

**Project Name:** IDENTIFICATION OF MICROBES CRITICAL TO THE ENHANCEMENT OF SEAGRASS RESTORATION

**Name of Organization:** Marine Laboratory, Sanibel-Captiva Conservation Foundation

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**ABSTRACT:**

The objective of this research was to examine the response of transplanted seagrasses to sediment manipulations in a controlled, laboratory setting. Results from previous research suggested that the sediment bacterial community carried a critical function to deter mortality in stressed *Thalassia testudinum* that was exposed to bare root transplant techniques. A benchtop microcosm greenhouse was established at Sanibel-Captiva Conservation Foundation Marine Laboratory where an experimental transplant was conducted. Bare root transplanted seagrass was exposed to one of four treatments; plus sulfide plus autoclave, minus sulfide plus autoclave, plus sulfide minus autoclave and minus sulfide minus autoclave. A control transplant simulated the plug technique where the plant was not removed from native sediment. Bare root transplants ( $1.1 \text{ mg dry weight day}^{-1}$ ) had less than half the rate of growth of the the plug control ( $2.5 \text{ mg dry weight day}^{-1}$ ), emphasizing the sensitivity of root disturbance in *T. testudinum*. Autoclaved sediments grew slower than native sediments in bare root transplants. Sulfide had no effect on plant growth or on the amount of extractable DNA from the sediment. The greatest amount of extractable DNA was in bare root transplanted treatments that had not been autoclaved. Bacterial species richness was significantly higher in the transplant sediments than in the control. However, community composition was similar in all treatments and control when analyzed with Terminal Restriction Fragment Polymorphism (TRFLP). Bare root transplant techniques decreased growth

rates by half of plugged transplants. Seagrass growth rates were significantly slower in autoclaved sediments than in native sediments, suggesting the importance of an intact bacterial community for restoration success. A shift in bacterial community composition among the treatments after the 51 day incubation could not be associated with observed differences in plant performance. Sulfide additions did not affect plant performance which could indicate that bacteria are using sulfide as an energy source. The community composition of bacteria would reflect selection for greater diversity of sulfate reducing bacteria, a pattern that was not observed. The functional role of bacterial communities in seagrass transplant performance remains unclear, yet the presence of an intact bacterial community is critical for plant survival.

## **INTRODUCTION:**

Widespread losses, declines in density, and changes in distribution of seagrass communities occur as the result of natural and anthropogenic activities (Short and Wyllie-Echeverria 1996). Natural stresses include disease (Muehlson et al. 1988, Durako and Kuss 1994), herbivory (Zimmerman et al. 2001), and changes in water column clarity due to algal blooms and particulate loading (Hall et al. 1999). Widespread seagrass losses have been attributed to anthropogenic activities (Duarte 1995) and have led to the development of water-quality targets (Dixon 2000, Corbett et al. 2006) for natural recovery of seagrass meadows. Attempts to restore seagrass habitats through bare-root vegetative transplants have met with limited success due to the complex relationship between seagrass health and environmental conditions (Thorhaug 1985, Lewis 1987, Molenaar and Meinesz 1995, Fonseca et al. 1998), including the biogeochemical aspects of the sediment (Koch 2001).

Seagrasses uptake nutrients primarily from the roots and, therefore, even when nutrients in the water column are not limiting, seagrass growth may be limited by the availability of nutrients in the sediments (Zimmerman et al. 1987). Nutrients in the sediments are derived primarily from organic matter in the sediments (Dennison et al. 1987, Fourqurean et al. 1992, Reusch et al. 1994). The small detrital particles and dissolved organic matter not only provide nutrients for seagrasses (Orth 1977), but support diverse sediment bacterial and invertebrate communities (Holmer et al. 2001). The activities of these communities strongly influence the pathways, pool sizes, and rates of organic matter remineralization in marine sediments (Aller and Aller 1998). Anoxic, anaerobic processes break down organic matter in the reducing layer, to low molecular

weight organic acids, alcohols and fatty acids (Ponnamperuma 1984, Lopez et al. 1995), some of which may be phytotoxic (Elliot et al. 2006). The products can serve as energy sources for chemolithotrophic bacteria and hydrogen donors for photoautotrophic bacteria. Alternatively, they can be completely oxidized by bacteria that use anaerobic terminal electron acceptors, such as  $\text{CO}_2$ ,  $\text{NO}_3^-$ , and  $\text{SO}_4^-$  (Blaabjerg et al. 1998, Smith et al. 2004).

The metabolic activities of seagrasses also modify sediment biogeochemical properties in the rhizosphere (Smith et al. 1988, Pregnall et al. 1984). Smith et al. (1988) showed that *Zostera marina*, L., pumps oxygen derived from photosynthesis through the lacunae to the roots, which leads to microaerobic zones in the rhizosphere. These microaerobic zones were localized at the root tips when exposed to light (Connell et al. 1999, Jensen et al. 2005).

Although previous reports have described the diversity of bacterial communities associated with seagrass meadows (Cifuentes et al. 2000, Weidner 2000, Bagwell et al. 2002, Kusel et al. 2006), there have been few attempts to manipulate sediment bacteria associated with seagrasses (Milbrandt et al., *in press*). The objective of this research was to grow *Thalassia testudinum* under controlled conditions in the laboratory and disrupt and manipulate the sediment to the effects of root zone manipulation on *Thalassia testudinum*, Banks ex. Koning, transplant performance.

## **MATERIALS AND METHODS:**

Experiments were conducted on a bench-top greenhouse established at the Sanibel-Captiva Conservation Foundation Marine Laboratory (Figure 1). The

greenhouse contained grow lights, a recycling seawater system, and six microcosms (53L). Microcosms were constructed by using Rubbermaid® tubs that were independently drained to a sump. The sump collected water, skimmed protein, and pumped water back to each microcosm. Each microcosm was not a closed system, rather all six microcosms were exposed to a single large volume of seawater. The artificial seawater (Instant Ocean®) was mixed according to the manufacturer's instructions to 33 PSU. Salinity was maintained at 33 PSU by adding water and Instant Ocean® every 4-6 days throughout the course of the experiment. In addition, a 9 kilogram carbon dioxide tank bubbled gas through a carbon dioxide reactor to elevate dissolved CO<sub>2</sub> levels. Dissolved carbon dioxide levels were monitored and made constant with a bubble counter and by measuring pH. The grow lights were powered by 4 lamp ARO Model 4LZ – VHO ballast with eight Coralife 95W fluorescent lamps. The tanks are lighted by three separate light fixtures to simulate high light conditions. Light conditions in the microcosms were measured with a Biospherical 4π hand-held PAR sensor.

*Thalassia testudinum* were collected from J.N. “Ding” Darling National Wildlife Refuge within a designated research study area on 18 January 2007. Native sediment was also collected at this time. Each plant was carefully extracted from the sediments with at least 2 cm of lateral rhizome and a healthy shoot structure. Plants were potted in native sediment and allowed to acclimate for 8 weeks, a period sufficient to demonstrate shoot growth. In order to simulate conditions that would occur in a seagrass restoration transplant, the plants were cleared of epiphytes and stored in seawater for 2 hours at ambient temperature. The plants were potted in 0.94 L deli containers made of non-

reactive clear plastic with one of two sediment types; native and autoclaved. Blade length was recorded along with temperature, salinity, dissolved oxygen and pH.

The treatment conditions were prepared during the 8 week acclimation period. Play sand was purchased from a local supply store and distributed on a stainless steel rack covered with aluminum foil. The distribution of sand on the rack was an approximately 4 cm rectangular slab. The sediment was steam sterilized in a Napco Model 8000 autoclave for 50 minutes at high temperature (130°) and high pressure (2.2 atm). The autoclaved sand was sub-sampled for DNA analysis and placed in 4 L plastic bags until the simulated transplant.

A new method was developed for administering porewater sulfide treatments. This method also served as delivery for the plus sulfide treatment (10mM). Ten mM was chosen based on a sulfide tolerance experiment (Erskine and Koch 2000) which demonstrated adenylate inhibition when exposed to a 10 mM Sodium Sulfide solution. Dialysis tubing was purchased from Fisher Scientific. The dialysis tubing retains and excludes molecules greater than 12,000 m.w.; this includes cells and sediment particles. However, it allows smaller molecules, like carbohydrates, amino acids, exoenzymes and ions to equilibrate with porewater. The tubing was tied at each end and filled with filtered artificial seawater to form a 3-4 cm sausage. Sausages were equilibrated in artificial seawater plus 10mM sodium sulfide, while the remaining sausages equilibrated with sterile filtered artificial seawater.

On 18 March 2007, three replicates of each of the following treatments were established; plus sulfide/plus autoclaved sand, minus sulfide/plus autoclaved sand, plus sulfide/native sand, minus sulfide/native sand, and a plugged control. The control was to

mimic a technique common in restoration to transport sediments along with the plant to not disturb the rhizosphere. Each pot contained three plants sediment, dialysis tube, and a plastic label. Each plant was marked through the basal sheath for measuring growth rates. The plants were rotated every 2-5 days during the experiment to ensure equal light and water quality conditions during the study period. The experiment was run for a total of 51 days before the plants were harvested for data analysis. A subsample of treated sediments were sub-sampled and stored (-60°C) for bacterial DNA analysis.

Survivorship, growth rate, shoot:root ratios, DNA yield, and bacterial community composition were recorded. Growth rate was measured with a syringe hole punch method (Zieman) which is the total area added by all leaves. The punch mark is located on each leaf and the distance from the mark to the meristem and the leaf width are recorded. The total area added per plant is divided by the number of days. A linear relationship ( $R^2 = 0.98$ ) between *T. testudinum* leaf area and mg dry weight (Milbrandt, unpublished data) was used in the plots. Shoot:root ratios were determined for each plant by measuring fresh weight, drying at 60°C overnight and reweighing.

Bacterial community composition and diversity was determined by terminal restriction fragment polymorphism (T-RFLP), as described in Schmitt-Wagner et al. (2003). DNA was directly extracted from sediment samples using the Mo Bio© (San Francisco, CA) Power Soil Extraction kit. Concentration of DNA from the extraction was determined from a 2 µL subsample on a NanoDrop 1000. Sediment wet weight was recorded for each DNA extraction to determine the DNA yield per g FW sediment. The bacterial-specific ribosomal genes (16S rDNA) were amplified with universal eubacterial primers (8F, 907R). The forward primer in the polymerase chain reaction (PCR) was 5'

modified with a Proligo© Well Red dye (Boulder, CO). Nucleotides that were not used in the PCR reaction were removed with an Invitrogen© PCR Purification kit (Carlsbad, CA). Purified PCR products were digested with 20 Units of Msp I and 20 Units of Hha I (New England Biolabs© , MA). Digested samples were loaded on a Beckman-Coulter© CEQ 8000 Gene Analyzer (Mou et al. 2005, Morris et al. 2005) for determining the fragment sizes generated in each sample. The total peak height of a T-RFLP profile was defined as the sum of the peak heights of all peaks greater than 50 bp. The relative height of a given peak was determined by dividing its peak height by the total peak height of the profile. When determining the number of distinct terminal restriction fragments (TRF), only those with a relative peak height greater than 1 % of the total peak height were used.

## **RESULTS:**

Temperature was maintained during the 51 day incubation period between 19 and 21°C, while salinity was kept between 29 and 30 psu. While artificial lighting provided high radiance, potted plants were rotated every 3-5 days to ensure equal irradiances in the mesocosms and avoid a container effect. In previous experiments where the experimental units were outplanted into the estuary, survivorship was the primary metric for assessment of transplant performance. However, there were no mortalities during the 51 day incubation, therefore growth rates and shoot:root ratios were used to determine the effect of the sediment manipulation on transplant performance. DNA yields and bacterial community composition were used to compare the effect of sediment manipulation on bacteria.

After the transplants had incubated for 51 days, the growth rates among the four treatments were significantly different than the control (Figure 3). The control had a

mean growth rate of 2.5 mg DW day<sup>-1</sup> while mean transplant growth was 1.1 mg DW day<sup>-1</sup>. Among the transplants, the highest mean growth rates were obtained with native sediments, where sulfide treated transplants in native sediments had a mean growth rate of 1.3 mg DW day<sup>-1</sup> and non-sulfide treated transplants grew at a higher rate of 1.5 mg DW day<sup>-1</sup>. The slowest growth rates were in autoclaved sediments where sulfide treated, autoclaved sediments had a mean growth rate of 0.90 mg DW day<sup>-1</sup>. Autoclaved sediments with no sulfide addition had the lowest growth rate of 0.67 mg DW day<sup>-1</sup>.

Shoot to root ratios varied, but not significantly among the transplants and control (Figure 4). Given that the shoot to root ratios were not controlled at the start of the incubation period, it is inconclusive whether sediment manipulation had any effect on shoot to root ratios.

DNA yield was not significantly different in transplants versus control. However, the mean values of all transplanted treatments do not demonstrate the variability among transplant treatments. The highest DNA yields were from native sediments that had been used in transplants (1255 ng g wet weight<sup>-1</sup>) in sediments in the control (870 ng g wet weight<sup>-1</sup>). The lowest DNA yields were extracted from autoclaved sediments. As observed in growth rates, sulfide treated transplants were not significantly different than non-sulfide treated, however there were significant differences between autoclaved and native sediments.

Species richness was significantly higher in transplanted sediments than in the control (Table 1). The lowest mean H' diversity and species richness was found in the control. Variability among individuals used to calculate mean values is the standard

deviation, which was much lower in the control. All transplanted sediments had mean species richness greater than 10 while the control was 6.

Bacterial species composition was compared among treatments with multivariate techniques. The results of a Bray-Curtis similarity analysis were plotted on a non metric Multi Dimensional Scaling, two dimensional plot (Figure 6). There were no discernable clusters observed in the NMDS plots, suggesting that the community composition was similar among treatments.

## **DISCUSSION:**

The primary objective of this study was to determine if disrupting sediments and accompanying bacterial community changed the response of transplanted *T. testudinum* when light, salinity and temperature were controlled in a laboratory setting. While transplanting seagrass offers the possibility of improving degraded habitats, varying levels of success have been reported (Sheriden et al. 1998, Bull et al. 2004, Fishman et al. 2004, Bell et al. *in press*). Tomasko et al. (1991) demonstrated that a greater number of short shoots was associated with greater survivorship of *T. testudinum* transplants and that the presence of the rhizome apical meristem improved transplant success. However, there is much more uncertainty associated with transplantation due to unknown and unpredictable linkages between seagrasses and their preferred habitat; e.g. grazers (Fonseca et al. 1996), and potentially unsuitable sediments (Koch 2001).

The laboratory offers potential advantages to a researcher because of the possibility of controlling otherwise variable parameters. Favorable growth conditions were obtained by providing high light, shallow microcosms, ideal for promoting *T. testudinum* growth. This was evidenced by the fact that no mortalities were reported

during the 51 day incubation, despite the bare root transplant treatment and the disruption of the rhizosphere and associated sediments. There are drawbacks to laboratory work, including the difficulty of controlling container effects. Container effects include variability in evaporation rates or variability in light intensity. Therefore, the plants were rotated every 3 to 5 days and the tanks were an open seawater system with a filter to ensure that salinity and temperature was uniform throughout the incubation.

Rather than repeated measurements of blade length and number during the course of the incubation, growth rates were only measured at the end of the incubation. Since plants were marked at the meristem at the start of the incubation, the marks were evident at the end of the integration and provided an integrated growth estimate over the entire incubation period.

Bare root transplantation had a significant effect on growth rates. While this may be a straightforward conclusion, there are very few reports of it in the literature. Survivorship is higher when seagrasses are plugged than when plants are bare rooted (Fonseca et al. 1996, Bull et al. 2004), and based on these observations the growth rates are nearly double when seagrasses are transplanted in plugs (with no disruption of the rhizosphere). While bare root transplanting techniques may be cheaper and logistically less challenging than plugs, the physiological stress placed on the plants can have a profound effect on growth and restoration success.

Native sediments outperformed the autoclaved sediments, as previously reported (Milbrandt et al. *in press*). Growth rates were significantly slower in autoclaved sediments than in native sediments. The amount of extractable DNA was also significantly higher in native sediments. The amount of extractable DNA has been used

as an indicator of microbial biomass (LaMontagne et al. 2002). While it is unclear what proportion of the extractable DNA is bacteria, the bacterial community in native sediments appears to be more developed. Plant and coral reef communities have highest species diversity under periods of intermediate disturbance (Connell 1978). One would expect species richness and Shannon diversity to be highest in the native sediments with a transplant, and lowest in the control and the autoclave treatment. The autoclave treatment and the native with transplant were not different and significantly higher than the control. Additional evidence supporting this hypothesis was not found in the two dimensional plot of community similarity. From this analysis the bacterial communities from each treatment were largely similar. The 16S rDNA had similar fragments when digested by Hha I. Other studies have demonstrated that Hha I does not generate as many fragments from a sample and therefore does not produce as many markers of community composition (Milbrandt, unpublished data) as other restriction enzymes such as Msp I. While the Msp I data was collected, it was not amenable to analysis because of unexpected reaction failures in the control. The Msp I reactions were re-run twice with similar results, a third re-run was planned but was not accomplished at the time the final report was prepared. Therefore, the bacterial community composition among the treatments after the 51 day incubation did not indicate a shift that could be associated with observed differences in plant performance.

Sulfide additions decreased the differences in growth rates had no effect on plant performance. Sulfide concentrations (10mM), delivered directly to the roots via dialysis tubing did not have an effect in either the autoclaved or the native sediments. Other work on the physiological response of *Zostera marina* (Holmer et al. 2005) and *T. testudinum*

(Erskin and Koch 1999) suggested that these concentrations negatively affected seagrass health. While it is possible that Charlotte Harbor populations are better adapted to high sulfide conditions, it is more likely that the method of delivery or the design of the treatment was inadequate. However, a distinctive black coloration to the autoclaved sediments was observed (Figure 7) in the high sulfide treatments suggesting the formation of pyrite (FeS). There were no signs of a white film or mat typically associated with sulfide oxidizing bacteria, such as *Beggiatoa*. The presence and intensity of pyrite formation is an indicator of the strength of sulfide treatments (Holmer et al. 2005). Given that gaseous sulfide concentrations associated with seagrass die-off areas in the Florida Bay were as low as 0.5 mM, the concentrations used would have been adequate. However, the chemical state of sulfide in the rhizosphere was not known due to complications in pore water sampling protocols. Sulfide can penetrate the lipid bilayer as H<sub>2</sub>S or as S<sup>2-</sup>. These two states are only possible in anoxic, reducing conditions. Theoretically, conditions in the rhizosphere are reducing so the conditions for formation of the chemical form detrimental to seagrasses were favorable. It is also possible that the sodium sulfide in solution were more readily available to sulfide reducing or sulfide oxidizing bacteria. The community composition of bacteria would reflect selection for greater diversity of sulfate reducing bacteria, a pattern that was not observed.

While the responses by seagrasses to sediment manipulation were expected, the methodologies for delivering the sulfide treatment and measuring porewater at the end of the 51-day incubation were largely experimental. Half of the dialysis tube “sausages” (Figure 8) were incubated in 10 mM sodium sulfide solution in sterile seawater for 24 hours prior to the transplant. The other half were incubated in the seagrass tanks in

seawater with no sodium sulfide added. The sausages were deployed in all treatments, except the control. Upon completion of the 51-day incubation, the sausages were no longer in the sediments because they had dissolved. Dialysis tubing is made from cellulose which makes it porous to small molecular weight ions and acts as a barrier to cells, proteins, and other large molecular weight compounds. Unfortunately, marine sediments contain bacteria that have the ability to digest cellulose with an enzyme called cellulase. The dissolution of the sausages did not affect the delivery of the sodium sulfide because the equilibration of the sausage with the porewater occurred immediately after the transplant. However, porewater conditions (especially sulfide concentrations) at the end of the experiment could not be measured because the sausage was dissolved by bacteria. One must consider the effects of cellulose addition on the bacterial community. It served as a food source and may have contributed to the growth of bacteria that use cellulose as a carbon source. This, however, would have occurred in the first 10-15 days of the incubation. It is not known what effect the cellulose had, but all treatments, except the control were subjected to the same amount. Therefore, it is unlikely that the dissolution of cellulose had any effect on seagrass performance, but it may have had an effect on community composition. This may partly explain the lack of differentiation among the treatments when each treatment was expected to have a unique bacterial community.

Success of seagrass restoration projects remain highly variable (van Keulen et al. 2003); therefore, predictive tools should be used to aid in improving success rate of large projects (Fonseca et al. 1996). Early indicators of transplant stress were detected in this laboratory experiment were detected between bare root transplants and plugged

transplants. The role of an intact, native bacterial community in seagrass performance remains elusive. While one objective of this research was to determine the specific biogeochemical functions attributed to plant health, the failure of the porewater analysis meant that the differences in plant performance could not be attributable to specific analytes. Cultivation of seagrass associated microbes under anaerobic conditions and hydroponic experimentation is likely required (Kusel et al. 1999). With further investigation, it may be possible to develop an inoculum or an anaerobic enrichment for bare-root transplants to decrease mortality during initial transplant shock.

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Table 1. Bacterial species diversity indices for Hha digested 16S rDNA.

Treatment	Species Richness	H' (Shannon Diversity)
SA	13 (6.0)	2.1 (0.6)
SN	10 (2.8)	1.9 (0.3)
NSA	12 (3.5)	2.1 (0.5)
NSN	16 (1.7)	2.4 (0.8)
Control	6.3 (0.6)	1.6 (0.7)

## FIGURE LEGEND:

Figure 1. Microcosm experimental set up at Sanibel-Captiva Conservation Foundation Marine Laboratory. Six Rubbermaid tubs hold approximately 5 deli containers, each containing 3 *Thalassia testudinum* collected from J.N. “Ding” Darling National Wildlife Refuge.

Figure 2. Map of the study area. Turtlegrass (*Thalassia testudinum*) was collected from the “transplant site” along the shallow water edge of its distribution. Plants were collected in a designated Research Study Area in J.N. “Ding” Darling National Wildlife Refuge.

Figure 3. Growth rates of seagrass in the experiment. This is the average daily growth rate during the 51 day incubation period. Abbreviations are as follows; SA, sulfide autoclave, SN, sulfide not autoclaved, NSA, no sulfide autoclaved, NSN, no sulfide not autoclaved, control, plugged transplant.

Figure 4. Shoot to root ratio of seagrass in the experiment. The bars are the mean dry weight shoot to root ratio, error bars are standard deviation at the conclusion of the 51 day incubation. Abbreviations are as follows; SA, sulfide autoclave, SN, sulfide not autoclaved, NSA, no sulfide autoclaved, NSN, no sulfide not autoclaved, control, plugged transplant.

Figure 5. Extractable DNA from the rhizosphere. Yield is reported as ng DNA per mg fresh weight, bars are mean values for each treatment with standard deviation bars. Abbreviations are as follows; SA, sulfide autoclave, SN, sulfide not autoclaved, NSA, no sulfide autoclaved, NSN, no sulfide not autoclaved, control, plugged transplant.

Figure 6. Non metric ordination of 16S rDNA community similarity of bacteria in the rhizosphere. Abbreviations are as follows; SA, sulfide autoclave, SN, sulfide not autoclaved, NSA, no sulfide autoclaved, NSN, no sulfide not autoclaved, control, plugged transplant.

Figure 7. Evidence of pyrite (FeS) in 10 mM sulfide treatments.

Figure 8. Dialysis tubing filled with sterile artificial seawater for determining pore water concentrations and for delivering 10 mM Sodium Sulfide.



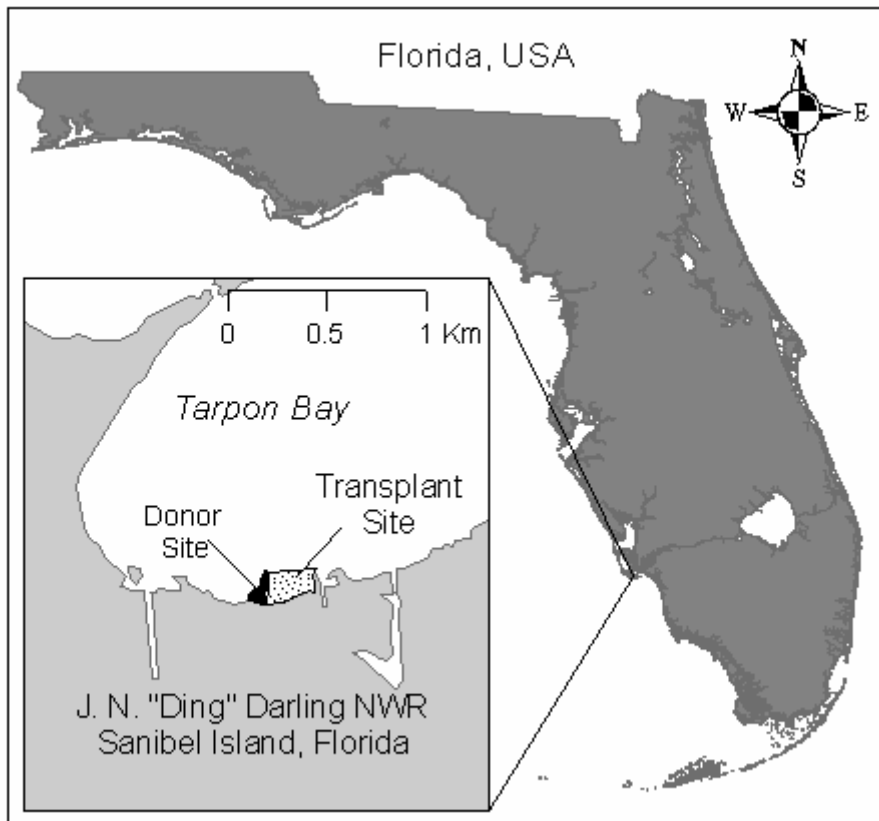


Figure 2

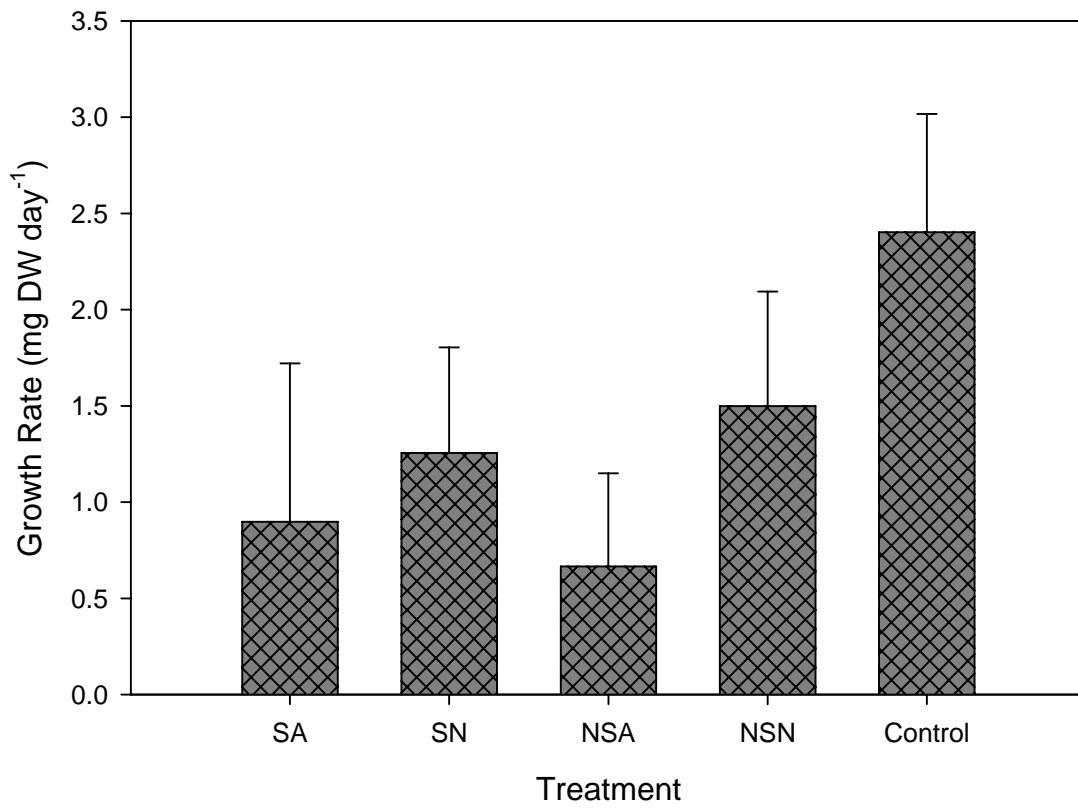


Figure 3

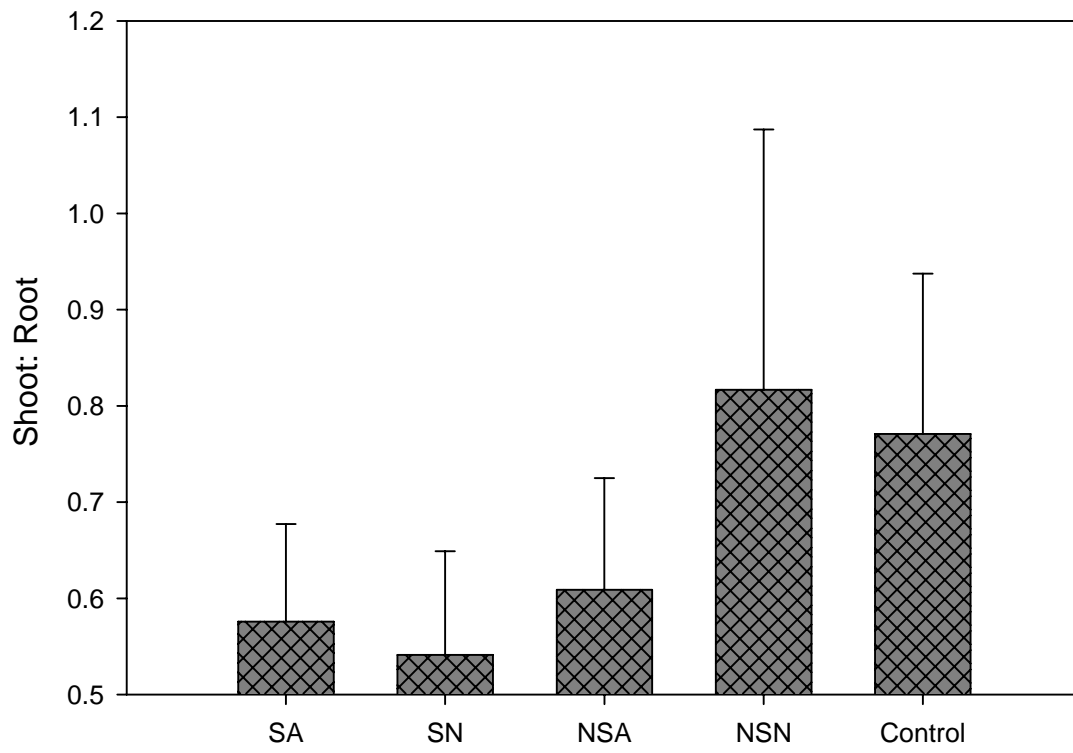


Figure 4

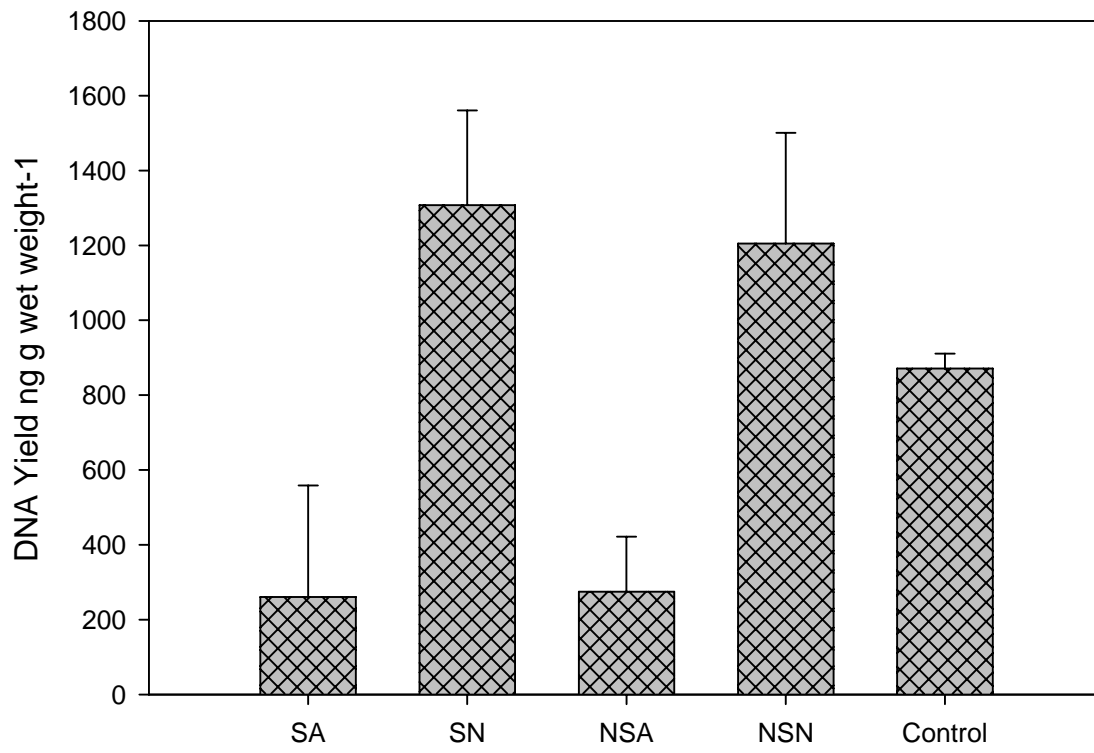


Figure 5

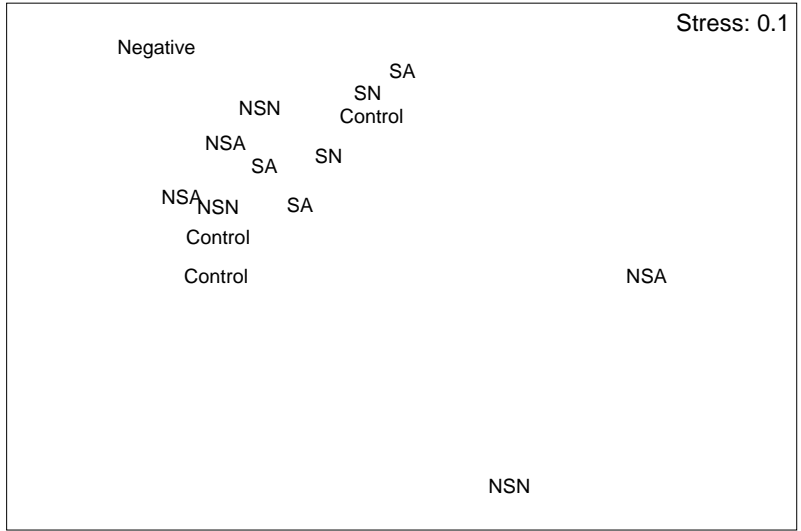


Figure 6



Figure 7



Figure 8