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Effects of sediment pore water sulfide on eelgrass (*Zostera marina*) health, distribution, and population growth in Puget Sound

Eelgrass-Sulfide Workshop

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Table of Contents

Factors of Sulfide Production in Seagrass Meadows 1
Historical Seagrass-Sulfide Interactions 2
Workshop Goals 3
Section 1: Chemical and Physiological Basis of Sulfide Intrusion 4
- Sulfide Intrusion Mechanisms 5
- Effects on Varying Life Stages 6
- Techniques, Measurements, and Methods 6
- Research discussion points 7

Section 2: Ecology of Sulfide Production in Benthic Sediments 8
- Bacterial Significance 9
- Case Studies 10
- Research Discussion Points 11

Section 3: Implications for Restoration and Management 12
- International Case Studies 13
- Puget Sound Regional Case Studies 14

References 16

Table of Figures (Possible Figure Ideas)

Figure 1: Sediment Chemistry? ....
Figure 2: Life Stages..
Figure 3: Measurement Techniques...
Figure 4: Presence of Sawmills in Relation to Eelgrass ....
Figure 5: Decline Map ....
Figure 6: Restoration Map ....

Workshop Goals

The purpose of the Eelgrass-Sulfide workshop was to develop a deeper understanding of seagrass-sulfide interactions in Puget Sound and worldwide. To accomplish this we:

1. Synthesized the current state of research through discussions and presentations;
2. Developed new seagrass-sulfide research ideas through collaboration;
3. Determined the implications and applications of sulfide research for seagrass restoration.

Throughout the workshop we considered the interaction of seagrass and sulfide worldwide and how this applies to the range of sulfide conditions throughout Puget Sound. The workshop participants reviewed seagrass-sulfide interactions, discussed new research ideas and management implications and initiated the creation of a database of sulfide ranges in Puget Sound and around the globe.

Factors of Sulfide Production in Seagrass Meadows

Seagrasses, rooted flowering plants that live in coastal marine waters, form extensive meadows worldwide and strongly influence the coastal environment (Orth et al. 2006; den Hartog and Kuo 2006). Eelgrass (*Zostera marina)*, one of approximately 60 seagrass species, is found throughout northern temperate marine waters and provides many ecological services. In the Salish Sea these services range from food supply and habitat for economically important species such as Dungeness crab (*Metacarcinus magister*) and salmon (*Oncorhynchus* spp.) to sediment stabilization (Thom 1987, Moore and Short 2006, Kenworthy et al. 2006) and nursery grounds for Pacific herring (*Clupea pallasii*) (Short and Wyllie-Echeverria 1996, Orth et al. 2006). However, seagrasses, and the services they provide are threatened due to anthropogenic stressors and environmental degradation (Short and Wyllie-Echeverria 1996; Waycott et al. 2009) leading to worldwide declines in eelgrass populations (Orth et al. 2006; Waycott et al. 2009). Puget Sound has approximately 22,000 ha of eelgrass (Christiaen et al. 2015). Limitations in light availability (Thom et al 2008), habitat loss, and hypoxic conditions have all been cited as reasons for this decline. Sulfide intrusion may be another factor in the loss of seagrasses worldwide.

The toxicant hydrogen sulfide is known to inhibit photosynthesis in seagrasses (Goodman et al. 1995, Lamers et al. 2013). Multiple studies have shown the presence of H_2S in eelgrass beds leads to diminished growth, increased die-backs, a decrease in plant biomass, and negative physiological effects (Bagarinao 1992; Pedersen et al. 2004; Borum et al. 2005, 2013; Korhonen et al. 2012; Lamers et al. 2013). The production of sulfide occurs through the anaerobic mineralization of organic matter. Microbes are a major player in organic matter mineralization and will utilize all available oxygen in the sediment for respiration (Burdige 2006, Mitsch and Gosselink 2007). Within a depth of a few millimeters, the available oxygen in the sediment is used and the microbes begin to exploit other potential sources of energy for respiration. In order of highest favorable energy output, the microbes utilize nitrate, manganese oxide, iron oxide, and sulfate (Fig. 1, Burdige 2006, Mitsch and Gosselink 2007). It is through sulfate reduction which sulfide is formed. Several species of sulfide exist in marine sediments, depending upon pH and the composition of other solutes, including the toxic form H_2S. To prevent toxic levels of H_2S from entering the plant, seagrasses typically exude oxygen from root tips whick oxidizes sulfide to sulfate (Fredricksen and Glud 2006). It is through photosynthetic processes and oxygen diffusion that seagrasses are able to produce or transport sufficient oxygen to sustain an oxidized rhizosphere. However, under anoxic conditions these barriers can breakdown allowing for the intrusion of H_2S into the roots (Pedersen et al. 2004).

Studies worldwide have implicated the damaging effect of sulfide intrusion as a reason for major seagrass die-off events (e.g., Borum et al. 2005, Borum et al. 2014). Borum et al. (2014), suggested sulfide intrusion as the main cause of the ‘fairy ring’ pattern seen in *Z. marina* dieback in Danish coastal zones during 2010. Low iron availability within the primarily carbonate sediments allowed for high levels of H_2S to be present in the sediment. In Florida, the tropical seagrass *Thalassia testudium*, experienced significant die-offs due to H_2S coupled with the presence of poor water column oxygen conditions (Borum et al. 2005). During 1987-1991, a large-scale die-off of *T. testudium* was reported in Florida Bay and high levels of sulfide working synergistically with other stressors were cited as the proximal causes of death (Carlson 1994). Large eelgrass die-offs due to high levels of pore water sulfide have not been recorded in Puget Sound though the presence of H_2S could be a barrier to successful restoration.

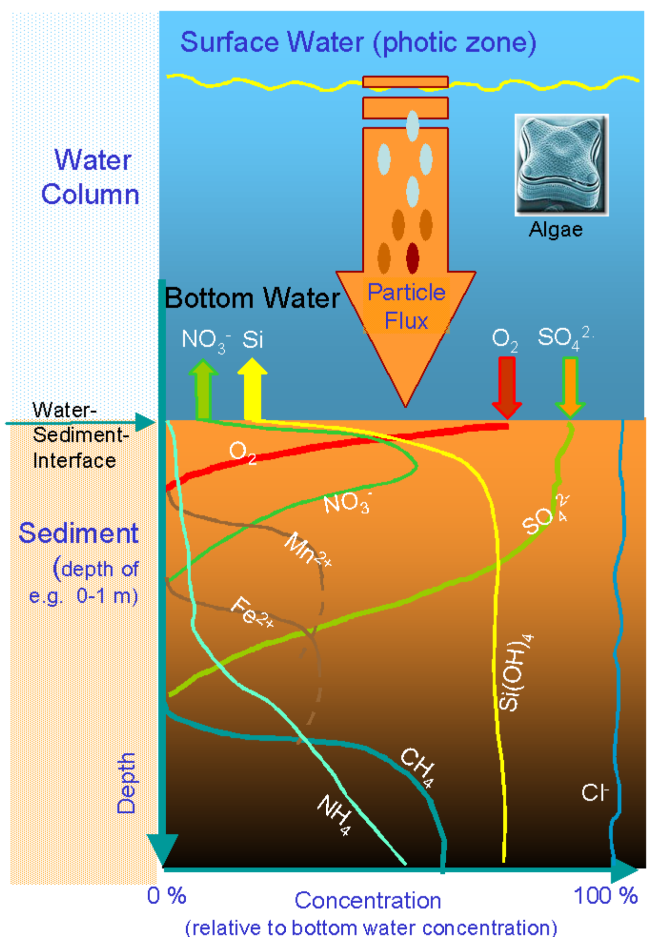

Figure 1. Pathways of organic-matter mineralization in sediments. Electron acceptors are used in order of decreasing free energy of reaction. Sulfate reduction produces sulfide as a byproduct.

Section 1: Chemical and Physiological Basis of Sulfide Intrusion

Sulfide Intrusion

Coastal marine waters are typically characterized by anoxic and reduced sediments and thus, seagrasses have adapted to survive in these conditions. Seagrasses defend their belowground biomass from anoxic conditions with the presence of aerenchyma tissue (Borum et al. 2006; Holmer and Hasler-Sheetal 2014). During photosynthesis, the oxygen produced by seagrass is either transported into the water column through the leaves or into the sediment through belowground tissues (Borum et al. 2006). The oxygen released into the sediment is particularly important as protection from various phytotoxins such as metal ions and reduced compounds (Borum et al. 2006). Typically, the seagrass is protected from phytotoxin intrusion at the root tip. The oxygen leaked into the rhizosphere will oxidize reduced compounds such as sulfide and Fe^{2+} (Borum et al. 2006) essentially creating a barrier from intrusion. The oxic conditions created by the leakage of oxygen through the root tips oxidizes sulfide into sulfate, a compound safe for seagrasses and readily used by bacteria. This allows roots to grow in anoxic conditions while preventing sulfide intrusion (Pederson et al. 2008).

During dark conditions, photosynthesis ceases leading to low oxygen levels in the plant. However, oxygen diffusion from the water column can provide necessary protection from sulfide intrusion. However, when oxygen concentrations in the water column decrease to 25% -30% then the oxidized barrier around the root tip is lost as anaerobic metabolism in the roots begins (M. Holmer, workshop presentation). The anoxic conditions produced from anaerobic metabolism can then lead to the intrusion of sulfide (Borum et al 2006, Holmer, Pederson 1994, Frederickson and Glud 2006, Korhonen et al 2012, Glaub et al 2005, Elliott et al 2006, Lee and Dunton 2000). The presence of these conditions is especially troubling given that eelgrass has been shown unable to withstand anoxic conditions when coupled with other stressors (Pulido and Borum 2010; Holmer and Bondgaard 2001) and that sulfide works similarly to cyanide resulting in 90% less energy efficiency and a net loss of 30 ATP (M. Holmer, workshop presentation).

Sulfide intrusion can occur through molecular mechanisms or be enhanced by environmental stressors (Holmer and Halser-Sheetal 2014). Molecular intrusion of sulfide can be traced through examination of the total sulfur in the plant tissue (Holmer and Hasler-Sheetal 2014). Holmer and Hasler-Sheetal (2014) concluded that in rhizomes and roots there was an increase in sulfur when higher concentrations of pore water sulfide were present. Sulfur was found to be present in the aerenchyma at high levels in plants exposed to high pore water sulfide compared to control treatments. Total sulfur in eelgrass is correlated with pore water sulfide (0 mM to 4 mM). When sulfate is taken up into the plant tissue through the water column or pore water, then it is metabolized to organic sulfur. If sulfide is taken up through the pore water, then it is metabolized to elemental sulfur as well.

It is likely that sulfide intrusion works synergistically with other environmental stressors to cause decreased growth and die-offs. In Florida Bay, it was thought the die-off of *T. testudium* was due to the presence of sulfide coupled with high temperatures, high salinity levels and *Labyrinthula* (Carlson 1994). Similarly, warmer temperatures and high sulfide levels led to a decline in *Posidonia oceanica* growth (Garcia et al. 2012). Furthermore, the presence of microbial mats altered the concentration of sulfide in sediments (Elliott et al. 2006).

What is the Poisonous Dose?

Related to understanding the mechanisms for sulfide intrusion is the question of the poisonous sulfide dose. This is not easily answerable as many factors influence the poisonous dose including exposure time, the species of sulfur present (H_2S or HS^-), the pH, the life stage of seagrass affected, and the species of seagrass. As previously mentioned, sulfide intrusion is far more likely to occur during dark than light periods due to the lack of oxygen present. Sulfate reduction can produce both H_2S and HS^-, but H_2S is far more toxic.

The different stages of eelgrass have a varying reliance on the presence of sulfide. *Z. marina* seed germination, for example, required high organic content sediment (Yang et al. 2013), which is typically associated with sulfide. Likewise, seedlings might benefit from low levels of H_2S; Dooley et al. (2013) found that at low doses (68 μM), *Z. marina* seedlings experienced increased photosynthesis. At high levels, however, seedlings had decreased photosynthetic capabilities and increased rates of mortality. Adult seagrasses are exposed to a range of H_2S values worldwide. It was suggested during the workshop that mature seagrasses can withstand H_2S doses up to 2 mM while seedlings experience mortality at 400 uM. Elliott et al. (2006) found that areas in Puget Sound with 0.716 mg/l H_2S experienced reduced growth and areas with 3.578 mg/l H_2S had no eelgrass present but instead were covered in bacterial mats. Dooley (2015) recorded a wide range of H_2S values throughout Puget Sound from near 0 mM H_2S in Shallow Bay to 10 mM H_2S in Westcott Bay.

Concentrations of pore-water sulfide reached 3 mM during the *T. testudium* die-off (Carlson et al. 2000). The sulfide toxicity threshold in *T. testudium* was found to be ~2 mM for acute exposure and ~1 mM for chronic exposure with the western basins having the highest sulfide concentrations as well as the most organic matter.

Methods of Sulfide Manipulation and Measurement

Many methods have been created to manipulate and measure pore water sulfide. In lab and field experiments, sulfide levels have been manipulated through the addition of glucose. Porous polyethylene tubes filled with glucose are placed into seagrass beds to alter the sediment sulfide concentrations (Carlson et al. 2000). Sulfide-embedded agar has been buried under mud, in which eelgrass has been planted in the lab (Goodman et al. 1995). For seed germination, Dooley et al. (2013) grew eelgrass hydroponically in liquid hydrogen sulfide that had been dissolved in deionized H_2O. Another method mentioned during the workshop was to bubble in sulfide through PVC pots.

Several methods have been employed to quantify pore water sulfide concentration. Diffusive Gradient in Thin-Films (DGTs) have been used recently in eelgrass sediments *in situ* to characterize the extent of sulfide, trace metals and other compounds around the roots and rhizomes (Deborde et al 2008; Cesbron et al 2014; Pages et al 2012). Sulfide probes that can be inserted directly into the sediment also allow for *in situ* porewater sulfide measurement. Also, porewater has been extracted using sediment cores, sippers, and peepers and taken back to the lab to determine sulfide concentration via ion-selective electrode or colorimetric methods. Howes et al. (1985) and Fuller (1994) describe the design and functionality of the sippers. Yet another methods utilizes peepers, which are inserted into the sediment, then filled with freshwater and allowed to come into equilibrium with the surrounding pore water.

Future Research areas discussed during the workshop

1. What are the differences between seagrass species with respect to sulfide toxicity?
2. Can we increase understanding of sulfide intrusion mechanisms through expanded metabolomics and genetics research?
3. What is the effect of sediment type on sulfide intrusion?
4. How do the different methods of sulfide measurement and manipulation compare?

Section 2: Ecology of Sulfide Production in Sediments

Sulfide Production and Microbial Mats

Microbial mats play a large role in the presence and health of eelgrass meadows (Holmer and Nielsen 2007; Elliott et al 2006). Microbial mats have been found within eelgrass habitat at many sites and overlay sediments containing high concentrations of sulfide (3-4 mM). In Commencement Bay, eelgrass is absent where microbial mats are present within otherwise favorable eelgrass habitat (Elliott et al 2006). The diversity of these microbial mats, however, is far greater than originally conceived. Originally thought to consist solely of *Beggiatoa* spp., it is now known that multiple bacterial species comprise the microbial mats (J. Elliot workshop presentation). These white mats composed of large, filamentous bacteria were identified using genetic analysis where it was determined 82% of the bacteria was proteobacteria (Elliott et al. 2006). Of these, 16% were classified as Gamma bacteria and 53% were classified as Epsilon bacteria. Both bacterial types are known sulfide oxidizers and contain gene sequences similar to those found in other high sulfide areas such as hydrothermal vents.

Sources of Organic Inputs

Production of sulfide has also been associated with sites of high organic input, including eutrophication, wood waste, and macroalgae. Eutrophication, the input of excess nutrients into a system, was a cause of *T. testudium* mortality (Carlson et al 2000). In a bird stake experiment, roosting stakes were added into *T. testudium* dominated meadows (Fourqurean et al 1995). The resulting guano increased the nutrient levels and subsequently increased the sediment sulfide concentrations to 2uM. This was enough to alter the community from *T. testudium* to *Halodule wrightii*, a seagrass species with higher sulfide tolerance (Carlson et al 2000). Eutrophication was also implicated as a major cause of seagrass loss in Denmark (Valdemarsen et al 2010).

In the Pacific Northwest, timber production and the resulting wood waste contamination have created areas with high levels of sediment sulfide. Locations in close proximity to lumber mills have no visible eelgrass present but do have high levels of sulfide and sulfur-oxidizing microbes whereas areas further from lumber mills often have eelgrass present (Elliott et al. 2006). It is clear that wood waste and its effects can last for decades after it enters the water steam. For example, Port Gamble, WA, which recorded 150 years of mill activity, has very little eelgrass present and little recruitment to the area. Here, the sediment consists mainly of sawdust, is strongly reducing and reaches high sulfide levels of 90-100 μM (McMillan 2015).

Future Research

1. Better quantify microbial diversity and the role of different microbial species in the development of sulfide.
2. How does type of organic pollution affect the development of sulfide?

Section 3: Implications for Restoration and Management

Understanding the mechanisms through which H_2S can impact seagrass meadows worldwide has important implications for seagrass restoration. Denmark experienced a 90% decline in eelgrass coverage since 1983 (Valdemarsen 2010), seagrasses in Florida have experienced multiple die-offs (Carlson et al 2000) and some declines appear to have occurred in Puget Sound (Thom and Hallum 1990). Various initiatives have begun worldwide to address these issues. The European Union has instituted multiple directives specifically to combat eelgrass decline including the Water Framework Directive, Marine Strategy Framework Directive, and the Habitats Directive (M. Holmer workshop presentation). The Seagrass Integrative Mapping and Modeling Program has been implemented in Florida to track the presence of seagrasses along the Florida coast (P. Carlson workshop presentation). In the Pacific Northwest, specifically Puget Sound, WA, an initiative started by the Puget Sound Partnership aims to increase eelgrass coverage by 20% by 2020.

Danish Restoration Case Study (Summary of M. Holmer workshop presentation)

In Denmark, the large decline in eelgrass had several ecosystem repercussions. With the lack of eelgrass, hydrodynamic conditions of the coastal waters changed leading to changes in nutrient dynamics, light conditions, and erosion and sedimentation patterns. These environmental changes prevented the recruitment of eelgrass even after a reduction in nutrient loading, the primary cause of the initial decline in eelgrass. Multiple steps were then taken to restore eelgrass in Denmark. Transplanting shoots had limited success as the mature plants were often uprooted due to decreased anchoring capacity of the sediment. Furthermore, seedlings were easily uprooted in muddy sediments further suggesting that recovery is limited by a lack in anchoring ability. Resuspension of the sediment also caused seedling disappearance and created increased turbidity, another barrier to successful restoration. Other issues in restoring eelgrass included the presence of lugworms and macroalgae. Lugworms mixed the sediment and forced seeds below a depth of 5 cm where eelgrass seeds will no longer germinate. The macroalgae damaged or uprooted seedlings and covered adult shoots. Thus, recovery is limited by light, high turbidity, and lack of anchoring capacity. Modeling is underway to determine areas where restoration is more likely.

Puget Sound Case Studies

In Puget Sound, organic material enrichment due to the timber industry is most relevant. Studies conducted at Milwaukee Dock, Commencement Bay (Elliot et al. 2006) and Port Gamble (McMillan 2015) indicate that buried wood waste results in long-term changes in sediment properties, including sulfide production, that causes local extinction of eelgrass. Given the large mass of wood waste (sometimes > 10m deep), eelgrass restoration in these areas would be quite costly.

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