Tolerance of *Phyllospadix scouleri* seedlings to hydrogen sulfide

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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 days. Our study confirms that *Phyllospadix* seedlings are also vulnerable to increasing sulfide loads.

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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; Elliott 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 (Phillips, 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; Elliott 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, 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 et al. (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).

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2. Materials and methods

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 °C 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 °C and 32 PSU, respectively, with nutrients added [$NaNO_3$ + Na_2HPO_4 + $MnCl_2 \cdot 4H_2O$ + ferric-sodium EDTA + H_3BO_3 + HCl] (Churchill, 1991).

All experiments were conducted axenically, and tested to confirm. Testing was conducted by removing 1 ml 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%.

2.2. H$_2$S production and measurement

H$_2$S 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 0.01 ml increments while stirring, until a pH of 7.2 is reached, resulting in a solution 0.5 M ± 25 mM (5%), as determined with a H$_2$S/Sulfide Probe (Sea & Sun Technology GmbH, Trappenkamp, Germany). Next, the 0.5 M H$_2$S 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$_2$O to the stock solution to make each treatment concentration. After dilution we confirmed sulfide concentration with the H$_2$S/sulfide probe.

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., 2015 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$_2$S solution. Due to the relatively short half-life of H$_2$S (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 °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$_2$S, 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) Q_{max}, the maximal photochemical efficiency of PSII (F_v/F_m). Q_{max} was calculated according to Krause and Weis (1991) equation: $F_v/F_m = (F_m - F_o)/F_m$; 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 Q_{max} 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$_2$S 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
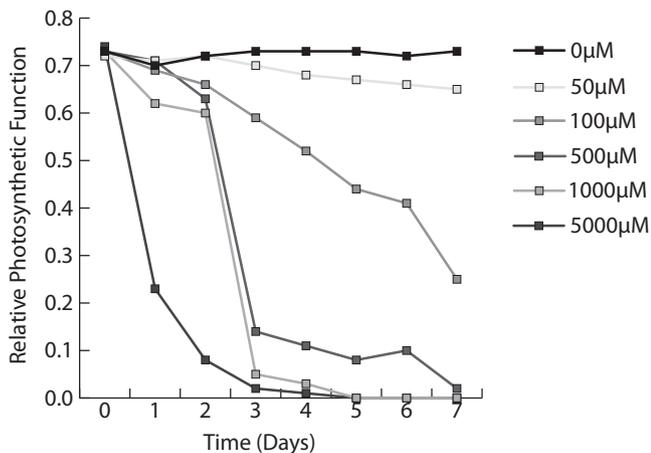

Fig. 1. Variation in Q_{max} ((F_v/F_m) indicator of photosynthetic function) of as a function of H$_2$S concentration and duration of exposure. 250 and 750 μM data were omitted due to being indistinguishable to 500 and 1000 μM, respectively.
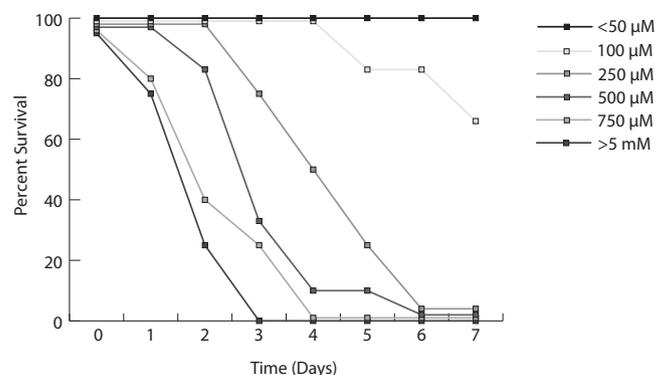

Fig. 2. Seedling percent survival for each H$_2$S treatment over time. LD$_{50}$ at 48 h is 430 ± 21.9 μM and at 7 days LD$_{50}$ = 86 ± 13.6 μM. Differences between treatments were significant ($P < 0.001$).

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.

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., 2013a,b) was compared to the values obtained in this study using a Wilcoxin rank sum test. The criterion for significance was set at $P < 0.05$.

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 days 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. 1). Using photosynthetic output as the measure of survivorship, there were significant differences between control and $\leq$100 μM treatment groups ($P < 0.001$); 50 μM was not different from that of the controls. Using a Michaelis Menten equation the LD_{50} boundary was assigned at 430 ± 21.9 μM ($r^2 = 0.97$) after 48 h and 86 ± 13.86 μM ($r^2 = 0.81$) after 7 days (Fig. 2). In addition, it was found that when *P. scoluri* seedlings were exposed to high levels of H_2S, Photosystem II decreased in activity whereas Photosystem I was maintained, which is very similar to the response of *Z. marina* seedlings (Dooley et al., 2013a). It was also noted that after exposure to high concentrations (10 mM), for a short period of time ($\sim$1 h), 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; $P = 0.02$.

4. Discussion

The experiments presented here provide evidence in support of the hypothesis that *P. scouleri* seedlings have a lower tolerance to sulfide (7 days LD_{50} = 86 μM) than *Z. marina* (7 days 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. 1), 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; Weiss 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).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2015.02.004.

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