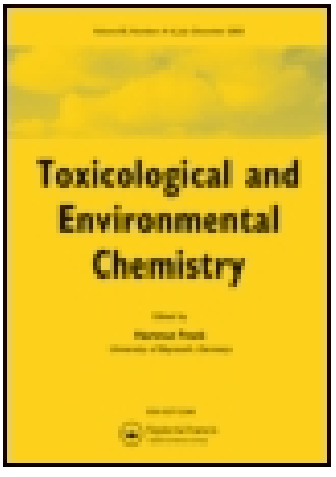

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Evolutionary legacy response observed in algae and bryophytes following hydrogen sulfide administration

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The study presented here examined the effects of administering hydrogen sulfide (H_2S) to several ancient extant plant species to determine the organisms' response to stress. Even though sulfur is an essential macronutrient required for growth and productivity, there are toxic compounds of this element that exert detrimental effects and produce physiological stress. It is speculated that the accumulation of H_2S, a lethal gas, may have been a major contributing factor in past mass extinction events, where the environment was fairly anoxic with fluctuating temperatures. The potential of this toxic compound to exist as an environmental stressor suggests that certain organisms may have adapted to survive these periods of mass extinctions. It is hypothesized that due to the abundant presence of H_2S in the past, ancient land plants may have an adaptive advantage that allowed them to survive and thrive. In this study, species of bryophytes and algae were exposed to specific concentrations of aqueous H_2S over a seven-day period and measured their photosynthetic capacity at timed intervals using a FluorCam. Studying the effects of this toxic gas on ancient plants is imperative to our understanding of sulfur's varying biological roles, and provides insight on the evolutionary phenotypic variations amongst plants and stress responses in order to survive mass extinctions. Results indicate that *Hypnum*, *Chlamydomonas*, and *Charophyta* are all able to tolerate significant quantities of H_2S and show resilience through increased photosynthetic capacity over a period of exposure, indicating a genetic and phenotypic legacy response.

Keywords: photosynthesis; plant toxicity; mass extinctions; lethality; hydrogen sulfide

Introduction

Predicting the response to climate change of any particular species presents a major challenge in ecology (Long et al. 2004), and in many cases a drastic change may result in extinction. While sulfur (S) is one of the six macronutrients necessary for growth and productivity within plants and ecosystems, there are many sulfur compounds that exert toxic effects. Sulfur and sulfide accumulation in marine sediments have been known to induce stress responses and physiological toxicity on organisms and the ecological community as a whole. Oxygen reduction and sulfide accumulation may have played a role in past mass extinctions (Kump, Pavlov, and Arthur 2005). Similar to these past mass extinctions, modern sulfide eruptions (Kaiho et al. 2006) and euxinic environments of inshore embayments that are produced by several factors [from biotic (decomposing biomass – algae blooms) to abiotic (hydrothermal vents) and anthropogenic activities], are exerting stress on ecosystem communities (Short and Burdick 1996; Kamp-Nielsen et al. 2001; Halun et al. 2002). In response to these changing conditions, organisms need to rely on

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not only their phenotypic variation but on genetic resilience in order to survive. Responding to these conditions is in part a legacy response.

Biological effects of sulfide and hydrogen sulfide (H_2S) are wide ranging (Attene-Ramos et al. 2006; Raven and Scrimgeour 1997). When compared to other plant hormones and toxins H_2S is similar to ethylene, nitrous oxide, and carbon dioxide (CO_2), because it is a tiny molecule, often referred to as a gasotransmitter (Lloyd 2006). As a gas, H_2S diffuses readily; moving like CO_2. H_2S diffusive properties and solubility in water enhances its movement throughout an organism, therefore it is able to exert its influence in minute quantities. Like other gasotransmitters, H_2S has the potential to act as a natural plant regulator. Theories regarding how H_2S regulates the organism depend on some aspect of DNA transcription or RNA translation, thus changing some factor of protein synthesis and, consequently, enzyme patterns. However, many other mechanisms are also likely to be in operation.

Previously, data suggested that the presence of H_2S exerted significant effects on the relative activity of the two photosystems (Dooley, Wyllie-Echeverria, et al. 2013). This was first observed by Oren, Padan, and Malkin (1979) in cyanobacteria, and our observations, suggest a similar pattern in an angiosperm – *Zostera marina*. At high concentrations (millimolar), Photosystem II (PSII) shuts down and Photosystem I (PSI) remains active (Oren, Padan, and Malkin 1979; Cohen et al. 1986; Chen et al. 2011; our study). Studies by Thompson and Kats (1978) and Chen et al. (2011) complemented by our findings suggest that these responses are conserved throughout the plant kingdom, and, furthermore – as to a mechanism, heat stress, and herbicides are often correlated with decrease of PSII and the failure of the electron transport chain (Berry and Björkman 1980; Weiss and Berry 1988; Havaux 1993). Interestingly at low doses (<100 μM) photosynthetic activity and leaf health may increase. It appears that chloroplast biogenesis is at least partially responsible for this phenomenon. More after observations suggested that plant growth may increase, and whole cells may undergo biogenesis (Dooley, Nair, and Ward 2013).

Due to the polarity of these effects, exposure to H_2S presents a fundamental question to evolutionary science and the mechanism of stress responses. The aim of this study is to test hypotheses relating to the general effects of H_2S on modern extant photosynthetic organisms representing diverse groups originating in deep geologic time. These experiments will show a unique response to H_2S that may be conserved throughout the tree of life.

Methods

Hydrogen sulfide preparation

Half-molar H_2S was made by dissolving 78.04 g anhydrous sodium sulfide into 2 l of double filtered deionized water (ddi-H_2O). Hydrochloric acid was then titrated into this solution in 0.01 ml increments while stirring until pH of 7.2 was reached, resulting in a solution 0.5 M $\pm$ 25 mM (5%) as determined by using an H_2S/Sulfide Probe (Sea & Sun Technology GmbH, Trappenkamp, Germany). The 0.5 M H_2S solution was then filtered with a filter with pore size 0.2 μM and stored in 250 ml flasks capped with nitrogen gas (in order to maintain stability). Each treatment concentration was made adding ddi-H_2O. After dilution, the H_2S/Sulfide Probe was used to confirm concentration and pH of solution was checked. In all experiments the following concentrations were prepared [0, 0.01, 0.1, 1, 3, 5, 7, 10, 25, and 50 mM].

Algae

Algal growth rate, biomass, and survival were evaluated in the Cattolico Laboratory at the University of Washington. Algae *Chlamydomonas* sp. were grown in 250 ml Fernbach flasks that contained 100 ml Coras as media (Bigelow et al. 2011). Media chemicals were of reagent grade. Cultures were maintained at 20 °C on a 12 h light: dark photoperiod (100 μE m^2 s^{-1} light intensity) with no CO_2 or agitation. Experiments were initiated by adding 1 ml of stock mother culture to each fresh sterile flask containing media. Cultures were returned to the growth chamber and left for 48−72 h until cultures reached a density of approximately 10^3 cells per ml. At this time and at every 24 h thereafter, experimental flasks were inoculated with H_2S in concentrations ranging from 0 to 50 mM. Cell counts were made daily at light 3 (L3) in the circadian photoperiod using an Accuri flow cytometer. Furthermore, 0.25 ml of sample was removed from each flask daily and observed under a microscope. Activity (movement) of cells was recorded for *Chlamydomonas*. Each treatment consisted of 10 replicates.

Macro organisms

To measure plant health and survivorship, 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 photon systems instruments (PSI) Qmax, the maximal photochemical efficiency of PSII (*Fv*/*Fm*) was calculated according to the Krause and Weis (1991), 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 (Force et al. 2003; Liu et al. 2006; Guo et al. 2008; Dooley, Wyllie-Echeverria, et al. 2013).

Cultures of Charophytes were isolated individually from a bath containing the stock, which was purchased from a biological supply company. Each specimen was haphazardly assigned to a 50 ml clear test tube. Each experimental treatment contained 10 distinct specimens, and each treatment was replicated four times ($n = 40$). Containers were filled with sterilized water from tap (Seattle Municipal). After 48 h all specimens were scanned using the Fluorcam and determined to be healthy. Thereafter, each was randomly assigned to a treatment group and time zero measurements were taken. Next, and throughout the experiment, H_2S solutions were made by diluting stock H_2S with sterile tap water until desired concentration was achieved. Solutions were replaced within the test tube daily. At the desired time increments, individual *Charas* were removed from the test tube, placed into petri dishes with the corresponding H_2S solution and scanned using the Fluorcam. Once scanned they were returned to the test tubes and placed back into the growth chambers and maintained at 20 °C on a 12 h light: dark photoperiod (100 μEm^2s^{-1} light intensity) with no CO_2 or agitation.

Hypnum moss and liverworts both had the same procedure. The cultures were obtained from the UW Botany Greenhouse Collections and screened for insects and disease. Once isolated, each was sterilized using a 10% bleach solution for 15 min, rinsed with di-H_2O and placed into water baths for approximately 10 min. The *Hypnum* was placed onto sunshine #6 soil (Sun Gro Horticulture Canada Ltd) and incubated at 20 °C on a 12 h light: dark photoperiod (100 μE m^2 s^{-1} light intensity). The liverworts were

kept in a solution and placed in a growth chamber under the same conditions. All specimens were kept under control conditions one week for stabilization.

After one week, samples of similar size were randomly extracted and separated into strands while wearing sterile nitrile gloves. Each sample was inspected for fractures and left in a deionized water bath for approximately 10 min. Succeeding the soak, each individual sample was placed in a 20 ml Falcon^{TM} Bacteriological Petri Dish. Following placement, each petri dish was randomly assigned with an H_2S treatment concentration. Thirty individual liverworts were evaluated per treatment group plus a control, likewise 80 specimens were in each Hypnum sp. treatment group.

The samples were then saturated with 10 ml of their respective H_2S concentration solutions. The capped petri dishes were randomly distributed across a counter and incubated at 20 °C on a 12 h light: dark photoperiod (100 μE m^2 s^{-1} light intensity). The samples were measured for their photosynthetic activity 1, 3, 6, and 12 h after exposure on the day of sample creation, and every 12 h thereafter for seven days. Due to the short half-life of the H_2S solutions used during experimentation (Napoli et al. 2006), the solutions for the samples were replaced following the daily photosynthetic measurements. During each interval between measurements, the samples were randomly distributed on the counter of storage.

Statistical test

Means from each treatment group were compared against the control by a paired *T*-test. Second, these data were 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. Using photosynthetic output as the measure of survivorship the LD_{50} boundary was determined by using a third degree polynomial best fit which was plotted on a saturation curve (Hoffman 1995). The criterion for significance was set at $P < 0.05$.

Results

Chlamydomona

Green microalgae Chlamydomona sp. had an LD_{50} of 3.3 mM after seven days of exposure ($R^2 = 0.88$) (Tables 1 and 2), 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.

Table 1. Percent survival by concentration (mM) after two days of exposure.

Organism	<0.5	1	3	5	7	10	25	50	LD_{50}
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 2. Percent survival by concentration (mM) after seven days of exposure.

Organism	<0.5	1	3	5	7	10	25	50	LD_{50}
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**

Chara

Chara sp. of the order of Charales LD_{50} was 6.6 mM ($R^2 = 0.8$) after seven days, with a maximum survival rate of 25 mM (Tables 1 and 2). 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 PSII was inhibited, whereas PSI remained active. This was reversible up to 48 h after exposure.

Liverworts

Liverwort *Ricctocarpus* sp. had a seven day LD_{50} of 2.7 mM ($R^2 = 0.86$) and an LD_{50} of 9.7 mM after 48 h (Tables 1 and 2). Liverwort Qmax maintained relatively stable levels throughout the experiments at the lower concentrations, however, at higher concentrations (>10 mM) 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 ($P < 0.001$) (Figure 1).

Moss

Hypnum sp., a common moss, experienced a relatively high LD_{50} of 7.8 mM ($R^2 = 0.9$) (Tables 1 and 2) after seven days with a maximum survival at 25 mM for long-term

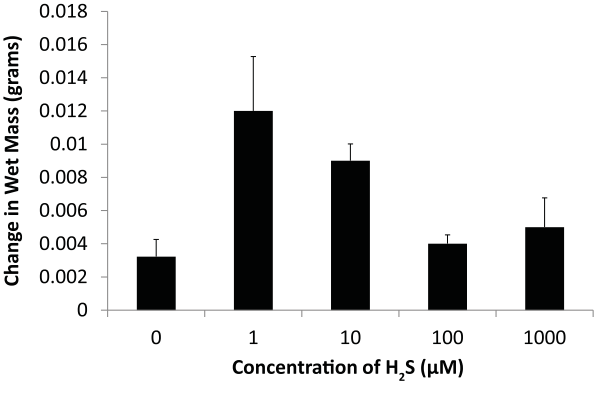

Figure 1. Change in wet mass of *Ricctocarpus* sp. with exposure to H_2S compared to control (0 μM). Concentrations (1−100 μM) were larger than the controls ($P < 0.001$).

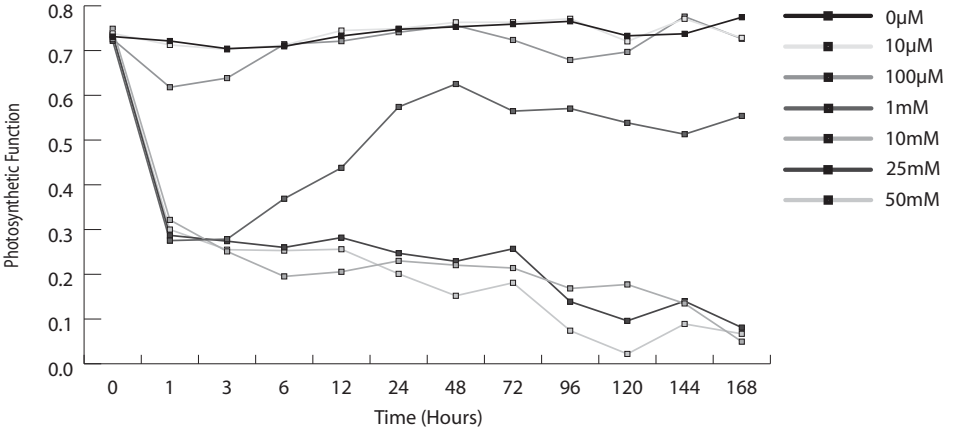

Figure 2. Measurements of Qmax (indicator of photosynthetic function) over time per treatment of H_2S in *Hypnum* sp. Both 0 and 10 μM have relatively comparable values. 100 μM experiences a depressed output for the initial 12 h but rebounds to slightly lower levels than previous exposure, 1 mM experienced the same depression in photosynthesis but is unable to fully recover, and $>$10 mM never experiences any rebound.

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 2) 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.

Discussion

The data described in this study suggest that as the physiological complexity [defined as the addition of macro structures such as stomata and/or specialized tissue(s)] of an organism increases and its relative origin in geological time [Supplemental 1 (available with the online version of this article)] decreases, its tolerance towards H_2S falls. This phenotypic tolerance may serve as an adaption in response to environmental stressors in geological history at which the individual organisms originated. Bryophytes are speculated to have originated in the Devonian Period (Beerling 2008; Kenrick and Davis 2004; Lang and Cookson 1935), which came before the Permian, when anoxic ocean environments were commonly attributed to the decomposition of sulfur bacteria, resulting in an abundance of gaseous H_2S, a potential kill mechanism for eukaryotic life (Knoll et al. 2007a).

Today, moss and liverworts are found in dark and moist environments where potential sulfide production may result from decaying organic matter. In terms of both previous high sulfide events and local sulfide exposure, it is highly advantageous for moss and liverworts to be able to tolerate H_2S and sulfides. The increased fitness attributed to this trait might have allowed moss to survive several mass extinction events and exist today.

Similarly, Charophytes and *Chlamydomonas* are also found in euxinic environments (Pröschold et al. 2001; Hoham et al. 2002), which would make it highly advantageous for the organisms to possess resilience to toxic sulfide. *Chlamydomonas* is typically found in brackish waters high in oxygen, whereas Charophytes are found in sulfidic lakes low in oxygen (Kovtun-Kante, Torn, and Kotta 2014).

Previous studies have shown that low levels of H_2S exposure generally are correlated with increases in plant size; however, in our study we only observed a significant change in liverworts. At lower quantities (between 1 and 10 μM) of H_2S exposure, there was an increase of wet mass by 0.012 and 0.09 g, respectively. At relatively higher concentrations (100 and 1000 μM), there was an observed elevation in wet mass, but tapered in comparison to lower concentrations. This suggests that at lower quantities, H_2S induced growth which has been noted in modern angiosperms (Chen et al. 2011; Dooley, Nair, and Ward 2013). Interestingly, it was observed that the Qmax values of *Hypnum* initially depress upon immediate exposure to H_2S, but after continuous daily exposure, the plant seemed to tolerate toxicity and its photosynthetic capacity rebounded. The earliest observed "bounce back" was witnessed approximately six hours after initial exposure. This is similar to the findings by Chen et al. (2011), which dealt with *Spinacia oleracea* seedlings. However, upon prolonged exposure over a period of several days, the bounce back effect diminished and the photosynthetic capacity of the organism steadily declined. Data from respiration measurements of *Zostera marina* plants in response to H_2S illustrate that the adverse effect did not decrease respiration, but rather inhibited photosynthesis. In these experiments, pre- and post-respiration rates were measured following exposure; data indicated that respiration in fact increased two- to four-fold when compared to pre-exposure. While photosynthesis is steadily reduced and inhibited, respiration is increased (Dooley, Wyllie-Echeverria et al. 2013).

Data suggest that H_2S influenced the activity of both photosystems. At higher concentrations, it appears that PSII activity ceased, whereas PSI remained active (Oren, Padan, and Malkin 1979; Cohen et al. 1986; Chen et al. 2011). The increase in Qmax observed in segments of the study may be attributed to the rise in PSI activity. The shutting down of PSII appears to be the mechanism of death observed at the end of experimentation, a similar phenomenon observed when plants are exposed to the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This inhibition of PSII suggests that one of the proteins along the electron protein complex is inhibited from acting. The protein that is most likely to be affected is in the cytochrome b6f complex, which is responsible for electron transport between PSII and PSI, and is often inhibited by toxins.

This study showed that select green plants may have evolved a mechanism to deal with sulfide and H_2S throughout time through a potential stress mechanism. This ability to tolerate H_2S suggests that there was a preferential survival of species for previous mass extinctions involved in sulfide blooms and euxinic environments. Green plants can typically inhabit high H_2S environments, whereas browns and reds macroalgae inhabit high oxygen environments with relatively lower sulfur content (Knoll et al. 2007b). The H_2S tolerances of red and brown algae, and haptophytes may be significantly lower [e.g. *Chrysochromulin*a sp. has an LD_{50} of 50 μM (Supplemental 2, available online)] than those of green plants, and a marine angiosperm, *Zostera marina*, a relatively recent (Cretaceous) and complex plant (angiosperm), has an LD_{50} of 300 μM (Dooley, Wyllie-Echeverria et al. 2013). The examples of *Chrysochromulina* sp. and *Z. marina* have tolerances magnitudes lower than those of the more basal linages tested.

This change in tolerance over time and complexity may be the result of any number of factors; however, the phenotypic resilience towards H_2S by the green linage illustrates its

resistance to H_2S which is a potential kill mechanism for several of mass extinctions (Ward et al. 2005; Kump, Pavlov, and Arthur 2005; Lamarque et al. 2006).

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Supplemental data

Supplemental data for this article can be accessed here.

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