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

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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 *Z. 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.

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 subtropical 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 (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 Scrimgeour, 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.,

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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 (<1 mM). 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 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).

2. Materials and methods

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 4 cm).

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 5 °C and 32 PSU until germination trials were initiated (Wyllie-Echeverria et al., 2003), five months after collection.

Nine hundred and 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 [$NaNO_3 + Na_2HPO_4 + MnCl_2{\cdot}4H_2O$ + ferric-sodium EDTA + H_3BO_3 + HCl] (Churchill, unpublished data); and held at 20 °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 h of PAR, 235 μmol m^{-2} s^{-1}, and temperature and salinity were maintained at 10 °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.

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; 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 (12–37 h depending on conditions, e.g. Napoli et al., 2006), treatment solutions were replaced every 12 h 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 (Fv/Fm). Qmax was calculated according to Krause and Weis (1991) equation: Fv/Fm = (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 h, seedlings were scanned again to determine Qmax. After 48 h, 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 6.8 mM H_2S for 1 h. 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 saturation curve was plotted (Hoffman, 1995). In 2011 the same procedural methods were used on three additional treatments (400, 500 and 800 μM) and a control in order to better define the LD_{50}.

3. Results

3.1. Field studies

In sites with stable *Z. marina* populations, average H_2S concentration was 0.052 ± 0.007 mM. In historic sites where *Z. marina*

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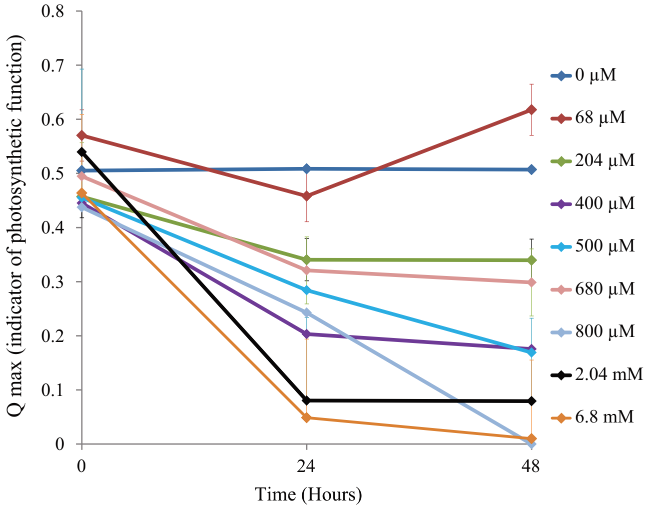

Fig. 1. Variation in Qmax (Fv/Fm) indicator of photosynthetic function) of as a function of H_2S concentration and duration of exposure. Note, both 2.04 and 6.8 mM Qmax is <0.1, thus seedlings are determined to be dead. The 204 and 680 µM treatments have suppressed photosynthetic function and 68 µM changed very little. SE bars shown.

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 ($P = 0.84$).

3.2. Lethality experiments

In all treatments, Qmax decreased in the initial 24 h (Fig. 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. 1), while Qmax was 0.34 ± 0.02 at 204 µM and 0.32 ± 0.09 at 680 µM. After 48 h 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 h, these seedlings had degraded substantially and were dead after 7 days (Fig. 2). In 204 µM and 680 µM treatments, measurements indicated that seedlings had lower Qmax after 48 h. And after 7 days only the 204 and 68 µM were actively photosynthesizing.

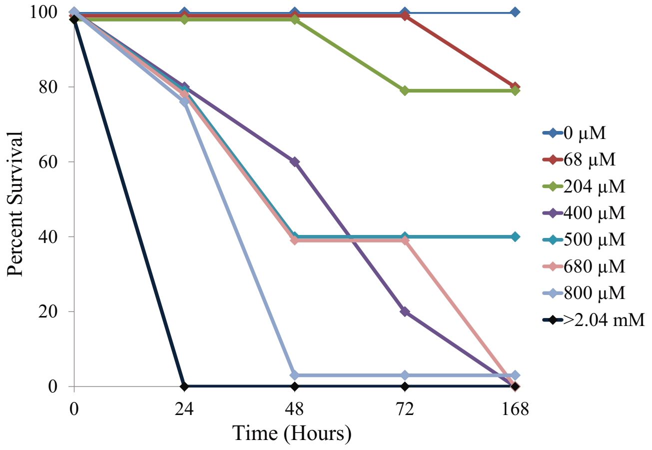

Fig. 2. Seedling percent survival for each H_2S treatment over time. LD_{50} at 48 h is 483 µM ($R^2 = 0.92$) and at 7 days $LD_{50} = 334$ µM ($R^2 = 0.78$).

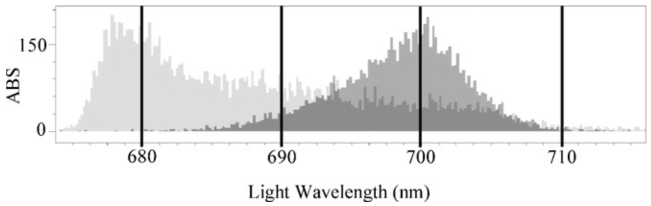

Fig. 3. An example of the irradiance spectrum observed in a plant both pre and post expose to 6.8 mM H_2S. Note in the pre-exposure spectrum (light gray), ABS (absorbance) is occurring in both 680 and 700 nm; however, the vast majority of spectrum is under 690 nm. This indicates that both Photosystem I and II are active in normal conditions, where Photosystem II is the dominant reaction. Notice that post 6.8 mM H_2S exposure there is a dramatic shift in the spectrum (dark gray, intermediate gray is overlap). There is almost zero activity below 690 nm, and the peak is at ~700 nm. This spectral shift is directly correlated with the activity of PSI. Lack of activity in the lower spectrum suggests that PSII is inactive.

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 h and by 48 h no photosynthesis was occurring, Qmax = 0.

After 7 days of recovery, all seedlings that were still alive (control, 68 and 204 µM) were exposed to 2.04 mM solution of H_2S for another 48 h. After 24 h all seedlings that were placed in 2.04 mM treatments Qmax decreased to <0.1 indicating no photosynthetic activity. However, after 48 h 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 h is 483 µM ($R^2 = 0.92$) and at 7 days $LD_{50} = 334$ µM ($R^2 = 0.78$) (Fig. 2). In addition, it was found that when *Z. marina* seedlings were exposed to high levels (>10 mM) of H_2S, Photosystem II decreased in activity whereas Photosystem I was maintained (Fig. 3). It was also noted that after exposure to high doses, for a short period of time (~1 h), this change in photosystem activity is reversible (100% reversion rate if only exposed 1 h) and after a 24-h recovery period photosynthetic activity returns to pre-exposed parameters (Qmax pre-exposure levels were 0.66 ± 0.13, after 1 h of exposure 0.23 ± 0.13, and given a 24-h recovery it rebounded to 0.55 ± 0.18).

4. Discussion

Both field observations and laboratory experiments, as described in this study, suggest that even relatively low concentrations of H_2S (400–500 µM) 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 Thompson and Kats (1978),

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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.

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