



UNIVERSITY OF AMSTERDAM

MSc Biological Sciences
Freshwater and Marine Biology
Research project

The facilitating effects of the seagrasses *Halophila stipulacea*, and *Halodule wrightii* on *Thalassia testudinum* through lowering pore-water sulfide levels

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42 ECTS Credits
September 2021- November 2022

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Abstract

Seagrass beds are among the world's most productive systems with many ecosystem services such as coastal protection. The global rate of seagrass loss is high and despite legislation, the rate of loss has not slowed down substantially. A significant stressor on the Caribbean coast is the Sargassum mats that wash ashore. The coastal waters become enriched in nutrients and oxygen-depleted, releasing toxic hydrogen sulfide gas. The sediment becomes hereby uninhabitable for the native seagrass *Thalassia testudinum*.

This study aimed to investigate whether there is a facilitating effect of *Holophila stipulacea* and *Halodule wrightii* on *T. testudinum* through sediment oxidizing properties. The following sub-questions were raised: (1) "Is there a difference in the growth rate of the seagrasses *H. stipulacea*, *H. wrightii*, and *T. testudinum* when they are transplanted to a post-SBT sulfide-rich environment?", (2) "Is there a difference in sulfide levels in the sediment next to transplanted *H. stipulacea*, *H. wrightii* and *T. testudinum* cores?", and lastly (3) "Is there a difference in the growth of individual *T. testudinum* shoots next to these transplanted cores?"

To test this, cores of all three species were taken from healthy seagrass beds and transplanted to a sulfide-rich area. (1) The relative growth rate of the cores was measured, and, (2) sulfide levels in the sediment next to the transplanted cores compared. Lastly, (3) the growth of individual *T. testudinum* shoots was measured when placed next to the transplanted cores.

This study showed (1) no difference in growth rates of the transplanted cores, and (2) temporarily elevated pore-water sulfide levels in the immediate surroundings of the transplanted cores. Lowest sulfide levels near the *H. wrightii* transplantation and (3) no difference in the growth of the individual *T. testudinum* shoots between treatments. Concludingly, it is unlikely that there is a plant-plant facilitating effect of *H. stipulacea*, *T. testudinum* and *H. wrightii* on *T. testudinum*. Transplantation of *T. testudinum* cores with *H. stipulacea* or *H. wrightii* is not a restoration measure that may facilitate *T. testudinum* in bare areas with high pore-water sulfide levels. Investigating the behavior of the sulfide levels over a longer period is advised since the duration of the experiment was too short to observe the equilibrium that arose.

Keywords: plant-plant facilitation, seagrass restoration, sargassum, pore-water sulfide, *Thalassia testudinum*, *Halophila stipulacea*, *Halodule wrightii*.

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1.0 Introduction

Seagrass beds are ecosystem engineers, as they create habitats by modifying their environment (Bos et al., 2007). The beds support ecosystem services, such as erosion control, playing a significant role in coastal protection, carbon sequestration, and the provision of crucial habitats for juvenile reef fish (Christianen et al., 2013; Dorenbosch et al., 2005). Despite the legislation on habitat improvement and environmental rehabilitation, the global reduction rate of seagrass has not slowed down substantially (Waycott et al., 2009; van Katwijk et al., 2016). Reintroduction and restoration efforts have, however, proved crucial to restoring their ecosystem services (van Katwijk et al., 2016).

One major stressor for seagrass in the Caribbean Sea is Sargassum (*S. fluitans* and *S. natans*) (Franks et al., 2011). Sargassum is a naturally occurring brown macroalga in the temperate part of the trophic oceans. The algae can live holopelagic, meaning never being attached to the ocean floor but instead floating. When drifting offshore, the complex seaweed mats provide habitat for seabirds, sea turtles and fish, and invertebrates. Besides, it acts as a nursery for many organisms of which some of commercial importance (Laffoley et al., 2011; Pendleton et al., 2014). The seaweed mats occur in large rafts in the Sargasso Sea in the North Atlantic. Since 2011, huge Sargassum blooms, called the Great Atlantic Sargassum Belt, have been emerging in the northern tropical Atlantic Ocean, caused by an abnormal current pattern transporting a large amount of Sargassum from the Sargasso Sea into the tropical Atlantic (Johns et al., 2020). Sargassum has now successfully colonized this new habitat, its presence in this dynamic part of the ocean displays a large spatial, seasonal, and interannual variability (Johns et al., 2020; Jouanno, Benschila, et al., 2021; Wang et al., 2019). This is due to the influence of wind, currents, and availability of nutrients from various sources, such as ocean upwelling, export from the Amazon, Congo, and Orinoco rivers, and deposition of Sahara dust (Johns et al., 2020; Jouanno & Benschila, et al., 2021; Jouanno & Moquet, et al., 2021; Oviatt et al., 2019; Pi et al., 2009; Skliris et al., 2022; Wang et al., 2019).

Currents take large quantities of the Sargassum mats to the Caribbean where they wash ashore and start to decompose. Consequently, the coastal area becomes turbid brown, highly enriched in nutrients and organic carbon. An increase in available organic carbon stimulates the metabolism of bacteria that reduce sulfate to its end product sulfide, in marine habitats, the most important electron acceptor after oxygen (Pedersen et al., 2004). As a consequence, the sediments become depleted of oxygen and toxic hydrogen sulfide gasses are released. These events are called Sargassum Brown Tides (SBT) and occur across the Caribbean. The rotting beaches with the hydrogen sulfide smells negatively affect tourism and put severe pressure on coastal ecosystems. Both seagrass fields, mangrove forests, and coral reefs are subjected to high levels of stress during an SBT (van Tussenbroek et al., 2017). If mangroves and seagrasses are able to provide sufficient leakage of photosynthetically produced oxygen from the roots, the plants can create a micro shield of oxygen around roots and rhizomes. This biogeochemical pathway counteracts sulfide toxicity (Pedersen et al., 2004; Pi et al., 2009).

Numerous ecosystem services are provided by many seagrass species across genera, and, in general, larger seagrasses with more complex structures offer more diverse services (Mtwana Nordlund et al., 2016). However, the ecosystem service of counteracting the accumulation of sulfides may likely be better performed by small, fast-growing species, probably due to the faster growth (Soissons et al., 2019). The seagrass beds in Lac Bay Bonaire are strongly influenced by the negative effects of the SBT (Leemans et al., in preparation). The bay is mainly inhabited by the native seagrass species *Thalassia testudinum* and since 2010 also by the invasive *Halophila stipulacea* that originated from the Red Sea. *Halodule wrightii*, *Syringodium filiforme*, and *Ruppia maritima* are also present but in smaller amounts (Debrot et al., 2018). Since its establishment, *H. stipulacea* competes with the native *T. testudinum* and has an advantage under eutrophic conditions (van Tussenbroek et al., 2016). The pioneer species *H. stipulacea* displays rapid growth, and its roots remain shallow. Moreover, personal observations showed that *H. stipulacea* was able to recolonize the sulfide-rich bare sediments after an SBT. From this, it is hypothesized that *H. stipulacea* can maintain high growth rates in sulfide-rich areas, but shallow rooting may prevent thorough oxygenation of the sediments. The fast-growing

native pioneer species *H. wrightii* has a shallow root system but roots deeper than *H. stipulacea*. From this, it is hypothesized that *H. wrightii* has stronger sediment oxygenating properties than *T. testudinum*, but lower growth rates due to higher exposure to sulfide by the deeper roots

Positive feedbacks are highly important within the seagrass beds (Maxwell et al., 2017). Since they beneficially influence their environments so much, seagrass restoration is often difficult because large transplants are necessary (van Katwijk et al., 2016). Facilitation of seagrass growth through suspension-feeding lucinid bivalves, which decrease sediment pore-water sulfide, has been broadly reported (Chin et al., 2021; Van Der Heide et al., 2012). For example, bivalves have been shown to have a positive effect on this oxygen micro shield in the sediment through sulfur-oxidizing gill bacteria. Furthermore, mechanical oxidation of the sediment through bioturbation of macrofauna is considered a possible pathway by which pore-water sulfide can be reduced (Jørgensen et al., 2019), or, the plant-plant grazing protection mutualism involving coralline algae (Leemans et al., 2020). However, there is no account of documentation of biochemical plant-plant facilitation. Here, it was hypothesized that facilitation of the climax species *T. testudinum* may occur by the pioneer species *H. wrightii* and *H. stipulacea* after an SBT has removed previous seagrass vegetation, by reducing sulfide levels enough for *T. testudinum* survival. Rather than focusing on natural pioneer-climax vegetation succession dynamics, we specifically test the hypothesis that co-transplantation of desired climax species *T. testudinum* with pioneer species *H. wrightii* or *H. stipulacea* may increase transplant success through sediment oxidizing activities.

This study aimed to investigate whether there is a facilitating effect of *H. stipulacea* and *H. wrightii* on *T. testudinum* through sediment oxidizing properties. The following sub-questions were raised: (1) “Is there a difference in the growth rate of the seagrasses *H. stipulacea*, *H. wrightii*, and *T. testudinum* when they are transplanted to a post-SBT sulfide-rich environment?”, (2) “In this sulfide-rich environment, is there a difference in sulfide levels in the sediment next to transplanted *H. stipulacea*, *H. wrightii* and *T. testudinum* cores?”, and lastly (3) “Is there a difference in the growth of individual *T. testudinum* shoots next to these transplanted cores, and if so, can it be explained by sulfide or other factors?”.

It was hypothesized that the highest sulfide tolerance and growth will be seen in *H. stipulacea* from its shallow rooting and due to the natural high capacity of *H. stipulacea* to accumulate sulfide (Apostolaki et al., 2018). Moreover, it is expected that transplanted *H. wrightii* express the most sediment sulfide oxidizing properties, because of its fast growth and deeper rooting. *T. testudinum* is expected to have the lowest tolerance and lowest oxidizing effects, because of its slow growth (Soissons et al., 2019). Accordingly, it was hypothesized that the growth rate of individual *T. testudinum* shoots was highest when transplanted together with *H. wrightii*.

Three complementary in situ experiments were carried out. To test the first hypothesis that *H. stipulacea* is the most sulfide tolerant as compared to *H. wrightii* and *T. testudinum*, cores of all three species were taken from healthy seagrass beds and were transplanted to a sulfide-rich degraded area. The relative growth rate was measured based on changes in their leaf density after 7 weeks. The second hypothesis, stating that *H. wrightii* is the best sulfide reducer of the three seagrasses, was tested by comparing sulfide levels in the sediment next to transplanted *H. wrightii*, *H. stipulacea*, and *T. testudinum* cores. To test the last hypothesis that there is a difference in the growth of the *T. testudinum* shoots depending on the seagrass species they are placed next to, the growth of individual *T. testudinum* shoots was measured when placed next to *H. stipulacea*, *H. wrightii*, and *T. testudinum* cores.

2.0 Materials & Methods

2.1 Study site

This study was carried out from mid-November 2021 to the beginning of January 2022. The in-situ experiment took place in Lac Bay on the east side of Bonaire in the Dutch Caribbean (7 km², 12°10'N, 68°15'W) (Figure 1). The depth of the inland bay varies between -0,5 m to -5 m (Engel S., 2017). The macrophyte community consists predominantly of the seagrasses *T. testudinum*, *H. wrightii*, *H. stipulacea*, and various macroalgae. Areas in Lac Bay are heavily impacted by SBT in March 2018 and again in March 2019. The wind and water currents carry the Sargassum to the Western part of the bay. Seagrass beds show a yearly decrease in abundance (Leemans et al., in preparation). Still, dense *T. testudinum* seagrass beds occur in shallow waters close to the border of the mangrove forest.

2.2. Baseline measurements

To gain insight into the present sulfide levels in the bay's sediments and to determine the experimental site and the donor sites, pore-water samples from the sediments were taken as baseline measurements from different locations in Lac Bay prior to the start of the experiment. A Sargassum-affected area, with sulfide levels exceeding 550 micromoles per liter, was chosen as the experimental site, and the unaffected seagrass beds with low sulfide concentrations were chosen as donor sites (Table 1). The sulfide levels in the experimental site were significantly higher than in all three donor seagrass beds (Appendix 1).

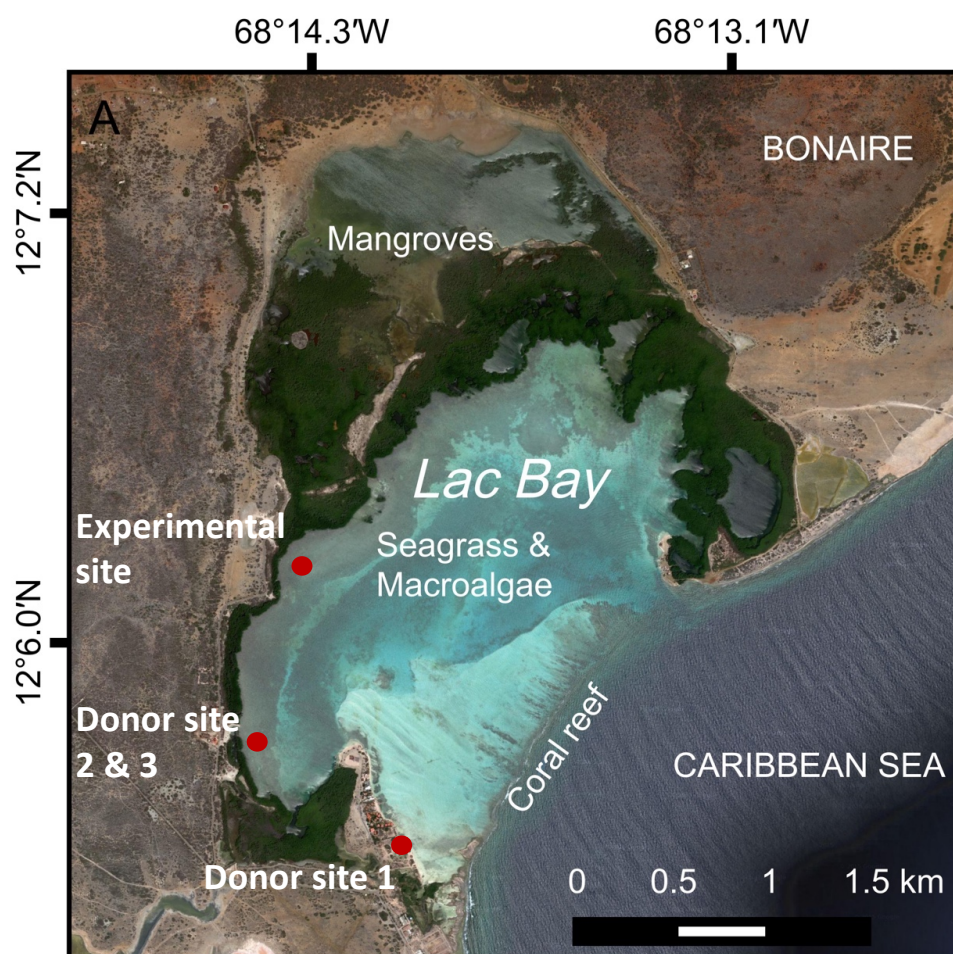


Figure 1. Distribution of seagrasses and mangroves in Lac Bay Bonaire, and locations of the donor sites and the experimental site.

Table 1. The baseline average level of sulfide ($\mu\text{mol/l}$) in the experimental site and three donor sites.

Location	Average	SD
Experimental site	560	256
Donor site 1 (<i>H. wrightii</i>)	86	82
Donor site 2 (<i>H. stipulacea</i>)	53	84
Donor site 3 (<i>T. testudinum</i>)	36	33

2.3 Experimental design

2.3.1 Experiment 1- Is there a difference in the relative growth rate between *H. stipulacea*, *T. testudinum*, and *H. wrightii* in a sulfide-rich environment?

Measuring the relative growth rate of H. stipulacea, T. testudinum, and H. wrightii cores after transplantation to a sulfide-rich environment.

Cores with either seagrass and sediment or only sediment without seagrass from a healthy seagrass bed were transplanted to the SBT-degraded experimental site. The cores were 15 cm in diameter and 30 cm deep. *H. wrightii* and *T. testudinum* were collected from the same donor site in Lac Bay and *H. wrightii* from a seagrass bed further to the south of Lac Bay (Figure 1). The cores were transported to the experimental site using floating buckets. The cores were placed in the sediment in one swift movement whilst still in the PVC containers used to take the core to maintain the core's shape. Then, the PVC container was slowly removed, and the edges of the cores were gently pressed down to equalize the height of the cores to the surrounding sediment if needed. This in-situ experiment used three different seagrass cores and two different sediment cores. The control had no core added. *H. stipulacea* and *T. testudinum* beds were located next to each other at the same location, therefore only one sediment core was used for these two species. The seagrass cores consisted of *H. stipulacea* (Hs), *T. testudinum* (Tt), and *H. wrightii* (Hw). Moreover, the sediment treatments consisted of sediment from the donor site of Hs and Tt (DHsTt) and sediment from the donor site of Hw (DHw). The control contained solely bare sediment from the experimental site (Co). Five replicas per seagrass treatment and the control, and 3 replicas per sediment treatment and control were used. Subsequently, to protect the grasses from herbivory by fish, cages of chicken wire (5mm x 5mm) were placed over the individual plots (Figure 2). To measure the decreasing light availability after placement of the cages, HOBO light data logger field devices were used. These showed a six times lower light availability after two days by epiphytal growth. Therefore, it was decided to clean the cages from epiphytes every day or every other day (Appendix 2 to 7).

The effect of high pore-water sulfide on plant health was assessed. Shoot density was used as a measure for relative growth rate. This was assessed by measuring leaf count at the start of the experiment in mid-November, and at the end of the experiment, in late December. The number of leaves present in a 5x5 cm quadrant within the core were counted. That meant counting the leaves whose shoots were rooted within the quadrant. Lateral growth was not assessed. The vertices of the quadrant were marked with skewers reassuring the exact corresponding sampling location upon revisiting (Figure 3).

To assess the effect of transplantation on the three seagrass species, five cores of each species were taken from the healthy seagrass bed and directly placed back in their original spot as site controls (CHs, CHw and CTt). The leaf density was measured using the same method as for the treatments in the experiment.

2.3.2 Experiment 3- Is there a difference in sulfide levels in the sediment near the *H. stipulacea*, *T. testudinum*, and *H. wrightii* cores in a sulfide-rich environment?

Measuring sulfide levels in sediment near the H. stipulacea, T. testudinum, and H. wrightii cores after transplantation to a sulfide-rich environment.

On one side of the seagrass cores, two individual *T. testudinum* shoots, also derived from the *T. testudinum* bed donor site, were placed at the edge of the cores (Figure 2). The individual *T. testudinum* shoots were around the same size and contained at least 5cm of the rhizome.

After setting up experiment one, four rhizon pore-water samplers were placed in each plot. One against the rhizome of each of the individual *T. testudinum* shoots and the remaining two on the other side of the core, two centimetres from the edge. The rhizon samplers were attached to a small piece of PVC tube that was anchored in the sediment. This ensured that the rhizons could be left in place by which the pore-water of the same spot could be sampled. The rhizons were placed at a depth of 7 cm to give the best proxy of the sulfide changes in the sediment. The sulfide concentrations of the pore-water samples were calculated by comparison to known sulfide concentrations of a standard curve. Pore-water was extracted at a 5 to 10 cm depth. Sulfide in the pore-water was measured using the permanent rhizon samplers every other week. Pore-water sulfide concentrations can heavily depend on the time of day, the rhizon samples were therefore always taken around the same time of day (Lee & Dunton, 2000).

2.3.3 Experiment 2- Is there a difference in the growth rate of individual *T. testudinum* shoots depending on the seagrass core they are placed next to?

Measuring the growth rate of individual T. testudinum shoots after transplantation next to seagrass cores in a sulfide-rich environment.

As an indicator of plant health, the leaf elongation rate of *T. testudinum* shoots was used. The leaf elongation rate was measured using a leaf puncturing method based on the plastochrone interval measuring method from Shorts & Coles (2001). Leaves were marked by puncturing two tiny holes with a needle right above the sheath. After three days, the distance between the punctured holes and the sheath was measured. Only the youngest leaf was punctured since the older leaf often fell off or discolored, in which cases the punctured holes could not be retrieved. When a new leaf emerged, the length of this new leaf was added up with the growth of the, by then, second youngest leaf. This was done because only measuring the second youngest leaf would distort the picture of the shoot's health. Field observations showed that sometimes the second youngest leaf would have grown close to nothing while the new leaf had grown many centimetres.

To assess the effect of transplantation on the individual *T. testudinum* shoots growth, ten individual *T. testudinum* shoots were gathered as site controls and placed in a bare sediment area next to the healthy seagrass bed where the sulfide levels were low.

2.4 Data analysis

To assess if the relative growth rates, the mean sulfide levels, and the growth rates of the individual *T. testudinum* shoots differed between treatments, a two-way analysis of variance (ANOVA) test was used. No non-parametric tests were used due to the more explanatory power of the ANOVA with small sample size. Log-transformed data, square-root-transformed data, and untransformed data were compared. The analysis was performed on the model with the most normally distributed residuals, as

determined by Shapiro-Wilk's test. A post hoc Tukey HSD test was used whenever significant differences were found. The level of significance that was used was $p \leq 0.05$. All analyses were carried out in Rstudio (version 4.0.5).

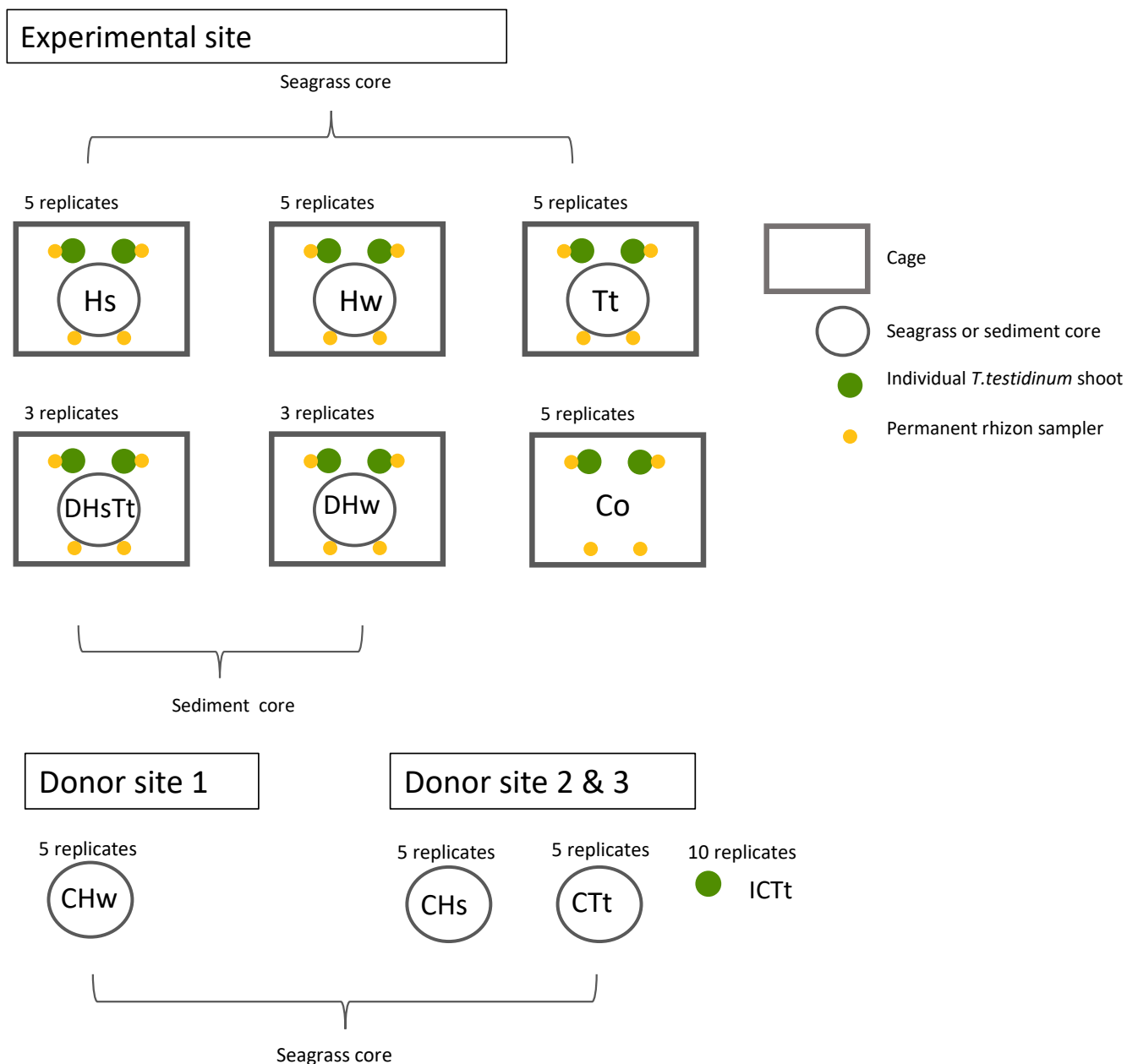


Figure 2. Schematic overview of the experimental design. The black circles represent the cores with the abbreviations of the treatments written within. In the experimental site: Hs indicates the *H. stipulacea* core, Tt the *T. testudinum* core, Hw the *H. wrightii* core. DHsTt indicates a sediment core without seagrass from the donor site of *H. stipulacea* and *T. testudinum*. DHw indicates a sediment core without seagrass from the donor site of *H. wrightii*. Lastly, the control has no core added and is indicated with the abbreviation Co. The green dots indicate individual *T. testudinum* shoots with a pore-water sampler placed against their rhizome. The yellow dots represent the rhizon pore-water samplers on both sides of the plot. Two of these samplers are placed against the rhizome to the individual *T. testudinum* shoots, the other two were placed on the other side of the core. In donor site 1 the site control plot of *H. wrightii* is represented by the abbreviation CHw, and, the site controls of *H. stipulacea* and *T. testudinum* by CHs and CTt respectively. The 10 individual *T. testudinum* shoots represent the site control of the individual *T. testudinum* shoot growth (ICTt).



Figure 3. Photos at the beginning of the experiment. Panel A, C, and E show plots of the Hw, Hs, and Tt cores, respectively. Panel B, D, and F show the placement of the quadrant used for measuring leaf density. The red arrows in panel A indicate the individual *T. testudinum* shoots and the red circles point out the above-ground part of the rhizon samplers on one side of the core.

3.0 Results

3.1 Difference in growth rate between *H. stipulacea*, *T. testudinum*, and *H. wrightii* in a sulfide-rich environment

Measuring the growth rate of H. stipulacea, T. testudinum, and H. wrightii cores after transplantation to a sulfide-rich environment

No significant differences in relative growth rate were found between the seagrass cores in the sulfide-rich environment (Hs, Hw, and Tt) and the control cores that were placed back within the donor meadows (CHs, CHw, and CTt) (ANOVA, $F= 1.13$, $df= 5$ and $p= 0.36$). Also, no differences in relative growth rate were found between the seagrass treatments (Figure 4). While all plants in the Hw treatment showed declining density, plants in CHw had highly variable responses where some cores showed some increase in density. The variance of the Tt treatment is higher, indicating a stronger decrease in growth for some transplanted cores and some cores showing a positive growth rate whilst the site control (CTt) showed more or less no increase and no decrease in growth.

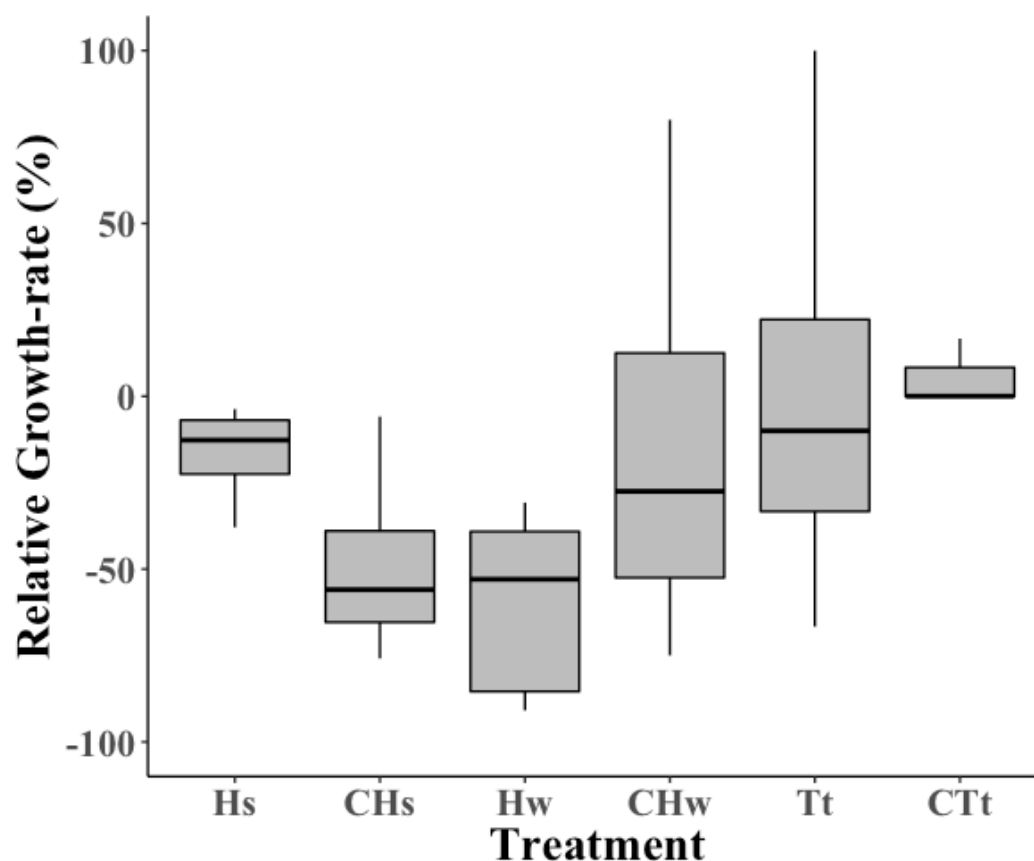


Figure 4. Boxplot of relative growth rate for the seagrass cores at the experimental site (sulfide-rich) (Hs, Hw and Tt) and in cores that were placed back at the donor site (CHs, CHw and CTt). Bold horizontal lines indicate the median, boxes show the interquartile range (IQR), and bars represent $Q1 -/+ 1.5 \times IQR$ or $Q3 -/+ 1.5 \times IQR$. Treatments did not differ significantly.

3.2 Difference in sulfide levels in the sediment near the *H. stipulacea*, *T. testudinum*, and *H. wrightii* cores in a sulfide-rich environment

Measuring sulfide levels in sediment near the H. stipulacea, T. testudinum, and H. wrightii cores after transplantation to a sulfide-rich environment.

Figure 5 shows differences in pore-water sulfide levels between all treatments and control. All seagrass-containing treatments show higher sulfide levels than the treatments only containing sediment. Significant differences were found in mean sulfide levels between treatments (ANOVA, $F = 5.22$, $df = 5$, $p < 0.005$). The pore-water sulfide levels in the Hs and Tt treatment were significantly higher when compared to sediment from their donor site DHsTt (Tukey HSD $p = 0.045$ and $p = 0.036$ respectively). Even though not significant, the treatments with sediments from the donor sites, DHsTt and DHw, show lower sulfide levels than the natural sulfide levels in the experimental site, Co.

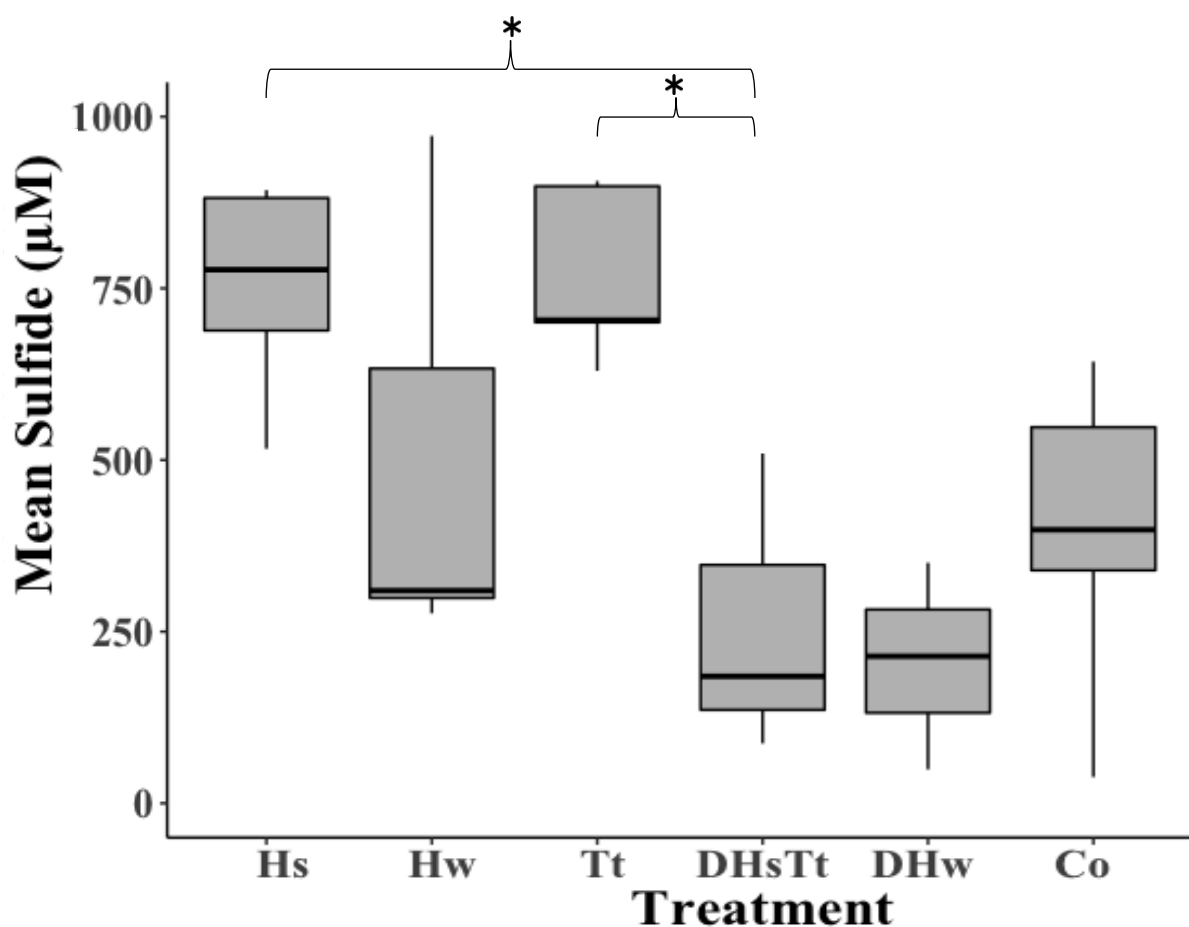




Figure 5. Boxplot of mean sulfide over four -time points for all the treatments: the addition of seagrass cores with *H. stipulacea* (Hs), *H. wrightii* (Hw), *T. testudinum* (Tt), and sediments cores from the donor site of the species *H. stipulacea* and *T. testudinum* (DHsTt), sediment cores from the donor site of the species *H. wrightii*, and a control at the experimental site without cores added (Co). Bold horizontal lines indicate the median, boxes show the interquartile range (IQR), and bars represent $Q1 \pm 1.5 \times IQR$ or $Q3 \pm 1.5 \times IQR$. Dots represent outliers. Significance is indicated with an asterisk, $* < 0.05$ (ANOVA).

Figure 6 displays the sulfide levels over a period of 40 days. The sediment treatments (DHsTt and DHw) and the control (Co) show only an increase in sulfide levels from the first measurement on day 6 to day 17. A slight decrease in sulfide levels is thereafter observed. The seagrass treatments (Hs, Hw, and Tt) show a decrease in sulfide levels between day 6 and day 14. A high peak in sulfide levels is seen after this, followed again by a strong decrease in sulfide levels (Table 2). The strongest reduction of sulfide level was observed in the Hs treatment, and the lowest sulfide level was observed in the Hw treatment. Cloud cover, whereby 0 means no clouds and 8 means total cloud cover, is highest during the last measurement. Cloud cover was positively significantly correlated to sulfide levels (SPEARMAN S = 87533, p = 0.008, rho =0.28) (Appendix 8).

Table 2. Mean and standard deviation of sulfide levels (in $\mu\text{mol/l}$) in the pore-water samples taken from next to the core for all treatments during the four different time points. First pore-water measurement at t=6. Six days after placement of cores in the study area at t=0. Cloud cover is indicated by cloud icon.

Treatment	measurement 1 t=6  3.25	measurement 2 t=14 1.5	measurement 3 t=26 3	measurement 4 t=39 7.75
Hs	332 ± 21	311 ± 36	1445 ± 165	733 ± 86
Hw	357 ± 87	286 ± 22	946 ± 189	462 ± 42
Tt	568 ± 68	410 ± 52	1080 ± 108	930 ± 107
	t=6  1.5	t=17 3	t=32 7.75	
DHsTt	47 ± 9	485 ± 54	262 ± 46	
DHw	26 ± 10	385 ± 5	259 ± 83	
Co	217 ± 226	595 ± 160	469 ± 464	

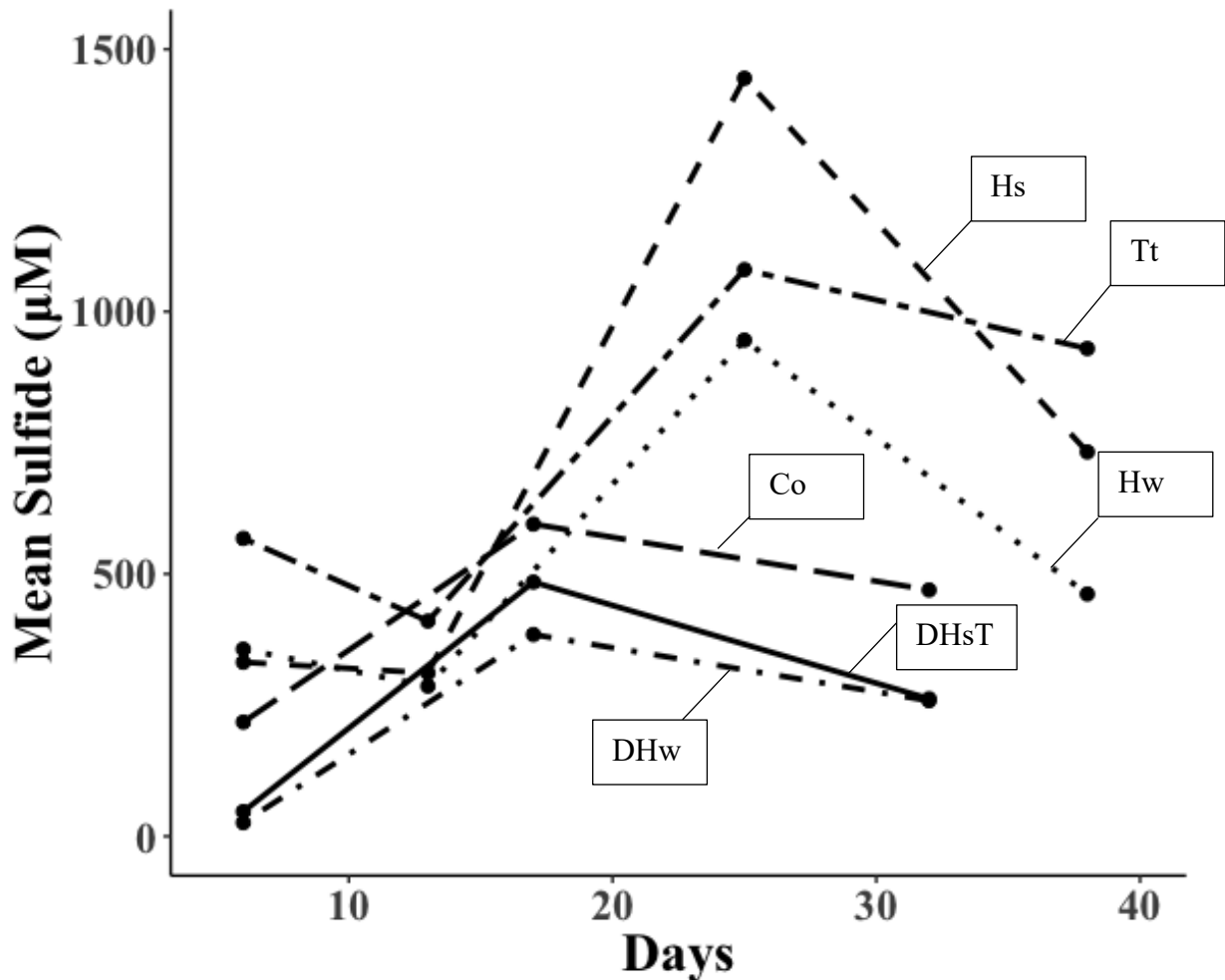


Figure 6. Line graph of mean sulfide levels (μM) over number of days in the field (days). The dashed line is the Hs treatment, the dotted line is the Hw treatment, the Twodash line is the Tt treatment, the dot-dash line is the DHw treatment, the solid line is the DHsTt treatment, and the long dash line is the Control treatment.

Figure 7 shows how the mean sulfide levels throughout the entire duration of the experiment are correlated to the relative growth rate of the cores. The Hw treatment proved to be the only treatment significantly correlated to sulfide levels (Table 3). The Hw treatment showed the largest variance in sulfide levels. The Hw treatment also showed the lowest relative growth rate of all species. The Tt treatment shows the strongest effect of sulfide levels on the relative growth, but it is also the only treatment showing any positive growth up to approximately 800 micromoles.

Table 3. Pearson's correlation between relative core growth rate and sulfide levels.

Treatment	t	df	cor	p-value
Hs	-2.15	2	-0.84	0.17
Tt	-1.65	3	-0.69	0.2
Hw	-3.91	3	-0.91	0.03 *

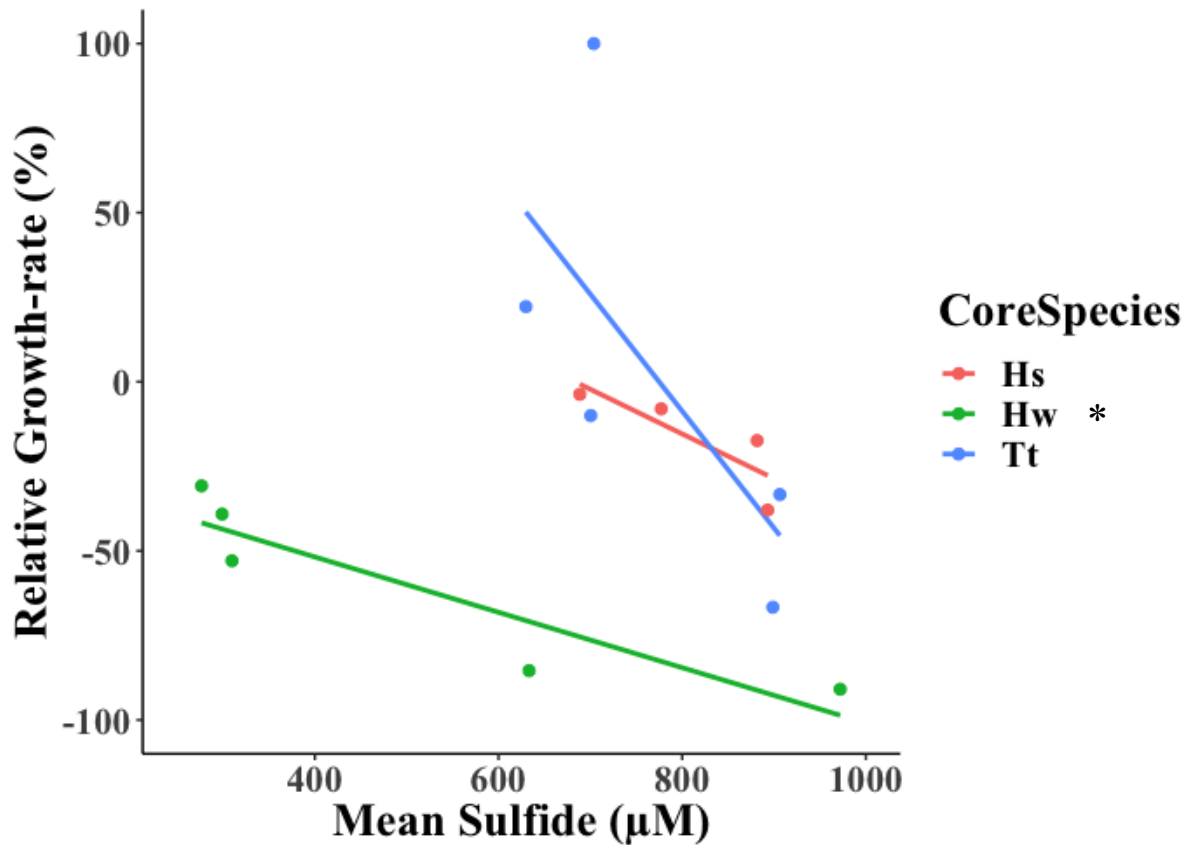


Figure 7. Regression lines of the relative growth rate of the cores of *H. stipulacea* (Hs), *H. wrightii* (Hw) and *T. testudinum* (Tt) measured by changes in leaf density of the different core treatments in percentages against mean sulfide levels in micromole. A significant correlation is indicated by an asterisk.

Every core contained four rhizons, two of which were placed next to a *T. testudinum* shoot and two that were not (Figure 2). A T-test was used to test whether or not the *T. testudinum* shoots influenced the sulfide levels next to the core. No difference was found in sulfide levels between the rhizons against the rhizome of the *T. testudinum* shoots and the rhizons at the other side of the core (Table 4). The rhizon samples could therefore be pooled, resulting in four rhizons sample points per plot instead of two.

Table 4. Results of the T-test testing for differences in sulfide levels between the rhizons placed against a *T. testudinum* shoot and the rhizons placed at the other side of the core.

Treatment	t-value	df	p-value
Hs	0.62	8	0.55
Hw	-0.18	8	0.87
Tt	0.23	8	0.83
DHsTt	0.06	4	0.95
DHw	-0.33	4	0.76
Co	-0.71	8	0.5

3.3 Difference in the growth rate of individual *T. testudinum* shoots depending on the seagrass core they are placed next to

Measuring the growth rate of individual T. testudinum shoots after transplantation next to seagrass cores in a sulfide-rich environment.

The shoot growth of the individual *T. testudinum* shoot next to the cores was measured (Figure 8). The site control consisted of measuring the growth of individual *T. testudinum* shoots in the donor site with low sulfide levels (Table 1). Significant differences between the treatments were found (ANOVA, $F = 5.3$, $df = 6$, $p < 0.001$). The site control treatment showed significantly higher growth rates than the Hw treatment (TukeyHSD $p < 0.001$). Moreover, the site control was also significantly higher than the Tt treatment (TukeyHSD $p = 0.02$). The DHw treatment was almost significantly lower than the site control (TukeyHSD $p = 0.06$). No differences were found between all the other treatments (Appendix 9). The shoot growth ranged from one cm per day to 5.5 cm per day with an outlier of around 8 cm per day. The graph shows the lowest mean growth rate in the Tt treatment and the ICTt the highest.

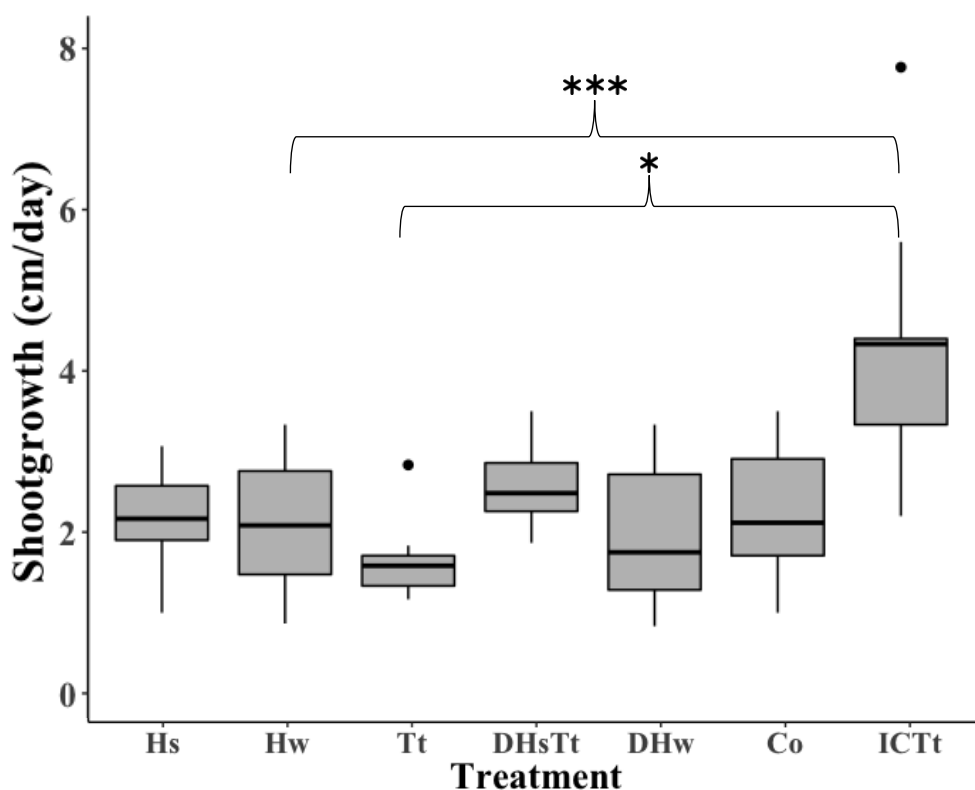


Figure 8. Boxplot of individual *T. testudinum* shoot growth in cm per day for all the treatments: Seagrass cores *H. stipulacea* (Hs), *H. wrightii* (Hw), *T. testudinum* (Tt), and sediment cores from donor sites of the species *H. stipulacea* and *T. testudinum* (DHsTt), and *H. wrightii* (DHs), the control where no seagrass cores were transplanted (Co), and the site control of individual *T. testudinum* shoots (ICTt) in donor site 2&3 (Figure 1). Bold horizontal lines indicate the median, boxes show the interquartile range (IQR) and bars represent $Q1 \pm 1.5 \times IQR$ or $Q3 \pm 1.5 \times IQR$. Dots represent outliers. Significance is indicated with an asterisk, $* < 0.05$, $** < 0.01$, $*** < 0.001$.

No significant correlations were found between the shoot growth and the sulfide levels for all treatments (Table 5). All treatments, except the Tt treatment, showed a negative correlation meaning that the growth decreased when the sulfide levels increased. The regression line in figure 8 shows a slight decrease in growth when the sulfide levels increase ($p = 0.44$ and $cor = -0.16$).

Table 5. Pearson's correlation between shoot growth of the individual *T. testudinum* shoots and sulfide levels per treatment.

Treatment	t-value	df	cor	p-value
Hs	-0.14	3	-0.08	0.9
Hw	-1.01	3	-0.5	0.39
Tt	0.43	3	0.24	0.7
DHsTt	-0.01	1	-0.01	0.99
DHw	-0.58	1	-0.5	0.67
Co	-0.04	3	-0.02	0.97

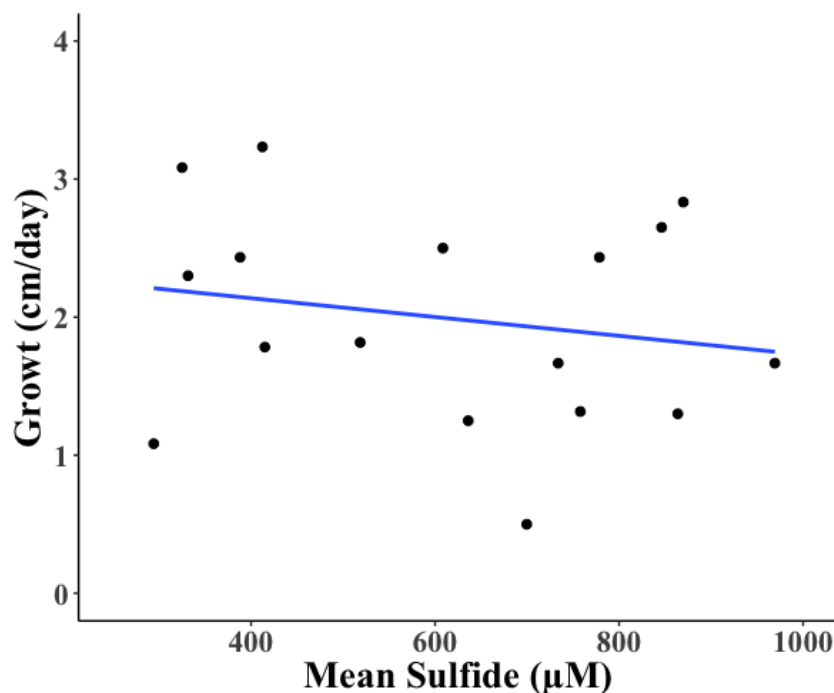


Figure 8. Regression line of the individual *T. testudinum* shoot growth (cm/day) against the mean sulfide levels (µM), $p = 0.44$ and $cor = -0.16$.

4.0 Discussion

4.1 The difference in growth rate between *H. stipulacea*, *T. testudinum*, and *H. wrightii*

This part of the study investigated the tolerance of *H. stipulacea*, *T. testudinum*, and *H. wrightii* to high pore-water sulfide levels. This was done by measuring the differences in the growth rate of the seagrasses after transplantation to a sulfide-rich environment at the beginning and at the end of the experiment. It was hypothesized that *H. stipulacea* expressed the highest growth rate through its proposed high tolerance to sulfide. This was however not observed in this study. Because no significant differences in core growth were found, it can be concluded that transplantation has no effect on the relative growth rate.

The growth rate of *H. stipulacea*, *T. testudinum*, and *H. wrightii* cores was measured after transplantation to a sulfide-rich environment. The site control of the Hs treatment showed a stronger decrease in relative growth rate than the Hs treatment at the sulfide-rich environment (Figure 4). A potential reason for this stronger decrease in relative growth rate, which is observed in the site control, is that the site control core was placed back in its original seagrass bed location. Hereby, the seagrasses in the core are not only exposed to the decomposition of the cut and damaged roots within the core, but also exposed to the decomposition of the cut and damaged roots from the neighbouring plants outside the core. This could lead to higher sulfide levels, in turn showing a stronger decrease in relative growth.

The Hw treatment at the sulphide-rich environment had a stronger decrease than its site control (CHw). This could intuitively be explained by the higher levels of sulfide at the experimental site as compared to the site control at the donor site

The site control of the Tt treatment (CTt) reacted as expected with no growth or decrease in growth as compared to the experimental, sulphide-rich environment. The Tt treatment at the experimental site showed a large variance with some cores doubling in the number of leaves and others losing half. Remarkably, the Tt treatment core expresses such a large variation in growth rate while the site control behaves according to the expectations, expressing almost no growth or decrease in growth. A possible explanation for this is that the absence of competition in the experimental site results in higher growth rates in some of the cores. (The site control plots were placed directly back into their original locations and possibly competing with the neighboring plants.) In contrast, other cores may have experienced an accelerated decline when parts of the *T. testudinum* in the cores died off. Literature suggests that dying areas of *T. testudinum* beds elevate pore-water sulfide levels (Carlson et al., 1994). This could indicate that *T. testudinum* die-off results in more sulfide stress on the remaining plants. This could further explain the high variability among cores of the Tt treatment. Another possible explanation for this is the way core growth was measured. Only the number of leaves per plot was counted, not the leaf area. It is fairly possible that the cores doubling in the number of leaves had admittedly more leaves, but smaller. This would result in a total smaller leaf area than the cores that grew fewer leaves but bigger in size. This is especially for the *T. testudinum* important since their leaves are larger and can vary much more in size than for *H. stipulacea* and *H. wrightii*.

4.2 Changes in pore-water sulfide and growth rate between *H. stipulacea*, *T. testudinum*, and *H. wrightii* transplants

This part of the research aimed to investigate whether *H. stipulacea*, *H. wrightii*, and *T. testudinum* differed in their effect on sulfide levels and whether growth correlates with sulfide levels. This was tested by measuring pore-water sulfide levels next to *H. stipulacea*, *T. testudinum*, and *H. wrightii* cores after transplantation to a sulfide-rich environment. Sulfide levels were generally higher near the seagrass cores than in the surrounding sediment or the sediment at the donor locations. This effect was the least for *H. wrightii*, in accordance with the hypothesis that they could have the most reducing effect on the sulfide levels. Growth was correlated to sulfide in the Hw treatment.

The sulfide levels were significantly higher near the Hs and the Tt cores when compared to the sulfide levels near the donor sediment core (DHsTt) (Figure 5), implying that cores with seagrass elevate sulfide levels in the surrounding sediment. This can most likely be attributed to the extra input of organic carbon into the sediment from roots that became damaged when taking the cores. Furthermore, literature states that *T. testudinum* beds have higher sulfide levels than bare sediment (Carlson et al., 1994). The lower, but not significant lower, sulfide levels near the sediment cores (DHsTt and DHw), compared to the natural sulfide levels in the experimental site (Co), suggests that sediments from the donor sites lower the sulfide levels in the surrounding sediment. These sediment cores likely have lower organic matter content because they were not affected by SBT. This showed that sediment, already containing low levels of sulfide, brings down the sulfide levels in the surrounding sediment. No significant differences were found in the sulfide levels between near the Hw core and DHw core, whilst there was

significance between the Hs core and DHsTt core and the Tt core and DHsTt core. There are multiple indistinguishable explanations for this absence in significance in the Hw treatment. For example, a higher sediment oxygenation capacity of *H. wrightii*, or, a lower organic carbon availability for decomposition in the root system of *H. wrightii*. However, other explanations could also be plausible.

During the baseline measurements, at the start of the experiment, the sulfide levels at the experimental site exceeded 550 micromoles per liter whilst during the experiment in the control plots (Co) the sulfide levels were around 250 micromoles per liter during the first measurement (Table 1 and 2). This difference in sulfide levels can likely be attributed to disturbances in the sediment during placement of the cores, the individual *T. testudinum* shoots, and the rhizon samplers. Oxygen from overlaying water will most likely have reacted with the sulfide in the sediment, resulting in the observed lower sulfide levels during the course of the experiment. It was only around day 14 that the original 550 micromoles per liter was again reached for the control.

While all the sediment treatments and the control showed an increase in the sulfide level between the first and the second measurement, the seagrass treatments all showed a decrease in sulfide levels between the first and second measurements (Figure 6). This initial increase of sulfide levels in the sediment treatments could be explained by the sediment returning to its natural high sulfide levels after the disturbance of placing the core, the individual *T. testudinum* shoots, and the rhizon samplers. Then, after this increase, a slight decrease is observed, probably caused by leakage of oxygen from the oxygen-rich sediment. For the seagrass treatments, the same initial increase in sulfide could be expected, instead, a decrease was observed. There are apparent mechanisms in place that counteract the initial increase in sulfide as seen in the control and the sediment treatments. The most obvious reason is the oxygen leakage through their root system. The high peaks in sulfide levels during the third measurement could be explained by the degradation of roots that died or became damaged during taking the cores. These damaged roots caused extra organic carbon in the sediment that in turn would be converted to sulfide. The following decreases in sulfide might be caused by a combination of the following: (1) oxygen leakage from the seagrass root systems (2) and the decomposition rate of the extra organic carbon slowing down because the most labile fraction was consumed at that point.

The Hs treatment showed both the highest measured sulfide levels but also the leading decrease in sulfide levels (712,2 $\mu\text{mol/l}$), while the Hw treatment showed the lowest sulfide levels measured and a second to strongest decrease in sulfide (483,9 $\mu\text{mol/l}$) (Figure 6, Table 2). Remarkably, sulfide levels near the Hw core are lower during the last measurement since the Hs and Hw treatment started out on almost the same sulfide levels.

Cloud cover is positively significantly correlated to sulfide levels. More cloud cover during the day of measurement results in higher sulfide levels. The relation between sulfide levels and light availability is well-known (Lee & Dunton, 2000), and does not show an exceptional pattern in this research (Table 2).

There was a negative correlation between relative growth rate and mean sulfide, as was expected based on literature that shows the toxic effects of sulfide on seagrasses (Koch et al., 2007). However, the trend was only significant for the Hw treatment (Table 3). It is in this study impossible to say whether lower sulfide levels are caused through oxidizing of the sediment by the seagrass or if the growth rate is higher because a specific part in the sediment has coincidental lower sulfide levels.

The Tt treatment is the only treatment showing positive growth at any sulfide level, this could indicate some resistance against sulfide in the sediment. This is in accordance with previous research where it was found that *T. testudinum* has a greater potential to sustain biomass under heat stress, even when the sulfide levels in the cores are two times higher than in the *H. wrightii* cores (Koch et al., 2007). Moreover, *T. testudinum* seems to be short-term tolerant to sulfide exposure up to 10mM (Koch & Erskine, 2001). There was however no indication of heat stress on the seagrasses during this experiment so sulfide tolerance of *T. testudinum* in comparison to *H. wrightii* is not likely.

The Hw treatment showed the largest variation in sulfide but the lowest growth rate (Figure 7). The lowest sulfide levels and highest mortality were measured at the Hw treatment, which can be explained by a possible trade-off. The *H. wrightii* seagrass might lower the pore-water sulfide levels at the expense of its growth rate. However, no literature has been found on this matter. When comparing the sulfide levels of individual cores, it was not found that Hw treatment cores, with a stronger decrease in sulfide levels after the initial sulfide peak, had higher growth rates.

4.23 The difference in the growth rate of individual *T. testudinum* shoots when placed next to transplanted cores

To investigate the possible facilitating effect of *H. stipulacea* and *H. wrightii* on *T. testudinum* transplants, the growth rate of individual *T. testudinum* shoot transplants was measured. This was done by determining the leaf elongation rate after transplantation of the shoots next to seagrass cores in a sulfide-rich environment (Figure 2). Individual *T. testudinum* shoot growth decreased when sulfide levels increased, which is in accordance with the expected effect of sulfide on individual *T. testudinum* shoots. However, the effect was not significant for the Hs, DHsTt, DHw, and Co treatments. The individual *T. testudinum* shoots did not express the most growth when placed next to *H. wrightii* which was in contrast to the hypothesis.

Notably, only the *T. testudinum* shoots next to the Hw and Tt cores show significant differences with the shoots at the site control (ICTt) (Figure 8). It was expected to observe significantly lower growth rates of the individual *T. testudinum* shoots at the Tt treatment as compared to the site control due to its weak sediment oxidizing properties. It is however surprising that also the Hw treatment showed significant lower growth rates of the individual *T. testudinum* shoots as compared to the site control (ICTt) since it was hypothesized that the Hw treatment would express the most plant-plant facilitation.

Since individual *T. testudinum* shoot growth was not significantly correlated to sulfide levels per core species (Table 5), the significantly lower growth rate near the Hw and Tt cores could also be caused by other factors. For example, competition between the individual *T. testudinum* shoots and the cores. Moreover, the sulfide levels are not significantly correlated to the individual *T. testudinum* shoot growth (Figure 8). The decrease in the regression line underlines the idea that higher sulfide levels result in lower growth rates of individual *T. testudinum* shoots. The positive, but not significant, correlation found between the Tt treatment and sulfide levels (Table 5) is however surprising. It is highly unlikely that higher sulfide levels result in a higher growth rate of individual *T. testudinum* shoots when they are placed next to other *T. testudinum* shoots, this is also not observed in the results. Maybe a core of *T. testudinum* is unable to facilitate individual *T. testudinum* shoots by lowering sulfide in the sediment but can facilitate the individual *T. testudinum* shoot through other unknown mechanism. No literature on possible other facilitating factors was found.

4.4 Suggestions for implementation and further research

In this study, only the sulfide levels in the pore-water were measured at the experimental site. Avenues for future research include measuring sulfide levels in the site control cores. This could give insight into the exact effect of damaged roots by transplantation on sulfide levels. The data that was gathered for this research was collected only 40 days after transplantation. Future research could be conducted on the long-term effects of the different treatments on individual *T. testudinum* shoots health and sediment sulfide levels. It would be interesting to observe the behavior of the sulfide levels in the sediment over a longer period since this study shows an initial peak in sulfide followed by a decrease. The duration of the experiment was too short to observe the equilibrium that arose. Furthermore, sulfide levels decreased strongly during the mid-day period, and therefore, the measured sulfide levels in this research might underestimate the actual peak sulfide levels during the night. For *T. testudinum*, it is, for example, known that the plants rely on the stored oxygen in their tissues during nighttime when there is no photosynthesis to sustain oxygen leakage from the root system (Lee & Dunton, 2000). To gain a more

representative picture of the sulfide levels, measurements during the night should be taken as well (Lee & Dunton, 2000). Lastly, to gain more information into shoot and core growth, future studies could take leaf area and lateral growth into account instead of only leaf density as was done in this study.

The negative effects of exposure to sulfide on *T. testudinum* are stronger when combined with other stressors, particularly high temperature and high salinity (Koch & Erskine, 2001). Investigating multi-interactive stressors is thus important for future restoration practices. Therefore, restoration measures should take these processes into account and plan the moment of restoration accordingly. It is also important to take *H. stipulacea*'s invasiveness into account if future research should show that larger-scale transplantation of *H. stipulacea* would facilitate *T. testudinum* recovery. *H. stipulacea* is an invasive species in Lac Bay, reintroduction of *T. testudinum* together with *H. stipulacea* might therefore not be desirable. The potential invasiveness of this non-native seagrass is most likely dependent on environmental conditions e.g., high nutrient concentrations (van Tussenbroek et al., 2016). Lac Bay has a high nutrient load, but *H. stipulacea* is not per se invasive and can function as a food source for the recovering green turtle population (Becking et al., 2014), and grow beneath the other seagrasses, increasing structural complexity.

5.0 Conclusion

This study shows that *H. stipulacea*, *H. wrightii* and *T. testudinum* cores temporarily elevate pore-water sulfide levels in their immediate surroundings, probably through the decomposition of extra organic carbon. *H. stipulacea* transplantation results in the highest fluctuations in surrounding pore-water sulfide levels while *H. wrightii* transplantation results in the lowest sulfide levels. No difference in the growth of the individual *T. testudinum* shoots between treatments is observed. Therefore, concludingly, it is unlikely that there is a plant-plant facilitating effect of *H. stipulacea*, *T. testudinum*, and *H. wrightii* on *T. testudinum*. Transplantation of *T. testudinum* cores with *H. stipulacea* or *H. wrightii* is not a restoration measure that may facilitate *T. testudinum* in bare areas with high pore-water sulfide levels.

6.0 Acknowledgment

I would like to thank my supervisor Luuk Leemans for all his feedback and supervision and my assessor Marieke van Katwijk for her guidance. Furthermore, I would like to thank Fons Smolders, Leon Lamers, Brigitta van Tussenbroek, Luuk Leemans, and Marieke van Katwijk for their input during the brainstorming sessions and STINAPA Bonaire for providing materials and granting access to the study area. And special thanks to Sabine Engel for being the point of contact on Bonaire. I would like to thank Ingmar Rondeel for working in the field with me and always keeping the spirit high.

7.0 Literature

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8.0 Supplementary material

The data repository is available on dropbox to third parties, please contact luukleemans@gmail.com to be granted permission.

The following supplementary material is available in this article:

Appendix 1. Baseline sulfide measurements.

Appendix 2. hobo light measurement lac 1 inside cage

Appendix 3. hobo light measurement lac 2 inside cage

Appendix 4. hobo light measurement lac 3 inside cage

Appendix 5. hobo light measurement lac 3 outside cage

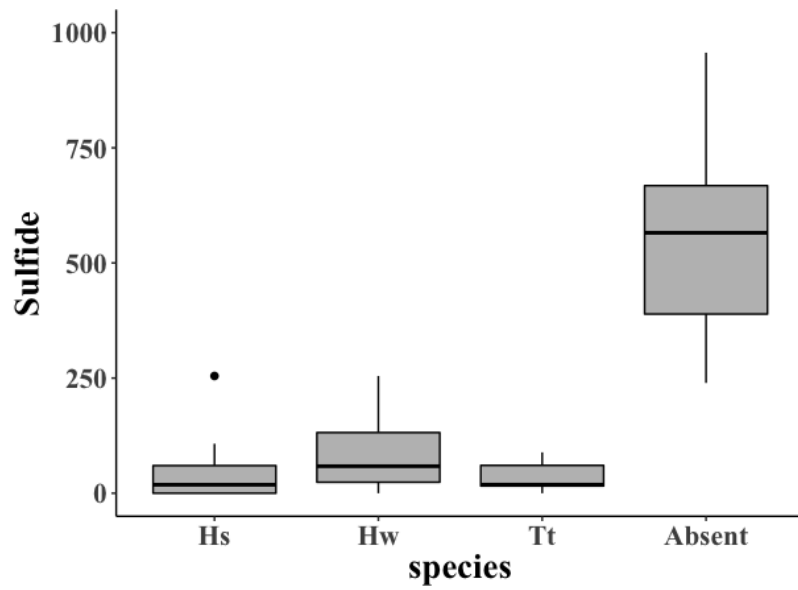
Appendix 6. hobo light measurement lac 5 outside cage

Appendix 7. hobo light measurement lac 6 outside cage

Appendix 8. Cloud cover correlation to sulfide

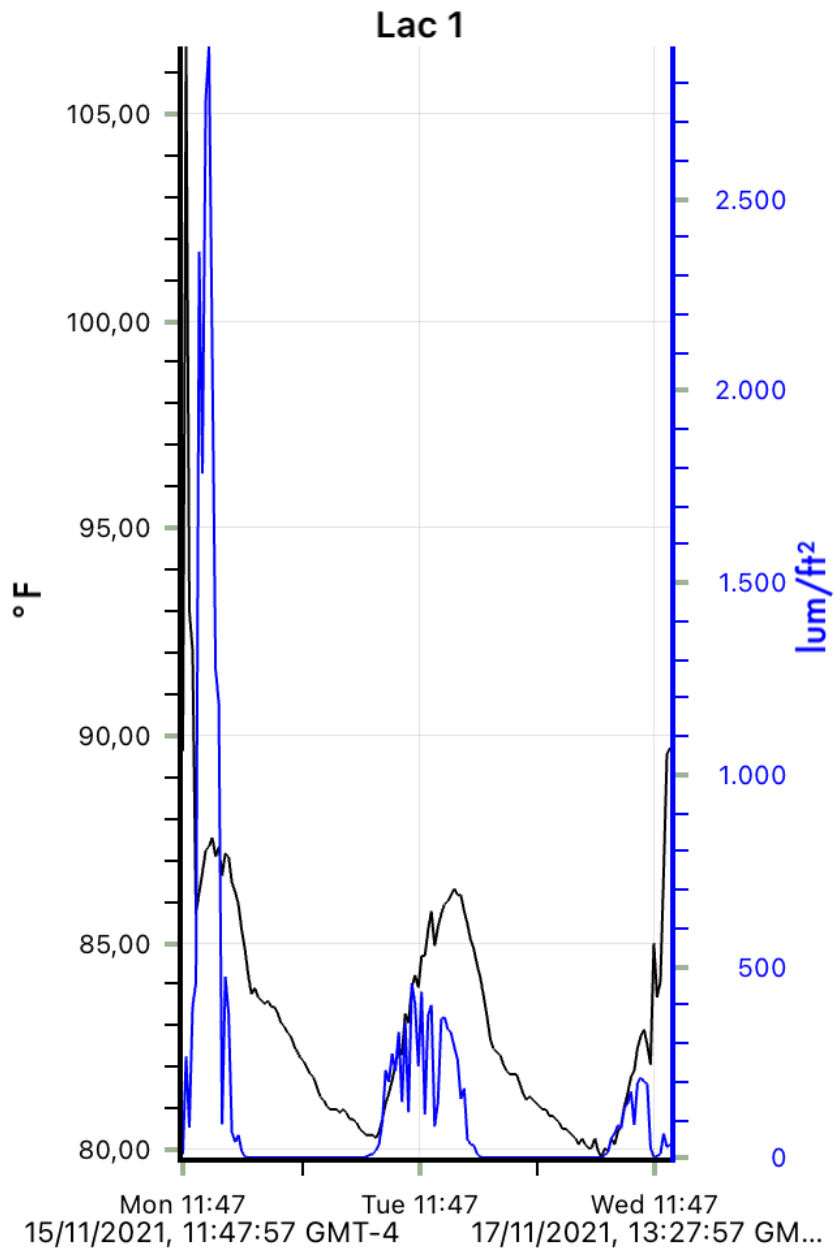
Appendix 9. results anova difference in shoot growth

Appendix 1. Baseline sulfide measurements. Hs, Hw and Tt are measurements at donor site, absent is sulfide level measurement at experimental site.

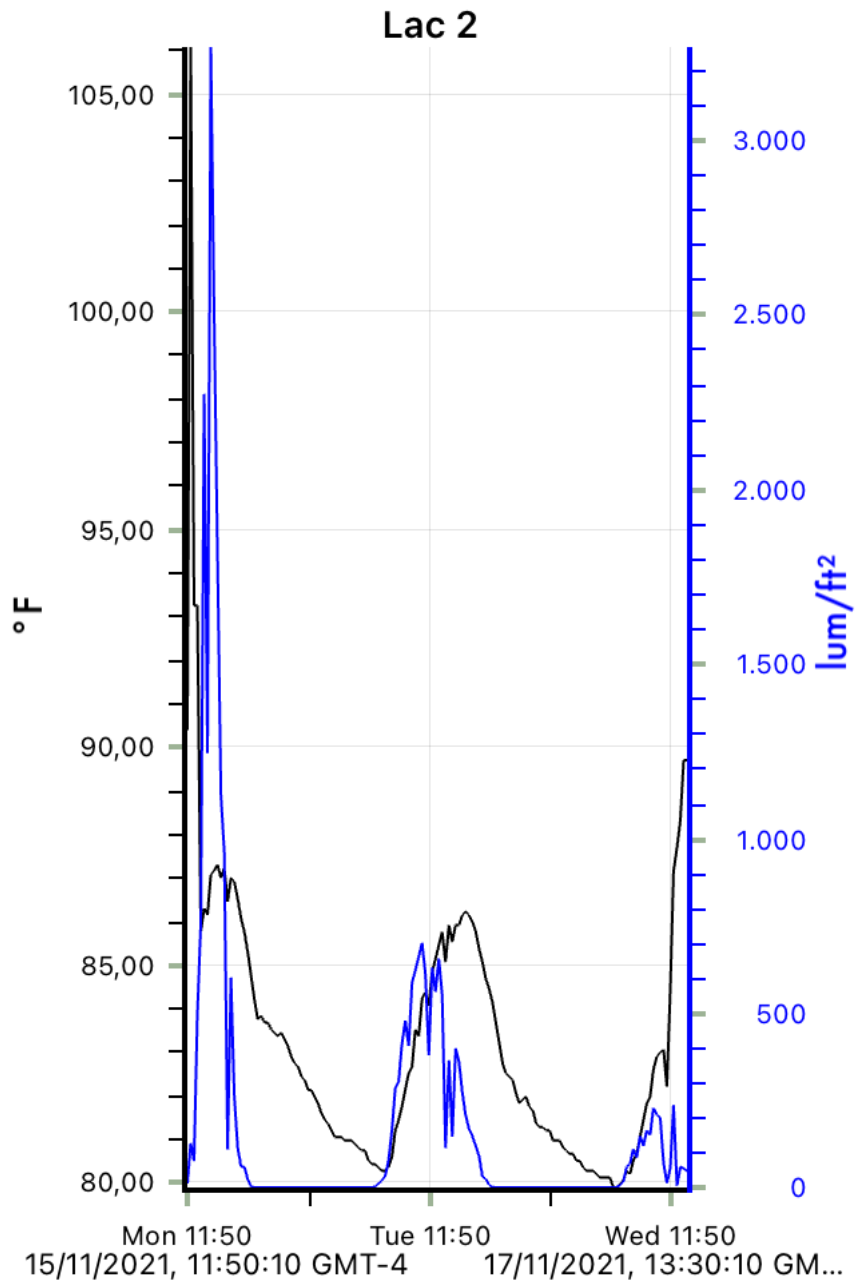


Kruskal-Wallis chi-squared = 15.79, df = 3, p-value = 0.001252

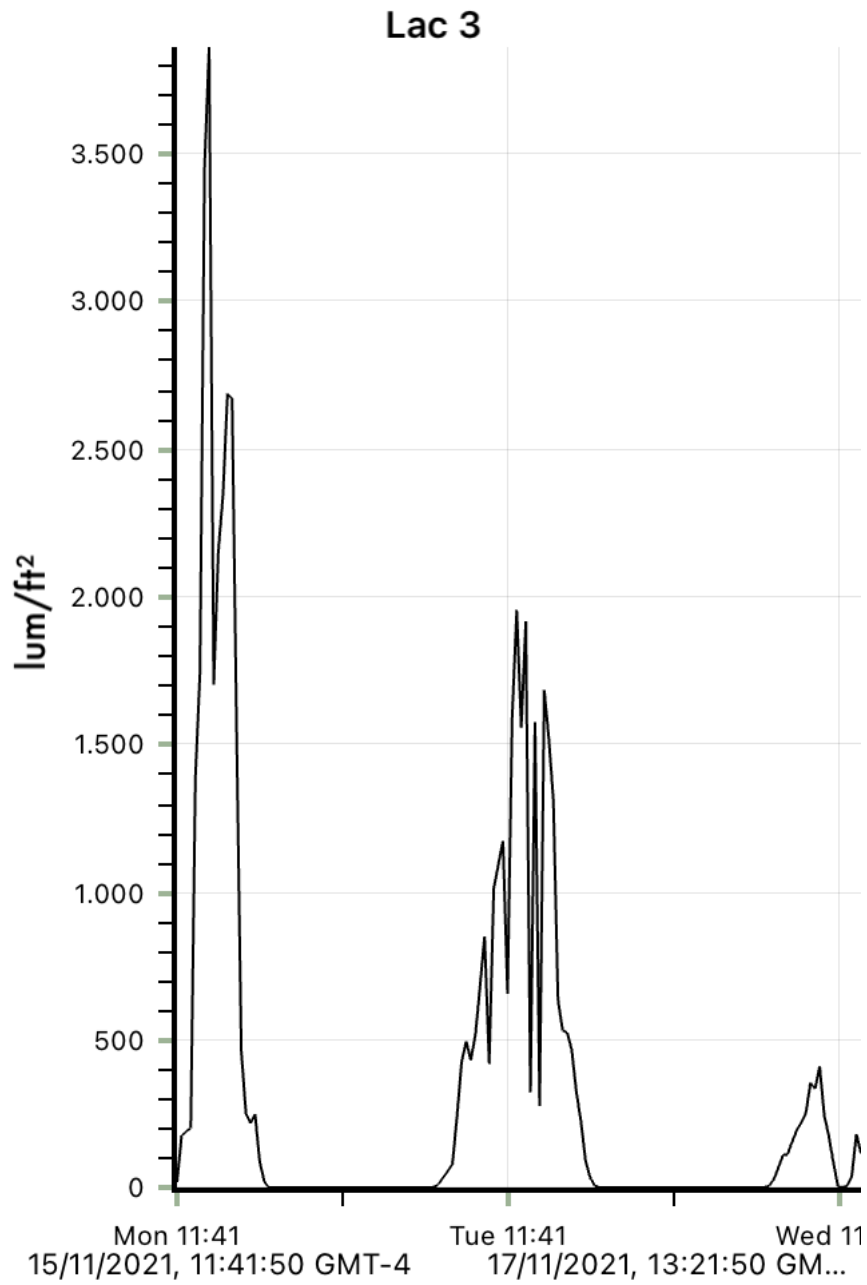
# Comparison	Z	P.unadj	P.adj
#1 Absent - Hs	3.5378302	0.0004034294	0.001210288
#2 Absent - Hw	2.5612346	0.0104300898	0.020860180
#3 Hs - Hw	-1.1795904	0.2381631625	0.285795795
#4 Absent - Tt	3.5844652	0.0003377696	0.002026618
#5 Hs - Tt	-0.0295785	0.9764032159	0.976403216
#6 Hw - Tt	1.1815249	0.2373942615	0.356091392



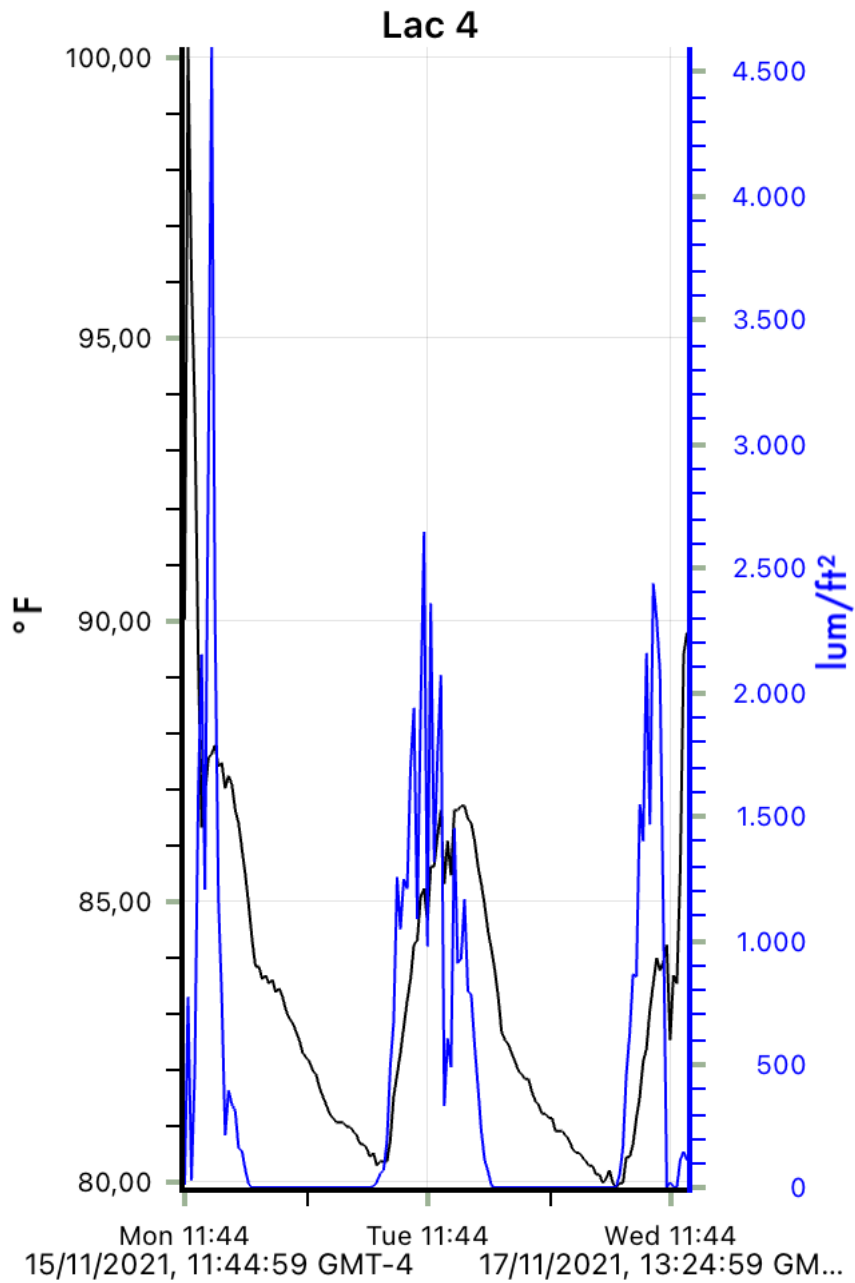
Appendix 2. hobo light measurement lac 1 inside cage over duration of 3 days



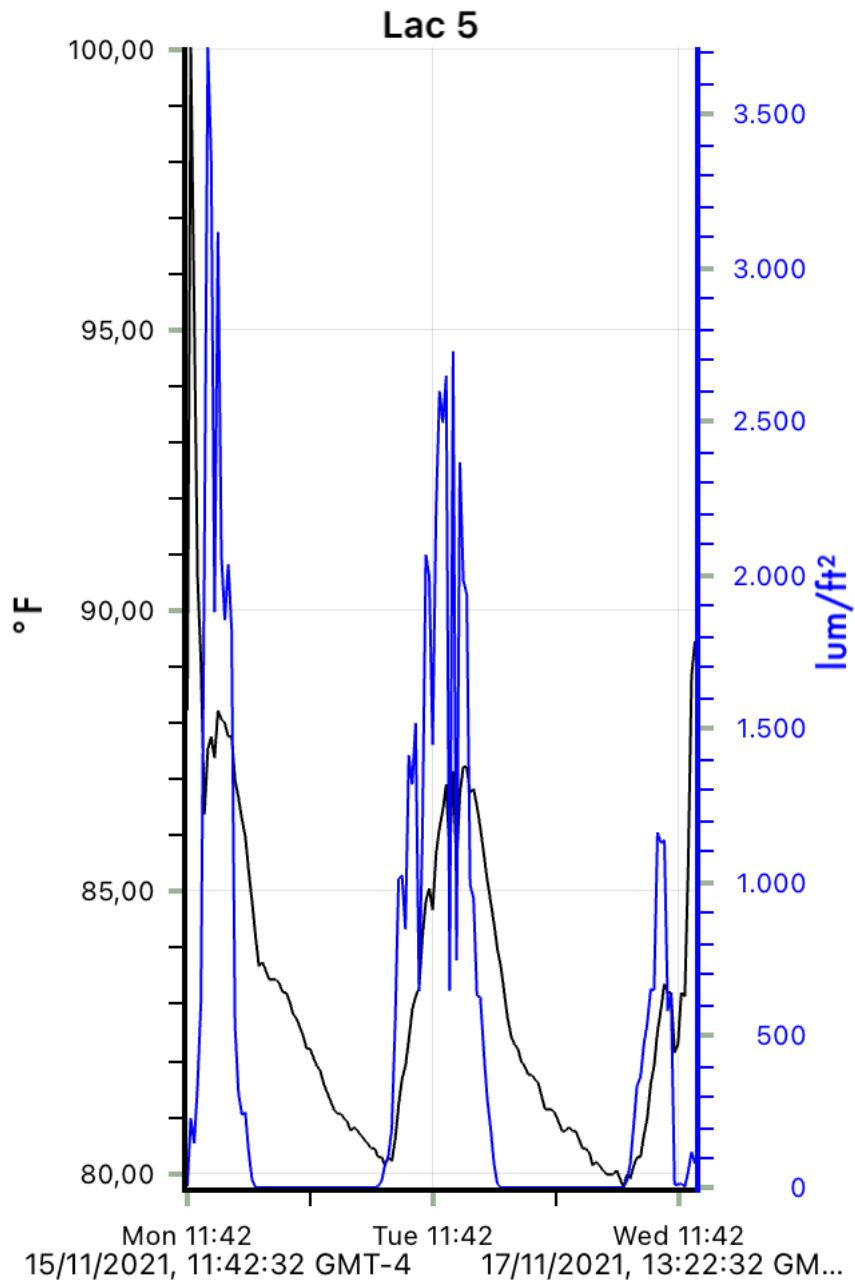
Appendix 3. hobo light measurement lac 2 inside cage over duration of 3 days



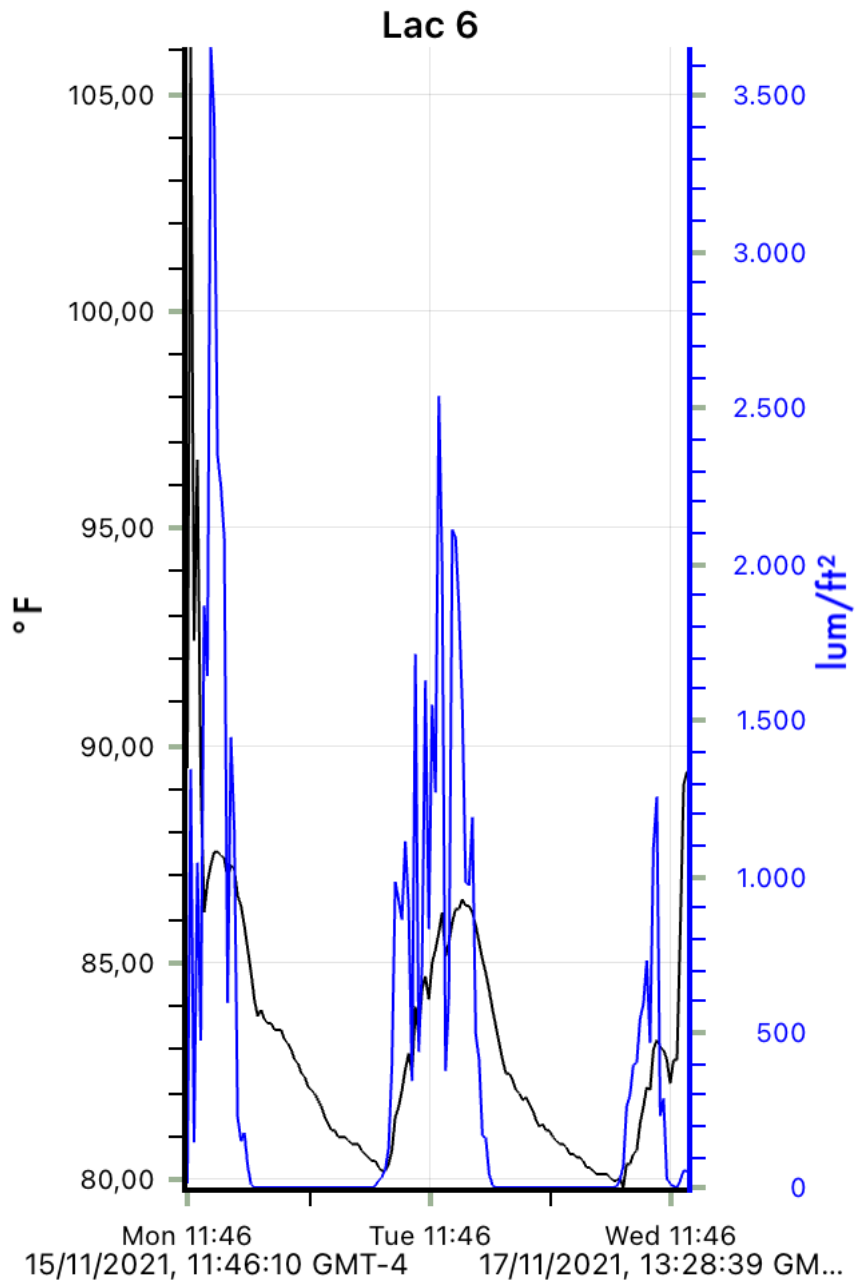
Appendix 4. hobo light measurement lac 3 inside cage over duration of 3 days



Appendix 5. hobo light measurement lac 4 outside cage over duration of 3 days



Appendix 6. hobo light measurement lac 5 outside cage over duration of 3 days



Appendix 7. hobo light measurement lac 6 outside cage over duration of 3 days

Appendix 8. results of spearman correlation cloud cover and sulfide levels.

S	87533
P-value	0.007639
Rho	0.2794718

Appendix 9. results anova difference in shoot growth

#	diff	lwr	upr	p adj
#Fo-PCo	-0.49283190	-1.3235965	0.33793267	0.5079834
#Hs-PCo	-0.66913077	-1.3641983	0.02593674	0.0649764
#Hw-PCo	-1.07221598	-1.7672835	-0.37714848	0.0006610
#So-PCo	-0.81185098	-1.6426156	0.01891359	0.0587178
#Tt-PCo	-0.88711604	-1.5821835	-0.19204853	0.0060171
#Co-PCo	-0.62711321	-1.3221807	0.06795430	0.0977030
#Hs-Fo	-0.17629887	-1.0863559	0.73375812	0.9957798
#Hw-Fo	-0.57938408	-1.4894411	0.33067291	0.4253783
#So-Fo	-0.31901908	-1.3364937	0.69845557	0.9510162
#Tt-Fo	-0.39428414	-1.3043411	0.51577286	0.8103879
#Co-Fo	-0.13428131	-1.0443383	0.77577568	0.9990718
#Hw-Hs	-0.40308521	-1.1912177	0.38504726	0.6697239
#So-Hs	-0.14272021	-1.0527772	0.76733678	0.9986905
#Tt-Hs	-0.21798527	-1.0061177	0.57014721	0.9730998
#Co-Hs	0.04201756	-0.7461149	0.83015003	0.9999976
#So-Hw	0.26036500	-0.6496920	1.17042199	0.9682762
#Tt-Hw	0.18509995	-0.6030325	0.97323242	0.9882380
#Co-Hw	0.44510278	-0.3430297	1.23323525	0.5642225
#Tt-So	-0.07526505	-0.9853220	0.83479194	0.9999677
#Co-So	0.18473777	-0.7253192	1.09479476	0.9945643
#Co-Tt	0.26000283	-0.5281296	1.04813530	0.9381823

Appendix 10. Results of ANOVA, difference in sulfide levels over time within treatments. The post hoc test of significant p-values are stated below the table.

Treatment	df	f-value	p-value
Hs	3	10.94	0.00416
Hw	3	1.406	0.278
Tt	3	6.149	0.00554
DHsTt	2	2.524	0.16
DHw	2	3.187	0.114

Co	2	1.687	0.234
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Treatment Hs

	diff	lwr	upr	p adj
#2-1	0.5274501	-6.642966	7.697866	0.9964811
#3-1	21.4542200	14.283804	28.624636	0.0000018
#4-1	10.0794895	2.474115	17.684864	0.0081138
#3-2	20.9267700	13.756354	28.097186	0.0000025
#4-2	9.5520394	1.946665	17.157414	0.0120241
#4-3	-11.3747305	-18.980105	-3.769356	0.0030911

Treatment Tt

#	diff	lwr	upr	p adj
#2-1	-3.5199972	-11.770575	4.730581	0.6233019
#3-1	6.7496123	-1.500966	15.000190	0.1301144
#4-1	6.5218538	-1.728724	14.772432	0.1490873
#3-2	10.2696095	2.019032	18.520187	0.0124986
#4-2	10.0418510	1.791273	18.292429	0.0146603
#4-3	-0.2277585	-8.478336	8.022819	0.9998154