

## Abstract

The effects of light reduction from organic matter and sulfide stress resulting from its decomposition by sulfate reducing bacteria in sediments can have significant impacts on shallow water angiosperms such as the seagrass, *Zostera marina* (eelgrass). Previous studies suggest that, as sulfide concentrations increase, seagrass photosynthesis may decrease. A reduction in light may have a confounding effect by limiting rates of photosynthesis and, thus, preventing adequate re-oxidation of sediments. The objectives of this experiment were to evaluate the interactive effects of different sulfide concentrations and light levels on the in situ photosynthetic activity of eelgrass using pulsed amplitude modulated (PAM) fluorometry. Eelgrass plants from a nearby estuary were transplanted into microcosms in a light and temperature environmental chamber under ambient field conditions for 31 days. In order to investigate the effects of light environment, half of the plants were shaded to approximately 10% of regular irradiance. Sediments remained ambient or were injected with two different amounts of sodium sulfide to modify sulfide concentrations resulting in a 3 by 2 factorial design. After 17 days of exposure elevated sediment sulfide concentrations and reduced light levels had significant, additive effects on fluorescence measurements (maximum ETR and maximum quantum yield). These results suggest that declines of seagrass observed in coastal systems may be related to a combination of stresses that produce a negative feedback cycle, limiting seagrass recovery.

## Introduction

The seagrass, *Zostera marina*, commonly known as eelgrass, thrives in shallow estuaries and serves as a habitat and nursery area for a variety of marine organisms, forming vital estuarine habitats in the Chesapeake Bay. Creeping rhizomes, a lack of stomata, aerenchyma, and linear, strap-shaped leaves filled with lacunae characterize the growth form of this perennial

angiosperm (Appendix 1). The lack of stomata prevents oxygen loss into the water column, while aerenchyma, tissue that transports oxygen from photosynthesis from the leaves to the roots (Armstrong et al., 1979), and lacunae, internal gas spaces located in the root system that store oxygen (Kuhn, 1992), are features of eelgrass that facilitate respiration of below ground tissues. Eelgrass has a relatively high light requirement in order to meet the demand for growth in highly reducing sediments (Kuhn, 1992).

Typically, sediments in eelgrass beds are very organic rich because of a high rate of organic matter deposition. Suspended populations of phytoplankton, macroalgae, and epiphytes can limit the amount of light available for seagrass photosynthesis, and their deposition in seagrass beds can increase sediment oxygen demand (Twilley et al., 1985). When oxygen supply becomes limiting in highly reducing sediment, anaerobic processes replace aerobic respiration (Koch et al., 1990). Under anoxic conditions, inorganic ions such as sulfate ( $\text{SO}_4^{-2}$ ) serve as terminal electron acceptors in oxidative phosphorylation (Hines et al., 1989). Due to an abundance of sulfate in seawater, the breakdown of deposited organic matter is largely accomplished by sulfate reducing bacteria that release free sulfide ions into the sediment environment (Howarth and Teal, 1979). Factors that may increase sulfide concentrations in seagrass sediments include increased rates of organic matter deposition, increased bacterial activity with temperature, or decreased rates of re-oxidation from oxygen diffusing from seagrass roots and rhizomes (Carlson et al., 1994).

Increased nutrient enrichment in the Chesapeake Bay has been related to a decline in eelgrass since the 1970's (Kemp et al., 1983). Nutrient enrichment can lead to increased production of phytoplankton and algae. Experimental studies have shown that reductions in

ambient light caused by high levels of phytoplankton and epiphytic algae are partially responsible for seagrass declines resulting from nutrient enrichment (Twilley et al., 1985).

Increases in sediment sulfide concentrations have also been implicated in the declines of seagrass. High sulfide concentrations observed before the widespread mortality of the seagrass *Thalassia testudinum* in Florida Bay suggest elevated sulfide concentrations may have triggered die-off episodes (Carlson et al., 1994). It has been determined that sulfide can inhibit plant growth and may increase mortality of the salt marsh macrophyte, *Spartina alterniflora* (Koch and Mendelssohn, 1989). One effect of sulfide accumulation in plant tissue is decreased activity of metallo-enzymes, which are components of the electron transport chain (Koch et al., 1990). Other effects include limitations on alcoholic fermentation (Koch et al., 1990), a negative carbon balance (Hines et al., 1989), and a reduction in nitrogen uptake (Smith et al., 1988).

The precise effects of elevated sulfide levels are unknown, but a reduction in adenosine triphosphate (ATP) production has been observed in salt marsh plants under sulfide stress (Koch and Mendelssohn, 1989). This suggests the deleterious effects of sulfide may be affiliated with the light dependent reaction of photosynthesis. In the light-dependent reaction of photosynthesis, excess light from the thylakoid membrane is re-emitted (as fluorescence) or dissipated (as heat). After photosystem II absorbs a photon, the photosystem reaches an excited level and is stable for less than  $10^{-8}$  seconds (Appendix 2). During this short period, charge separation within the reaction center results, constituting the primary photochemical step of photosynthesis (Bolhàr-Nordenkamp and Oquist, 1993). After charge separation, the excited electrons are accepted by a membrane protein and enter the electron transport chain. After the secondary plastoquinone acceptor processes electrons, fluorescence peaks because an expenditure of light is released. After the light dependent reaction, a negligible amount of light is released as fluorescence as a

result of regressing rates of photochemical quenching and non-photochemical quenching. In photosynthesis, the quantum yield of fluorescence is equal to the quotient of fluorescence over the sum of de-excitation via photochemical quenching, via fluorescence, and via heat.

Previous research has suggested that short-term increases in sediment sulfide levels can affect eelgrass photosynthesis (Copeland, 2001). Over a longer term, decreased seagrass photosynthesis may lead to a negative feedback cycle, where increases in sediment sulfide result in decreased rates of seagrass photosynthesis. Subsequently, the seagrass roots diffuse less oxygen into the sediments, lowering rates of sulfide re-oxidation, increasing sulfide levels, and leading to even lower rates of seagrass photosynthesis until plant death. Low light levels may increase this effect by providing less light for photosynthesis.

Recent advancements in instrumentation now permit the in situ assessment of photosynthetic activity in marine plants (Ralph, 1998), using a submersible pulse-amplitude modulated (PAM) fluorometer (Walz, Germany; Appendix 3). Since environmental stresses can affect the PSII reaction centers (Ralph, 1999), fluorescence can be used as a tool in quantifying sulfide stress response. The power of the PAM technique is inherent in its ability to detect photosystem efficiency non-invasively, and its potential for measuring plant stress may be useful in providing a rapid assessment of the health of seagrass systems for management purposes.

The PAM fluorometer provides information about the electron-transport rate, quantum yield, and photochemical efficiency of marine plants. This instrument uses a fiber optic cable to provide a consistent flow of red light on the area of the leaf isolated with a leaf clip; the associated fluorescence is known as  $F_o$ . A white light of  $6,000 \mu\text{mol photons sec}^{-1} \text{ m}^{-2}$  then fills all functioning photosystems, and the instrument measures the corresponding fluorescence curve. The peak of this curve is known as the maximum fluorescence ( $F_m$ ), which is the implied point

where all operating photosystems are processing light. The ratio of the variation of  $F_o$  and  $F_m$  to  $F_m$  is known as the quantum yield. A rapid light curve (RLC) consists of nine consecutive yield measurements at increasing light intensities between 0 and  $1,200 \mu\text{mol photons sec}^{-1} \text{ m}^{-2}$  (Appendix 4). Since RLCs consist of yield measurements at increasing light intensities, they contain information on induction as well as saturation characteristics of photosynthesis.

The objectives of this experiment were to quantify and to evaluate the combined effects of increased sulfide concentrations and light reduction on the light-dependent reactions of photosynthesis in eelgrass over a 30-day period and, consequently, to determine the general photosystem health of the plants. It was hypothesized that increasing sulfide concentrations would increase photosystem stress, and photosystem efficiency and capacity would decrease. Additionally, it was hypothesized that light reduction would limit photosynthesis, further decreasing photosynthetic performance and compounding the sulfide stress. The null hypotheses tested were that there would be no significant effects of sulfide concentration or light modification on photosystem health. The independent variables were sulfide concentration and light level; the dependent variables were the various fluorescence measurements.

#### Methods and Materials

On October 12, 2001, eelgrass plants were collected from the York River estuary (water temperature  $20^\circ\text{C}$  and salinity 25 psu). Cores, including whole plants, roots, and sediments were immediately transplanted into 5- inch by 6-inch pots. Afterwards, they were immersed in fresh water for thirty minutes to kill potentially damaging invertebrates. They were then placed in 100-liter, aquariums filled with filtered, continuously aerated seawater at 25 psu in an environmental chamber for acclimation at a temperature of  $25^\circ\text{C}$ , under a 14 h light: 10 h dark cycle with a saturating irradiance of  $500 \mu\text{mol photons sec}^{-1} \text{ m}^{-2}$  (approximately 33% of noon

irradiance). On November 2, 2001 twenty-four pots of eelgrass were randomly paired and placed in one of twelve, 2-liter aquariums (Appendix 5) at 25°C and 25 PSU. A porewater sipper was placed into the sediment of each pot (Appendix 6). The sippers were flushed with nitrogen gas and vacuum-evacuated to limit oxidation of sediment pore water after placement. The constants in this experiment were: type of plant (eelgrass), location (environmental chamber), origin of sample (nearby estuary), salinity of filtered seawater (25 psu), temperature of aquariums during the experiment (25°C), and type of aquarium.

Initial fluorescence measurements were made on November 13, 2001 to determine the baseline photosystem health of the eelgrass before the application of the light and sulfide treatments. In situ measurements were taken using the second-youngest leaf of randomly chosen shoots within each pot. Care was taken to ensure the leaves were not damaged by extraneous factors such as excessive handling, and the non-destructive measurements were repeated on the same leaves throughout the study. On each sampling date, RLC measurements were taken from an area of the leaf isolated with a leaf clip; following a 10-minute dark adaptation, maximum quantum yield was measured. The maximum electron transport rate (ETR) was calculated from the rapid light curve as the mean of the two highest ETR values.

On November 13, light and sulfide treatments were applied to the 24 pots of eelgrass. The light treatment consisted of either high light (HL;  $500 \mu \text{ mol photons sec}^{-1} \text{ m}^{-2}$ ) or low light (LL; shaded to  $50 \mu \text{ mol photons sec}^{-1} \text{ m}^{-2}$  with neutral density screening) conditions. The sediment sulfide treatments ranged from ambient sulfide (AS) to intermediate sulfide (IS) to high sulfide (HS). The HS treatment was attained by enriching sediment with a solution of 5 grams of sodium sulfide ( $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ ) dissolved in 25 mL of seawater (Goodman et al., 1995). The solution was injected through the bottom of the core with a hypodermic syringe at eight

locations. Similarly, a solution of 2.5 g sodium sulfide dissolved in 25 mL seawater was injected into sediments of plants in the IS treatment. AS treatments were injected with seawater only. There were four pots per light and sulfide treatment combination.

The in situ fluorescence responses and sediment sulfide levels were measured on November 17, November 25, November 30, December 8, and December 14. Sediment sulfide levels were determined by sampling 2.5 mL of porewater for sulfide from each pot by vacuum extraction and fixed directly into 2.5 mL of zinc acetate solution to minimize oxidation to sulfate. Sulfide concentrations were determined spectrometrically after Cline (1969).

Two-way analysis of variance was used to determine the levels of significance of the sulfide and light treatments on each measured parameter at each sampling date as well as among dates. Individual means were compared, a posteriori, using Fishers Test of relative significance. Additionally, changes in fluorescence measurements were compared to average measurements of sediment sulfide concentrations using a linear regression model. Statistical analyses were calculated using StatView™SE+ (Abacus Concepts, Inc., Berkeley, CA) statistical software.

## Results

Injections of varying concentrations of Na<sub>2</sub>S produced significant changes in the pore water sulfide levels when compared to pretreatment levels (Appendix 7). Mean AS concentrations were relatively low (<200 μM) with a gradual increase over the study period and were similar (p>0.05) among HL and LL treatments. IS concentrations (both HL and LL) were initially higher than (p<0.01) AS concentrations for four days, declining to similar levels five days later on Nov. 25. Although IS concentrations decreased to tolerable levels, sulfide concentrations in both HS treatments were always significantly higher (p<0.01) than IS and AS,

steadily decreasing throughout the experiment from a maximum after 4 days to a minimum after 31 days. HL sulfide levels were generally significantly ( $p < 0.01$ ) lower than LL sulfide levels.

On Nov. 14, prior to exposure of the eelgrass to sediment sulfide manipulations, the various measures of photosynthetic performance (Max ETR and Quantum Yield) demonstrated no significant differences ( $p > 0.10$ ) among the pots (Appendices 8, 9, 10, 11). Quantum yields were in the range of 0.7, which indicated relatively non-stressed photosystems (Ralph, 1998).

Sulfide amendments (Appendix 7) had significant effects on photosynthetic performance. By 12 days of exposure on Nov. 17, HL HS treatments significantly decreased ( $p < 0.02$ ) photosystem efficiency, or MQY, from an initial 0.68 to 0.61. Although MQY for LL HS plants did not decrease over this period (0.72 to 0.72), they failed to improve efficiency to compensate for reduction in light levels, while LL IS (0.69 to 0.73) and LL AS (0.70 to 0.74) were able to adapt. As a result of this impairment, MQY remained significantly higher in IS and AS than HS ( $p < 0.05$ ). After 17 days of exposure on Nov. 30 and through the end of the experiment, maximum ETR of HS plants was significantly lower ( $p < 0.05$ ) than IS and AS, which were not significantly different ( $p > 0.10$ ) than each other.

Beginning on Nov. 17 and through the remainder of the treatment period (Appendices 8 and 10), the effect of light environment on Max ETR and MQY was significant ( $p < 0.05$ ; Appendices 9 and 11). After 31 days on Dec. 14, Max ETR was significantly reduced for HS treatments when compared to IS and AS under HL and, especially, under LL. On the same day, MQY was significantly lower for HS (HL: 0.53, LL: 0.64) than IS (HL: 0.69, LL: 0.76) and AS (HL: 0.70, LL 0.73). Photosynthetic capacity (max ETR) was significantly lower in LL than in HL throughout the experiment, reflecting the reduction in light available for photosynthesis. MQY, or photosystem efficiency, was significantly higher when comparing LL with HL

( $p < 0.05$ ) throughout the exposure period, demonstrating the plants' adaptation to lower light levels. The interaction of light and sulfide stresses was generally not significant ( $p > 0.05$ ; Appendices 9 and 11) indicating that the effects were cumulative. This suggests that the effects of light stress compounded that of sulfide stress.

Regression models of average sediment sulfide concentrations indicated a significant ( $p < 0.001$ ; Appendix 12) correlation with the net change in MQY over the treatment period ( $R^2 = 0.63$ ); however, the regression with Max ETR was determined to be insignificant.

### Discussion and Conclusions

The photosystem stress response of eelgrass to reductions in light and increases in sediment sulfide measured using the PAM fluorometer indicated that both can be significant sources of plant stress, and that the stress on one compounds the stress of the other. Therefore the null hypotheses stating there would be no significant effects of sulfide or light treatments on fluorescence measurements ( $p < 0.05$ ) were rejected. The high sulfide levels were consistently higher in low light than in high light treatments, corresponding with the findings of Kuhn (1992). He found, when roots are subject to increasing levels of sediment sulfide and ambient light is insufficient, the oxygenation of below ground tissues and the subsequent re-oxidation of sediments may be substantially reduced. Consequently, the role of underground tissue in re-oxidizing sediments is a vital process in the eelgrass ecosystem.

Unlike previous experiments that only evaluated either sulfide stress or light reduction over a period of two weeks, this experiment more accurately simulated the natural environment by investigating their interactive effects over a month. Plants adapted to light reduction by increasing photosystem efficiency and decreasing photosystem capacity. While this adaptation is temporarily beneficial, due to the high light requirements of eelgrass, plants were unable to

maintain this type of photosynthesis for an extended period of time, especially under high sulfide conditions. Photosystem efficiency and photosystem capacity significantly decreased when sediment sulfide exceeded 1000  $\mu\text{M}$ . The relationships between sediment sulfide levels and photosystem efficiency found here may help explain loss of seagrass in Florida Bay (Carlson et al., 1994). There, initial increases in organic matter production and deposition in the seagrass beds due to nutrient enrichment may have led to increases in sulfide levels that promoted photosystem stress and plant dieback. This dieback coupled with reductions in ambient light levels from algae and epiphytes promoted even higher sulfide levels that led to continued dieback and limited recovery.

Since a single application of sediment sulfide in this experiment caused a detrimental effect on seagrass over time, sulfide stress from a sustained period of organic matter deposition may be a more severe problem than tested here. Therefore a series of injections over a given period of time would more accurately address this problem in a controlled experiment. Other measures of plant health such as oxygen production, nitrogen uptake, and carbon balance might be useful for isolating the effects of sulfide on marine plants. The PAM fluorometer provided valuable measures of seagrass stress, but due to natural variation in fluorescence, more treatment levels are essential to determine more precise relationships between sulfide concentrations, photosynthetic responses, and seagrass health.

#### Literature Cited

- Armstrong, R.S. 1979. Bottom Oxygen and Stratification in 1976 and Previous Years. NOAA Office of Marine Pollution Assessment, Rockville, MD.
- Bolhàr-Nordenkamp, H. R., and G. Oquist. Chlorophyll Fluorescence as a Tool in Photosynthesis Research. London, England: Chapman and Hall, 1993.

- Carlson, P. R., L. A. Yarbrow, and T.R. Barber. 1994. Relationship of Sediment Sulfide to Mortality of *Thalassia testudinum* in Florida Bay. *Bulletin of Marine Science* 54(3):733-746: 733-746.
- Cline, J. D. 1969. Spectrophotometric Determination of Hydrogen Sulfide in Natural Waters. *Limnol. Oceanogr.* 14: 454-456.
- Copeland, C. C. 2001. The Effect of Varying Sulfide Concentrations on the Photosynthetic Activity of *Zostera marina*. Virginia Junior Academy of Science. 19 p.
- Goodman, J. L., K. A. Moore, and W. C. Dennison. 1995. Photosynthetic Response of Eelgrass (*Zostera marina* L.) to Light and Sediment Sulfide in a Shallow Barrier Island Lagoon. *Aquatic Botany* 50: 37-47
- Hines, M.E., S.L. Knollmeyer, and J.B. Tugel. 1989. Sulfate Reduction and Other Sedimentary Biogeochemistry in a Northern New England Salt Marsh. *Limnol. Oceanogr.* 34(3): 578-590.
- Howarth, R. W. and J. M. Teal. 1979. Sulfate Reduction in a New England Salt Marsh. *Limnol. Oceanogr.* 24(6): 999-1013.
- Kemp, W. M., R. R. Twilley, J.C. Stevenson, W.R. Boynton, and J.C. Means. 1983. The Decline of Submerged Vascular Plants in Upper Chesapeake Bay: Summary of Results concerning Possible Causes. *Mar. Tech. Society. Journal.* 17(2): 78-89.
- Koch, M.S. and I.A. Mendelsohn. 1989. Sulfide as a Soil Phytotoxin: Differential Responses in Two Marsh Species. *Journal of Ecology* 77:565-578.
- Koch, M.S., I.A. Mendelsohn, K.L. Mckee. 1990. Mechanism for the Hydrogen Sulfide-Induced Growth Limitation in Wetland Macrophytes. *Limnol. Oceanogr.* 35(2): 399-408

- Kuhn, W.A. 1992. The Interaction of Light and Sediment Sulfide Levels on Eelgrass Growth Response. Master's Thesis, University of MD, MEES.
- Ralph, P. J. 1998. Photosynthetic Response of Laboratory-cultured *Halophila ovalis* to Thermal Stress. Marine Ecology Progress Series 171: 123-130.
- Ralph, P. J. 1999. Photosynthetic Response of *Halophila ovalis* Hook f. to Combined Environmental Stress. Aquatic Botany 1329:1-4
- Smith, R.D., A.M. Pregnall, and R.S. Alberte. 1988. Effects of Anaerobiosis on root Metabolism of *Zostera marina* (eelgrass): Implications for Survival in Reducing Sediments. Mar. Biol. 98:131-141
- Twilley, R. R., W. M. Kemp, K. W. Staver, J. C. Stevenson and W. R. Boynton. 1985. Nutrient Enrichment of Estuarine Submersed Vascular Plant Communities 1. Algal Growth and Effects on Production of Plants and Associated Communities. Mar. Ecol. Prog. Series 23: 179-191

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(Appendix pages are placed here.)