Bay that had abnormally low levels of H_2S compared to surrounding stations. Based on the coordinates of these locations it is suspected that these sites were capped through an ecologically restorative process involving the removal of sediment, addition of activated charcoal and other elements. The findings on these two stations provide a plausible method of lowering high sulfide in sediments.

Key words: eelgrass, Puget Sound, eutrophication, pore water, sulfides

3.1 Introduction

Puget Sound and the encompassing Salish Sea have many islands and inlets with hundreds of kilometers of shoreline, many of which have extensive seagrass meadows (Wyllie-Echeverria and Ackerman 2003). Seagrass meadows provide food and habitat for animals, stabilize shorelines, and help to control erosion (Phillips, 1984; Duarte, 2002; Short et al., 2011). However, seagrass populations in many of these locations, and globally have undergone extensive reductions and in some cases localized extinctions (Short and Wyllie-Echeverria, 1996; Orth et al., 2006; Short et al., 2011; Cullen-Unsworth 2013). Because of the financial and ecological benefits provided by seagrass habitats (Orth et al., 2006) Washington State has recently engaged in a restorative campaign to add 20% more meadows by 2020 (Thom et al., 2014).

Seagrasses often grow in sediments rich in organic matter containing sulfur and sulfide rich compounds (Kamp-Nielsen et al., 2001; Frederiksen et al., 2006a,b; Mascaro et al., 2009; van der Heide et al., 2011; Govers et al., 2014a,b). These sediments are composed of iron and other binding agents which allows for a high storage capacity of these compounds. Sediment types my influence seagrass performance in ways other than through chemical interactions. Increased organic loading creates soft porous sediments which reduces the anchoring ability of seagrass shoots (Koch 2001). Organic loading also limits light availability through dark-colored sediments, which absorb light rather than reflecting it back to photosynthesizing shoots (Goodman et al., 1995; Halun et al., 2002; Cambridge et al., 2012). Living in these sediments and within eelgrass meadows are invertebrate assemblages and complex microbial communities (Danovaro et al., 1994; Elliott et al., 2006; van der Heide et al., 2012; Christiaen et al., 2013), which further modify sediment chemistry (Govers et al., 2014).

Stressors to seagrass range among anthropogenic disturbance (pollution, dredging, boating, etc.) (Short and Wyllie-Echeverria, 1996), climate change (Garcia et al., 2013), disease (Groner et al., 2014) or any combination of these (Raven and Scrimgeour, 1997; Pedersen et al., 2004; Frederiksen et al., 2006; Orth et al., 2006; Mascaro et al., 2009), which may lead to population changes and ecosystem shifts (Duarte, 2002). *Zostera marina* (eelgrass) and other seagrasses, have undergone extensive reductions globally (Waycott et al., 2009; Short et al., 2011). There are indications that eelgrass populations have experienced reductions in Washington State (Wyllie-Echeverria et al., 2003, 2010, Ferrier and Berry 2010; Groner et al., 2014), however the trends are not as well developed as they are globally (Department of Natural Resources, 2015). Around Puget Sound and near Hood Canal the increases in wood waste, high organic matter and decomposition have been correlated to gaps in eelgrass (Elliott et al., 2006).

High organic matter in the sediment is a plausible explanation for increases in sulfide and hydrogen sulfide levels within the sediment. In situ experiments have helped to explain how these changes affect the individual plants and whole communities. Studies have shown that seedlings are highly vulnerable to even relatively low levels of H_2S (Van der Heide et al., 2011 and 2012; Lamers et al., 2013; Govers et al., 2014; Dooley et al., 2013 and 2015). H_2S is known to affect the mitochondria of eukaryotic cells, however recent studies have alluded to the effect that H_2S is a phytotoxin (Oren et al., 1979; Cohen et al., 1986; Ralph, 1999; Dooley et al., 2013; Martin and Maricle, 2015). It has been determined that moderate levels (>10mM) of H_2S disrupts photosynthetic function and more specifically photosystem II (Dooley et al., 2013; Holmer and Hasler-Sheetak, 2014). Ultimately the reduction of photosynthesis may reduce energy output, hampering the plants ability to grow and survive (Penhale and Wetzel, 1983; Goodman et al., 1995; Erskine and Koch, 2000; Pedersen et al., 2004; Koch et al., 2007; Mascaro et al., 2009; Korhonen et al., 2012). These physiological experiments provide evidence for a plausible hypothesis as to why seagrass meadows would decrease under conditions of augmented H_2S.

Here, we present H_2S concentrations as well as an array of other environmental data collected from several sites across the San Juan Archipelago and surrounding areas within the Salish Sea, Washington State USA, in the fall of 2014. The sites were specifically selected to include recent seagrass declines and management interventions to modify sediment properties, as well as more stable populations. We examine characteristics of intertidal sediments in relation to the native eelgrass, *Zostera marina*.

3.2 Methods

3.2.1 Sites

During the low tide series (ranging between 0 - + 1.3m) of the late 2014 summer (August and September) we sampled 85 stations at eight different sites within the San Juan Archipelago and surrounding areas within the Salish Sea, Washington State, USA. (Table 1/Figure 1: Fidalgo Bay, Padilla Bay, Picnic Cove, Shallow Bay Sucia, Beach Haven, Fisherman Bay, Westcott and Garrison Bay). Sites were chosen based on having recent extant seagrass populations (Ferrier and Berry, 2010; Wyllie-Echeverria et al., 2010). Westcott, Garisson and Fisherman Bays were included because of potential problems with eelgrass, including Westcott Bay where eelgrass disappeared in 2003, and Fisherman Bay where it is declining with a potential outbreak of the seagrass wasting disease (Muehlstein and Wyllie-Echeverria, in prep). All sites were in small embayments with mean grain size, at all sites, of 0.147 ± 0.06 (SD) mm (n = 5) (table 2), measured using a Ro-Tap Sieve Shaker (Dooley et al., 2013). During sampling atmospheric temperatures were approximately 24°C, with negligible wind, few clouds and high sun.

3.2.2 Data Collection

Water column temperature, pH, salinity, dissolved oxygen, and chlorophyll content were measured with a Hach Hydrolab DS5 (OTT Hydromet). Hydrolab measurements were obtained by lowering the probe in to the mid-water and the data were recorded for 10 seconds. Total sulfide and biologically active hydrogen sulfide levels in the surface sediment were measured using an H_2S/Sulfide Probe (Sea and Sun Technology). Probes were calibrated to manufacturer specifications (OTT Document number 55.495.000.BE, 02-0511; AMT Analysenmesstechnik 2002, Würdemann et. al, 2014). Before sampling the H_2S/Sulfide Probe was compared to a known standard, measurements were within 3.0 ± 1% of expected values. H_2S concentrations when water depth was 0.3 to 4 meters, and in the top 4 cm of the sediment surface. At each station, H_2S was measured three times by: (1) dropping the probe into the surface, (2) allowing

the probe to measure the H_2S content for approximately 10 seconds and (3) raising and re-dropping in approximately the same location.

Eelgrass was assessed categorically for cover and disease occurrence, as well as quantitatively for Normalized Difference Vegetation Index (NDVI), which is an important indicator of chlorophyll content in plants, with a PlantPen (PSI: Photon System Instruments). NDVI was measured approximately ⅔ of the way up on the longest leaf on 5 shoots per station when eelgrass was present. Shoot density was assigned using an approximant density of maximum (>75% cover), high (50-74% cover), moderate (25-49% cover), sparse (<25% cover), and none (0% cover). Shoot density was determined quantitatively at five sites. In False Bay, Picnic Cove, Shallow Bay, and Shoal Bay 10 randomly selected stations were sampled a long a 100 m transect was sampled for shoot density in 0.25 m^2 quadrats. In Fisherman Bay five randomly selected stations were sampled within 1 m^2 quadrats. At each station shoot density, the length of the longest leaf on each shoot and the presence and length of all visible lesions due to wasting disease, *Labyrinthula sp.,* were recorded. A subset of shoots was sampled for histological analysis to confirm field diagnosis by Dr. M. Groner (University of Prince Edward Island). Disease prevalence and methods are presented in Groner et al., (2014).

At Fisherman Bay, Westcott Bay, Beach Haven, and Fidalgo Bay sediment samples were obtained and analyzed for % organic matter (%OM). Sediment samples were obtained by dropping a 25 lb. ponar style grab sampler into the mud at select stations within each site. Samples were placed into Ziploc bags and placed on ice. Once back at the Northwest Indian College samples were first centrifuged for five minutes at 4ºC, at 4680RPM. Next samples were dried at 105ºC for 24 hours in dehydrating oven. Samples were then transferred to a porcelain crucible and weighed for pre-LOI (loss on ignition) weight. Samples were then heated at 475ºC

for 18 hours, then cooled and reweighted to obtained post-LOI. % OM was calculated using: (pre LOI – post LOI)/pre LOI = *n*; *n* X 100 = %OM. Three replicate samples were analyzed per station, and there were four to six stations per site.

3.2.3 Statistics

Data analyses were carried out in R (R version 2.14.2). Two H_2S measurements were computed from the raw data collected using the H_2S/sulfide probe: peak and average value. Peak value was the maximum value recorded, usually the first or second value once the probe reached the sediment. Average value was calculated by taking the sum of all measurements while in the sediment, and dividing it by the number of measurements; both average and peak value(s) are listed in the results. Temperature, salinity, pH, and dissolved O_2 were evaluated with a linear mixed effects model with ANOVA, based on measured variables as fixed effect and site as random effect.

Concentration of H_2S (both peak and average values) and % OM were computed as continuous variables, and evaluated with a linear mixed effects model. H_2S concentration (continuous data) was compared to station eelgrass density (which were computed as ranked categories), for each site, using a logistic regression model using the glm (generalized linear model) function while neglecting site level random effects. Fidalgo and Portage Bays were evaluated individually as well in order to remove the non-independence among samples when all stations are included simultaneously without site as a random effect. Statistics and P-values are listed when appropriate. The criterion for significance was set at $P<0.05$.

3.3 Results

Station averages of T, pH, %OM, H_2S concentration and the presences or absence of eelgrass and disease over each site are listed in table 2. Individual station data, by site, are listed in tables 3-10. Temperature, pH, dissolved oxygen and salinity had little variation between stations or sites ($P>0.05$). Concentrations of H_2S corresponded with %OM in the sediment (Figure 2, Tables 12-15: %OM by station for Westcott Bay are listed in table 12, Beach Haven in table 13, Fisherman Bay in table 14, and Fidalgo Bay in table 15.) ($F= 9.135$, $df = 14$, $P<0.01$). Eelgrass categorical density was inversely related to H_2S (figure 3) ($F=8.728$, $df =81$, $P<0.001$). Within both Fidalgo Bay and Portage Bay, a cove of Bellingham Bay, eelgrass categorical density was inversely related to H_2S (Figure 3, 5 and 6) (Fidalgo Bay, $F=15.4$, $P<0.01$, $df =34$: Portage Bay, $F=17.9$, $P<0.001$, $df =24$).

At sites with high eelgrass cover, very low levels of H_2S were detected. Sites with no seagrass present had concentrations far exceeding the known lethal limit for seedling survival (e.g. Dooley et al., 2013 and Dooley et al., 2014). Sites that have been locally extinct for several years (e.g. Westcott Bay) had concentrations well into the mM (peak observation 3.8mM), and far exceeding the known lethal limit for mature plants [LD_{50} = 2.0mM at 48h) (Figure 6; Dooley unpublished data; methods similar to those in Dooley et al., 2013 and 2015)].

3.4 Discussion

Seagrass biometrics can be strongly related to environmental conditions (e.g. Duarte, 2002; Holmer et al., 2001, 2002, 2003; Cambridge et al., 2012; Walser, 2014; Marbà et al., 2015, Ruesink et al., 2015). In the present study, we included sites that have experienced reductions and/or local extinctions. Here we provide details on Puget Sound seagrass stands. We found that in sites with large robust *Z. marina* populations, the sediment had a low %OM and low levels of

H_2S. However, in sites where populations have gone locally extinct or a meadows was drastically reduced in size (e.g. Westcott Bay), H_2S levels and %OM were significantly higher, and the H_2S levels were above the known lethal limit for *Z. marina* seedlings (Dooley et al., 2013). There may be several reasons to explain seagrass loss, but based on the %OM we can hypothesize that %OM increases (Grossman et al., 2007), along with other environmental relationships, may be the cause.

Many factors affect organic content of seagrass stands. For example it is known that organic content experience substantial temporal and spatial variability even within small geographic range (Koch, 2001; Ruesink et al., 2015). Sites, such as Beach Haven, which have low %OM and H_2S, may experience larger hydrodynamic energy compared to more sheltered sites with high H_2S and %OM (e.g. Westcott Bay). Possible transition sites (e.g. Fidalgo and Fisherman Bay) have a large gradient of sulfide concentrations over a similar sized area. We found eelgrass stands to have higher shoot densities when the concentration of H_2S and %OM is lower towards the inlet of Fidalgo Bay. This may be a function of greater hydrodynamic energy than the shallow south end of the bay where H_2S and %OM increase.

Changes in %OM in the sediment have been previously correlated to wood-debris from mills (Elliott et al., 2006). Fidalgo Bay, like many other locations around Puget Sound, has had a large mill. Custom Plywood once existed on the west side of the bay, opposite to the current refinery. Within our sampling we detected two stations with low concentrations of H_2S but surrounded by higher concentrations. In the 2000's a large clean up took place in these two stations and records suggests that both of these were caped (ww.re-sources.org/programs/fidalgo). Capping involves the isolation and removal of the contaminated material and the addition of gravel, sand, and charcoal which absorbs the volatiles. The presence

of these two stations, surrounded by high concentrations provides a possible mitigation technique. The capping of sites and transplantation of seagrass to these locations may lower sulfides and create more oxygenated sediment (Frederiksen and Glud, 2006).

Never the less, the measurements presented here add support to the hypothesis that H_2S and other sulfides may be involved in the reduction of seagrass meadows and possibly the inhibition of seedling recruitment. While there is strong support for this hypothesis one cannot say that it is causation. It may be as likely that the H_2S/sulfides appear due to the loss of the seagrass. Because of this and other factors further hypothesis testing and experiments are needed.

However, based on these measurements and historical events we propose a general mechanism (figure 7) for the cause of current and local future extinction events in these embayments. Throughout geologic history as the temperature increases, oxygen levels decrease, this has allowed for sulfide and hydrogen sulfide intrusion (Kump et al., 2005; Ward, 2006). This may occur in local sediments and possibly in the water column if temperatures continue to increase. These increases may decrease the health of the seagrass in these locations. This only exacerbates the reduction in fitness caused by other conditions, anthropogenic and natural, that the organisms are experiencing (Garcia et al., 2013; Groner et al., 2014). Together these variables may affect seedling recruitment, and the physiological health of mature plants, providing evidence for a mechanism of lethality and ultimately extinction of the seagrass meadow(s). Understanding the causes of these reductions is extremely important for restoration and maintaining current stands for ecosystem services (Lavery et al., 2013; Cullen-Unsworth et al., 2013), and ultimately for predicting how future climate change will interact within the system (Marbà et al., 2015).

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Table 3.1: Site locations and number of stations recorded

Site Name, Location	GPS		Number of stations
	North	West	
Beach Haven, Orcas Island	48°41'30.82"N	122°57'10.38"W	3
Shallow Bay, Sucia Island	48°45'36.27"N	122°54'54.11"W	6
Westcott Bay, San Juan Island	48°35'51.70"N	123° 8'57.96"W	5
Garrison Bay, San Juan Island	48°35'5.76"N	123° 9'18.83"W	3
Fidalgo Bay, Anacortes	48°28'50.85"N	122°35'31.99"W	35
Padilla bay, Anacortes	48°28'56.80"N	122°32'39.39"W	5
Fisherman Bay, Lopez Island	48°30'33.94"N	122°55'16.01"W	5
Bellingham bay	48°42.989”N	122°37.549”W	25

Table 3.2: General site summary

Site Name, Location	Conditions		% organic Matter	Hydrogen Sulfide		Seagrass		
	°C	pH		average	peak	Present	Disease	NDVI
Beach Haven, Orcas Island	13.4 ± 0.4	8 ± 0.05	1.04 ± 0.08	0.9 ± 0.48	7.0 ± 1.81	Y	N	6.9 ± 0.4
Shallow Bay, Sucia Island	17.5 ± 0.7	8.3 ± 0.06		7.0 ± 1.27	23.2 ± 2.01	Y	N	5.7 ± 0.8
Westcott Bay, San Juan Island	15.2 ± 1.2	7.9 ± 0.1	5.13 ± 0.46	11.2 ± 1.53	31.7 ± 3.82	N	N/A	N/Aº
Garrison Bay, San Juan Island	15 ± 1.4	7.9 ± 0.05	N/A	9.3 ± 2.23	19.9 ± 2.79	N	N/A	N/Aº
Fidalgo Bay, Anacortes	14.1 ± 0.2	7.8 ± 0.04		14.7 ± 1.06	23.9 ± 1.19	Y	Y	5.91
Padilla bay, Anacortes	15.4 ± 0	8.1 ± 0.01		11.2 ± 3.1	41 ± 6.7	Y	N	6.14 #
Fisherman Bay, Lopez Island+	15.2 ± 0.3	8.2 ± 0.2	1.5 ± 0.19	16.8 ± 1.14	27.5 ± 4.96	Y	Y	5.05 ± 0.2
Bellingham bay	12.4 ± 0.1	6.7 ± 0.02	N/A	24.6 ± 1.26	54.22 ± 2.44	Y	N	N/A*

'+ station 5 the probe may have malfunctioned and data was not counted into average

*specimens were too deep to record

only 1 NDVI recorded

ºseagrass is locally extinct

Table 3.3: Bellingham Bay station measurements

	Conditions		Hydrogen Sulfide		
Station	T °C	pH	Average	Peak	NDVI
1	12.8	6.6	18.65	30.81	N/A
2	12.8	6.7	17.49	21.3	N/A
3	12.8	6.7	28.66	40.41	N/A
4	12.8	6.7	10.88	15.47	N/A
5	12.8	6.6	18.66	23.49	N/A
6	12.8	6.6	22.41	44.76	N/A
7	12.8	6.7	24.31	35.26	N/A
8	12.8	6.7	29.15	47.98	N/A
9	12.2	6.7	19.76	27.97	N/A
10	12.1	6.8	24.43	38.27	N/A
11	12.2	6.9	17.18	29.68	N/A
12	12.1	6.8	31.02	53.68	N/A
13	12.2	6.8	14.94	22.22	N/A
14	12.2	6.8	23.90	42.83	N/A
15	12.2	6.7	25.18	32.40	N/A
16	12.1	6.6	25.71	45.76	N/A
17	12.7	6.8	30.95	52.17	N/A
18	12.2	6.9	24.41	38.40	N/A
19	12.2	6.7	18.33	27.11	N/A
20	12.2	6.7	29.48	49.08	N/A
21	12.6	6.7	31.09	52.07	N/A
22	12.6	6.8	30.72	54.22	N/A
23	12.6	6.8	30.89	43.11	N/A
24	12.2	6.7	32.81	58.87	N/A
25	12.2	6.7	34.08	54.44	N/A

Table 3.4: Westcott Station Measurements

	Conditions		Hydrogen Sulfide		
Station	T°C	pH	Average	Peak	NDVI
1	18.2	8.1	10.2	17.0	N/A
2	18.2	8.1	10.1	15.9	N/A
3	12.9	7.8	16.2	31.7	N/A
4	12.9	7.4	12.5	20.3	N/A
5	13.8	8.0	6.9	8.3	N/A

Table 3.5: Garrison Bay Station Measurements

	Conditions		Hydrogen Sulfide		
Station	T°C	pH	Average	Peak	NDVI
1	17.4	8.0	6.8	21.3	N/A
2	15.1	8.0	7.2	14.5	N/A
3	12.5	7.8	13.7	23.9	N/A

Table 3.6: Beach haven Station Measurements

	Conditions		Hydrogen Sulfide		
Station	T °C	pH	Average	Peak	NDVI
1	13.5	8.1	1.8	7.0	7.4
2	13.9	8.0	0.9	2.6	6.9
3	12.7	7.9	0.1	0.9	6.1

Table 3.7: Sucia Island Station Measurements

	Conditions		Hydrogen Sulfide		
Station	T°C	pH	Average	Peak	NDVI
1	18.0	8.5	4.5	13.7	3.8
2	17.5	8.4	6.5	14.5	5.1
3	14.5	8.2	4.0	9.2	6.8
4	14.6	8.3	5.0	10.4	7.0
5	13.8	8.1	11.0	14.6	N/A
6	13.8	8.1	10.6	23.2	N/A

Table 3.8: Fisherman Bay Station Measurements

	Conditions				Hydrogen Sulfide		
Station	T °C	pH	Sal (ppt)	O_2 (mg/L)	Average	Peak	NDVI
1	15.8	7.6	29.5	5.6	19.0	27.0	N/A
2	14.3	8.4	30.5	9.3	6.0	15.0	4.51
3	14.9	8.5	29.2	7.2	33.0	42.0	5.52
4	16.0	8.7	29.6	8.2	9.0	26.0	4.62
5*	14.9	7.8	28.7	12.4	40.0	144.0	5.55

Sulfide probe may have malfunctioned

Table 3.9: Padilla Bay

	Conditions		Hydrogen Sulfide		
Station	T °C	pH	Average	Peak	NDVI
1	15.42	8.05	4	4	N/A
2	15.42	8.05	18	29	N/A
3	15.4	8.1	5	10	N/A*
4	15.4	8.05	10	26	N/A*
5	15.4	8.05	19	41	6.14

*eelgrass was present but too deep to sample, appeared to be diseased

Table 3.10: Fidalgo Bay

	Conditions						Hydrogen Sulfide	
Station	T °C	pH	Sal (ppt)	Turbidity (NTU)	O_2 (mg/L)	Chlorophyll (ug/L)	Average	Peak
1	14.6	8.0	26.8	9.3	8.5	11.4	11.4	18.4
2	17.7	8.0	26.3	7.0	8.7	10.9	15.8	22.5
3	14.8	8.0	29.4	9.7	8.8	14.5	11.7	23.0
4	14.8	8.1	29.5	2.3	9.2	13.9	9.0	19.1
5	15.5	8.2	29.7	19.4	10.6	25.1	18.4	30.0
6	15.7	8.0	29.7	12.6	8.4	37.9	16.6	26.2
7	16.3	8.0	29.5	2.5	8.2	15.7	14.7	19.9
8	15.8	8.0	29.7	2.7	8.3	12.0	16.1	31.7
9	15.5	8.1	26.5	4.3	8.3	3.7	15.1	28.0
10	15.4	8.2	28.9	4.4	9.2	5.1	19.8	29.5
11	15.0	8.2	28.6	4.2	9.0	3.8	13.1	26.0
12	14.7	8.1	28.8	7.4	9.4	9.7	14.5	26.4
13	15.2	8.1	28.5	4.2	9.1	3.2	18.3	27.2
14	12.9	7.4	26.5	N/A	6.8	N/A	19.4	19.4
15	13.0	7.6	26.5	N/A	6.8	N/A	21.0	27.0
16	13.4	7.7	26.5	N/A	7.3	N/A	30.0	37.0
17	13.4	7.7	26.5	N/A	6.9	N/A	15.0	20.0
18	13.4	7.7	26.5	N/A	7.0	N/A	2.0	6.0
19	13.5	7.6	26.5	N/A	6.4	N/A	25.0	29.0
20	13.8	7.6	26.5	N/A	6.7	N/A	10.0	20.0
21	13.8	7.7	26.5	N/A	6.9	N/A	19.0	28.0
22	13.9	7.7	26.5	N/A	6.9	N/A	11.0	17.0
23	13.8	7.7	26.5	N/A	7.0	N/A	8.5	23.0
24	13.6	7.7	26.5	N/A	7.1	N/A	11.0	28.0
25	13.4	7.8	26.5	N/A	6.9	N/A	5.0	14.0
26	13.2	7.7	26.5	N/A	7.6	N/A	9.0	17.0
27	13.0	7.7	26.6	N/A	7.4	N/A	5.0	9.0
28	12.9	7.8	26.6	N/A	7.4	N/A	10.0	15.0
29	12.9	7.7	26.6	N/A	7.5	N/A	9.0	14.0
30	13.0	7.7	26.6	N/A	7.5	N/A	17.0	25.0
31	13.0	7.7	26.6	N/A	7.4	N/A	14.0	25.0
32	12.8	7.7	26.3	N/A	7.1	N/A	15.0	17.0
33	13.0	7.7	26.6	N/A	7.1	N/A	11.0	14.0
34	12.9	7.7	26.6	N/A	7.2	N/A	24.0	27.0
35	12.9	7.7	26.6	N/A	7.0	N/A	28.0	36.0

Table 3.11: Shoot densities (Wyllie-Echeverria unpublished data)

Table 1. San Juan Archipelago *Zostera marina* Density Transects (Mean shoot density/0.25 m^2)										
Location	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Total shoots										
False Bay, San Juan Island	13.2	11.7	6.1	11.4	6.5	15.8	4.6	16	20.1	9.2
Picnic Cove, Shaw Island	30.2	57.1	12.3	1.8	2.2	8.9	13	0.7	8.5	1.7
Shallow Bay, Sucia Island	13	29.4	19.1	25.7	21.1	39.3	13.4	2.9	0.9	0
Shoal Bay, Lopez Island	6.3	15.1	14.5	21.8	14.9	18.1	7.5	12.7	12.8	14.5
Sterile shoots										
False Bay, San Juan Island	13.2	11.5	5	9.2	4.5	14.9	3.9	16.7	19.4	8.6
Picnic Cove, Shaw Island	28.3	53.9	12.3	1.5	2.1	8.8	12.1	0.7	8.2	1.7
Shallow Bay, Sucia Island	12.2	28.2	16.5	24.9	19.3	38.3	12.2	2.9	0.9	0
Shoal Bay, Lopez Island	6.3	15.1	14.4	21.6	14.6	18.1	7.5	12.7	12.8	14.5
Flowering shoots										
False Bay, San Juan Island	0	0.2	1.1	2.2	0.2	0.9	0.7	1	0.4	0.7
Picnic Cove, Shaw Island	1.9	3.2	0	0	0.1	0	0.3	0	0.3	0
Shallow Bay, Sucia Island	0.8	0.6	2.6	0.8	1.8	1	1.3	0	0	0
Shoal Bay, Lopez Island	0	0	0.1	0.2	0.2	0	0	0	0	0
Seedlings	0	0	0	0	0.1	0	0	0	0	0
False Bay, San Juan Island	0	0	0	0	1.8	0	0	0	0.3	0
Picnic Cove, Shaw Island	0	0	0	0.1	0	0.1	0	0	0	0
Shallow Bay, Sucia Island	0	0	0	0	0	0	0	0	0	0
Shoal Bay, Lopez Island	0	0	0	0	0	0	0	0	0	0

Table 3.12: % organic matter Westcott Bay

Site	Soil Pre LOI	Soil Post LOI	%OM
W001-1	4.8744	4.5617	6.4151
W001-2	5.8218	5.457	6.2661
W001-3	5.2806	4.9422	6.4084
W002-1	4.7791	4.4993	5.8547
W002-2	4.623	4.3578	5.7365
W002-3	4.6631	4.3859	5.9445
W003-1	5.899	5.5406	6.0756
W003-2	5.7284	5.3844	6.0052
W003-3	5.4762	5.1395	6.1484
W004-1	3.2517	3.0727	5.5048
W004-2	2.9597	2.7918	5.6729
W004-3	3.2201	3.0374	5.6737
W005-1	5.3938	5.2964	1.8058
W005-2	6.4909	6.3771	1.7532
W005-3	4.6891	4.6116	1.6528
		Average %OM	5.1278
		without W005	5.9755
		Std Dev	1.7749

Table 3.13: % organic matter Beach Haven

Site	Soil Pre LOI	Soil Post LOI	%OM
BH001-1	5.7933	5.7447	0.8389
BH001-2	5.3335	5.2878	0.8568
BH001-3	6.0348	5.9819	0.8766
BH002-1	6.2559	6.1881	1.0838
BH002-2	4.4481	4.402	1.0364
BH002-3	4.5322	4.4813	1.1231
BH003-1	4.0817	4.0202	1.5067
BH003-2	4.8822	4.8167	1.3416
BH003-3	4.3914	4.3288	1.4255
BH004-1	5.2163	5.1746	0.7994
BH004-2	6.4212	6.3716	0.7724
BH004-3	5.6231	5.5795	0.7754
		Average %OM	1.0364
		Std Dev	0.2639

Table 3.14: % organic matter Fisherman Bay

site	Soil Pre LOI	Soil Post LOI	%OM
L001-1	5.9613	5.8924	1.1558
L001-2	5.0753	5.0078	1.321
L001-3	4.7797	4.7197	1.2553
L002-1	7.7701	7.6708	1.278
L002-2	12.4667	12.3111	1.2481
L002-3	11.0243	10.8776	1.3307
L003-1	4.2071	4.1411	1.5688
L003-2	4.807	4.7347	1.5041
L003-3	4.8079	4.7358	1.4996
L004-1	4.5315	4.4569	1.6463
L004-2	4.5567	4.4831	1.6152
L005-1	4.9585	4.8818	1.5468
L005-2	5.9953	5.8893	1.7681
L005-3	4.9333	4.8499	1.6906
L006-1	5.1875	5.1062	1.5672
L006-2	5.2408	5.1522	1.6906
L006-3	4.0435	3.9738	1.7238
		Average %OM	1.4947
		Std Dev	0.1924

Table 3.15: % Organic Matter Fidalgo Bay

Fidalgo Bay Organic Matter	
station	
SB 1	1.9637
SB 2	2.0689
SB 3	2.0381
site AVG	**2.0236 %OM**
St.6 1	5.1116
St.6 2	5.1026
St.6 3	5.1349
site AVG	**5.1164 % OM**
St.7 1	4.3715
St.7 2	4.1366
St.7 3	4.053
site AVG	**4.1870 % OM**

Figure 3.1: Map indicating site locations.

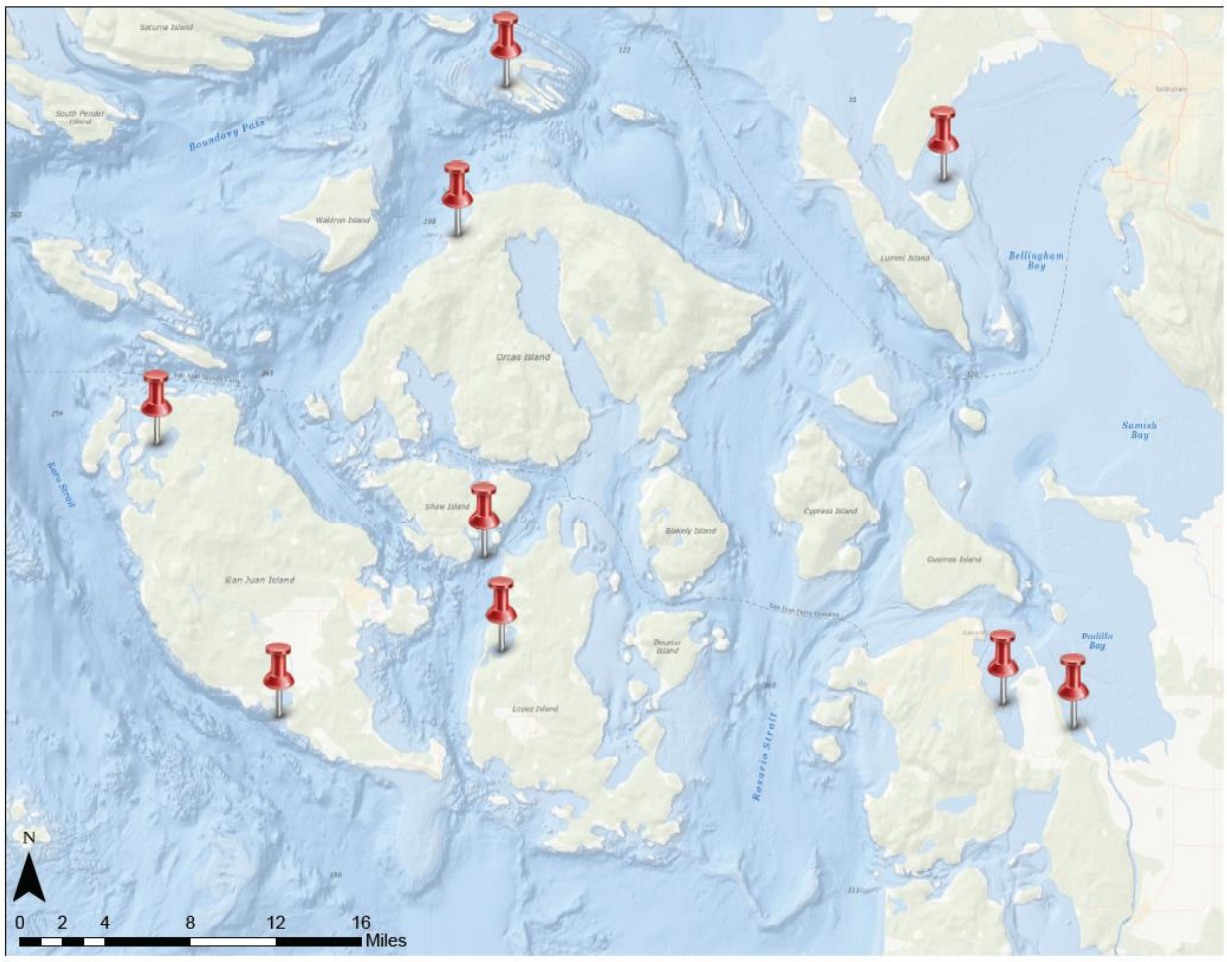


Figure 3.2: H_2S concentration vs eelgrass density. LD_{50} values of seedlings and mature *Z. marina* plants are shown.

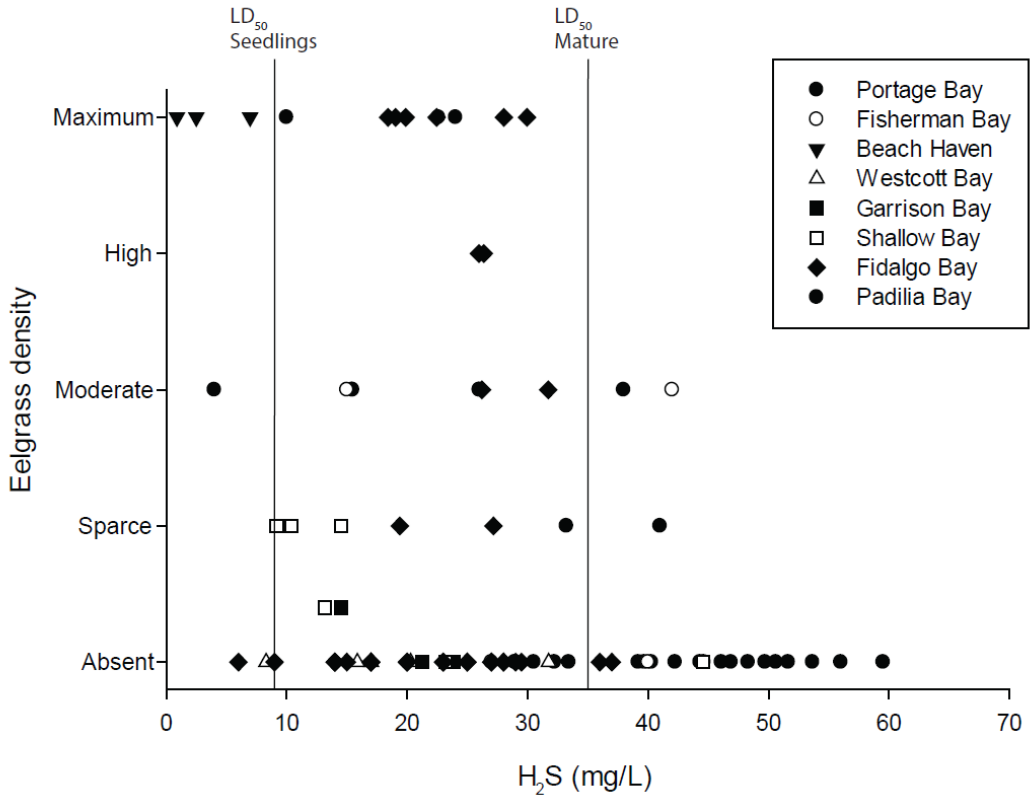


Figure 3.3: % organic matter vs. H_2S concentration. SE bars are shown.

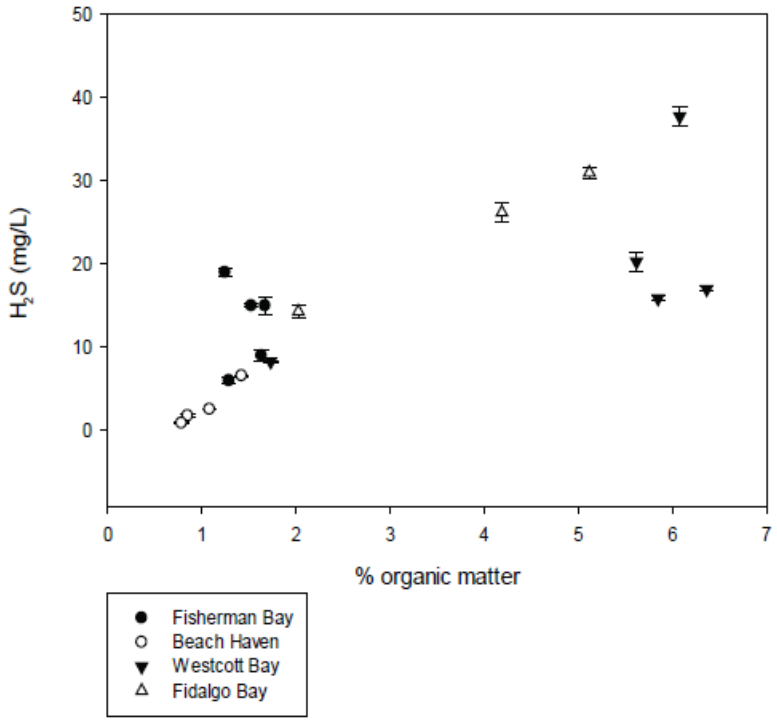


Figure 3.4: Organic matter and H_2S concentrations by site overview

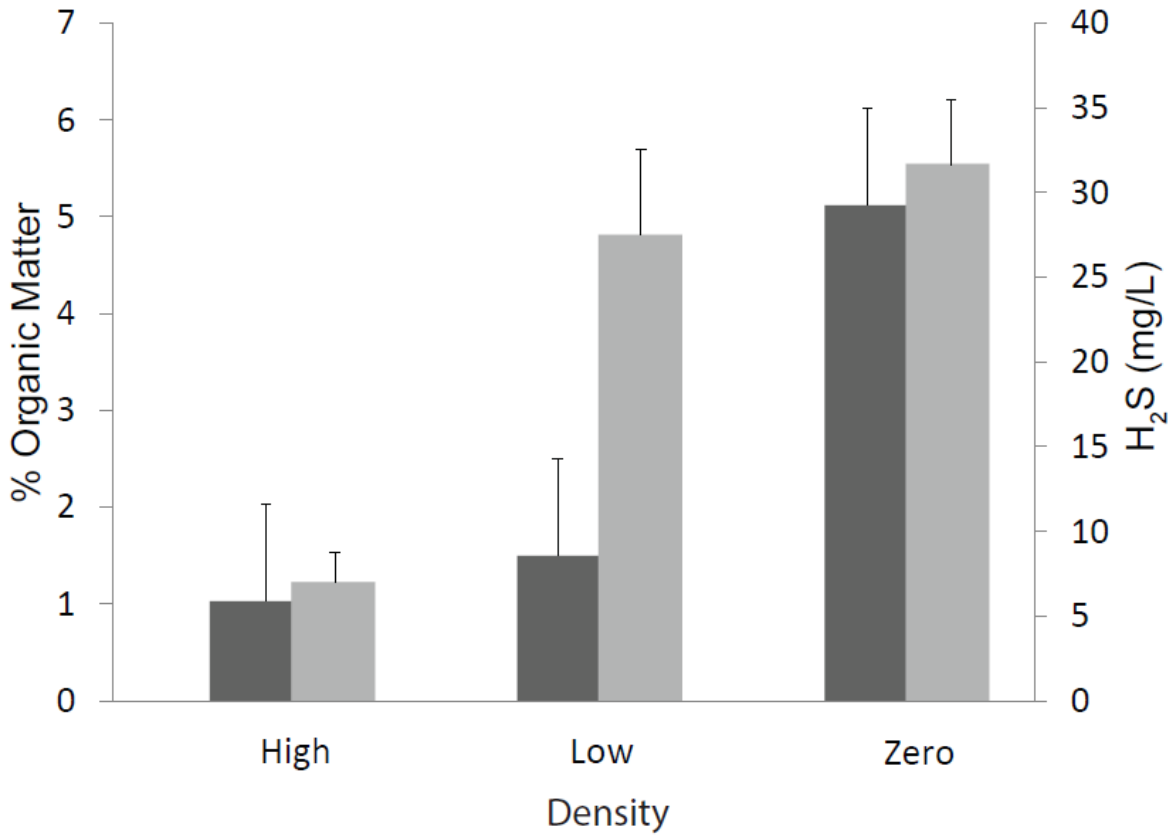


Figure 3.5: Percent survival of mature *Zostera marina* plants in H_2S (concentrations in µM). LD_{50} at 48hrs is 2000µM. Treatment groups are statistically different from each other (P<0.001).

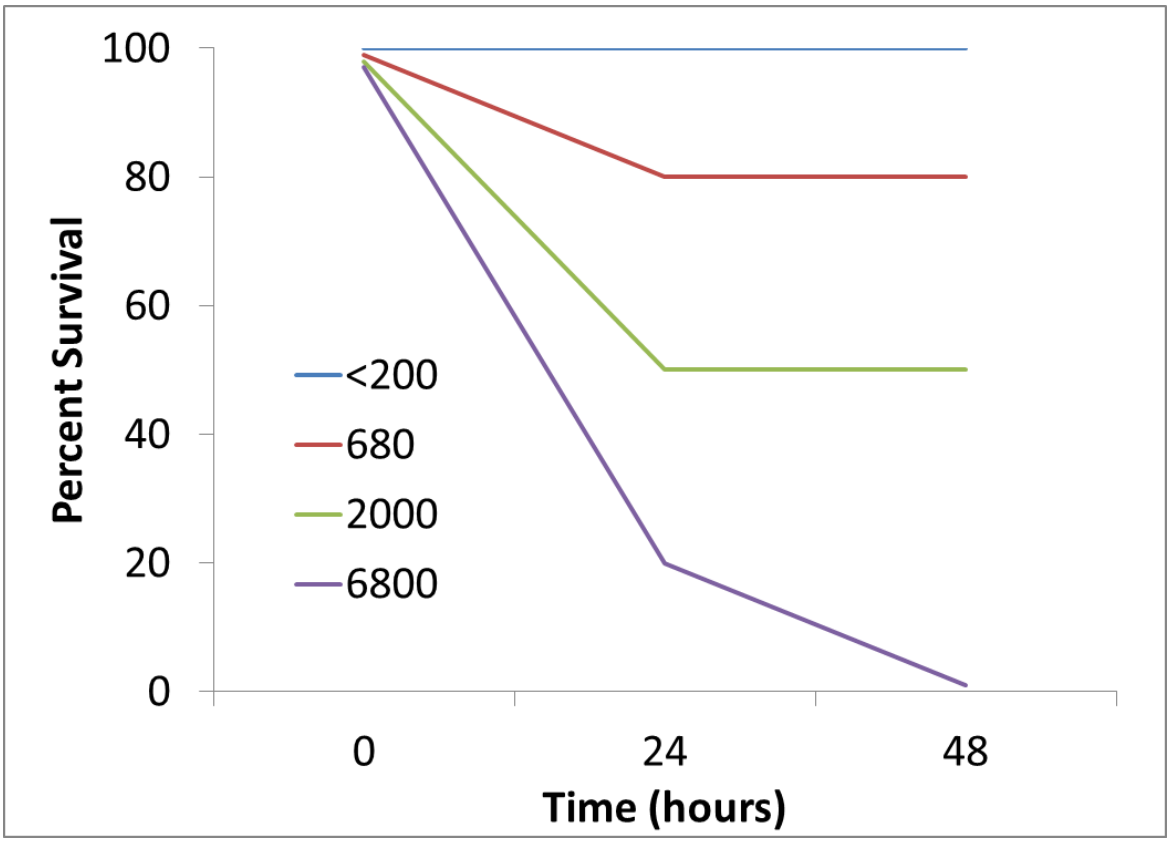


Figure 3.6: Map illustrating concentrations of H_2S (mg/L) in Portage Bay.

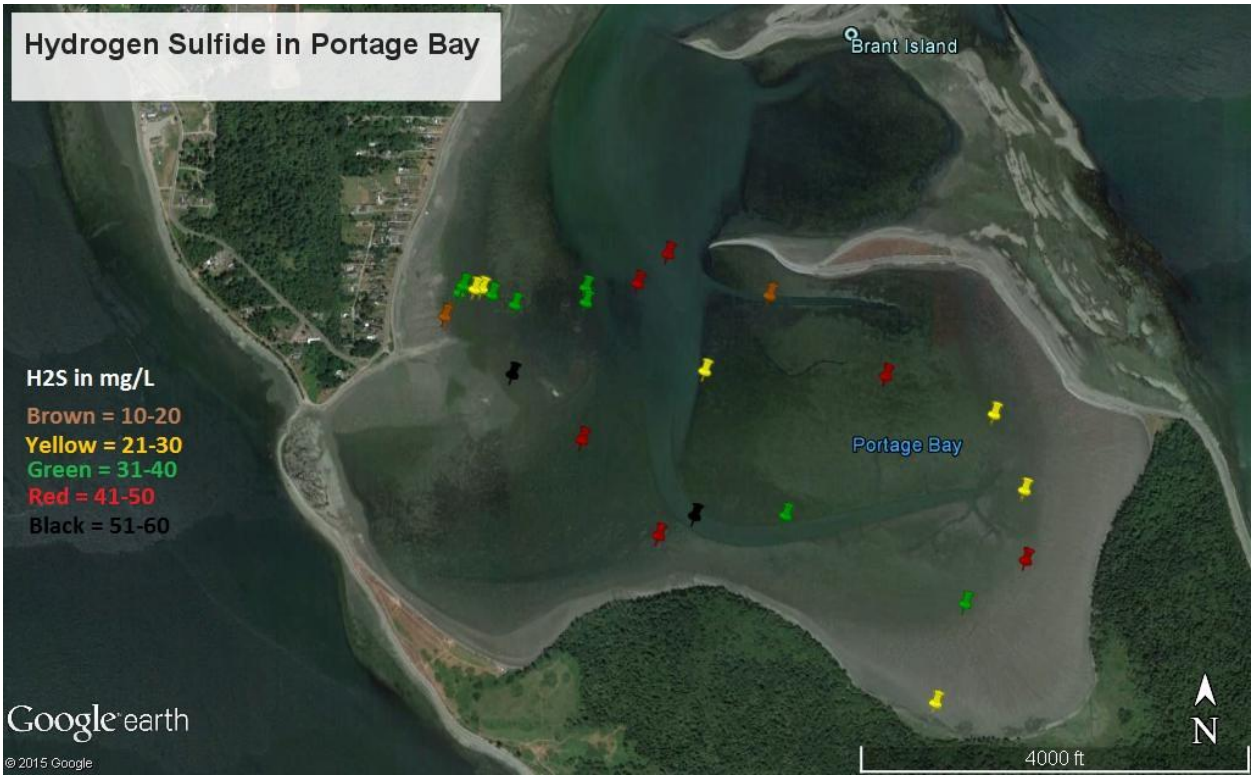


Figure 3.7: Concentrations of H_2S (mg/L) in Fidalgo Bay.

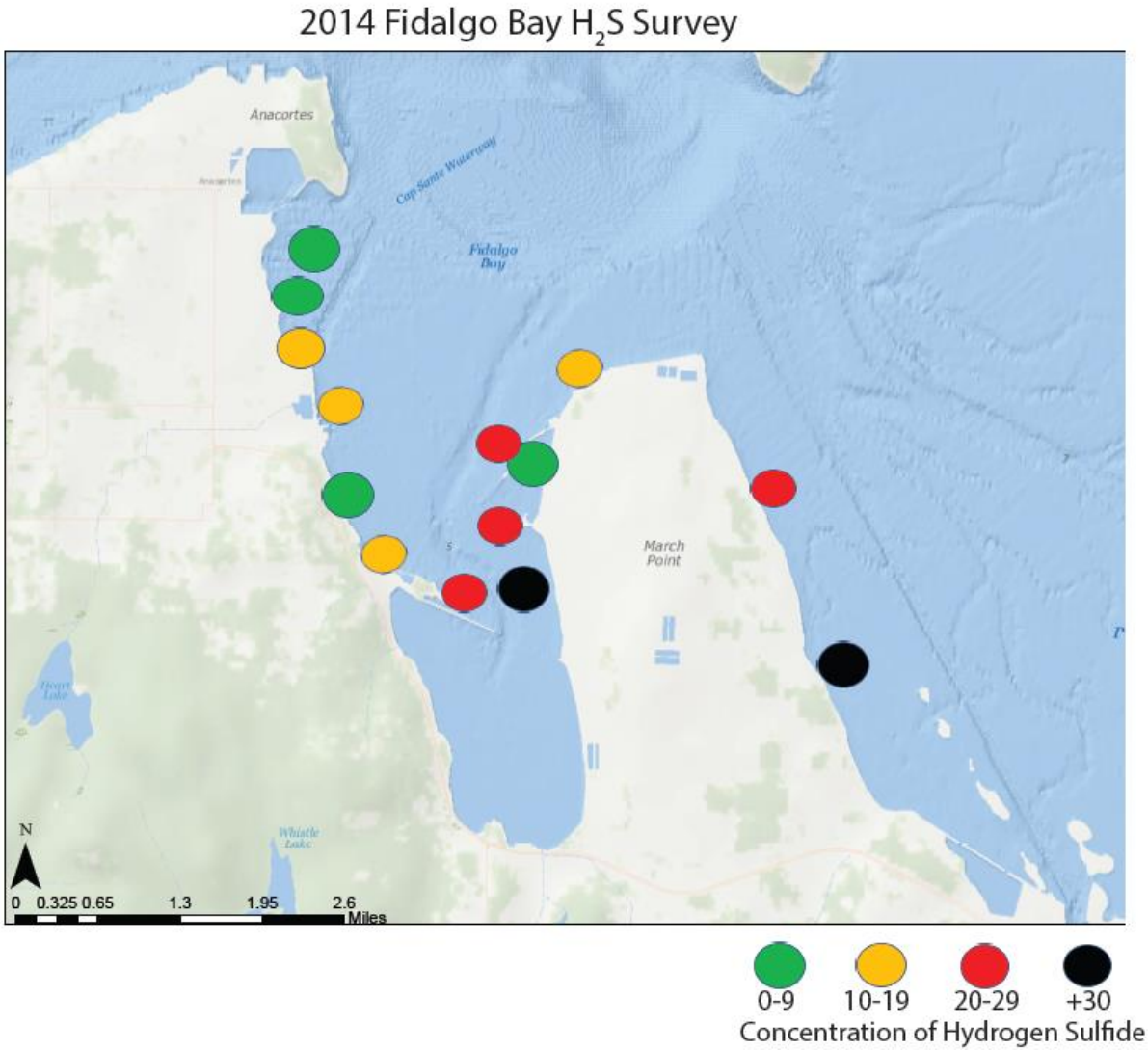


Figure 3.8:

Concentration of H_2S in a sample after each measurement with the H_2S probe. SD Bars are shown.

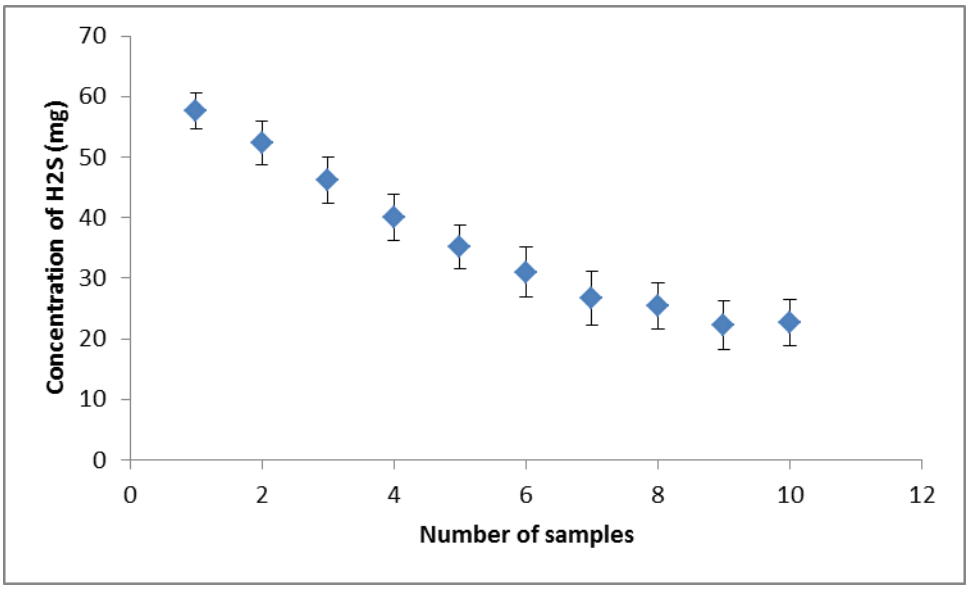
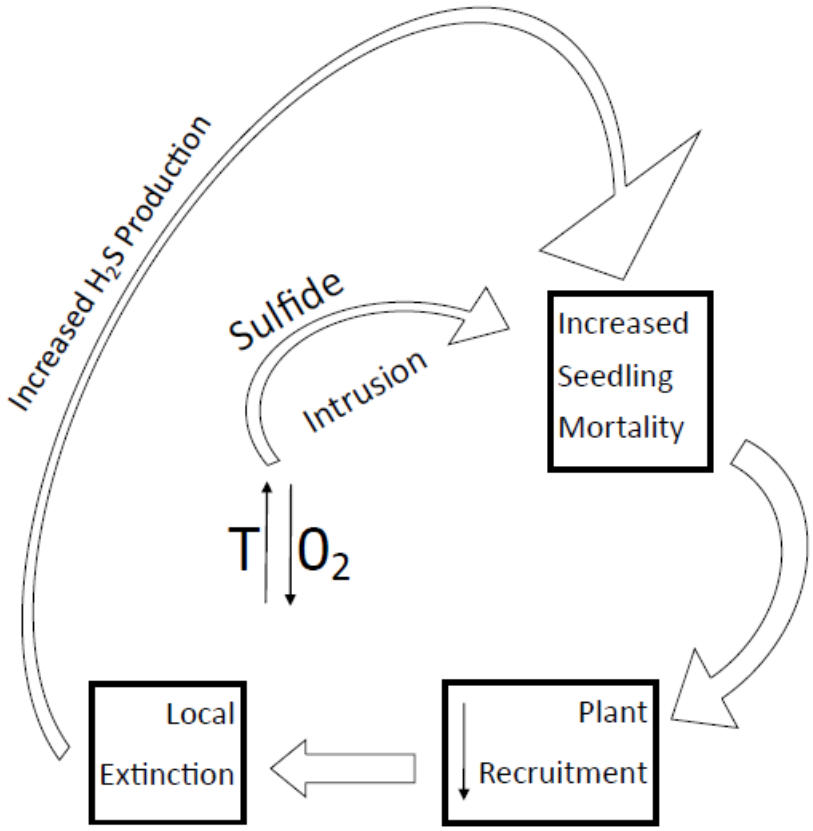


Figure 3.9: Summary figure



Chapter 4

Tolerance and response of *Zostera marina* seedlings to hydrogen sulfide

Chapter 4 introduces the physiological responses of Zostera marina, seedlings to hydrogen sulfide. Portions of this chapter were originally published in the Journal of Aquatic Botany in collaboration of Sandy Wyllie-Echeverria, Peter D. Ward and Mark Roth. **Dooley, F.,** Wyllie-Echeverria, S., Roth, M., & Ward, P. 2013. Tolerance and response of Zostera marina seedlings to hydrogen sulfide. Aquatic Botany. 105: 7-10.

Abstract

Populations of *Zostera marina* L., the common seagrass of Pacific Northwest shallow marine environments, has undergone local extinction in coastal embayment's where it has traditionally existed. Because the habitat created by these plants is important for near-shore productivity and biodiversity, declining populations and local extinctions can have serious ecosystem consequences. One possibility for the failures of population increase and re-colonization of embayment's with complete loss is an increase in sediment H_2S. We designed experiments to test the influence of various H_2S concentrations on *Z. marina* seedlings. To do this we immersed seedlings in five different concentrations of H_2S (68 μM, 204 μM, 680 μM, 2.04 mM and 6.8 mM) in 2010, and three additional concentrations (400 μM, 500 μM and 800 μM) in 2011. Treated seedlings were consistently killed above 680 μM. In addition, high doses

(680 μM, 800 μM, 2.04 mM and 6.8 mM) of H_2S caused depression of photosynthetic output, as well as causing Photosystem II to become inactive whereas Photosystem I remained active. At low doses of H_2S (68 μM) it appears that photosynthesis increases. Our observations also suggest that this plant may adapt to lethal H_2S concentrations if subjected to multiple, but gradually increasing sub-lethal H_2S concentrations. These results suggest that *Zostera marina* seedlings are consistently killed at concentrations of hydrogen sulfide found in localities that have experienced declines and local extinctions, and ultimately can be used to explain the lack of re-colonization in these sites.

4.1 Introduction

Zostera marina L. (eelgrass), like other seagrass species, provides an environment which increases biodiversity and productivity in shallow marine estuaries (Kenworthy et al., 2006). *Z. marina* is important to a wide variety of animals as food or shelter during some part of their life cycle (Moore and Short, 2006). However, seagrass beds have experienced declines and local extinctions (Short and Wyllie-Echeverria, 1996; Waycott et al., 2009).

Currently, *Z. marina* grows in sub-arctic, temperate and sub-tropical environments in the northern hemisphere (Short and Coles, 2001; Wyllie-Echeverria and Ackerman, 2003), and the genus has been present since the Cretaceous (McCoy and Heck, 1976; Phillips and Menez, 1988). These sites are colonized by the combined strategy of creeping rhizomes and seed dispersal that result in large contiguous underwater "prairies" which harbor a large diversity of other organisms (Moore and Short, 2006). Seagrass beds are found in sediments which are often

anoxic and rich in sulfide compounds (Pedersen et al. 2004; Frederiksen et al. 2006; Mascaro et al., 2009).

Increases in H_2S is caused by several factors, biotic (decomposing biomass – algae blooms) to abiotic (hydrothermal vents). Human activities, such as increasing organic and nutrient loading, have provided conditions in which H_2S production is increased (Short and Burdick 1996; Kamp-Nielsen et al. 2001; Halun et al. 2002). Indications, based on field studies in Commencement Bay in Puget Sound (Elliott et al., 2006; and our sampling), have suggested that hydrogen sulfide concentrations may control *Z. marina* expansion and re-colonization in these regions (Goodman et al. 1995; Holmer and Bondgaard, 2001; Plus et al. 2003; Pedersen et al., 2004). Additionally, new research into past mass extinctions coincide with increased sulfur loads in most marine systems (Berner and Ward, 2006; Ward, 2006).

The toxicity of hydrogen sulfide (H_2S) has been studied for over 200 years (Lloyd, 2006); however the effects on plants have only recently been described (Chen et al., 2011). In eukaryote cells hydrogen sulfide is toxic because it inhibits cytochrome oxidases at concentrations as low as 1 -10 µM (Fenchel and Finlay, 1995; Raven and Scrimageour, 1997). High levels of hydrogen sulfide have shown to be a cause of toxicity within the plant cells and ultimately cause death in several species (Lloyd, 2006). Studies involving mature *Z. marina* plants have correlated high levels of hydrogen sulfide with diminished health of the shoots due to intrusion of sulfide into tissues (Goodman et al., 1995; Erskine and Koch, 2000; Pedersen et al., 2004; Mascaro et al., 2009). H_2S tissue toxicity is associated with the inhibition of growth (Erskine and Koch, 2000) and the reduction of photosynthetic activity at high concentrations (<1mM). It has been shown in other plant species that at high concentrations photosynthetic activity within the chlorophyll changes (Oren et al., 1979; Cohen et al., 1986; Chen et al., 2011).

Z. marina has physical structures that help prevent sulfide toxicity (Penhale and Wetzel, 1983). This plant forms aerenchyma, which transport oxygen to the roots when the plant is photosynthesizing (Mascaro et al., 2009), which in turn reacts with H_2S to produce SO_4^{2-} and H_2O; thus diminishing the negative effects of H_2S in the rhizosphere (Pedersen et al., 2004; Koch et al., 2007). Still, seedlings still may be impacted. Seedling rhizomes are small when compared to that of mature plants, photosynthetic capacity may be limited, and seedlings may be less resilient, possibly making this a critical stage in re-establishment (e.g., Plus et al., 2003).

The objective of this study was to assess experimentally the relationship between H_2S concentrations and *Z. marina* seedling health. We describe a series of laboratory experiments and *in-situ* field measurements designed to evaluate the toxicity of H_2S on *Z. marina* seedlings. We use this evaluation to understand ongoing die offs, as a possible factor causing mass extinctions (Short et al., 2011).

4.2. Materials and Methods

4.2.1 Field studies

To parameterize lethality experiments, measurements of H_2S concentrations in and around *Z. marina* stands were taken in the San Juan Archipelago, Washington State; USA. It has been noted that there has been a reduction in size and number of previously long-lived stands in this region (Wyllie-Echeverria et al., 2010). In the late summer and early fall of 2008, 33 stations at 4 sites were sampled using a Submersible H_2S/Sulfide Probe (Sea and Sun Technology

GmbH**,** Trappenkamp, Germany). Sites were chosen based on the local extinction or extant presence of *Z. marina* (Ferrier and Berry, 2010; Wyllie-Echeverria et al., 2010). All sites were in small embayments with fine sediments (mean grain size at all sites was 0.147 ± 0.06 mm), measured using a Ro-Tap Sieve Shaker. H_2S concentrations were measured on the sediment surface (top four centimeters).

4.2.2 *In vitro* studies: Seed germination and culture preparation

Generative shoots of *Z. marina* were collected at False Bay (48°29'11N, 123° 4'28W), San Juan Island in the late summer of 2009. Shoots, containing seeds, were transported to the laboratory immediately following collection and placed in containers serviced by flowing seawater at the Friday Harbor Laboratories (FHL), University of Washington. Sixty days later, container contents were sieved and all seeds from the collected shoots were retained, placed in scintillation vials in batches of 100, and stored in the dark at 5º C and 32 PSU until germination trials were initiated (Wyllie-Echeverria et al., 2003), five months after collection.

Nine hundred seventy five seeds were obtained, sterilized with a 25% bleach solution (Churchill, 1991; Wyllie-Echeverria et al., 2003), placed into individual test tubes filled with a sterile, 20 PSU seawater with nutrients added [$NaNO_3$ + Na_2HPO_4 + $MnCl_2.4H_2O$ + Ferric-sodium EDTA + H_3BO_3 + HCl] (Churchill unpublished data); and held at 20^{o}C to force germination (Phillips, 1972). Once the seeds germinated (ranging in time from two days to almost six weeks) they were transferred to a submerged, closed, sterile seawater tank located in an environmental chamber at the Department of Biology, University of Washington. The seedlings were supplied a daily minimum of 6 hours of PAR, 235 µmol $m^{-2}s^{-1}$, and temperature

and salinity were maintained at 10^{O}C, and 32 PSU respectively. Air was bubbled into the tank and nutrients (Churchill media) were added weekly. The seedlings were held in these conditions until experiments began.

A total of 60 seeds germinated and developed foliage leaves; this is similar to a seedling production rate that is found in nature (e.g., Cabaco and Santos, 2010). Thirty-five of the best quality seedlings, based on general observations of condition, were selected and moved to the Fred Hutchinson Cancer Research Center (FHCRC) for the H_2S experiments.

4.2.3 Lethality experiments

Seedlings were randomly assigned into one of six categories; a control and five treatment groups, each with five replicates. Concentrations were derived from observations of H_2S concentrations in the field listed in Table 1; 68 µM, 204 µM, 680 µM, 2.04 mM and 6.8 mM. Seedlings were placed in petri-dishes filled with 25 ml of seawater, with nutrients added, plus the corresponding H_2S solution. Due to the relatively short half-life of H_2S (12 to 37 hours depending on conditions, e.g. Napoli et al. 2006), treatment solutions were replaced every twelve hours to maintain the corresponding concentrations.

Determining plant health when using H_2S is extremely difficult, traditional respiratory and photosynthetic measurements using O_2 electrodes are not applicable because the H_2S creates an environment in which the O_2 is removed. To measure the health of seedlings we used fluorescence, a measurement of photosynthesis. Before the seedlings were placed into solutions, general observations (e.g. color, leaf and root condition) were recorded, and each was laid flat

and scanned using the Z100 Kinetic Multispectral Fluorescence Imaging FluorCam System by PSI, to get a baseline reading for post-exposure comparison. Two photosynthetic measurements were taken using the FlourCam. (1) Qmax, the maximal photochemical efficiency of PSII ($\frac{Fv}{Fm}$). Qmax was calculated according to Krause and Weis (1991) equation:$\frac{Fv}{Fm} = \frac{Fm-Fo}{Fm}$; and (2) the overall absorbance spectrum of the leaf was recorded.

While in treatment, seedlings were returned to the incubator and held in pre-exposure environmental conditions. At 24 and 48 hours, seedlings were scanned again to determine Qmax. After 48 hours, all seedlings were evaluated and survivors were returned to sterile test tubes and returned to the incubator for 1 week. After 7 days these seedlings were scanned again, and surviving seedlings were placed into treatments of 2.04 mM liquid H_2S solution. Twenty-four and forty eight hours later, seedlings were scanned using the FluorCam. Five additional seedlings were scanned and then exposed to 6.8mM H_2S for one hour. Twenty-four hours post exposure each seedling was re-scanned in order to evaluate the effects of short term acute exposure. We assigned Qmax values of <0.2 as non-photosynthetic, 0.2-0.3 as marginal health, 0.3-0.5 as low function but healthy, and >0.5 as healthy and of good photosynthetic function (after: Force et al., 2003; Liu et al., 2006; Guo et al., 2008). Statistical analysis was computed in R. To distinguish differences between field sites a multinomial GLM model with three variables as factors was created. To identify the LD_{50} a 3rd degree polynomial best fit was plotted on a saturation curve (Hoffman, 1995). In 2011 the same procedural methods were used on three additional treatments (400, 500 and 800µM) and a control in order to better define the LD_{50}.

4.3 Results

3.1 Field studies

In sites with stable *Z. marina* populations, average H_2S concentration was 0.052 ± 0.007mM (Table 1). In historic sites where *Z. marina* became locally extinct, H_2S concentration averaged 2.7 ± 0.4 mM, and sites with known declining populations averaged 2.8 ± 0.4 mM. We compared the concentrations of H_2S by the type of *Z. marina* present (declining, none present, present) and it was determined that sites with and without eelgrass were significantly different (ANOVA $\alpha = 0.05$; $P<0.001$). The comparison between extinct sites and ones that were declining was not different (P=0.84).

4.3.2 Lethality experiments

In all treatments, Qmax decreased in the initial 24 hours (Fig. 4.1). In the 2.04 and 6.8 mM treatments the Qmax was below 0.1, indicating no photosynthetic activity. In 68 µM concentration, Qmax was only slightly lower than the control (Fig. 4.1), while Qmax was 0.34 ± 0.02 at 204 µM and 0.32 ± 0.09 at 680 µM. After 48 hours the Qmax of 2.04 and 6.8 mM treated seedlings remained below 0.1 and seedling tissue was turning brown and deteriorating. Within 72 hours, these seedlings had degraded substantially and were dead after 7 days (Fig. 4.2). In 204 µM and 680 µM treatments, measurements indicated that seedlings had lower Qmax after 48 hours. And after 7 days only the 204 and 68 µM were actively photosynthesizing. Interestingly, the 68 µM increased its Qmax to 0.56 ± 0.17, which was significantly higher than the controls ($P<0.01$). The 400 and 500 µM treatments showed similar responses in their Qmax as the 204 µM treatment. The 800 µM treated seedlings showed low Qmax (0.24 ± 0 .07) after 24 hours and by 48 hours no photosynthesis was occurring, Qmax = 0.

After 7 days of recovery, all seedlings that were still alive (control, 68 and 204µM) were exposed to 2.04 mM solution of H_2S for another 48 hours. After 24 hours all seedlings that were placed in 2.04 mM treatments Qmax decreased to <0.1 indicating no photosynthetic activity. However, after 48 hours the seedlings that had been placed previously in the 68 µM treatment, exhibited a borderline Qmax value of 0.15. One seedling having a value of 0.59, compared to 0.046 twenty-four hours earlier; this value is above those found in the control group. Throughout the experiment Qmax values for seedlings in the control treatment were 0.5 ± 0.02 indicating a strong and stable photosynthetic response.

Using a saturation curve the LD_{50} boundary at 48 hours is 488µM ($R^2 = 0.96$) and at 7 days LD_{50} = 333µM ($R^2 = 0.84$) (Fig. 4.2). In addition, it was found that when *Z. marina* seedlings were exposed to high levels (> 10mM) of H_2S, Photosystem II decreased in activity whereas Photosystem I was maintained (Fig. 4.3). It was also noted that after exposure to high doses, for a short period of time (~1hr), this change in photosystem activity is reversible and after a 24-hour recovery period photosynthetic activity returns to pre-exposed parameters (Table 4.2).

4.4. Discussion

Both field observations and laboratory experiments, as described in this study, suggest that even relatively low concentrations of H_2S (400-500µM) appear to significantly increase mortality in *Z. marina* seedlings. For example, in embayments where *Z. marina* was not recovering, we found levels of H_2S that where higher than the LD_{50} concentrations observed in our laboratory experiments.

Our data suggest that the presence of H_2S has significant effects on the relative activity of the two photosystems. This was first observed by Oren et al., (1979) in cyanobacteria, and our observations, suggest a similar pattern in an angiosperm -seagrass. At low doses (68 µM) photosynthetic activity (Qmax) and leaf health increases, this is similar to findings by Chen et al., (2011) in experiments with *Spinacia oleracea* seedlings. It appears that chloroplast biogenesis is at least partially responsible for this phenomenon. However in higher concentrations Photosystem II shuts down and Photosystem I remains active (Oren et al., 1979; Cohen et al., 1986; Chen et al., 2011; our study). Studies by Thomson and Kats (1978), Chen et al., (2011), and ours suggest that these responses are conserved throughout the plant kingdom.

It is possible that higher concentrations of H_2S could reduce photosynthetic oxygen output, and lacunar down flux to the roots and rhizomes to the extent the toxic sulfide intrusion would take place (Goodman et al., 1995; Erskine and Koch, 2000; Pedersen et al., 2004; Koch et al., 2007; Mascaro et al., 2009). This reduction and influx of toxic sulfide into the tissues could cause decreased seedling health and ultimately results in death (Goodman et al., 1995; Erskine and Koch, 2000; Pedersen et al., 2004). Therefore our data suggest that increases in sediment H_2S can explain why *Z. marina* seedlings have been unable to successfully re-establish in sites that have experienced declines.

Acknowledgments

We would like to thank H. G. Greene, Mike Morrison and Loren Ballanti for their efforts. We are grateful for the logistic support provided by Fred Hutchinson Cancer Research Center, University of Washington and the Friday Harbor Laboratories. Figure assistance was provided by the Design Help Desk at the University of Washington. The Design Help Desk is funded by the National Science Foundation REESE program Grant No. 1008568. The authors declare no competing financial interest; funding was provided NSF #0910196. Correspondence and requests for materials should be addressed to F.D.D. (fdd@u.washington.edu).

Table 4.1 Sediment H_2S concentrations by site with, without or declining seagrass

Site	H_2S mM Conc.	Seagrass Present
Fisherman Bay	2.653	Declining
Fisherman Bay	0.842	Declining
Fisherman Bay	1.205	Declining
Fisherman Bay	1.205	Declining
Fisherman Bay	3.677	Declining
Fisherman Bay	4.371	Declining
Fisherman Bay	2.042	Declining
Fisherman Bay	1.936	Declining
Fisherman Bay	4.404	Declining
Fisherman Bay	4.383	Declining
Picnic Cove	4.424	Declining
Picnic Cove	4.457	Declining
Picnic Cove	1.769	Declining
Picnic Cove	0.438	Declining
Picnic Cove	4.412	Declining
Westcott Bay	0.666	No
Westcott Bay	3.459	No
Westcott Bay	3.480	No
Westcott Bay	3.415	No
Westcott Bay	3.427	No

Westcott Bay	0.066	No
Westcott Bay	0.354	No
Westcott Bay	4.230	No
Westcott Bay	1.293	No
Westcott Bay	2.791	No
Westcott Bay	3.907	No
Westcott Bay	4.230	No
Westcott Bay	3.613	No
Shallow bay	0.060	Yes
Shallow bay	0.033	Yes
Shallow bay	0.047	Yes
Shallow bay	0.047	Yes
Shallow bay	0.074	Yes

Table 4.2 Evaluation of short term high level (6.8mM) H_2S exposure.

	Qmax			
Plant	Pre-Exposure	One Hour Exposed	24 Hours Post Exposure	Photosystem Recovery
1	0.76	0.32	0.61	Yes
2	0.64	0.21	0.71	Yes
3	0.69	0.04	0.32	Yes
4	0.77	0.39	0.38	Yes
5	0.45	0.21	0.71	Yes

Figure 4.1 Variation in Qmax ($(\frac{Fv}{Fm})$ indicator of photosynthetic function) of as a function of H_2S concentration and duration of exposure. Note, both 2.04 and 6.8 mM Qmax is <0.1, thus are determined to be dead. The 204 and 680 µM treatments have suppressed photosynthetic function and 68µM changed very little. SE bars shown.

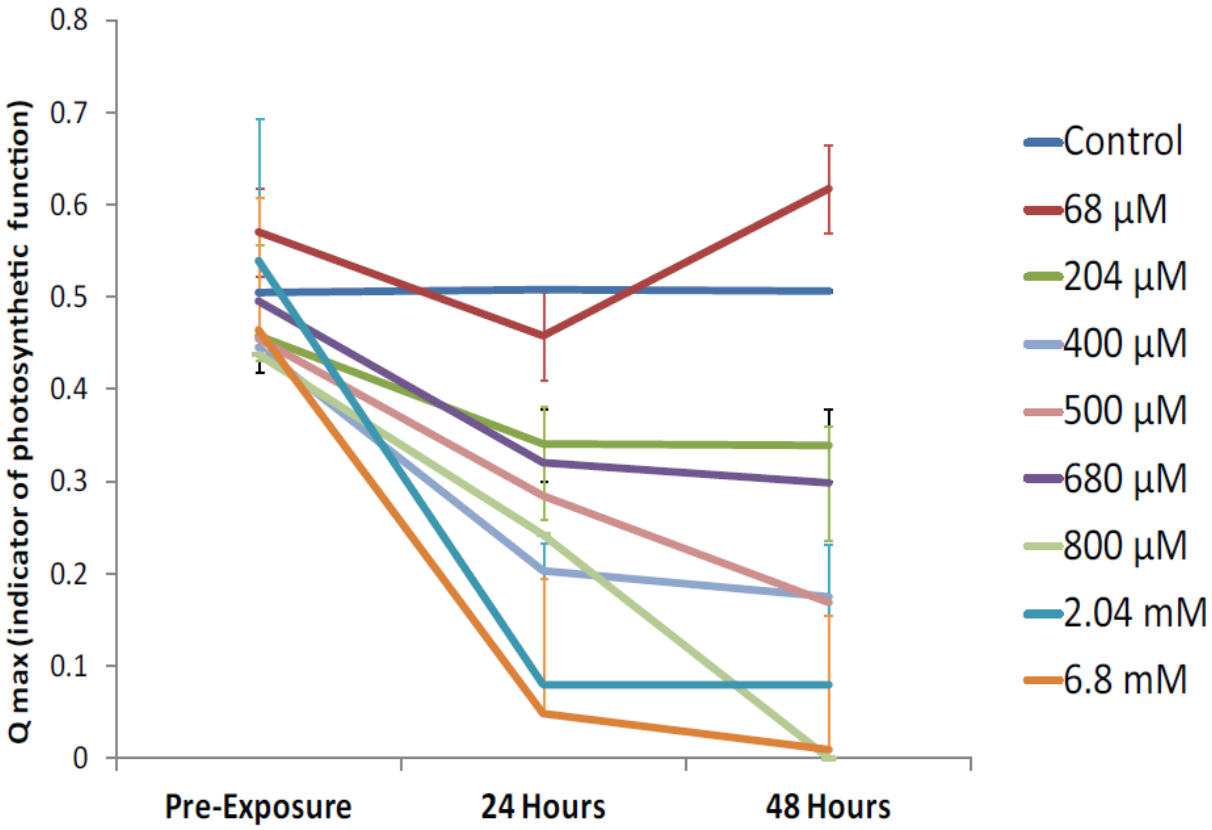


Figure 4.2 Seedling percent survival for each H_2S treatment over time. LD_{50} at 48 hours is 488µM (R^2 = 0.96) and at 7 days LD_{50} = 333µM (R^2 = 0.83).

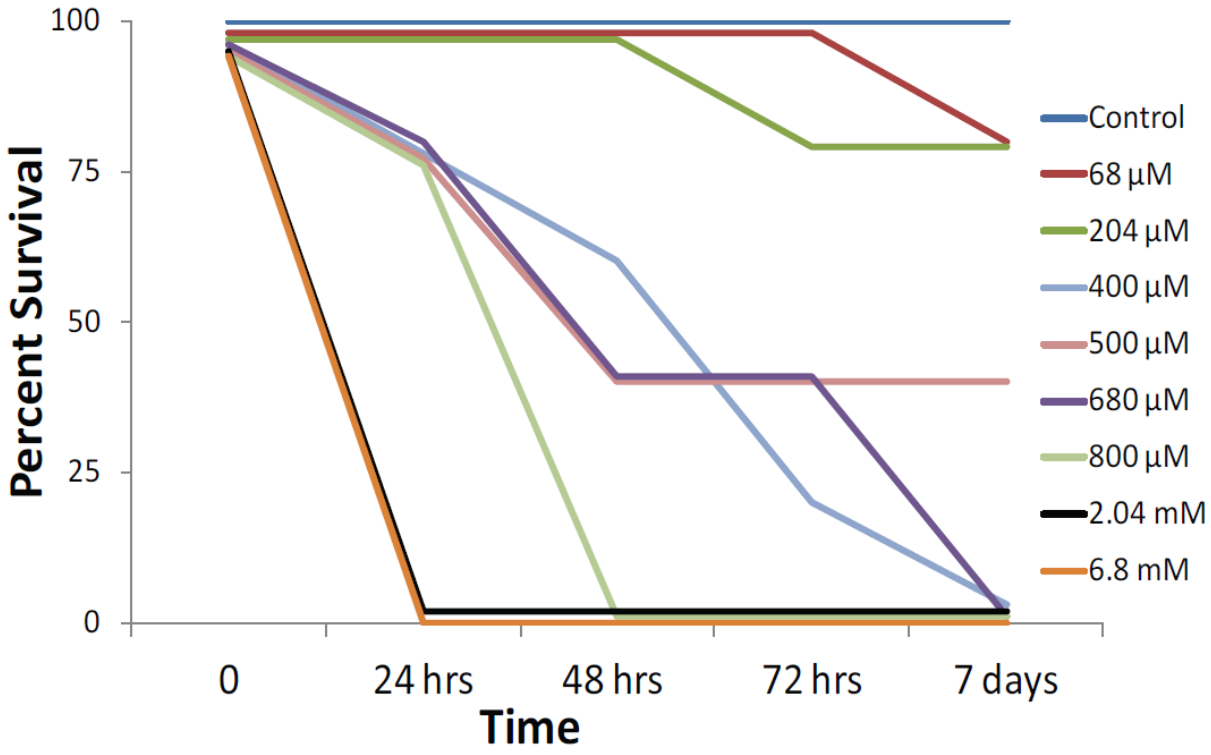
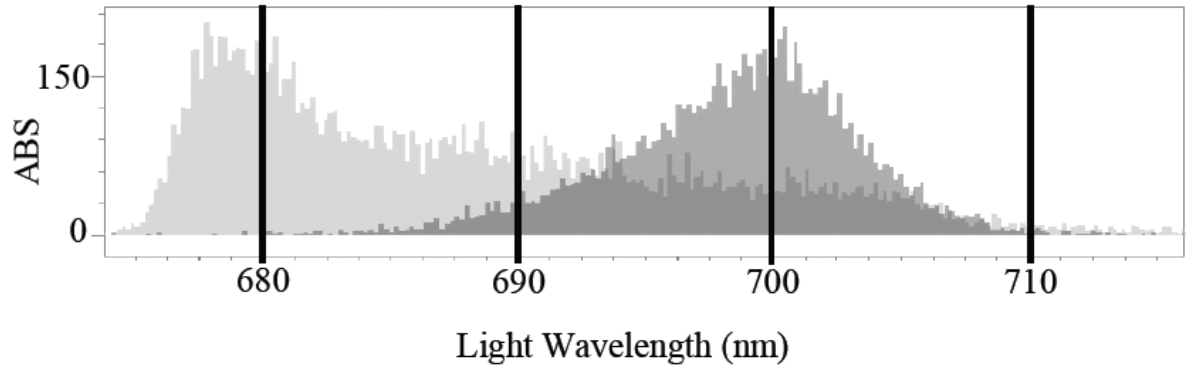
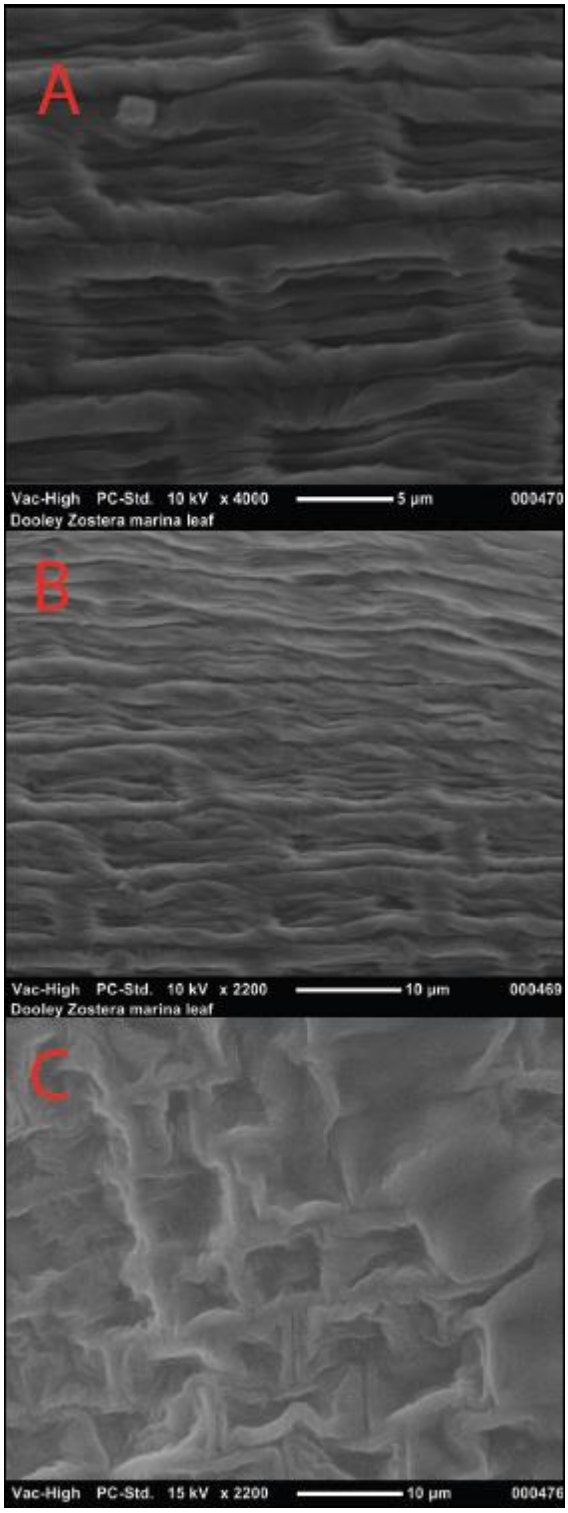


Figure 4.3 An example of the irradiance spectrum observed in a plant both pre and post expose to 6.8mM H_2S. Note in the pre-exposure spectrum (light gray), ABS (absorbance) is occurring in both 680 and 700 nm; however, the vast majority of spectrum is under 690nm. This indicates that both photosystem I and II are active- in normal conditions, where photosystem II is the dominant reaction. Notice that post 6.8mM H_2S exposure there is a dramatic shift in the spectrum (dark gray, intermediate grey is overlap). There is almost zero activity below 690nm, and the peak is at ~700nm. This spectral shift is directly correlated with the activity of PSI. Lack of activity in the lower spectrum suggests that PSII is inactive.



Supplemental 4.1: Leaf structure under microscopy. (A) normal leaf structure (0µM H2S); (B) leaf exposed to 100µM; (C) leaf exposed to 1000µM.



Chapter 5

Tolerance of *Phyllospadix scouleri* seedlings to hydrogen sulfide

Chapter 5 introduces the physiological responses of *Phyllospadix scouleri* seedlings to hydrogen sulfide. Portions of this chapter were originally published in the Journal of Aquatic Botany in collaboration of Sandy Wyllie-Echeverria, Eric Gupta, and Peter D. Ward. **Dooley F.,** Wyllie-Echeverria, S., Gupta, E., Ward, P. 2015. Tolerance of Phyllospadix scouleri seedlings to hydrogen sulfide. *Aquatic Botany.* 123: 72-75.

Abstract

Phyllospadix scouleri is a common seagrass along the rocky intertidal coast of the Pacific Northwest. Previously we established a correlation between increased sulfide and hydrogen sulfide (H_2S) and *Zostera marina* seedling senescence. While *Z. marina* grows in soft sediment environments, here we evaluate the possibility that *P. scouleri* may experience similar decreases in health when exposed to increasing H_2S loading. To do this, seedlings were immersed in various concentrations of H_2S, in axenic media, and photosynthetic and respiratory output was measured. We found that at high doses (mM) of H_2S Photosystem II was inhibited whereas Photosystem I remained active. At lower levels, total photosynthetic output decreased with increasing H_2S concentrations. Using these data we produced an LD_{50} of 430 µM at 48 h and 86

μM at 7 d. Our study confirms that *Phyllospadix* seedlings are also vulnerable to increasing sulfide loads.

Key Words extinctions, lethality, photosynthesis, seagrass, seedlings, climate change

5.1 Introduction

Seagrass populations around the world and locally within the Pacific Northwest have undergone extensive reductions in abundance and recently the health of large meadows has also started to decrease (Short and Burdick, 1996; Orth et al., 2006; Short et al., 2011). Washington State has recently engaged in a restorative campaign to regain 20% of its meadows by 2020 (Thom et al., 2014), however based on our and others' studies this may be difficult and more populations may go extinct (Wyllie-Echeverria and Ackerman, 2003; Elliot et al., 2006; Waycott et al., 2009). Explanations for the recent decline are wide ranging from anthropogenic activities to localized climate change (Short and Wyllie-Echeverria, 1996), and a combination of causes (Raven and Scrimgeour, 1997; Pedersen et al., 2004; Frederiksen et al., 2006; Orth et al., 2006; Mascaro et al., 2009).

Research has suggested that while individual seagrass plants may be able to tolerate a wide range of temperature (Bulthuis, 1987), salinities (Philips, 1972), and pH (Invers et al., 1997), a combination of these environmental changes may decrease the individual health and ultimately mortality. Moreover, we and several authors (Short and Wyllie-Echeverria, 1996; Elliot et al., 2006; Waycott et al., 2009) have found that in sites with high mortality hydrogen sulfide levels are found to be higher than in sites with healthy populations. In-situ laboratory

experiments have shown sulfides to be toxic to *Zostera marina* seedlings and other seagrasses (Lamers et al., 2013; Govers et al., 2014) which has probably led to their decline (Dooley et al., 2013a). Accumulation of sulfides in the rhizosphere may have several causes and the mechanism is fairly well studied (Van der Heide et al., 2011 and 2012).

Species inhabiting shallow coastal waters with little exchange such as lagoons could be expected to have developed accumulative and or adaptive mechanisms (Holmer and Bondgaard, 2001). The Van der Heide (2012) report on the symbiosis shows that microbial communities, in conjunction with invertebrate organisms (Lamers et al., 2013), affect the seagrass tolerances to these influxes of S. In well-flushed waters, however, S accumulation may be less severe, and Marbà et al. (2005) have shown that eutrophication effects are limited on rocky shores because of the high-water exchange. Therefore we hypothesize that *Phyllospadix scouleri*, a seagrass inhabiting rocky shores (Cooper and McRoy, 1988; Park and Lee, 2010), will have a higher sensitivity to S than *Z. marina*.

Our objective was to experimentally assess the relationship between H_2S concentrations and *P. scouleri* seedling health, and compare results to those obtained for *Z. marina* (Dooley et al., 2013a).

5.2 Materials and Methods

5.2.1 Seed germination and culture preparation

Generative shoots of *P. scouleri* were collected at Cattle Point, San Juan Islands, USA, in the summer of 2013. Shoots, containing seeds, were transported to the Friday Harbor Laboratories, University of Washington, and placed into containers serviced by flowing

seawater. Between 30 and 90 days later, container contents were sieved and all seeds were retained, and stored in the dark at 5 ºC and 32 PSU until germination (Dooley et al., 2013a, b).

In January 2014, seeds were sterilized with a 25% bleach solution for 20 min. Fifty seeds were placed into a 500 ml flask with 300 ml of sterile seawater. Once the seeds germinated (ranging in time from two days to almost six weeks) they were transferred to a submerged, closed, sterile seawater tank located in an environmental chamber at the Department of Biology, University of Washington. The seedlings were supplied a daily minimum of 6 h of Photosynthetically active radiation (PAR), 235 µmol $m^{-2}s^{-1}$, at a pH of ~8.1, and temperature and salinity were maintained at 10^{O}C and 32 PSU respectively with nutrients added [$NaNO_3$ + Na_2HPO_4 + $MnCl_2.4H_2O$ + Ferric-sodium EDTA + H_3BO_3 + HCl] (Churchill, 1991).

All experiments were conducted axenically, and tested to confirm. Testing was conducted by removing 1mL of media and rubbing plants with sterile swab and placing contents into an agar petri-dish (streaking isolation) with sterile bacterial culture medium (e.g. 0.1% peptone) and seawater. Petri dishes were cultured three days at 10°C. In practice axenic usually means 'without demonstrable unwanted prokaryotes or eukaryotes', however in reality there is no way of demonstrating that a micro-algal culture is completely axenic. Therefore, any colonies detected were counted and declared to be axenic if infection was under 5%.

5.2.2 H_2S production and measurement

H_2S was made by dissolving 60.04 g of sodium sulfide nonahydrate into 500 mL of double filtered deionized-water (Roth personal communication). Twelve molar hydrochloric acid was then titrated into this solution in 0.01mL increments while stirring, until a pH of 7.2 is reached, resulting in a solution 0.5M ± 25mM (5%), as determined with a H_2S/Sulfide Probe

(Sea & Sun Technology GmbH, Trappenkamp, Germany). Next, the 0.5 M H_2S solution was filtered (0.2 µm pore size) and stored in 250 mL flasks capped with nitrogen gas to maintain stability. We added di-H_2O to the stock solution to make each treatment concentration. After dilution we confirmed sulfide concentration with the H_2S/Sulfide Probe.

5.2.3 Lethality experiments

Seedlings were randomly assigned into eight categories [0, 50, 100, 250, 500, 750, 1000, and 5000 µM] each with six replicates. Concentrations were selected based on field measurements (Dooley et al., in review) and were prepared using the above method. Individual seedlings were placed in 250 ml flasks containing 200 ml of sterile seawater, with nutrients added, plus (or –) the corresponding H_2S solution. Due to the relatively short half-life of H_2S (Napoli et al., 2006), treatment solutions were replaced every 24 h to maintain the corresponding concentrations. While in treatment, seedlings were returned to the environmental chamber and held under standard conditions similar to the natural habitat which the seeds were harvested: 6 h of 235 µmol $m^{-2}s^{-1}$, at a pH of ~8.1, and temperature and salinity were maintained at $10^{O}C$ and 32 PSU (Dooley et al., 2013a)

To measure seedling health, length, wet mass and overall condition was recorded. Because dead or necrotized plants may appear to be healthy (are still green), and because traditional respiratory and photosynthetic measurements using O_2 electrodes are not applicable when using H_2S, fluorescence was measured. Fluorescence was measured by laying each seedling flat and then scanning it using the Z100 Kinetic Multispectral Fluorescence Imaging FluorCam System by Photon Systems Instruments (PSI). Two photosynthetic measurements were taken using the FluorCam. (1) Qmax, the maximal photochemical efficiency of PSII ($\frac{Fv}{Fm}$).

Qmax was calculated according to Krause and Weis (1991) equation: $\frac{Fv}{Fm} = \frac{Fm-Fo}{Fm}$; and (2) the overall absorbance spectrum of the leaf (Dooley et al., 2013a).

At 24 h and every 24 h thereafter for a total of seven days, seedlings were scanned using the FluorCam. Using the assigned Qmax values of <0.2 as non-photosynthetic, 0.2-0.3 as marginal health, 0.3-0.5 as low function but healthy, and >0.5 as healthy and of good photosynthetic function (after: Force et al., 2003; Liu et al., 2006; Guo et al., 2008; Dooley et al., 2013a, Gupta et al., 2014), we established a relationship between health and photosynthetic function. After seven days, seedlings were removed from the H_2S treatment and returned to pre-exposure conditions. One week later seedlings were re-assessed.

To determine if respiration was also affected, individual seedlings were placed into OX1LP-50 mL Dissolved Oxygen Package (DOP) by Qubit, and held at 10°C. The dissolved oxygen package includes an O_2 electrode within the water-jacketed cuvette used for measurements of O_2 consumption, which was calibrated using a two-point system. Individual healthy non-exposed seedlings were placed into the DOP for 12 h while in the dark, and the quantity of dissolved oxygen in the solution was recorded. After 12 h, the seedling was transferred to a 50 mL cuvette containing 10 mM of H_2S and it was kept in this solution for 12 h (235 µmol $m^{-2}s^{-1}$ PAR). After 12 h, of exposure, the seedling was rinsed with sterile seawater and placed back into the DOP in the dark for another 12 h. Pre and post exposure respiration measurements were compared. A 12:12 light cycle was chosen based on preliminary studies and for equal parts exposure and recovery.

5.2.4 Statistical Tests

Means from each treatment group were compared using a Dunnet Test. To identify the $LD_{50,}$ a Michaelis Menten equation was plotted and to establish significance between the different treatment groups and control a GLM model with an anova was developed. Finally, the LD_{50} from the *Z. marina* study (Dooley et al., 2013) was compared to the values obtained in this study using a Wilcoxin Rank Sum Test. The criterion for significance was set at $P<0.05$.

5.3 Results

Length and mass of the treated and untreated (control) seedlings did not significantly differ. Treated seedlings did not appear visually any different from those of the control for the first 72 h. After 7 d the seedlings in H_2S treatments which had low or no photosynthetic response were turning brown. Photosynthetic activity of the seedlings maintained comparable levels until H_2S was greater than 50 µM, thereafter photosynthetic output decreased and ultimately ceased after 72 h in concentrations >500 µM (Fig. 5.1). Using photosynthetic output as the measure of survivorship, there were significant differences between control and ≤100µM treatment groups ($P<0.001$); 50µM was not different from that of the controls. Using a Michaelis Menten equation the LD_{50} boundary was assigned at 430 ± 21.9 µM ($r^2 = 0.97$) after 48 h and 86 ± 13.86 µM ($r^2 = 0.81$) after 7 d (Fig. 5.2). In addition, it was found that when *P. scoluri* seedlings were exposed to high levels of H_2S, Photosystem II decreased in activity whereas Photosystem I was maintained, which is very similar to the response of *Z. marina* seedlings (Dooley et al., 2013a). It was also noted that after exposure to high concentrations (10 mM), for a short period of time (~1h), this change in photosystem activity appeared reversible. After a 24 h recovery period, 83% of the seedlings returned to a photosynthetic output of >0.5. Also, these data suggest that respiration

does not decrease, but rather increases two to four fold that of pre-exposure; pre-exposure average = 0.82 ± 0.57 mg/L O_2 per h, post-exposure = 2.54 ± 1.09 mg/L O_2; P=0.02.

5.4 Discussion

The experiments presented here provide evidence in support of the hypothesis that *P. scouleri* seedlings have a lower tolerance to sulfide (7 d LD_{50} = 86 µM) than *Z. marina* (7 d LD_{50} = 334 µM); the difference which was significant (P<0.01). As stated earlier one would expect this based on the environment in which the two naturally inhabit. *P. scouleri* seedlings should respond more negatively, which it did during these trials. However we found that the response occurred more instantaneously in *Z. marina* seedlings compared to *P. scouleri* where it took up to 48 h to observe an inhibition response. While this was unexpected, one explanation is the difference in root mass. In our experiment, *Z. marina* seedlings had a larger root mass (and consequently more root tips) than *P. scouleri* seedlings (supplemental fig. 5.3), therefore in a natural habitat *Z. marina* seedlings may uptake H_2S at a greater rate due to the increased root surface area.

The photosynthetic measurements observed here is analogous to those found in other studies (Ralph, 1999) where the presence of H_2S had significant effects on the photosynthetic apparatus. Photosynthetic output of photosystem II (PSII) was inhibited as concentrations approached mM, whereas PSI remained active. Likewise overall photosynthetic output was lowered as concentrations increased. Inhibition of PSII has been observed in cyanobacteria with H_2S (Oren et al., 1979; Cohen et al., 1986) and is known to be less tolerant to heat stress than PSI (Berry and Björkman, 1980; Weis and Berry, 1988) and more vulnerable to toxicity and heavy

metal stress (Chen et al., 2011). However these experiments suggest that respiration is not as adversely effected by the presence of H_2S at the levels tested here. While it is known that H_2S is highly toxic to the electron transport chain, here we found that at these levels respiration may increase. This could be due to any number of factors ranging from tissue trying to repair to the presence of reactive oxygen species. Regardless the end product may be the same. Increased respiration may result in the reduction of remaining oxygen in the system resulting in more anoxia and stress. It is possible that high concentrations of H_2S could reduce photosynthetic oxygen output, and lacunar down flux to the roots and rhizomes to the extent the toxic sulfide intrusion would take place (Penhale and Wetzel, 1983; Goodman et al., 1995; Erskine and Koch, 2000; Pedersen et al., 2004; Koch et al., 2007; Mascaro et al., 2009). This reduction and influx of toxic sulfide into the tissues might result in decreased seedling health and ultimately resulting in death (Goodman et al., 1995; Erskine and Koch, 2000; Plus et al., 2003; Pedersen et al., 2004).

Acknowledgments

We would like to thank Elizabeth Vogt, Olga Vitruk and Crystal Davis for their efforts in the laboratory and LouAnne Wyllie for assistance in the field. We are grateful for the logistic support provided by University of Washington and the Friday Harbor Laboratories. The authors declare no competing financial interest. Correspondence and requests for materials should be addressed to F.D.D. (fdd@u.washington.edu).

Figure 5.1: Qmax over time for *P. scouleri* seedlings

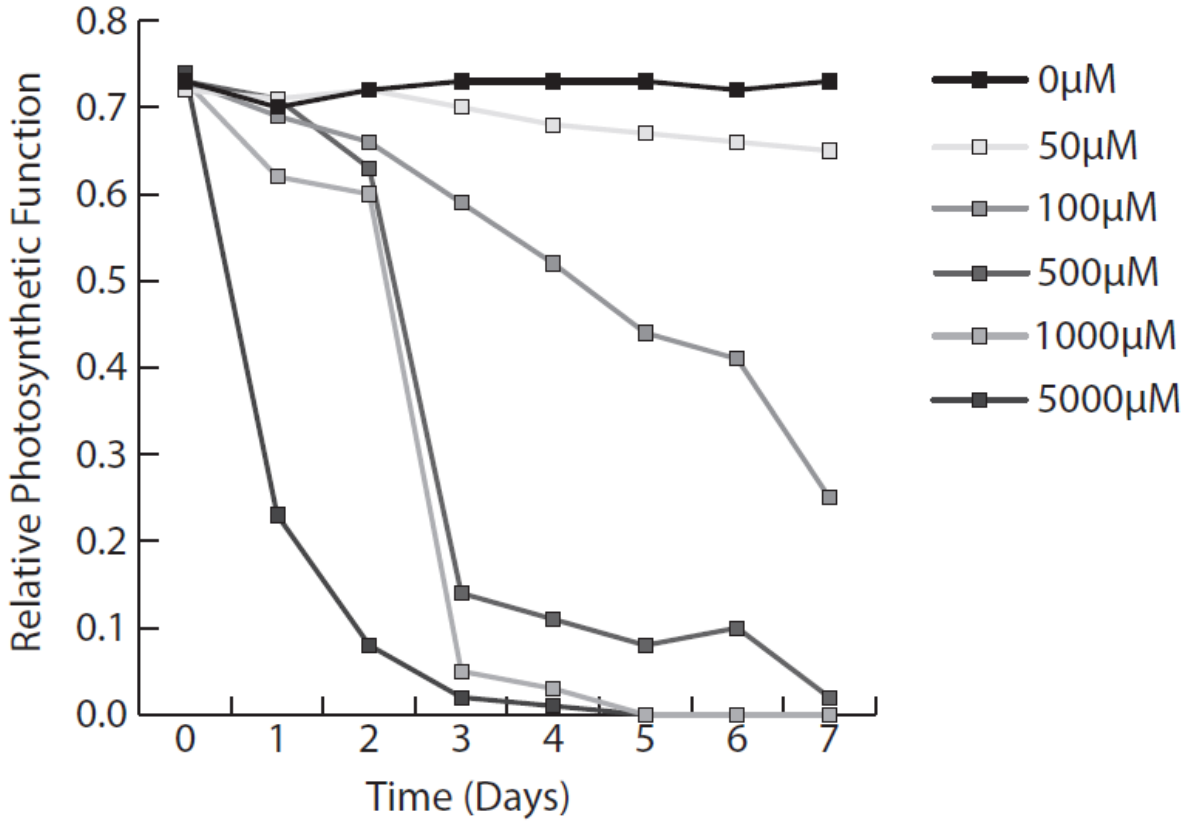
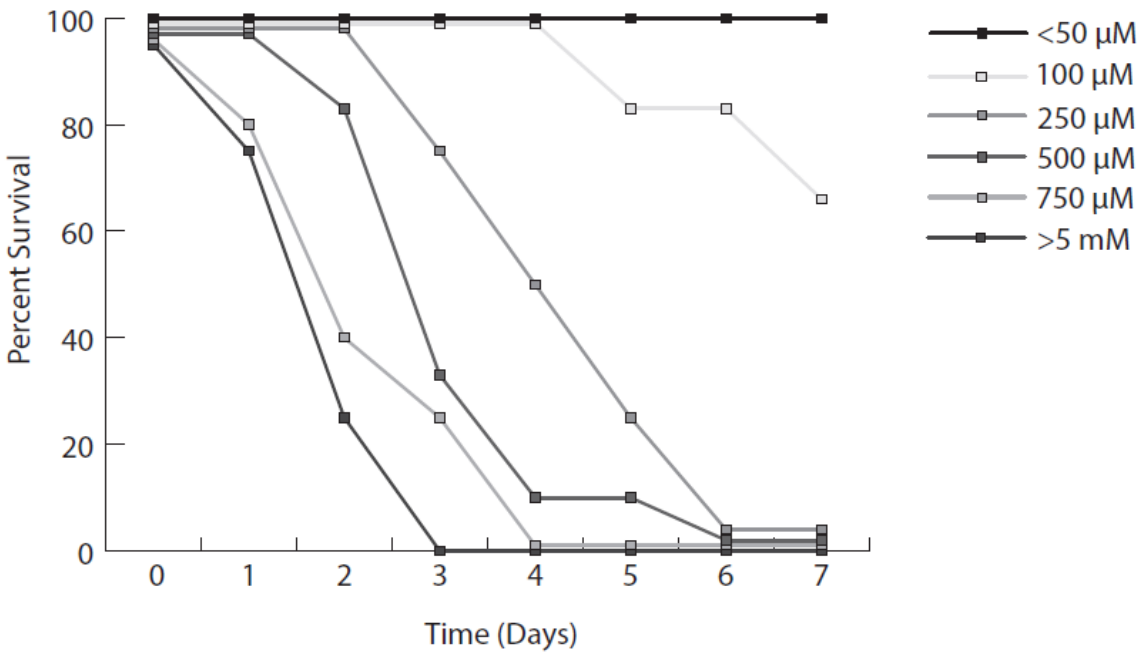
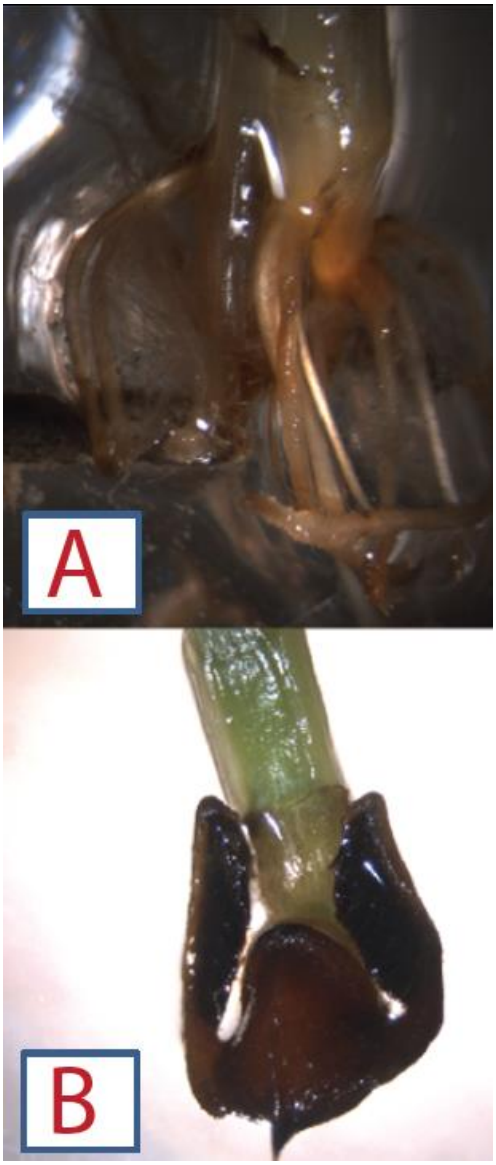


Figure 5.2: LD_{50} for *P. scouleri* seedlings



Supplemental Figure 5.1: Diagram illustrating root comparison between *Z. marina* and *P. scouleri*



Chapter 6

Unusual effects - Hydrogen Sulfide causes unusually fast growth in crop plants and disparity among lethal concentrations

Chapter 6 introduces the topic of unusual physiological effects observed in angiosperms when exposed to various concentrations of hydrogen sulfide. Portions of this chapter were originally published in the Journal of PLOS ONE in collaboration of Suven Nair, and Peter D. Ward. Dooley, F., Ward, P., & Nair, S. 2013. Hydrogen Sulfide causes unusually fast growth in crop plants; PLoS ONE 8(4): e62048. doi:10.1371/journal.pone.0062048. Additional portions were published in the Journal of Toxicological and Environmental Chemistry in collaboration with Eric Gupta and Peter Ward. Gupta, E., **Dooley, F.,** Ward, P. 2014. Evolutionary Legacy Response Observed in Algae & Bryophytes Following Hydrogen Sulfide Administration. J. Toxicol. Enviro. Chem. DOI:10.1080/02772248.2014.944353.

Abstract

Exposing either roots or seeds of multicellular plants to extremely low concentrations of dissolved hydrogen sulfide at any stage of life causes statistically significant increases in cellular and tissue growth rates, as well as increased biomass including higher fruit yield, in spite of the observation that individual cells in treated plants were smaller (~13%) than those of controls. Germination success and seedling size increased in, bean, corn, wheat, and pea seeds also

increases while time to germination decreases. These findings indicated an important role of H_2S as a signaling molecule that can affect rate of growth throughout the plant, in all plant species yet tested. The increased crop yields reported on here has the potential to effect the worlds agricultural output. Additionally over the course of experiments it was determined that there is a disparity among the lethal concentrations among the different taxa tested outside of angiosperms.

6.1 Introduction

The biological effects of hydrogen sulfide (H_2S) have received increasing attention in recent years, not only as a putative kill mechanism during past mass extinctions *(1-4)*, but as an important signaling molecule in both aerobic and anaerobic organisms (*5-13*). Hydrogen sulfide has recently been added to nitric oxide (NO) and carbon monoxide (CO) as a newly categorized group of biologically active gases termed gasotransmitters and gasomediators (*7, 14*). The origin of these dual activities remains unknown, but it may be that these varied signaling and biological mediating capabilities are remnants of biological responses by life either evolving and inhabiting the highly sulfidic and anoxic environments of most or all aquatic habitats during the Archean and Proterozoic Eras, when sulfide, "Canfield Oceans" were global, or are responses to the return to such oceanic conditions during the Phanerozoic "Greenhouse Extinctions", when the oceans changed from mixed to stratified with anoxic, sulfide rich bottoms. Since all organisms on Earth evolved from cells that initiated in sulfide rich environments, and that the current relationship between mitochondria and its surrounding cytoplasm, which entails H_2S oxidation by the mitochondria and H_2S generation by the cytoplasm might, in fact, echo a syntropic association between a deep time sulfide oxidizing purple bacterium and a sulfide producing host.

Today, H_2S causes a wide variety of vital effects across the "Tree of Life", from metabolic inhibition (*15*), to energy source (*16-20*), to coordination of developmental growth programs in yeast (*21-22*) and perhaps higher organisms as well. These findings led Lloyd (*16*) to propose that H_2S is as an important signaling mediator in most or all prokaryotic clades. Yet, in spite of these findings, only recently have there been analogous investigations into the potential signaling role of H_2S in eukaryotes. To date, only studies on its effect on the functioning of the electron transport chain in animals, and separately on its inhibition of physiological processes enabling endothermy in birds and mammals have been conducted.

Studies into the effects of sulfur and sulfide compounds on plants are still few in number (*13,25-26*), and of these, most have concerned the lethal effects of gaseous hydrogen sulfide on plants (*13,26*). From these it is now known that H_2S causes inhibition of photosynthesis at high concentrations (*25, 27-29*) and that it can decrease the time to germination (*31-32*), but also increases the resilience to drought and heavy metal toxicity (*30-33*). There is also recent, emerging evidence of a possible signaling role as well, for stomatal apertures (*11-12, 26*), and in promoting chloroplast biogenesis (*25*).

To ascertain the potential role of H_2S in that of higher plants, we conducted a series of experiments designed to evaluate sub-lethal levels. We show here that micromolar concentrations of hydrogen sulfide dissolved in water and taken up by either seeds or roots have significant effects on important aspects of plant physiology and life history, and may be an important new way of increasing human crop yields.

6.2 Materials and Methods

Hydrogen Sulfide Preparation

Half-molar hydrogen sulfide was made by dissolving 78.04 grams of anhydrous sodium sulfide into 500 mL of double filtered di-water. Hydrochloric acid was then titrated into this solution in 0.01mL increments while stirring until pH of 7.2 was reached, resulting in a solution 0.5M ± 25mM (5%) as determined by using H_2S/Sulfide Probe (Sea & Sun Technology GmbH, Trappenkamp, Germany). The 0.5M H_2S solution was then filtered and stored in a 500 mL flask filled with nitrogen gas to maintain stability.

Each treatment concentration was made adding ddi-H_2O. After dilution the H_2S/Sulfide Probe was used to confirm concentration.

Hydroponic Seed and Seedling Trials

Seeds from four common crop species *Phaseolus vulgaris* (bean), *Pisum sativum* (pea), *USU-Apogee* (Space Wheat), and *Zea mays L*. (corn) were exposed to five levels (0 µM, 10 µM, 100 µM, 500µM and 1mM, ± 5µM error) of liquid hydrogen sulfide dissolved in ddi-H_2O. Germination rates as well as the quantity of growth was measured and compared to a control (0µM H_2S), for a seven day growth period. Thirty seeds, of each species were placed into 100ml petri-dishes filled with 50ml of solution. Due to the relatively short half-life of H_2S when in solution (*40*), treatment liquids were replaced daily. Petri-dishes containing seeds were randomly placed on a shelf serviced by 12 hours of ~100 µmol $m^{-2}s^{-1}$ of light daily. Temperature was ambient room temperature (~23^{O}C). Every day at the same hour, the number of seeds which germinated was recorded. At 72 and 168 hours, the length of the fresh seedling was recorded using digital calipers

Seedlings of the same species of pea and bean used in the germination trials were also germinated hydroponically in 250mL containers (seeds in this case untreated with H_2S) Seeds were wrapped with a clean paper-towel and mounted to a glass slide. The slide, containing seed, was placed into the container and immersed in 75ml of ddi-H_2O. The containers were randomly placed on shelves serviced by grow lights provided with 12 hours of ~100 µmol $m^{-2}s^{-1}$ of light daily. Water was replenished weekly. Temperature was ambient room temperature (~$23^{O}C$).

Upon germination, seedlings were grown for fourteen days and then randomly selected for treatments. Twenty-Five seedlings were selected for each treatment and a control (0µM, 5µM, 10 µM, 100µM, 1mM, 10mM and 50mM). Before exposure, and 24, 48 & 168 hours, photosynthetic output (Qmax of the formula Fv/Fm) was measured with a Z100 Kinetic Multispectral Fluorescence Imaging FluorCam System by P.S.I. Qmax, the maximal photochemical efficiency of PSII (Fv/Fm) was calculated according to Krause and Weis (*41*) equation: $\frac{Fv}{Fm} = \frac{Fm-Fo}{Fm}$. At the same time, measurements of length of both the shoot and root, and wet mass of the plant were taken and recorded (using digital calipers). A liquid H_2S solution of 5 µM, 10µM, 100µM, 1mM, 10mM, and 50mM was then applied to the root each day. All plants were raised under similar environmental conditions ~100 µmol $m^{-2}s^{-1}$ of light daily, ambient room temperature (~$23^{O}C$) and relative humidity. In addition, we measured the pH of each treatment in order to rule out pH change as the reasoning for growth differences.

Growth to maturity in soil

One seed, of *USU-Apogee*, was placed in each of the four corners of a half-gallon pot filled with 400 grams of sunshine #6 soil. Each seed was submersed one cm from the surface of the soil. Six pots, each containing four seeds, were placed into each treatment. Treatments

consisted of 0, 1, 10, 100 and 500 µM H_2S solutions (diluted with ddi-H_2O). Additionally, each treatment consisted of two sub treatments; a three day and weekly exposure regimen.

Seeded pots were watered, either weekly or every three days, with 300 milliliters of the corresponding treatment solutions (0, 1, 10, 100 and 500 µM). Seeded pots were haphazardly distributed in the University of Washington Botany Greenhouse. Each week the length of the shoot, number of leaves, and developmental level, was measured. At the conclusion of nine weeks all plants were mature and had produced fruit. Each plant was carefully removed from the soil with root mass intact, and gently washed to remove the dirt. Whole plants were patted dry with a paper towel and weighed to the nearest 0.1grams. The roots and the fruit were independently removed from the plant and weighed to the nearest 0.1grams. Finally, the length of the plant was measured (to the nearest 0.5 cm).

Leaf Disks

Sixty-four, 6.05 ± 0.03 millimeter diameter, leaf disks were cut from growing leaves on a bean plant, which was grown in UW Botany Greenhouse. Disks were floated in a deionized water bath. Individual disks were haphazardly selected from this water bath, measured with digital calipers and put into labeled petri-dishes. Numbers associated with the labeled petri-dishes were randomly selected using a number generator and sixteen individual leaf disks were placed into treatments (0, 1, 10 and 100µM of H_2S + ddi-H_2O soln.) by a second individual. The first person, who took measurements, was unaware of which disks were in what treatment throughout the experiment. Initial disk size per treatment was statistically compared using ANOVA, to determine if the sample was truly random. Twenty-four and forty-eight hours after

exposure, leaf disks which were determined to be random were re-measured, using digital calipers, and the growth rate was compared.

Images (taken from Leica dissection scope camera) from an additional replicate of 10 disks per treatment, using the same double blind methods, were analyzed with Mitotic Image Plus 2.0 (Motic China Group Co.). Measurements consisted of: precision diameter (the largest distance across the leaf using two points); 3-point circle (3 points selected at random to form a circle of best fit); 5-point circle (5 points selected at random to form a circle of best fit); area in field of view using background exclusion. Measurements were compared to that of the calipers and differences were compared by using theoretical calculations: i.e. a theoretical area was calculated using the diameter (πR^2; R=D/2) as determined by the calipers and compared to that of the measurements obtained above.

Individual cell size was measured using tissues obtained from the leaf disk experiments. Cell size was measured using a 40X lens on compound microscope with camera attached. Images obtained from the microscope were analyzed using Motic Images Plus 2.0. Diameter and area of all cells, in the field of view, were measured; a total of 1590 cells.

Survivorship

To measure plant health and survirorship, individuals were weighted to the nearest 0.01 g, color and overall condition was recorded. However, because a dead or necrotized tissue may appear to be fine (have decent color) photosynthetic measurements were employed to determine if tissue was still functional. Relative photosynthetic capacity (Qmax of the formula Fv/Fm) measured with a Z100 Kinetic Multispectral Fluorescence Imaging FluorCam System by P.S.I. Qmax, the maximal photochemical efficiency of PSII (Fv/Fm) was calculated according to the Krause and

Weis equation: $\frac{Fv}{Fm} = \frac{Fm - Fo}{Fm}$. Qmax values of <0.2 were described as non-photosynthetic, 0.2-0.3 as marginal health, 0.3-0.5 as low function but healthy, and >0.5 as healthy and of good photosynthetic function (after: Force et al., 2003; Liu et al., 2006; Guo et al., 2008; Dooley et al. 2013b). Cultures were grown under standard conditions as listed above.

Statistical Test

Means from each treatment group were compared against the control by a paired T-test. Secondly, these data was inputted into R (R version 2.14.2) and treatments were compared as factors using a linear regression model with ANOVA. Both T and F statistics are listed when appropriate.

6.3 Results

Hydroponic Seed and Seedling Trials

Both root systems and seeds of *Phaseolus vulgaris* (*Bean*), *Pisum sativum* (Pea), *USU-Apogee* (Space Wheat), and *Zea mays L*. (Corn) were exposed to variable concentrations of H_2S dissolved in deionized water. Concentrations of H_2S varying from 10-100µM caused absolute stem and leaf growth (both length and mass) in seedlings to be significantly higher ($F = 10.86$, df 96, $P<0.001$) for all treated plants than growth in controls of the same species (Figure 1). Maximum growth rates for beans was 18.78 ± 1.49 cm at 10µM which was significantly higher than controls which was 8.8 ± 1.26 cm ($T = 3.8062$, df 49, $P<0.001$). Additionally increases in wet weight were highest (0.951 ± 0.16g ($T = 5.46$, $P<0.001$; $F = 9.58$, df 96, $P<0.001$)) in 10µM in beans. The 5µM treatment was not much different to the 10µM treatments with 15.24 ± 1.27 cm and 0.928 ± 0.10g, but was still significantly different from the controls ($F = 10.49$, df 96,

P<0.001). For peas, maximum length change (11.3 ± 0.66 cm) occurred at 100µM (Figure 1) and was the only treatment that was statistically different (T = 3.4035 P<0.01 & F = 3.59, df 1, P<0.01) when compared to controls that was 6.72 ± 0.8 cm. However, maximum change in wet mass occurred at 5µM with an observed value 0.223 ± 0.04g, statically different from controls which were and 0.04 ± 0.05g (F=4.494, df 96, P<0.01). Plants treated with higher than 1mM experienced decreased growth rate, with mortality at >20mM.

In addition to increasing growth rates at a finite and relatively narrow concentration of H_2S, we found that seeds treated with H_2S were statistically different from those of the controls in times to germination (Figure 2), as well as overall ontogeny and length of the plant after seven days. We found a range of concentrations that both increased the rate of seed germination (Table 1), and then brought about elevated growth for up to a week following germination of the treated seeds.

Growth to maturity in soil

During the growth to maturity trials, it was observed that the overall length of the mature space wheat plants, which were exposed to H_2S, were slightly longer, however not statistically different than that of the controls (37.1 ± 2.2cm for 0 µM (controls), whereas 1 µM was 37.5 ± 4.1cm, 10 µM was 38.4 ± 3.6cm, 100 µM was 37.2 ± 4.6cm, and 500 µM was 37.2± 4.7cm). This difference between treatments is amplified when only exposing the plants to H_2S every seven days (36.6 ± 2.1cm for 0 µM (controls), whereas 1 µM was 38.6 ± 5.2cm, 10 µM was 39.9 ± 2.1cm, 100 µM was 40.3 ± 3.6cm, and 500 µM was 40.2± 1.8cm; P= 0.08; F = 2.6). The overall mass of the plant, roots and fruit were independently larger than the controls for all plants exposed to H_2S. The mass of the entire plant was 7.0 ± 0.3g for 0 µM (controls), whereas 1 µM

was 8.3 ± 0.6g, 10 µM was 9.9 ± 0.5g, 100 µM was 8.8 ± 0.7g, and 500 µM was 10.8 ± 0.8g. Each was statistically different from the controls (df = 84, F= 4.81; P<0.01). Likewise, the mass of the roots was bigger in treated plants (1 µM was 3.8 ± 1.6g, 10 µM was 4.5 ± 0.8g, 100 µM was 4.7 ± 1.0g, and 500 µM was 4.7 ± 0.4g) compared to that of the controls (2.8 ± 0.6g for 0 µM). Beyond the macro plant, the fruit of the plant was also larger in the treated plants: 0.95 ± 0.1g for 0 µM (controls), whereas 1 µM was 1.1 ± 0.0g, 10 µM was 1.1 ± 0.1g, 100 µM was 1.1 ± 0.1g, and 500 µM was 1.5 ± 0.2g (Figure 3).

Finally, it was observed that there were differences in the results based on the specifics of timing of treatments. In general, plants exposed to the H_2S every seven days experienced more growth than those exposed every three days. However, even the plants exposed every three days were significantly larger than the controls.

Leaf disk trials

Beyond macro plant growth, leaf disks, which were exposed to H_2S, experienced growth which was significantly larger than those of the controls (F = 5.16, df 63, P<0.01). Mean change in growth was 0.17 ± 0.02mm for controls; 1µM: 0.36 ± 0.01mm; 10µM: 0.26 ± .03mm; and 100µM: 0.32 ± 0.01mm. Finally, when we compared the cells from the leaf disks it was determined that the cell size (diameter and area) was significantly smaller (~13% and 18%) than those of the controls (F = 66.7, df 1590, P<0.001). Mean diameter of controls was 8.38 ± 0.07µm; 1µM: 7.4 ± 0.09µm; 10µM: 7.1 ± 0.06µm; and 100µM: 7.3 ± 0.05µm.

Photosynthetic activity

Throughout the experiments, photosynthetic activity (Qmax - a measurement of maximum photosynthetic output) of all plants was monitored using a Fluorcam. Qmax maintained relatively comparable levels (to untreated controls) until the 50mM concentration, however values showed an increasing trend; not statistically significant. At higher concentrations (>50mM), significant decrease in Qmax was observed and differences in reflected spectra were apparent. In addition, high (<10mM) but sub-lethal levels caused photosystem (PS) II to shut down, whereas PS I remained active through the experimental period through and up to plant senescence.

Survivorship

Chlamydomona - Green microalgae *Chlamydomona sp*., had an LD_{50} of 3.3 mM after 7 days of exposure ($R^2 = 0.88$) (Table 2 & 3), with significant difference between treatment and control groups. Select individuals survived in higher concentrations (5-7 mM) for a limited time. Stable growth patterns were observed up to 1 mM for the entire length of experiments. Movement of flagella was inhibited in concentrations of >10 mM after only a few seconds. If flushed with fresh media within 1 h movement could restart.

Chara - *Chara* sp., of the order Charales LD_{50} was 6.6 mM ($R^2 = 0.8$) after 7 days, with a maximum survival rate of 25 mM (Table 2 & 3). All plants could survive a short term exposure of 50 mM with little effect if flushed with fresh media after 24 to 48 h. Individuals had a varied response but it appears that if most expelled their Ca^+ reserves with exposure after 24 h, this was visually observed however not measured. If exposed to concentrations >10 mM it appeared as if

photosystem II (PSII) was inhibited, whereas PSI remained active. This was reversible up to 48 h after exposure.

Liverworts - Liverwort *Ricctocarpus sp*. had a 7 day LD_{50} of 2.7 mM ($R^2 = 0.86$) and an LD_{50} of 5.7 mM after 96 h (Table 2 & 3). Liverwort Qmax maintained relatively stable levels throughout the experiments at the lower concentrations, however, at higher concentrations (>10mM) it appears as if PSII was inhibited thus reducing Qmax significantly to that of the controls. Both mass and length in the lowest concentrations (1-100 µM) were significantly larger than the controls ($P< 0.001$) (Table 2 & 3).

Moss - Hypnum sp, a common moss, experienced a relatively high LD_{50} of 7.8 mM ($R^2 = 0.9$) (Table 2 & 3) after 7 days with a maximum survival at 25 mM for long term exposure, however its short term tolerance was not as great as Charophytes. Like all macrophytes it appeared as if PSII was inhibited. More over there appears to be an adaptive response at all concentrations of H_2S. This was not concentration dependent as H_2S volatized, as the concentrations were constant. This response (Figure 6) appears to demonstrate resilience to H_2S by the plant through some mechanism. Initially, with exposure, photosynthetic activity is significantly inhibited to that of the control, but with time photosynthetic activity rebounds; at concentrations <100 µM they were no different to that of the control. The maximum photosynthetic output post exposure is dose dependent decreasing with increases in concentration.

6.4 Discussion

In recent years experiments have shown that hydrogen sulfide causes an array of biological effects (13, 33-37) and in this study we add to that growing list. Our experiments are the first to show that the application of extremely narrow concentrations (at taxon specific levels)

of liquid H_2S produces two separate kinds of increased growth rates in plants: time to seed germination; absolute mass of tissue in roots, stems, and leaves. Unlike the results in a previous study (*33*), our applications had no adverse effects on any of the treated plants: for example we observed no lesions or tumors were observed during the duration of the studies reported here. Enhanced growth occurred up to seven days after treatment, followed by a return to growth rates equivalent to the controls, unless re-exposed. This study also shows that H_2S is the *only* chemical necessary to produce such large differences in growth rates in these species.

Regardless of the physical, "macro-" changes in the plant, it is clear that the causes of these results are occurring on a cellular level. Our observation that cells increase in number rather than size suggests that the H_2S molecules are provoking cellular division through some kind of signaling. The increased photosynthetic activity shown in a previous study (*29*) is either caused by increasing the photo-efficiency in the existing chloroplasts, or by increasing the absolute number of chloroplasts per area (25).

Because hydrogen sulfide has 'clandestic messenger' like properties (*13*), this combination of multiple cellular effects (changes in photosynthetic activity and cellular growth rate) from the plant's contact with the relatively few number of H_2S molecules is reasonable. We hypothesize that administered H_2S could be regulating a hormonal pathway (*11,26*) or actively effecting a transcription factor involved in cellular replication (*39*), and not just increasing growth rate as a byproduct of the addition of sulfur as a nutrient ("fertilizer"), as is seen through addition of large concentrations of phosphates or nitrates. In any event, it is clear that H_2S is affecting the basic biology of the plants.

The origin of these effects is open to speculation. However, it may be that an increase in growth rates of some plants at the early onset of an increasing atmospheric or aqueous H_2S load was an evolutionary response to the short term and catastrophic changes in global atmosphere during the Phanerozoic, and perhaps before. Previous work on H_2S toxicity (Dooley et al., 2012, and unpublished observations made during the research described here) has shown that toxicity (as recognized by LD_{50} curves) decreases with plant size, even in the same species. Here we show that tolerance over time is effected by multiple characters but that origin and environmental conditions of ancestors directly effects those of the modern extant species (figure 8). The mechanism observed here might be that simple: at the first recognition of oncoming H_2S concentrations, survivability would be affected by overall plant size. Rapid growth would be selected for. While these results are early in development, these findings may provide beneficial use to agriculture and biofuels.

Acknowledgements

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Table 6.1

Percent Germination over time by concentration of H_2S

Zea mays L. (corn)					
Time (Hours)	0(µM)	10(µM)	100(µM)	500(µM)	1(mM)
24	0	0	0	0	0
48	3.3	10	26.6	5	0
72	23.3	40	46.6	45	40
168	90	90	93	60	75
USU-apogee (wheat)					
24	80	95	90	55	60
48	90	100	95	85	65
72	95	100	95	95	100
168	95	100	95	100	100
Pisum sativum (pea)					
24	0	6.6	10	5	5
48	50	73.3	63.3	30	25
72	73.3	90	76.6	85	65
168	80	96.6	90	85	65
Phaseolus vulgaris (bean)					
24	3.3	6.6	10	10	5
48	3.3	10	13	15	15
72	16.6	26.6	30	75	55
168	93.3	90	100	100	95

Table 6.2:

Organism	Percent Survival by Concentration (mM) after two days of exposure								
	<0.5	**1**	**3**	**5**	**7**	**10**	**25**	**50**	**LD50**
Charophyte	100	100	100	100	100	96	20	33	**20.5**
Chlamydomonas	100	75	90	75	10	30	10	1	**7.5**
Hypnum	100	100	100	70	80	64	70	10	**27.8**
Ricctocarpus	100	100	100	100	33	33	10	0	**9.7**

Table 6.3:

Organism	Percent Survival by Concentration (mM) after seven days of exposure								
	<0.5	**1**	**3**	**5**	**7**	**10**	**25**	**50**	**LD50**
Charophyte	100	100	80	20	20	40	2	0	**6.6**
Chlamydomonas	100	70	10	4	3	0	0	0	**3.3**
Hypnum	100	95	90	50	50	12	10	0	**7.8**
Ricctocarpus	100	100	66	25	0	0	0	0	**2.7**

Figure 6.1: In both *Pisum sativus* (pea plants) *and Phaseolus vulgaris* (bean plants), increased growth as measured linear growth (stems plus roots) is observed with all levels of H_2S exposures. * signifies a data point as being ≤ 0. SD bars shown.

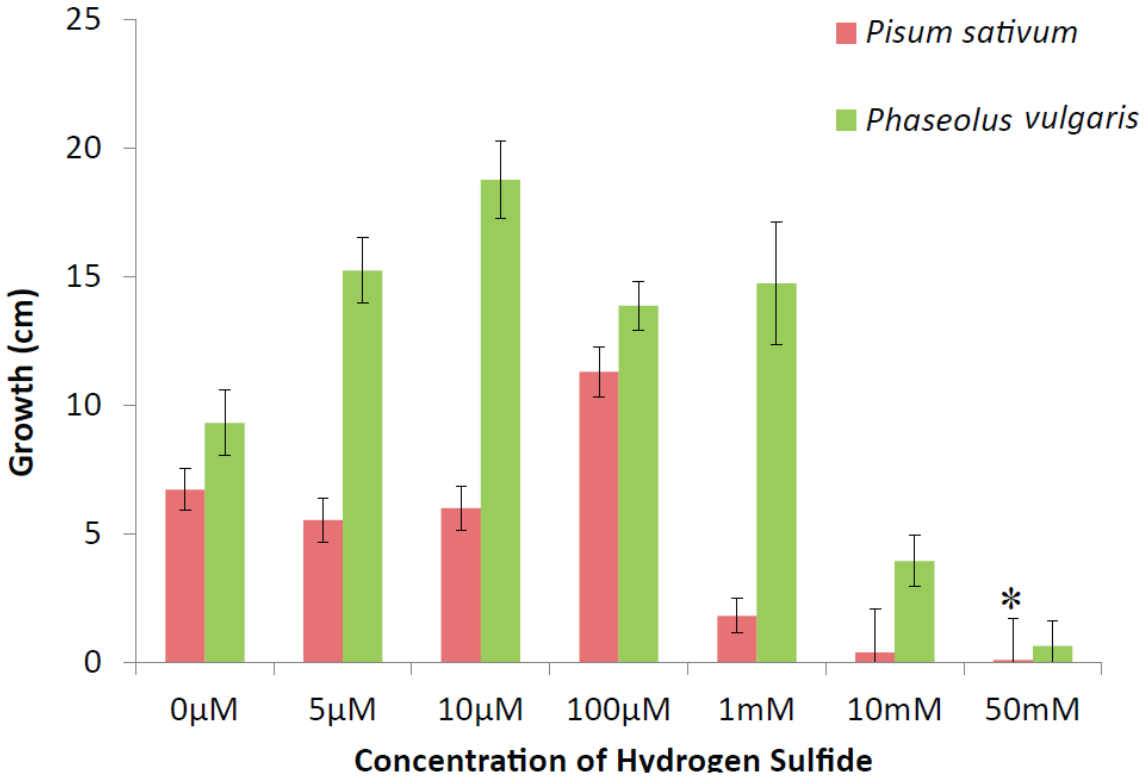


Figure 6.2: This image shows the germination of a USU-Apogee (Space Wheat) seed taken over 119hrs at 16hr intervals. The top panel shows the control seed germination while the bottom displays the H_2S exposed seed. Note at 119hrs the control is less developed then the exposed.

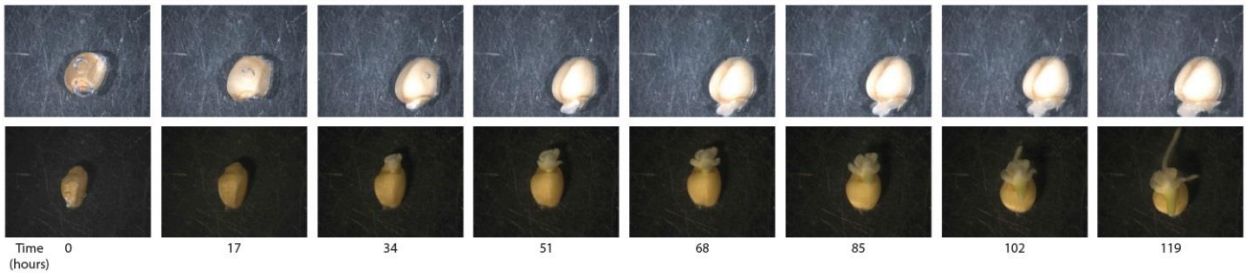


Figure 6.3: Average fruit yield per wheat plant. SD bars shown.

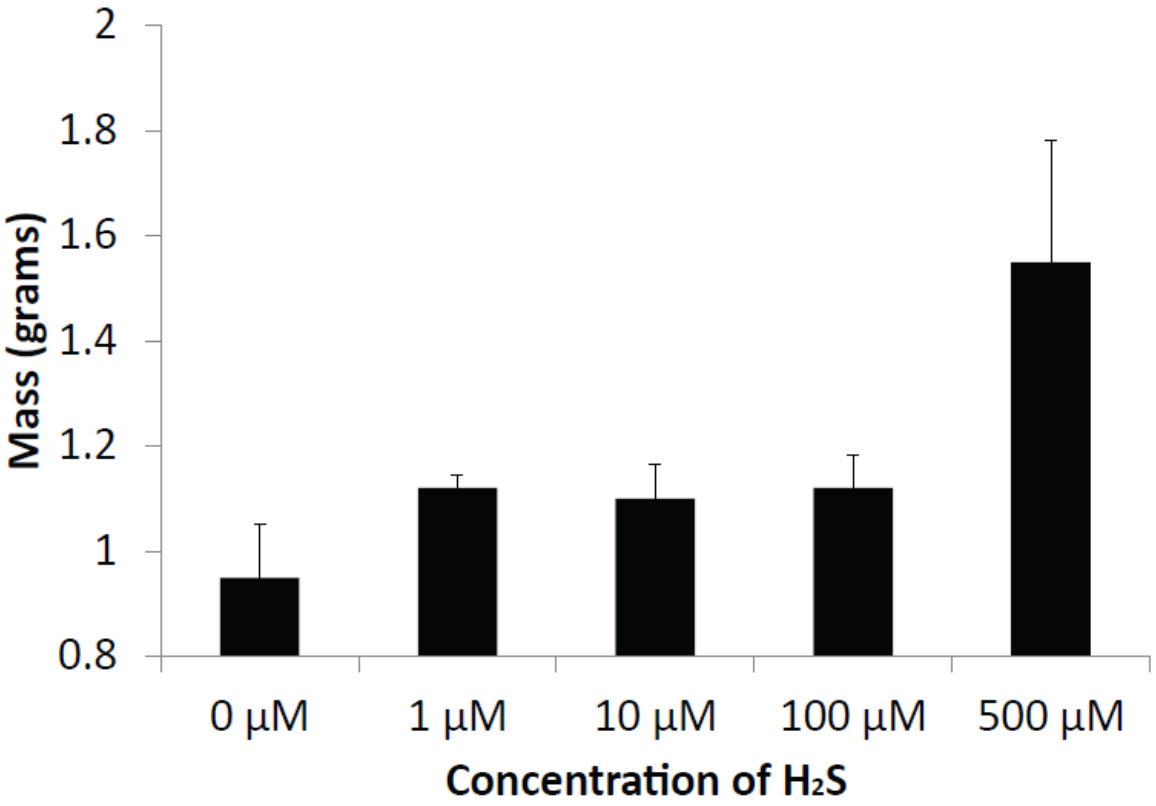


Figure 6.4: Image depicting space wheat trials. Containers with moss are the ones exposed to H_2S.

Figure 6.5: Percent moss cover with weekly and three day treatments.

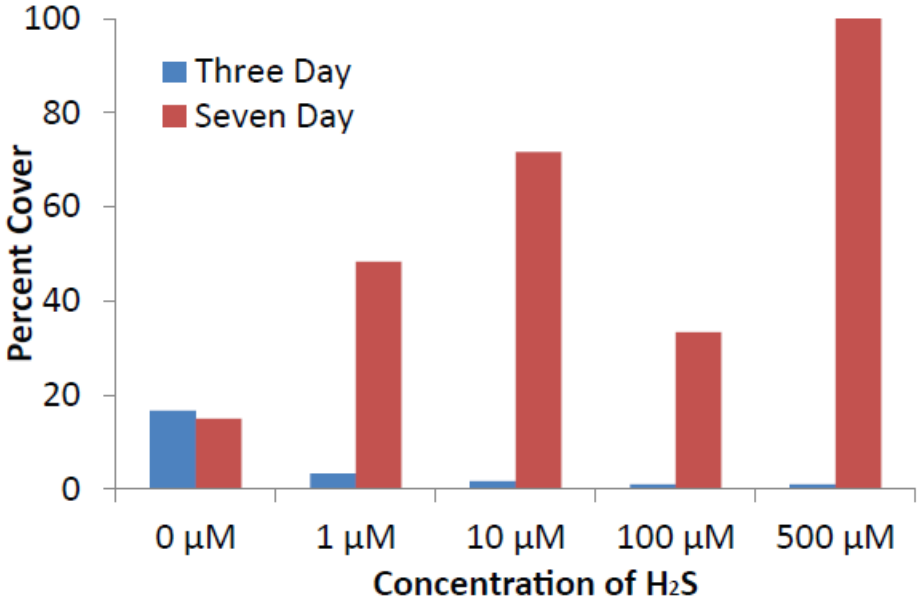


Figure 6.6: Measurements of Qmax (Indicator of photosynthetic function) over time per treatment of H_2S in *Hypnum sp*. Both 0 and 10 µM have relatively comparable values. 100 µM experiences a depressed output for the initial 12 h but rebounds to slightly lower levels than previous exposure, 1 mM experienced the same depression in photosynthesis but is unable to fully recover; >10 mM never experiences any rebound.

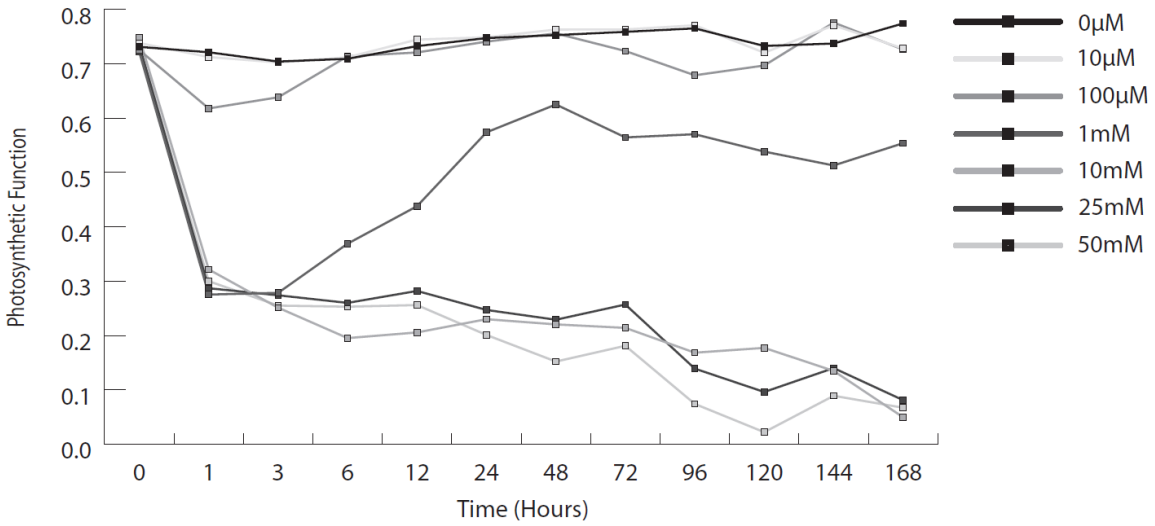


Figure 6.7: Geologic time scale with appearance of species

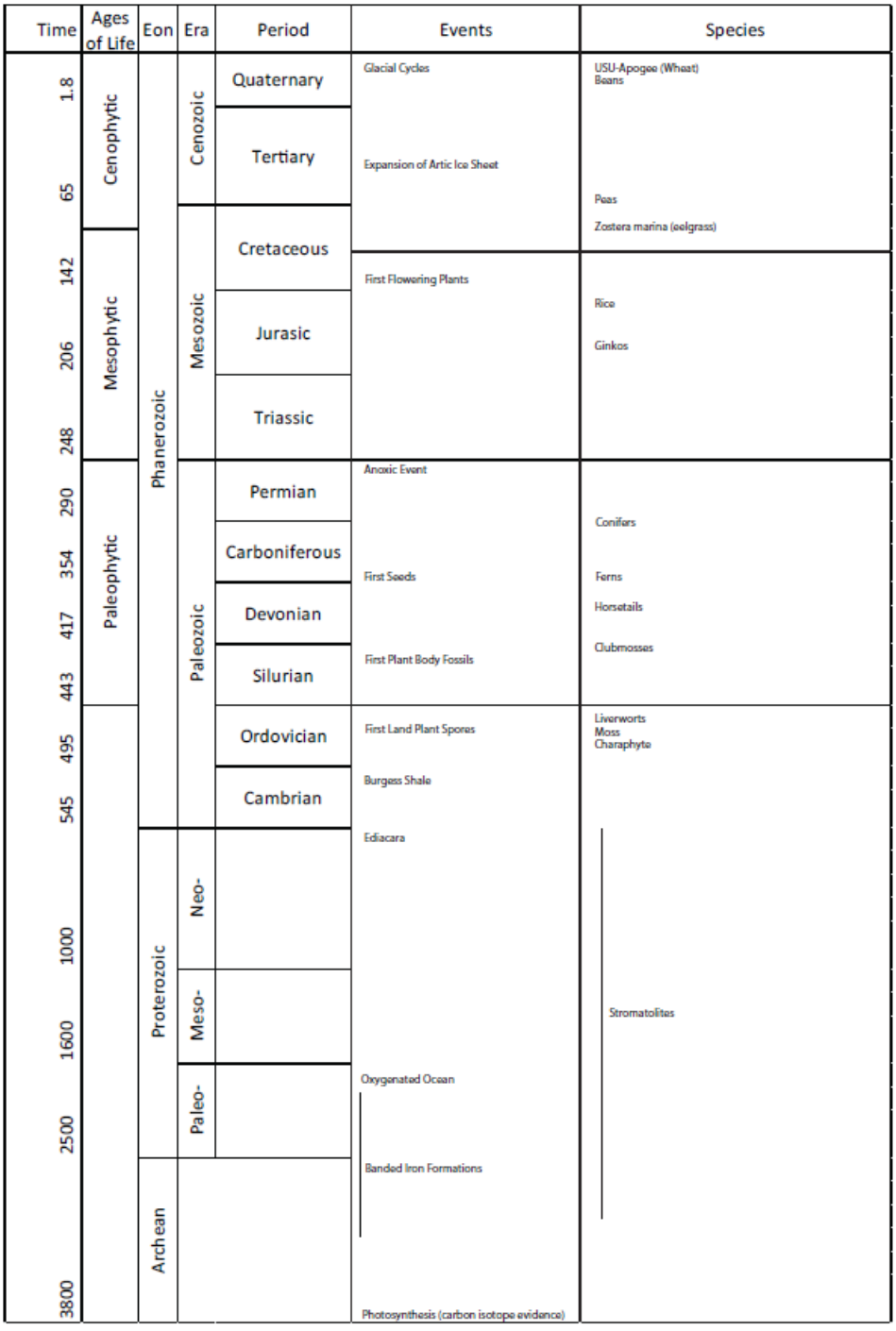
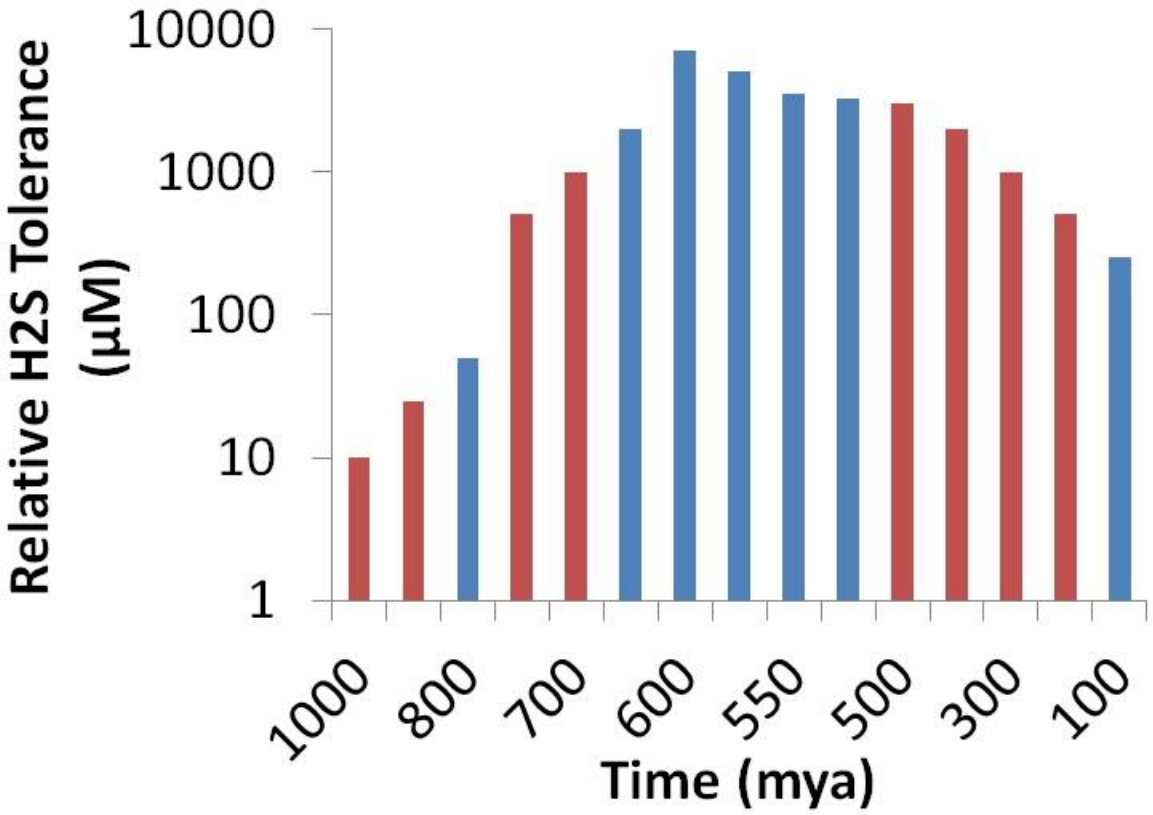


Figure 6.8: Relative H_2S tolerance over time



Chapter 7

Conclusion and synthesis of work

In this thesis I described how *Z. marina* reproduces, its seed production and viability rate, and how seedlings, including those of *P. scouleri*, respond to H_2S. Additionally, I explored the environmental conditions found in locations where *Z. marina* is and was present, and in sites which are undergoing change. I explored how anthropogenic activities and climate change factor in in explaining, the reductions and extinctions locally and globally among seagrass populations, as well as the unusual and evolutionary effects that H_2S play on these and other organisms. However, there are many other factors and co-factors that need to be explored.

Here, I found that increased levels of H_2S effects seedling and mature plants survivability and fecundity (fig. 7.1), however these toxicological effects are only exacerbated by the reduction in fitness caused by other environmental conditions (Garcia et al., 2013), anthropogenic activities, and natural processes that the system is exposed to (Raven and Scrimgeour, 1997; Pedersen et al., 2004; Frederiksen et al., 2006; Orth et al., 2006; Mascaro et al., 2009). Globally *Z. marina* populations have experienced wasting disease. In fisherman bay wasting disease is highly prevalent (Groner et al., 2014). In these circumstances the disease has already reduced the plants fitness, allowing for other environmental conditions to act as a kill mechanism if the disease already has not done so.

Likewise, temperature, salinity, turbidity and heavy metal toxicity all have components to individual and meadow fitness. Most *Z. marina* populations can survive large temperature

changes for short periods, and only exhibit decreased health (and death) after many days (figure 1.2), however with sulfide intrusion this stress is amplified; likewise with salinity and turbidity. Increases in salinity are known to negatively affect the plant physiology (Philips 1972), but it is highly unlikely that this would kill or interact with the sulfides. Turbidity on the other hand, decreases the already small amount of light that the seedlings and mature plants receive, decreasing the amount of photosynthesis, thus the amount of oxygen within the tissues which prevents sulfide toxicity (Penhale and Wetzel, 1983; Goodman et al., 1995; Erskine and Koch, 2000; Pedersen et al., 2004; Koch et al., 2007; Mascaro et al., 2009; Korhonen et al., 2012).

Beyond just the macro-environmental variables there are biological ecosystems and microbial changes that are at play too. Very rarely a system is isolated and only one organism effected. Throughout the physiological experiments tested in this thesis, I only explored the relationship on individuals under euxinic conditions. In nature this would be hard-pressed to find. In figure 7.2 (personal communication Wyllie-Echeverria S and Roth, M) we see the different relationships among the different macro-groups of bacteria found in different locations of sediment, often containing *Z. marina*. There appears to be a change in the micro-fauna when seagrass is present vs declining or extinct (Danovaro et al., 1994; Elliott et al., 2006; van der Heide et al., 2012; Christiaen et al., 2013). This may be in part responsible for the development of H_2S.

All together these variables directly affect seedling recruitment, plant physiology and provide evidence and a mechanism for lethality and extinction within the seagrass meadows (Fig. 7.1). From these experiments and field measurements we can conclude that additional experiments are needed to test the hypothesis that H_2S inhibits seedling recruitment entirely. In the experiments conducted here I started with seedlings and exposing seeds to H_2S, I found that

while some may break dormancy none would produce foliage. However these were only conducted in petri-dishes and for short period of time. Future experiments to elicit this question are needed. Ideally chambers containing seeds, mud, ± mature plants and a method for H_2S dispersal (Fig. 7.2) are to be set up and evaluated for one year under standard conditions. I have designed this experiment under with the hope of evaluating it as a PI next year.

In conclusion, the data and experiments presented here provide support for the hypothesis that increases in H_2S are a mechanism for lethality and reductions in seagrass stand. Future experiments proposed above will help to further evaluate H_2S production, seedling recruitment and ecosystem structure, under the overarching hypothesis in chapter 1.

Figure 7.1: Hypothesis model.

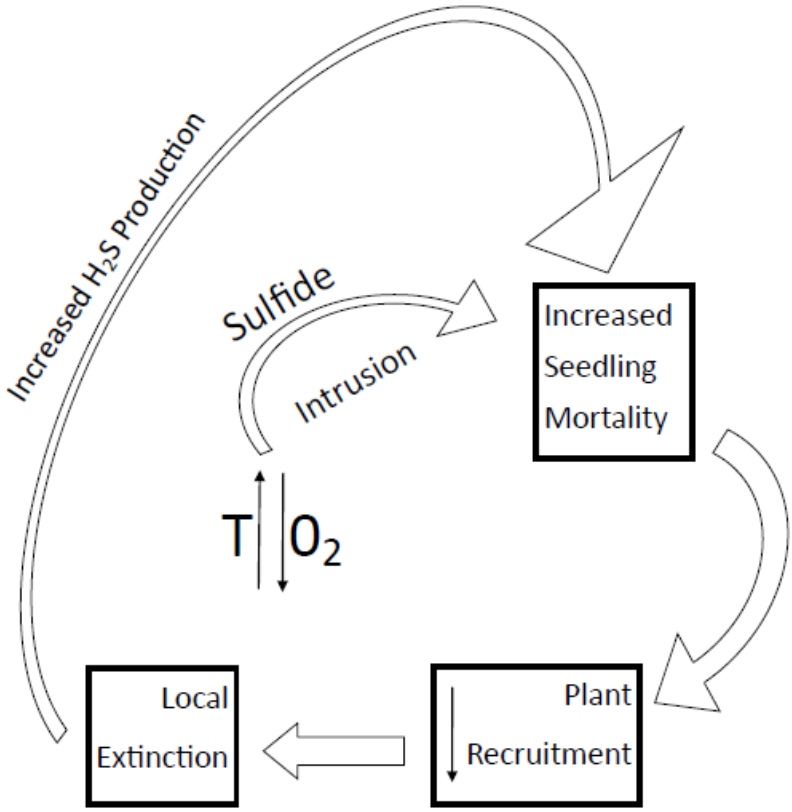


Figure 7.2: Bacterial community distribution by location.

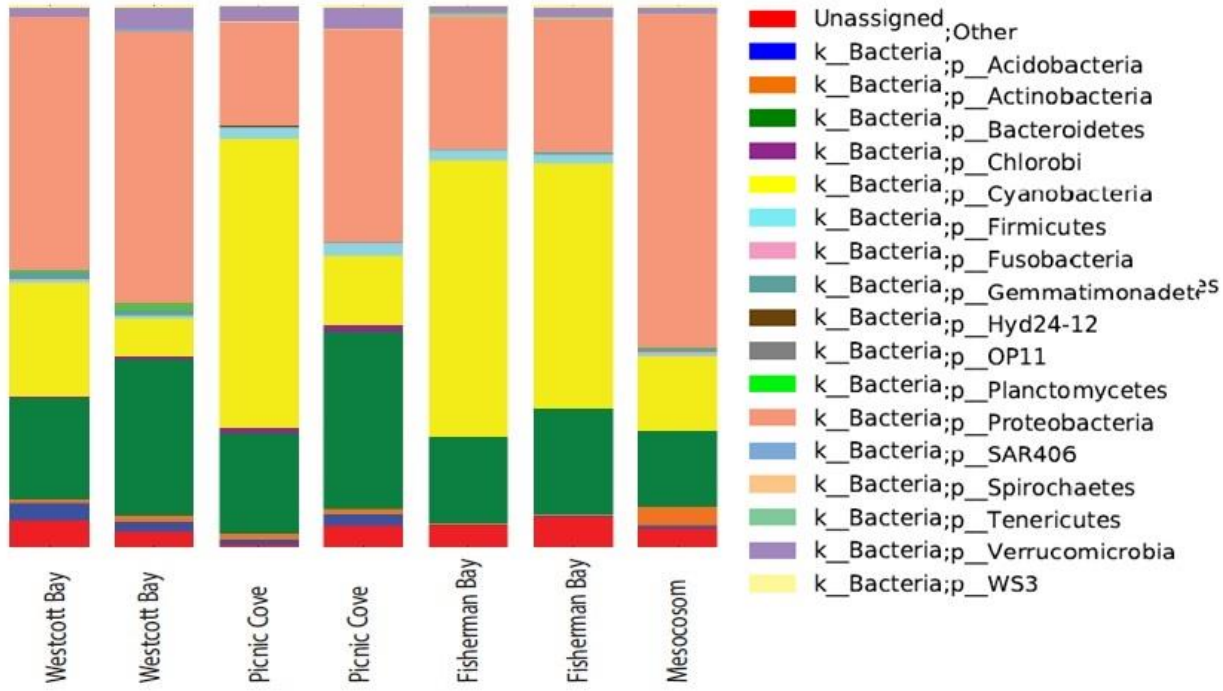


Figure 7.3: Future experimental set up

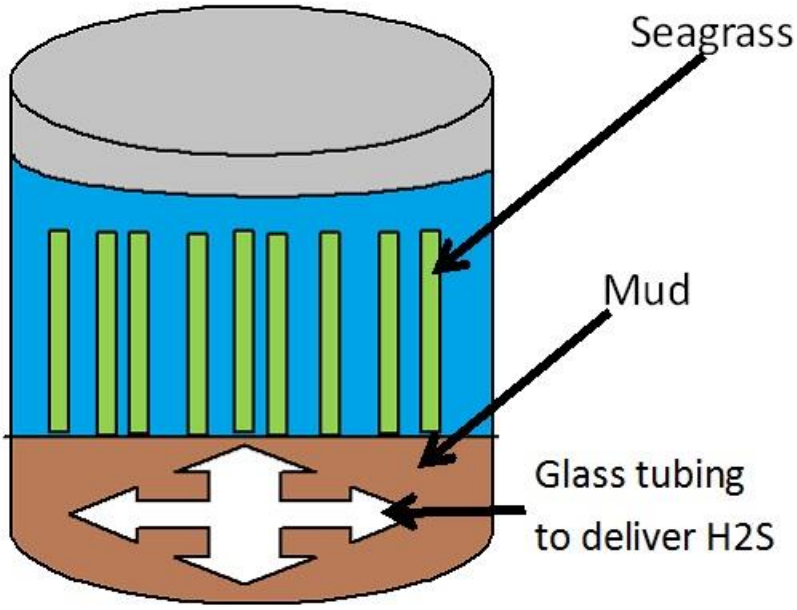


Fig. 1: Control set up with high density of mature shoots

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Teaching Assistant: University of Washington, Seattle: September 2010-June 2012
Classes: Biology 280 – New History of Life [instructor rating 4.2: very good - excellent]
Biology 200 – Introductory to Biology – Cellular and Molecular
Biology 280 – Introductory to Biology – Evolution
Biology 119 – Introduction to Human Physiology
Biology 446 – Biology of Algae

Research/Work Experience:

Research Technician: University of Washington, Seattle: July 2010 to Present

Research Technician: Seagrass Laboratory, Friday Harbor WA: September 2009 to July 2010

Volunteer Research Technician: University of Washington, Seattle January 2009 to June 2009

Volunteer Lab Help: University of Washington, Seattle: January 2008 to June 2008

EHL Instructor: American Red Cross, Everett: January 2005 to Present

Publications:

2015

Dooley F., Wyllie-Echeverria, S., Gupta, E., Ward, P. 2015. Tolerance of Phyllospadix scouleri seedlings to hydrogen sulfide. *Aquatic Botany.* 123:72-75

Dooley, F., Wyllie-Echeverria, S., Licata, E., Barr, S., Vitruk, O., et al. 2015. The influence of hydrogen sulfide on the distribution of seagrass in and around the San Juan Archipelago. Estuarine, Coastal and Shelf Science. (In-Review)

Vandepas, L., **Dooley, F**., Swalla, B., Ward, P. 2014. Phylogenetic analysis of Nautilus pompilius. (Submitted to Evolution)

2014

Barord G.J, **Dooley F.**, Dunstan A., Ilano A., Keister K.N, et al. (2014) Comparative Population Assessments of *Nautilus* sp. in the Philippines, Australia, Fiji, and American Samoa Using Baited Remote Underwater Video Systems. PLoS ONE 9(6): e100799. doi: 10.1371/journal.pone.0100799

Gupta, E., **Dooley, F.,** Ward, P. 2014. Evolutionary Legacy Response Observed in Algae & Bryophytes Following Hydrogen Sulfide Administration. J. Toxicol. Enviro. Chem. DOI:10.1080/02772248.2014.944353

2013

Dooley, F., Wyllie-Echeverria, S., Roth, M., & Ward, P. 2013. Tolerance and response of Zostera marina seedlings to hydrogen sulfide. Aquatic Botany. 105: 7-10.

Dooley, F., Wyllie-Echeverria, S., & Van Volkenburgh, E. 2013. Long-term seed storage and viability of Zostera marina. Aquatic Botany. 111: 130-134

Dooley, F., Ward, P., & Nair, S. 2013. Hydrogen Sulfide causes unusually fast growth in crop plants; PLoS ONE 8(4): e62048. doi:10.1371/journal.pone.0062048

PRE-2011

Dooley F., Wyllie-Echeverria S., & Greene H.G. (2011) Garrison Bay Overwater Structure Survey; National Parks Service, San Juan Island WA

Dooley, F. (2009) The Effects of Temperature on *Zostera marina* Seedling Development and Determination of Seed Viability; University of Washington Research Symposium Pg; 76

Dooley, F. (2008) Short Communication: The effects of temperature on *Zostera marina* seedling development; FHL Class Publication, San Juan Islands

Patents:

- Patent (serial number 61/584,966)
  - Plant growth accelerated by Hydrogen Sulfide

Grants Awarded:

Travel Award, Second European Conference… 2013

Graduate School Fund for Excellence and Innovation Travel Award 2013

WRF-Hall Fellowship Fall 2012

Mary Gate's Scholar 2008

Presentations:

Second European Conference on the Biology of Hydrogen Sulfide, Exeter England, 2013

UW Graduate Symposium, Seattle WA, 2012

Salish Sea Conference Poster presentation, Vancouver BC, 2011

Friday Harbor Laboratories Open House Poster Presentation, Friday Harbor WA, 2009 & 2010

Undergraduate Research Symposium, Seattle WA, 2009

Media Appearances:

Discovery News: H2S and Plants: http://news.discovery.com/earth/plants/deadly-gas-can-act-as-fertilizer-130423.htm

UW News: H2S and Plants: http://www.washington.edu/news/2013/04/17/a-key-to-mass-extinctions-could-boost-food-biofuel-production/

Fiji TV: Nautilus Research:
http://www.fijitv.info/yahoo_site_admin/assets/docs/7.41124949.wmv

The Daily: Work with WSU: http://dailyuw.com/news/2012/oct/08/collaboration-instead-competition/
The Daily: H2S and Plants: http://dailyuw.com/news/2012/jul/17/extinction-sustenance/

Affiliations/Volunteering:

American Red Cross, Snohomish County Chapter 2005-present

Professional Bowlers Association 2007-present

Professional Collaborations:

Mark Roth – Fred Hutchinson Cancer Research Center

Amit Dhingra – Washington State University

Joseph Kirschvink – CalTech

Lee Kump – PennState

Peter Ward - University of Washington

John Baross - University of Washington

Elizabeth Van Volkenburgh – University of Washington

Rose Ann Cattolico - University of Washington

Rusty Rodriguez – USGS and University of Washington

Sandy Wyllie-Echeverria – Friday Harbor Laboratories

Adam Summers - University of Washington

Billie Swalla - University of Washington

Toby Bradshaw - University of Washington

Joel Elliott – University of Puget Sound

H. Gary Greene - Moss Landing Marine Laboratories

Mentored Undergraduate Students:

Suven Nair+; Eric Gupta+; Olga Vitruk+; Tessa Marx; Kirsten Rarich; Racheal Jappert; Sarah Davis; Mathew Summers*; Shannon Russell#

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#Winner of Washington Space Grant Fellowship