

**Rare occurrences of free-living bacteria belonging to
Sedimenticola from subtidal seagrass beds associated with
the lucinid clam, *Stewartia floridana***

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Abstract

Lucinid clams and their sulfur-oxidizing endosymbionts comprise two compartments of a three-stage, biogeochemical relationship among the clams, seagrasses, and microbial communities in marine sediments. A population of the lucinid clam, *Stewartia floridana*, was sampled from a subtidal seagrass bed at Bokeelia Island Seaport in Florida to test the hypotheses: (1) *S. floridana*, like other lucinids, are more abundant in seagrass beds than bare sediments; (2) *S. floridana* gill microbiomes are dominated by one bacterial operational taxonomic unit (OTU) at a sequence similarity threshold level of 97% (a common cutoff for species level taxonomy) from 16S rRNA genes; and (3) the dominant OTU retrieved from *S. floridana* gill tissues represents less than 1% of all sediment and pore water OTUs from the *S. floridana* habitat. Population densities for *S. floridana* at Bokeelia ranged from 0 to 2354 individuals per cubic meter and were significantly higher with high seagrass coverage compared to bare sediments.

Sediment and pore water microbial communities were dominated by Delta- and Gammaproteobacteria. Over 97% of 16S rRNA gene sequences recovered from five *S. floridana* gill specimens, as well as gills of two other lucinid clams recovered from Bokeelia, *Ctena orbiculata* and *Lucinisca nassula*, were closely related to the previously described gammaproteobacterium, *Sedimenticola*, and one *Sedimenticola* OTU dominated the tissue communities. OTUs affiliated with *Sedimenticola* were also shared by sediment, pore water, and all host tissues, but represented < 0.5% of all the OTUs from free-living bacterial communities. The results from this study provide tentative identification of the endosymbiont of several lucinid clams from one habitat, and characterize the abundance of putative endosymbiont OTUs in the free-living environment, which has not been done previously for *S. floridana*.

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Section 1: Introduction

Although vegetated marine sediments represent less than 2% of coastal sediments worldwide, shallow marine vegetation accounts for 84% of benthic gross primary production and 45% of all oceanic carbon burial on continental shelves (Duarte *et al.* 2005). This highly productive biome supports a diverse array of ecologically and economically significant organisms (Bostrom & Bonsdorff 2000; Martínez *et al.* 2007). High levels of habitat complexity and biodiversity increase coastal ecosystem resilience and resistance to disturbance (Duffy 2006; Gartner *et al.* 2015; Montefalcone *et al.* 2015). Physically and chemically complex habitats provide a wide variety of niches that support high levels of biodiversity, and redundancy of ecological function within highly diverse communities of organisms provides resilience to species losses and physical or chemical alterations in the habitat (Duffy 2006; Montefalcone *et al.* 2015).

In the last century, approximately 30% of global seagrass beds have been lost (Waycott *et al.* 2009), predominately due to human modification of coastal habitats, over-exploitation of commercialized organisms, and the introduction of invasive species (Harley *et al.* 2006; Martínez *et al.* 2007; Halpern *et al.* 2008). The annual economic value of the resources and services provided by these ecosystems has been estimated to be as high as \$299-823 trillion/km² (Costanza *et al.* 1997; Turpie *et al.* 2003; Martínez *et al.* 2007). Understanding ecological processes in coastal ecosystems is integral to the development and application of management strategies aimed at maintaining these resources and services.

Ecosystem resistance and resilience to natural disturbances in the geological past may have benefited from processes mediated by symbiotic relationships, with one such symbiotic system being among marine angiosperms like seagrasses, lucinid bivalve clams, and sulfur-

oxidizing bacteria (van der Heide *et al.* 2012). In this case, marine angiosperms provided lucinid clams a habitat with protection from benthic predators and with geochemical conditions suited to their relationship with endosymbiotic sulfur-oxidizing bacteria (Stanley 2014). This may have allowed lucinid clams to persist and diversify through the end Cretaceous mass extinctions (Stanley 2014).

The “three-stage” symbiotic system is driven by the production of hydrogen sulfide and other reduced gases within vegetated sediments and pore water by sulfate-reducing and other heterotrophic and chemoorganotrophic microbes (Fenchel & Reidl 1970; Jorgensen 1982; Holmer & Nielsen 1997; Stewart *et al.* 2005; Reynolds *et al.* 2007; van der Heide *et al.* 2012). Although most marine plants release oxygen from their rhizomes and can create an oxic microenvironment that shields a plant from sulfide, sulfide may accumulate in undisturbed sediments to toxic levels that can inhibit vegetation growth and cause die-offs (Calleja *et al.* 2007; Dooley *et al.* 2013). The long-term success of shallow marine vegetation requires the removal of sulfide from the sediments (Holmer *et al.* 2003; Calleja *et al.* 2007). Reynolds *et al.* (2007) studied populations of the lucinid clams *Ctena orbiculata* and *Luciniscia nassaula* in southern Florida and found that lucinid clams consumed sulfide at rates ranging from 11-59 mmol m⁻² d⁻¹ or as much as 2-16% of sulfide produced in seagrass beds (Reynolds *et al.* 2007).

Lucinids live in marine sediment at the oxic-anoxic interface, and their ability to live in these habitats is attributed to chemosymbiosis with bacteria (Stewart *et al.* 2005; Dubilier *et al.* 2008; Duperron *et al.* 2013). Some lucinids, such as *S. floridana*, are also capable of anaerobic respiration (Anderson 1995; Larabee 1998). All lucinids studied to date have a reduced digestive system and large eulamellibranch gills (Allen 1958) that harbor endosymbiotic bacteria (Fisher & Hand 1984; Schweimanns & Felbeck 1985; Distel & Felbeck 1987), which makes the

Lucinidae the most diverse group of chemosymbiotic bivalves (Taylor & Glover 2006; Taylor *et al.* 2011; Stanley 2014).

The endosymbiotic bacteria occur in bacteriocytes beneath the ciliated epithelium within gill tissues (Reid & Brand 1986; Larabee 1998; Taylor & Glover 2000). Fisher and Hand (1984) conducted enzymatic assays with fresh *S. floridana* (Olssen & Harbison 1953) gill tissue homogenates collected from St. Joseph's Bay, Florida. Fisher and Hand (1984) observed that bacteria fixed CO₂ and synthesized organic carbon via the Calvin-Benson cycle based on high levels of ribulose 1,5-bisophosphate carboxylase and phosphoribulokinase activities, and oxidized reduced sulfur compounds based on assays of adenosine phosphosulfate reductase and adenosine triphosphate sulfurylase. Energy dispersive X-ray analysis indicated *S. floridana* gill tissue contained elemental sulfur (Fisher & Hand 1984), which is common within symbiotic sulfur bacteria in other animal hosts (Shulz-Vogt 2011). Rhodanese, an enzyme that converts cyanide into thiocyanate by reaction with sulfide, was present in the gill tissue homogenates but not quantifiable, and nitrate reductase, indicative of ammonia generation, was also detected (Fisher & Hand 1984; Reid & Brand 1986). These early studies showed that *S. floridana* contains an active endosymbiotic community capable of a chemoautotrophic sulfide metabolism.

Meta-analysis of published literature reveals that lucinids have been encountered in 97% of tropical seagrass meadows, 90% of subtropical meadows, and 50% of temperate meadows that have been surveyed (van der Heide *et al.* 2012). The association with vegetation may reflect the shared evolutionary history of lucinids and marine angiosperms (Stanley 2014). Based on population density, grams of gill tissue per individual, and chemoautotrophic primary productivity rates, *S. floridana* and its endosymbionts were estimated to contribute up to 400 g C/m²/year to the gross carbon fixation of the seagrass biome (Fisher & Hand 1984). This rate

has led many to consider the activity of lucinids in shallow marine habitats to be critical to overall ecosystem function (Barnes & Hickman 1999; Reynolds *et al.* 2007; van der Heide *et al.* 2012; Stanley 2014), particularly because population densities can be as high as 1500 individuals/m² (Fisher & Hand 1984; Zabbey *et al.* 2010).

Although *S. floridana* endosymbionts appear to be well-characterized enzymatically and microscopically (e.g., Fisher & Hand 1984), only scarce genetic data are available (Reid & Brand 1986; Distel & Felbeck 1988; Distel *et al.* 1994) and there is a relatively poor understanding of how endosymbionts are distributed within lucinid habitats, not just for *S. floridana* but also for other lucinids. Thus far, previous studies have demonstrated that *S. floridana* endosymbionts belong to a monophyletic clade that corresponds to Lucinoidea symbionts within the Gammaproteobacteria (Distel *et al.*, 1988, 1994; Durand & Gros, 1996; Durand *et al.*, 1996; Brissac *et al.*, 2011). Prior research also suggests that endosymbionts are acquired from the environment through horizontal transmission, which means that if geochemical conditions within the habitat become unfavorable for the bacteria, then lucinid growth and survival may become compromised, which would then affect associated vegetation within the same habitat (Gros *et al.* 2003; Green-García & Engel 2012).

Several seagrass-lucinid-bacteria systems have been studied in the context of “three-stage” symbiosis (Reynolds *et al.* 2007; van der Heide *et al.* 2012), but there remains a need to understand how diversity and functional changes among lucinid populations, microbial communities, and marine vegetation may impact overall ecosystem functions and evolution in changing marine habitats (Knutsen 1981; Waycott *et al.* 2009; Hendriks *et al.* 2010; Range *et al.* 2011; Bauer *et al.* 2013). The significance of a “three-stage” symbiosis was evaluated from a series of mesocosm experiments with the lucinid *Loripes lacteus* and the seagrass *Zostera noltii*

from Banc d'Arguin, Mauritania (van der Heide *et al.* 2012). In these experiments, *Z. noltii* was grown under four conditions, (1) in the presence of *L. lacteus*, (2) in absence of *L. lacteus*, (3) in sediments to which sulfide was injected, (4) in the presence of *L. lacteus* and sediments subjected to sulfide injection. Shoot and root biomass of *Z. noltii* were lower in sediments subjected to sulfide injection, but higher in the presence of *L. lacteus* regardless of sulfide addition (van der Heide *et al.* 2012). The condition of *L. lacteus* individuals was estimated after trials by ratio of dry flesh to dry shell, and it was found that the flesh to shell ratio was highest in the presence of seagrass when sulfide was injected and second highest when sulfide was injected without the presence of seagrass (van der Heide *et al.* 2012). These experiments showed that seagrasses and lucinid clams benefit from one-another's presence, but did not assess the distribution and diversity of bacteria within the seagrass beds and as endosymbionts. Therefore, the motivation for this thesis research was to expand our understanding of “three-stage” symbiotic associations among seagrass, lucinids, and bacteria. The study took place in a coastal seagrass beds off the coast of Bokeelia, Florida (Fig. 1), where *S. floridana* is the dominant lucinid species present. The diversity and distribution of the associated free-living bacterial communities, including the potential for specific associations with certain seagrass species, was also assessed.

Hypotheses

The thesis has three main hypotheses:

- 1. *S. floridana* will be found in greater abundance in sediments with high seagrass coverage than in bare sediments.** Recent publications support an evolutionary radiation of lucinids that has been coincident with marine angiosperms and suggest there is a beneficial relationship between lucinids and seagrasses (van der Heide *et al.* 2012; Stanley 2014).

2. *S. floridana* gill endosymbiont diversity does not vary between individual hosts at more than a 97% sequence similarity threshold (i.e., belong to the same species). Currently available 16S rRNA gene phylogenies place the *S. floridana* symbiont in a monophyletic clade of Gammaproteobacteria that also contains other lucinid endosymbiont sequences (Brissac *et al.* 2011). However, recent evidence suggests that some lucinid species may have more than one endosymbiont species that differs genetically at the 97% sequence similarity level (Green-García & Engel 2012). Several species of bivalves and oligochaete worms host multiple species of endosymbiotic thiotrophic and methanotrophic bacteria (Fisher *et al.* 1993; Nakagawa & Takai 2008). Having genetically identical endosymbionts would support hypotheses that the environment of a host controls and selects the endosymbionts and that hosts allow themselves to be colonized by select groups of bacteria (Gros *et al.* 2003; Brissac *et al.* 2011; Gros *et al.* 2012).

3. Sequences obtained from the environment as free-living bacteria that are phylogenetically identical to *S. floridana* endosymbionts are rare and are found at less than 1% of the free-living bacterial community. Free-living representatives for most chemosymbiotic associations have yet to be identified (Gros *et al.* 2003; Duperron *et al.* 2013). Among the chemosymbiotic associations known, lucinids are suspected to have free-living forms of their endosymbiotic bacteria (Gros *et al.* 2003; Nyholm & McFall-Ngai 2004; Green-García & Engel 2012; Ainsworth *et al.* 2015). Identifying the occurrence of free-living bacteria that are phylogenetically identical to lucinid endosymbionts is important to understanding the lucinid-bacteria chemosymbiotic systems and what potential limitations there are for lucinid growth and establishment in a habitat.

Results of this study include the genetic identification and characterization of the endosymbiont bacteria for *S. floridana*, definition of the physical, geochemical, and biotic environment of the seagrass-lucinid-bacteria system, and evaluation of the potential distribution of free-living symbionts within the habitat. This study fills a gap in knowledge regarding the distribution of free-living members of bacterial endosymbiont groups and provides 16S rRNA gene reference libraries for several species of lucinid clam, and a seagrass biome.

Section 2: Materials and Methods

Site Description and Sampling Strategy

Bocilla Island Seaport is a privately owned fishing pier located in the city of Bokeelia, near Fort Myers and on the northern end of Pine Island, Florida (N26.710 W82.164, Fig. 1). Field research was completed in late July and early August, 2014, and was permitted by the Florida Fish and Wildlife Conservation Commission (permit SAL-14-1599-SR). The permit stipulated benthic sediment sampling when seagrasses were present and restricted the number of lucinid specimens that could be collected for molecular genetics and other research. At the time of sampling, the coastline was reinforced by a concrete breakwall that extended along the parking lot of the seaport. Riprap extended along the rest of the shoreline. Ecological data and samples were taken systematically every five meters along five different 50-meter long transects from the shoreline out into the Gasparilla Sound (Fig. 2).

Oceanographic and meteorological data were measured in the field or obtained from NOAA monitoring station 8725520, Fort Myers. All of the sampling sites were subtidal during and leading up to the period of field work (Fig. 3). Air temperature ranged between 20 and 35°C, and water temperature ranged from 27 to 35°C during and leading up to the field work period. There were two severe weather events reported in the Lee County area during the weeks leading up to field work, including an E0 tornado and a severe thunderstorm. There was not visible evidence of disturbance at the Bokeelia study site related to these events.

Three different seagrass species were identified on each transect, including *Halodule wrightii*, *Syringodium filiforme*, and *Thalassia testudinum*, and they covered 49.3% of the total study area. Vegetation coverage was visually estimated at each sample point. Estimates were made by the same one or two individuals throughout the study, so as to limit the introduction of

inter-observer bias. A 0.01 m³ volume of sediment collected by hand shovel and sieve was sampled for live clams and articulated clam shells, and other infauna were identified and recorded. We were not able to take sediment cores in the seagrass, as stipulated by the FWS permit. Homogenized sediment samples from the top 20 cm were also collected for grain size analysis, loss on ignition total organic carbon estimates, and nucleic acid extractions. Sediment samples were put into sterile Whirl-Pak bags, transported on ice, and frozen to -20°C within hours of collection.

Live clams were harvested from two of the five transects and were stored and transported on ice prior to dissections done within several hours of collection. Articulated shells of recently deceased clams were collected to obtain ontogenic and potential predation information for the area. Gills and feet from live specimens were preserved separately from the shells and other internal organs, and stored in 75% molecular grade ethanol prior to freezing at -20°C. The size of each of the *S. floridana* clam shells was measured to the nearest 0.01 cm from commissure to hinge, the longest anterior-posterior distance of the shell, with Vernier calipers. Tissues and shells are vouchered as cataloged specimens in the South Dakota School of Mines and Technology, Museum of Geology, under the accession number SDSM 2014-003.

Aqueous Geochemistry

Water chemistry measurements were taken from at least two different points per transect (Fig. 2). Pore water was pumped using a stainless steel piezometer that penetrated the upper 30 cm into the sediments connected to a Geotech Geopump™ peristaltic pump that was set to the lowest pump setting. Nonreactive Geotech silicone tubing was used. Sediments and pore water were allowed to equilibrate and pore water temperature, pH, and conductivity were

monitored to ensure that ocean waters had not permeated the piezometer sampling hole during sampling. These methods were consistent with those described in Green-García & Engel (2012). Ocean and pore water pH, temperature, conductivity, and total dissolved solids were measured in the field using standard electrode methods (APHA 1998). Physicochemistry was monitored during pore water extraction to verify that ocean waters did not penetrate the piezometer sample hole. Dissolved oxygen and sulfide concentrations were measured from raw pore water using Chemetrics (Calverton, VA) colorimetric methods and a field spectrophotometer (APHA 1998). Pore water samples were passed through 0.22 μm polyethersulfone membrane Sterivex (EMD Millipore, MA, USA) filters prior to filling separate high-density polyethylene (HDPE) bottles prepared for dissolved anions, cations, alkalinity and total organic carbon analyses. Up to 0.5 liters of pore fluids were passed through each Sterivex filter following purging of the tubing with new water. Filters were purged of water then placed on ice prior to freezing to -20°C within hours of collection. Cation bottles were acid-washed with 10% HCl prior to use, and filtered water was preserved with trace metal grade nitric acid after collection. Alkalinity was manually determined in the field by end-point titration to pH 4.3 using 0.1 N sulfuric acid. Dissolved anions (except carbonate species) and cations were measured by using dual Dionex (Thermo Scientific) ICS-2000 ion chromatographs. Pore fluid dissolved non-purgable organic carbon (NPOC), total inorganic carbon (TIC), and total nitrogen (TN) were measured on a Shimadzu NPOC/TC/TN analyzer. Dissolved organic carbon (DOC) is reported as the difference between dissolved NPOC and TIC.

Sediment Properties

Frozen sediment samples were thawed to room temperature prior to removing 8 gram subsamples, which were dried at 110 °C for 24 hours prior to sieving into the grain size classes, >2.0 mm, 1.00 mm, 500 µm, 250 µm, 125 µm, and <63 µm. Additional 3 gram subsamples were dried at 110 °C and then subjected to six hours of combustion at 550 °C, prior to reweighing to determine the percentage of organic matter per sediment sample (Luczak *et al.* 1997).

Nucleic Acid Extractions

Frozen sediments thawed to room temperature were separated into triplicate aliquots of 0.25 g for extraction of total environmental nucleic acids using Power Soil DNA extraction kits (MO BIO Laboratories, Carlsbad, CA), following manufacturer instructions. Nucleic acids were extracted from the Sterivex filters used in filtering water samples with MO BIO Power Water DNA extraction kits, also following manufacturer instructions (Padilla *et al.* 2015). Total nucleic acids from lucinid tissues were extracted using a Qiagen DNeasy Blood & Tissue kit (Venlo, Limburg, Netherlands), following manufacturer instructions. A foot sample was examined to identify potential contamination during dissection, as the expectation was that no bacterial 16S rRNA genes would be associated with the foot tissue. These extraction methods were used in order to be consistent with Green-García & Engel (2012).

DNA quality and quantity for each of the extractions was assessed by PCR using 16S rRNA gene primers 8F and 1510R (Green-García & Engel 2012) by measure of absorbance maxima ratios at 260:280 nm, and 260:230 nm for dsDNA using a NanoDrop 2000 spectrophotometer (Thermo Scientific). PCR products from all samples were examined on TBE agarose gels stained with ethidium bromide.

Libraries of 16S rRNA gene amplicons were generated from 60 μ L homogenates of each sample (i.e., a total of 3 extraction eluates for each sediment sample, 2 extraction eluates for porewater, and 2 extraction eluates for tissues). The V1-V3 hypervariable region of the 16S rRNA gene was amplified by Roche 454 FLX Titanium pyrosequencing instruments and reagents at the Molecular Research Laboratory (Sweetwater, TX), according to previously described methods (Dowd *et al.* 2006). Libraries have been uploaded to NCBI's Sequence Read Archive under Bioproject ID PRJNA302122.

16S rRNA Gene Sequencing, Sequence Processing, and Analyses

Gene sequence data were denoised using a translation of the Pyronoise algorithm (Quince *et al.* 2009) within the bioinformatics suite, mothur (Schloss *et al.* 2009; Schloss *et al.* 2011). Following alignment, sequences were screened to eliminate sequences shorter than 200 base pairs. The average length of 16S rRNA sequences was 275 base pairs. Chimeras were removed within mothur by self-reference using the program UCHIME (Edgar *et al.* 2011). The taxonomic reference file, SILVA 102, was used to align and classify sequences (Quast *et al.* 2013). Non-bacterial sequences including chloroplasts, mitochondria, and Archaea were removed prior to analysis with the R package, phyloseq (McMurdie & Holmes 2013). The basic mothur pipeline I used can be found in Appendix C.

Operational taxonomic units were determined in mothur by average linkage clustering. Each group of sequences, sediments, pore water, and tissues, were first clustered separately in order to characterize the bacterial communities of each sample type. Pore water sequences and sediment sequences from locations where pore water was sampled were clustered to observe differences in bacterial diversity between environmental sample types. In order to characterize

the abundance of lucinid endosymbionts in the free-living environment, lucinid tissue sequences were clustered with sediment and pore water samples that contained sequences related to the genus *Sedimenticola*, a dominant OTU found within lucinid tissues.

Chao 1, Shannon, and Simpson's alpha diversity indices were calculated for each sample using *mothur*. For further analyses, OTU counts per sample were transformed to relative abundance of sequence counts per sample within *phyloseq* (McMurdie & Holmes 2013). In other words, OTU abundance was corrected for incidence based on the total number of sequence reads per sample.

Statistical Analyses

Generalized linear ANOVA models were used to test for variation in *S. floridana* population density between ranks of total seagrass coverage and ranks of each species of seagrass were run with Statistical Analysis Software (SAS Institute, SAS Studio v.9.4). Seagrass coverage values, recorded as percentage of sample site coverage, were transformed for the ANOVA as follows: 0% coverage = Rank 0, 0% < Rank 1 < 33% coverage, 33% < Rank 2 < 66% coverage, 66% coverage < Rank 3 (Elzinga *et al.* 1998). Correlation matrices for geochemistry, seagrass coverage, and *S. floridana* abundance were also generated in SAS (Appendix C).

Microbial community analyses were conducted using the packages *vegan* and *phyloseq* (McMurdie & Holmes 2013; Oksanen *et al.* 2015). Pore water and sediment samples from the same sample points were plotted in non-metric multidimensional space (NMDS), visualized with the package *ggplot2* (Wickham 2009). Significant environmental vectors were identified with *vegan*'s "envfit" function. Envfit identifies environmental variables with significant correlation coefficients by permutative regression and produces coordinates that can be used to project the

environmental vectors on an ordination (Oksanen *et al.* 2015). These vectors are projected to reflect correlation between microbial communities and associated environmental variables.

Heatmaps were constructed for the top twenty most abundant OTUs from sediment samples, porewater samples, clam tissue samples, and a subset of samples including both sediment and porewater samples. Bray-Curtis dissimilarity matrices for OTU abundance were produced using vegan's 'vegdist' and 'hclust' functions and plotted with gplots (Warnes *et al.* 2015).

Section 3: Results

Oceanographic and Chemical Characteristics

The field study took place over five days, during which time local air temperature varied between 25-35°C, ocean water temperatures were 30-34°C, pore water temperatures were 28-33°C, ocean water pH was 8.0-8.5, pore water pH was 7.0-8.3, ocean salinity was 26.4 ppt, and pore water salinity was 25.6-28.2 ppt (Table 1). There were no extreme weather events reported for the study area (Lee County) during fieldwork.

Ocean and porewaters were sampled at 11 different locations in the study area (Table 1, 2). The ocean water field pH was higher (average 8.19 ± 0.28 , $n = 9$) than porewater collected at the same location (average 7.71 ± 0.30 , $n = 11$), although water temperatures were similar to each other, with ocean water being slightly cooler (average $30.24^{\circ}\text{C} \pm 1.7^{\circ}\text{C}$, $n = 9$) than porewater (average $30.44^{\circ}\text{C} \pm 0.7^{\circ}\text{C}$, $n = 11$), but the values were not significantly different from each other. One representative ocean water sample was collected for the study, but temperature and pH of the ocean water were measured at each sample point (average temperature $30 \pm 1.9^{\circ}\text{C}$, $n = 9$, and average pH 8.19 ± 0.2 , $n = 9$). Field ocean and porewater conductivity values were within a standard deviation of each other (ocean 43.75 mS/cm, porewater 44.52 mS/cm, Table 2).

Grain size distributions of sampled sediments from the 50-m transects ($n = 50$) were characteristic of fine-grained sand, dominated by Phi (Φ) 3 (125 μm) which made up an average of $67.42\% \pm 6.73\%$ of the grain size distribution. Low amounts of organic matter as TOC were measured in the sediments, with an average of $0.70\% \pm 0.43\%$ ($n = 50$).

Vegetation, Geochemistry, and Lucinids

Seagrasses covered 49.3% of the study area and the remainder was underlain with bare sand ($n = 50$). The seagrasses *H. wrightii* (27% of the area) and *S. filiforme* (18.1% of the area) dominated sample location coverage, and *T. testudinum* was only observed in small patches that amounted to 4% of the area. In addition to the seagrasses, other fauna and flora identified at the Bokeelia location included the sponge, *Tedania ignis*, and several species of gastropods. Rays and dolphins were also observed at the site.

Most live *S. floridana* were 20 mm in height from commissure to hinge (Fig. 4) and were retrieved from 20 cm or less below sediment surfaces. In total, lucinid population densities at Bokeelia ranged from 0 to 2354 individuals per cubic meter (Table 3). Lucinid taxa retrieved were dominated by *S. floridana*, which were recovered from bare sand and underneath seagrasses. Other lucinid clams that were retrieved included *Lucinisca nassula* from transect #009 where 69 *S. floridana* were counted, and *Ctena orbiculata* from transect #010 where 85 *S. floridana* were counted.

The first hypothesis posed for this research was that *S. floridana* will be found in greater abundances in sediments with higher seagrass coverage than in bare sediments. Generalized linear ANOVA tests were used to detect variation in abundance of *S. floridana* between ranks of seagrass coverage in general and by species of seagrass (Appendix B). There was significant variance in the abundance of *S. floridana* between ranks of total seagrass coverage (F-value = 3.78, $p = 0.0173$, $\alpha = 0.05$, Fig. 5) and between coverage ranks of *H. wrightii* (F-value = 4.30, p -value = 0.01, $\alpha = 0.05$). Spearman's correlation coefficient indicated that *S. floridana* population densities and total seagrass coverage, as well as *H. wrightii* coverage, were positively correlated (Spearman's $\rho = 0.41$, $p = 0.003$, $\alpha = 0.05$, and $\rho = 0.44$, p -value = 0.001, $\alpha = 0.05$).

respectively, Fig. 5, Appendix C). Consequently, although clams were recovered from all ranks of vegetation coverage, more clams were retrieved from sediments with the highest seagrass coverage. Aside from seagrass coverage, the only other environmental parameter significantly correlated with *S. floridana* population density at Bokeelia was pH ($\rho = 0.83$, $p = 0.002$, Fig. 6 Appendix D).

Bacterial Communities

Sediment samples yielded 1300-11000 16S rRNA gene sequence reads after denoising pyrosequence data (Table 4 and 5), for a total of 287514 sequence reads that were condensed into 80267 OTUs at a 97% similarity cutoff. Pore fluid samples yielded 2000-5700 sequences per sample, for a total of 49754 sequences that were condensed into 17206 OTUs (Table 6). Lucinid tissue 16S rRNA gene sequence yield was low, 20-700 sequences, totaling 2649 for 345 OTUs (Table 6). *S. floridana* foot tissue was exceptionally low with 52 sequences and 4 OTUs, but *C. orbiculata* and *L. nassula* feet produced 388 and 308 sequences for 125 and 85 OTUs, respectively (Table 6). Comparison of observed and Chao 1 estimated numbers of OTUs by sample indicates that large portions of the free-living community of bacteria may remain unsampled (Table 5, 6, Fig. 7). Chao 1 indices for the lucinid gill tissues indicate poor sampling of the lucinid tissue microbiome (Table 6).

Sediment and Pore Water Bacterial Diversity

Several Delta- and Gammaproteobacteria OTUs dominated the sediment samples, representing at 30-50% of all OTUs, and a Bacteroidetes OTU represented up to 20% of all OTUs in some samples (Figure 6). Unclassified OTUs represented 41.3% (+/-8%) of all sediment samples and were considered to indicate that there is novel diversity yet to be described

(Wintzingerode *et al.* 1997; Rappe & Giovannoni 2003). The bacterial community appeared generally similar at all sample points excluding samples 009-35, -40, and -45, for which several OTUs within the Deltaproteobacteria made up over 80% of all OTUs.

From the pore water samples, Proteobacteria represented 10-80% of OTUs at most sampling locations, and Bacteroidetes represented up to 80% of OTUs at one point, with Cyanobacteria and Firmicutes representing 5-10% of OTUs (Figure 7). Alpha diversity indices indicated that the pore fluids were also undersampled and were generally less diverse than sediment samples (Table 6).

Clustering the subset of sediment samples associated with porewater sample points revealed distinct differences in the composition of sediment and porewater bacterial communities (Figure 8). Each group of samples is dominated by distinct OTUs associated with Gammaproteobacteria and Deltaproteobacteria that represent up to 20% of all OTUs in any sample (Figure 8). An OTU within the Firmicutes represents over 20% of all OTUs taken from one pore water sample. These clusters are also evident in NMDS space by samples or taxa (Figure 9).

Lucinid Tissues

Gill tissues were sampled from five *S. floridana* specimens collected from the #006 and #010 transects. Additionally, one specimen of *L. nassula*, and one *C. orbiculata* were retrieved from transects #009 and #010. Bacterial 16S rRNA gene sequences from the gills of all the lucinids were dominated by an OTU that classified as *Sedimenticola*, a genus within an unclassified order-level clade within the Gammaproteobacteria class. *Sedimenticola*-like sequence reads formed over 97% of all OTUs in each gill sample (Figure 10, Table 7). The foot of one *S.*

floridana, yielded a low count of this OTU, which is consistent with the assumption that this tissue is infection-free. However, large numbers of *Sedimenticola*-affiliated OTUs were retrieved from the feet of the *C. orbiculata* and *L. nassula* specimens and were shared with *Sedimenticola*-OTUs found in the gills of all the clams. All foot samples yielded OTUs representing several other taxonomic groups including an *E. coli*, several Alphaproteobacteria, and other Gammaproteobacteria (Figure 10, Table 7). The presence of these OTUs in the feet could indicate that nonspecific infection of tissue occurred in some lucinid clams, which has been observed for chemosymbiotic mussels (Wentrup *et al.* 2013). Several unclassified OTUs belonging to the Alpha- and Gammaproteobacteria, and to several unclassified phyla, were found in the gill tissues to represent up to 6% of all gill OTUs in any sample (Figure 10, Table 7). The second hypothesis for this research, that *S. floridana* gill endosymbiont diversity would not vary between individual hosts at more than a 97% sequence similarity threshold, is supported if only the *Sedimenticola*-like OTU is considered to represent the true endosymbionts. Over 99% of all *Sedimenticola*-type sequences belonged to one OTU within gill tissues of all lucinid clams (Figure 11). This evidence supports the hypothesis of a monospecific culture of gill endosymbionts. Lucinid feet may harbor more diverse communities of closely-related *Sedimenticola*-type sequences, and other taxa, as indicated by the formation of up to four different OTUs within *C. orbiculata* and *L. nassula* feet.

Local Distribution of *Sedimenticola*-like Sequences

The third hypothesis tested was that bacterial 16S rRNA sequences obtained from the environment would be phylogenetically identical to sequences obtained from *S. floridana* and presumed to be endosymbionts; however, these free-living bacteria would exist at less than 1%

of the free-living bacterial community. Because the dominant OTU in lucinid gill tissues was affiliated with *Sedimenticola*, sediment and pore water samples in which similar sequences had been detected were clustered with tissue samples to identify shared OTUs. Several OTUs were shared between lucinid tissues, sediments, and porewater samples, and the dominant one was a *Sedimenticola*-like OTU (Fig. 14). This OTU represented less than 1% of all OTUs from sediment or pore water samples, thereby supporting the third hypothesis.

Section 4: Discussion

Lucinid clams represent a taxonomically diverse and ecologically significant component of marine ecosystems as part of a three-stage symbiotic relationship between seagrasses, lucinid clams and their endosymbionts, and free-living microbial communities (Fisher & Hand 1984; Johnson *et al.* 2002; Reynolds *et al.* 2007; Meyer *et al.* 2008; Taylor *et al.* 2011). Previous research has established a significant relationship between lucinid clams, seagrass, and pore water sulfide concentrations (Reynolds *et al.* 2007; van der Heide *et al.* 2012; Stanley 2014). Van der Heide *et al.* (2012) demonstrated that there is strong relationship between the availability of sulfide and oxygen in seagrass beds and the growth of lucinid clams, and Reynolds *et al.* (2007) found that lucinid clams may remove 2-16% of sulfide produced in seagrass beds in which concentrations of sulfide were as high as 2000 μM . Field research has demonstrated that lucinid clams utilize isolated pockets of soluble sulfide or rely on oxidizing conditions created by their inhalant tubes to release sulfide from insoluble metal-sulfides stored in surrounding sediments (Cary *et al.* 1989; Dando *et al.* 1994). In this study, the maximum *S. floridana* population density increased with increasing seagrass coverage, but was not dependent on sulfide concentration. Soluble sulfide concentrations at Bokeelia were relatively high (9 – 100 μM) with respect to the Cary *et al.* (1989) and Dando *et al.* (1994) studies (0 – 8 μM), but much lower than observed during the Reynolds *et al.* (2007) survey. It is possible that sulfide was not a limiting factor to *S. floridana* growth and survival at this site. Dissolved sulfur species reach concentrations of 10-200 μM within the hemolymph and gills of *S. floridana* (Anderson 1995; Larabee 1998). If such quantity of sulfide is available ubiquitously in the pore fluids of a site, the distribution of a lucinid population may be controlled by factors other than sulfide availability.

There was no correlation between pH and *S. floridana* population density at Bokeelia. Although much work has been done to elucidate the relationship between lucinid clams and sulfide (Jackson 1969; Jackson 1972; Schweimanns & Felbeck 1985; Reid & Brand 1986; Taylor & Glover 2006; Reynolds *et al.* 2007; van der Heide *et al.* 2012; Stanley 2014), little, if any, has been done to examine the impact of changing pH on lucinid clams. Although the increase in global ocean pH due to absorption of anthropogenic CO₂ threatens all marine biodiversity, pH changes mediated by anthropogenic disturbance of coastal freshwater hydrology and ecosystem structure pose more immediate threats to coastal habitats than open ocean acidification (Knutsen 1981; Salisbury *et al.* 2008; Hendriks *et al.* 2010; Duarte *et al.* 2013). The chemical environment of coastal habitats is dynamic and affected by a variety of factors including ecosystem metabolism, anthropic ecosystem restructuring, freshwater hydrology, and pollution (Duarte *et al.* 2013). These processes produce significant pH changes that occur on a much shorter time scale than acidification by pCO₂ which has dominated most discussions of ocean acidification (Duarte *et al.* 2013). Ecosystem metabolic effects on pH are mediated by rates of biological production or respiration that may fluctuate seasonally and diurnally, and by rates of calcification (Duarte *et al.* 2013). Physical and chemical anthropogenic disturbance of coastal ecosystems can cause ecosystems to undergo significant restructuring. Removal of foundational plant species and keystone prey or predators species can cause trophic cascades that may ultimately tip the trophic status of a system and the balance between biomass production and respiration, or the capacity to serve as a CO₂ sink or source (Carpenter *et al.* 1985; Duarte *et al.* 2013). Freshwater discharge from groundwater, stream channels, or surface runoff can create strong gradients of high pCO₂ and low pH, but may also contribute alkalinity from weathered carbonate rocks thereby increasing the buffering capacity of coastal waters (Salisbury *et al.*

2008; Duarte *et al.* 2013). Bivalves construct their shells from calcium carbonate, and active populations of bivalves can have the effect of lowering alkalinity in surrounding waters (Salisbury *et al.* 2008; Duarte *et al.* 2013).

Several studies have been done on commercially significant bivalves in order to estimate the effect of coastal or open ocean acidification on bivalve growth and survival (Bamber 1987, 1990; Barros *et al.* 2013). Bamber (1987) exposed experimental populations of Venerid carpet-shell clams, *Venerupis decussata*, to seawater ranging in pH from 3.5 to 8.2. Shell dissolution was observed at $\text{pH} \leq 7.55$, shell and tissue growth decreased at $\text{pH} < 7$, and clams exposed to $\text{pH} \leq 6.5$ experienced over 50% mortality (Bamber 1987). Similar experiments were conducted on the commercial bivalve molluscs *Ostrea edulis*, *Crassostrea gigas*, and *Mytilus edulis*, and it was concluded that seawater of $\text{pH} < 7$ is generally intolerable to bivalve molluscs (Bamber 1990). Recent experiments were performed on the oyster, *C. gigas*, in which pH was adjusted to reflect modeled global changes in seawater pH ($\Delta\text{pH} = -0.4$ and $\Delta\text{pH} = -0.7$) (Barros *et al.* 2013). Reductions in pH of less than one pH unit resulted in severe decreases in *C. gigas* larval hatching rates and larval growth rates, as well as up to 100% mortality of larva (Barros *et al.* 2013). A coastal environment may experience similar or more extreme deviations in pH on a regular basis, but many coastal organisms are adapted to survive temporarily adverse chemical conditions (Ellington 1993; Pynnonen & Huebner 1995; Range *et al.* 2011; Duarte *et al.* 2013). At the Bokeelia site, pH ranged from 7.0-8.3. The fluctuation of pH was not monitored during sampling, and sampling occurred across tidal periods, but previous research strongly supports $\text{pH} \leq 7$ as a boundary for bivalve success (Bamber 1987, 1990; Barros *et al.* 2013).

Although biogeochemical research has been conducted on microbial activity in vegetated marine sediments for many years, this study represents one of few recent analyses of a

16S rRNA gene taxonomic library with geochemical data from such an environment. Recent studies utilizing 16S rRNA gene cloning and pyrosequencing have found that Proteobacteria often dominate microbial communities in shallow marine sediments, with the remainder of the community composed of OTUs from Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes (Green-García & Engel 2012). Sediment and pore water microbial communities at Bokeelia varied with increasing pore water concentrations of total nitrogen and dissolved oxygen, where pore water communities appear to be associated with higher amounts of dissolved oxygen and total nitrogen than sediment communities (Fig. 12). It is well known that microbial communities in marine sediments vary along vertical redox gradients, within biofilms, and between microniches in porewater and sediment (Fenchel & Reidl 1970; Jorgensen 1982; Elsgaard & Jorgensen 1992; Vos *et al.* 2013). The organisms inhabiting the pore water or existing nearer to the sediment surface generally are exposed to higher concentrations of oxygen (Todorova *et al.* 2014; Bourque *et al.* 2015). Pore water and sediment samples in this study represent homogenized chemical environments and microbial communities from the top 15-20 cm of the seagrass beds and should not truly reflect differences in vertical position, but it is likely that the NMDS plot (Fig. 12) depicts differences in oxygen tolerance and nitrogen requirements or utilization between sediment and porewater groups.

Gill tissue samples from all specimens of *S. floridana*, as well as specimens of *L. nassula* and *C. orbiculata*, share an OTU that is classified as a member of the *Sedimenticola*, a genus of Gammaproteobacteria. Over 97% of all 16s rRNA gene sequences recovered from the gills are included in one OTU (Table 7, Figure 12). Focused clustering of just those sequences revealed that there were multiple unique *Sedimenticola* OTUs within the feet and gills, but that over 99% of *Sedimenticola*-type sequences within gill tissues formed a single OTU (Figure 12).

Up to four *Sedimenticola*-type OTUs were formed from sequences of *C. orbiculata* and *L. nassula* feet tissues. A shared OTU is also present in sediment and pore fluid samples, though its total abundance represents less than 1% of any sample (Table 8).

Sedimenticola spp. have been cultured from marine or estuarine sediments and form a monophyletic clade with unnamed sulfide-oxidizing symbionts from marine invertebrates (Narasimarao & Haggblom 2006; Carlstrom *et al.* 2015; Flood *et al.* 2015). Cultured representatives of *Sedimenticola* include several strains of *Sedimenticola selenatireducens*, specifically strains -AK4OH1 (Narasimarao & Haggblom 2006), -CUZ (Carlstrom *et al.* 2015), -NSS (Carlstrom *et al.* 2015), and -SIP-G1 (Flood *et al.* 2015). Strain -AK4OH1 couples the oxidation of aromatics to the reduction of selenate and/or nitrite and nitrate (Narasimarao & Haggblom 2006), whereas strains -CUZ and -NSS are perchlorate- and chlorate-reducers, respectively (Carlstrom *et al.* 2015) that cannot grow by sulfide metabolism (Flood *et al.* 2015). *Sedimenticola thiotaurini* strain SIPG1 has been described recently and oxidizes reduced sulfur compounds during the autotrophic reduction of CO₂ (Flood *et al.* 2015). It also converts sulfur compounds into thiotaurine, which has been hypothesized to be a non-toxically shuttled from the bacterial cell to an animal host (Flood *et al.* 2015).

Free-living representatives of lucinid clam endosymbionts have previously been detected by fluorescence in-situ hybridization microscopy (FISH) (Gros *et al.* 2003), but this is the first study to tentatively identify representatives of free-living lucinid symbionts via 16S rRNA pyrosequencing. Using FISH probes specific to the 16S rRNA gene of the *Codakia orbicularis* endosymbiont, Gros *et al.* (2003) were able to identify several representative organisms within sediment samples from *T. testudinum* beds. They demonstrated that these representatives were the endosymbionts by conducting an experiment in which juvenile

specimens of *C. orbicularis* were introduced to sterilized sediments or crude sediments collected from their sampling site (Gros *et al.* 2003). Juveniles in the negative control remained aposymbiotic, while those in the crude sediment were infected with the endosymbiont (Gros *et al.* 2003). Single cell flow cytometry analyses revealed that the *C. orbicularis* endosymbiont contains up to four copies of its genome (Caro *et al.* 2007). The multigenomic state is characteristic of bacteria with rapid doubling times, often those that respond quickly when the nutritional status of their environment becomes hospitable (Thorsen *et al.* 1992; Caro *et al.* 2007). Such a characteristic would be valuable to organisms that spend much of their time in a dormant state awaiting hospitable conditions. Many bacteria within the free-living communities may cycle between dormant and active states as their microenvironment changes (Jones & Lennon 2010). Future work should investigate the possibility of a dormant reservoir of potential lucinid endosymbionts in lucinid habitats.

This study affirms previous research that demonstrated co-occurrence of lucinid clams and seagrasses and supports the three-stage model of ecosystem interactions between lucinid clams, seagrasses, and microbial communities. The gill endosymbionts of the lucinid clam, *S. floridana*, were tentatively identified as several OTUs of the genus *Sedimenticola* within the Gammaproteobacteria. Tentative *S. floridana* endosymbionts were found as less than 1% of the free-living microbial community, but as over 97% of the microbial community of gill tissue extracted from *S. floridana*, *C. orbiculata* and *L. nassula*. The observation that symbiont OTUs are shared between tissue samples and the sediment and pore water samples supports previous research that indicated lucinid clams are capable of limiting the colonization of their gills to specific bacteria that are encountered rarely in the free-living environment. Additionally, the presence of the numerous different OTUs in the feet of *C. orbiculata* and *L. nassula* indicates

that some lucinid clams may not be able to precisely regulate the colonization of symbionts throughout their tissues or prevent infection by other closely-related organisms.

Several other ecological parameters remain largely unconsidered in contemporary studies of lucinid clams, including the dynamics of predation, inter- and intraspecies competition for resources, and local or global ocean acidification. It has been posited that seagrass beds give the clams protection from predators that surf the benthos (Stanley 2014). However, burrowed lucinids may be protected from predators other than gastropods that burrow to similar depths, simply by virtue of the overlaying sediment (Jackson 1972). Little effort has been made to date to document the life history of most lucinids, including *S. floridana*, leaving major gaps in the ability to interpret interactions between lucinids and the ecological and chemical environments where they occur. To our knowledge, the impact of pH on lucinid clam populations has not been previously reported. In dynamic coastal environments and under conditions of global increases in $p\text{CO}_2$, it is important that future studies of lucinid clams consider the impact of pH alkalinity on lucinid diversity and functional role in coastal ecosystems.

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Appendices

Appendix A

Table 1. Field chemistry of pore fluids and a representative ocean water sample from Bokeelia, Florida. DOC presented as IC-NPOC.

Sample ID	Distance (m)	pH	Alk (mM)	Temp C	Salinity (ppt)	DO (μM)	H ₂ S (μM)	IC (mg/L)	NPOC (mg/L)	TN (mg/L)	DOC (mg/L)
LUC14.006.5	5	7.5	176.2	29.9	28.2	134.7	88.9	32.9	5.4	2.0	27.6
LUC14.006.20	20	7.8	125.2	29.1	27.9	197.8	28.1	28.3	4.2	0.8	24.1
LUC14.007.15	15	7.8	167.9	31.9	26.6	204.1	100.4	34.3	5.9	1.5	28.4
LUC14.007.40	40	8.3	145.7	30.6	26.5	206.7	9.4	27.2	4.9	1.1	22.3
LUC14.008.10	10	7.8	157.1	30.1		90.6	45.4	28.8	4.5	1.0	24.3
LUC14.008.30	30	7.5	146.4	30.3	27	82.5	26	28.3	4.2	0.9	24.0
LUC14.009.20	20	7.7	143.5	30.1	26.8	82.8	88	0.2	0.0	0.0	0.2
LUC14.009.40	40	7	148.6	30.8	26.6	97.2	39.3	28.8	4.7	0.7	24.0
LUC14.010.10	10	7.8	163.2	30.8	25.6	105.9	103.7	31.1	4.7	1.1	26.4
LUC14.010.20	20	7.9	153.2	31.3	26.8	93.1	64.3	29.1	4.3	0.8	24.8
LUC14.010.50	50	7.8	156.2	31.7	27.4	129.7	76.6	30.0	4.0	0.9	26.0
Ocean		8.2	147.4	31.1	26.4	206.3	0	22.4	4.9	0.4	17.6

Table 2. Pore fluid and ocean water ions.

Sample ID	F (mM)	Cl (mM)	NO2 (mM)	SO4 (mM)	Br (mM)	NO3 (mM)	PO4 (mM)	Ca (mM)	K (mM)	Mg (mM)	Na (mM)	NH4 (mM)
LUC14_006_5	0.03	387.3	BDL	19.4	0.5	BDL	BDL	12.5	11.5	62.3	508.6	1.7
LUC14_006_20	0.04	528	BDL	26.4	0.7	BDL	BDL	12.7	11.9	64	524.6	1.3
LUC14_007_15	0.03	533.7	BDL	27	0.7	BDL	BDL	10.2	9.6	52.4	439.7	BDL
LUC14_007_40	0.04	376.4	BDL	17.5	0.5	BDL	BDL	11.5	10.8	58.1	474.5	BDL
LUC14_008_10	0.04	517.4	BDL	26.2	0.6	BDL	BDL	9.1	8.6	48.1	415.8	BDL
LUC14_008_30	0.04	527.7	BDL	26.9	0.7	BDL	BDL	12	11.3	60.6	497.3	1.3
LUC14_009_20	0.05	531.2	BDL	27	0.7	BDL	BDL	10.4	9.8	53.6	450.9	BDL
LUC14_009_40	0.03	371.9	BDL	17.6	0.5	BDL	BDL	10.3	9.7	53.1	447.6	BDL
LUC14_010_10	0.04	376.3	BDL	17.8	0.5	BDL	BDL	13.1	12.2	65.5	535.8	1.5
LUC14_010_20	0.04	522.5	BDL	26.7	0.8	BDL	BDL	11	10.4	55.4	455.8	1.2
LUC14_010_50	0.04	541.1	BDL	27.5	0.8	BDL	BDL	10.4	9.8	53.3	449	BDL
Ocean	0.04	517.6	BDL	26.5	0.7	BDL	BDL	11.7	11	59	483.6	BDL

Table 3. Seagrass coverage ranks (0 = bare sand; 1 = 33% or less; 2 = 33-66%, 3 = 66-100%) for each transect position, and calculated population densities of live and articulated *S. floridana* per cubic meter.

Transect	Position	Seagrass	<i>H. wrightii</i>	<i>S. filiforme</i>	<i>T. testudinum</i>	<i>S. floridana</i> count	Articulated <i>S. floridana</i> count	<i>S. floridana</i> population density	Articulated <i>S.</i> <i>floridana</i> population density
6	5	0	0	0	0	0	1	0	94
6	10	3	3	0	0	25	2	2354	188
6	15	3	3	0	0	12	8	1130	753
6	20	0	0	0	0	3	2	283	188
6	25	1	1	0	0	5	5	471	471
6	30	3	0	3	0	14	2	1318	188
6	35	3	0	3	0	19	0	1789	0
6	40	1	1	0	0	21	3	1978	283
6	45	1	1	0	0	5	0	471	0
6	50	1	0	1	0	3	0	283	0
7	5	0	0	0	0	6	1	565	94
7	10	0	0	0	0	3	6	283	565
7	15	3	3	0	0	26	5	2449	471
7	20	3	3	0	0	10	5	942	471
7	25	1	1	0	0	3	1	283	94
7	30	0	0	0	0	11	6	1036	565
7	35	3	0	3	0	13	4	1224	377
7	40	3	0	3	0	5	0	471	0
7	45	2	0	1	2	0	5	0	471
7	50	3	0	2	2	0	6	0	565
8	5	0	0	0	0	1	0	94	0
8	10	3	3	0	0	6	1	565	94
8	15	2	2	0	0	4	0	377	0
8	20	3	3	0	0	3	0	283	0
8	25	1	1	0	0	12	0	1130	0
8	30	2	2	0	0	3	10	283	942
8	35	0	0	0	0	4	0	377	0
8	40	3	0	3	0	0	0	0	0
8	45	3	1	1	3	8	3	753	283
8	50	1	1	0	0	3	26	283	2449
9	5	0	0	0	0	9	0	848	0
9	10	0	0	0	0	2	3	188	283
9	15	0	0	0	0	2	0	188	0
9	20	0	0	0	0	1	0	94	0
9	25	0	0	0	0	1	0	94	0
9	30	1	1	0	0	9	0	848	0
9	35	3	0	3	0	10	3	942	283
9	40	3	2	2	1	7	2	659	188
9	45	3	1	1	0	8	1	753	94
9	50	3	3	1	0	20	1	1883	94
10	5	0	0	0	0	8	0	753	0
10	10	3	3	0	0	12	2	1130	188
10	15	3	3	0	0	3	3	283	283
10	20	3	0	3	0	7	7	659	659
10	25	3	3	0	0	18	3	1695	283
10	30	0	0	0	0	6	12	565	1130
10	35	2	2	0	0	7	2	659	188
10	40	3	3	0	0	15	1	1413	94
10	45	1	0	1	0	4	4	377	377
10	50	1	1	0	0	5	0	471	0

Table 4. Summary of sequence processing. Counts are reported by sample type since they were processed in bulk by sample type.

Sample group	Raw sequence count	Sequence count after trimming	Average seq. length after trimming (bp)	Number of sequences after screening for length, filtering out gaps, removing non-unique sequences	Chimeric sequences	Final sequence count	No. OTUs (97% cutoff)
Sediment (n=49)	295627	296613	270.61	287514	0	287514	80267
Porewater (n=11)	274472	55888	266.92	52175	2421	49754	17206
Tissue samples (n=10)	269889	6427	290.22	2659	10	2649	345

Table 5. Final sequence and OTU counts after pre-processing sediment samples. Alpha diversity indices include Chao 1 (estimated number of OTUs) and Shannon and Simpson indices of richness/evenness. *indicates outliers

Sediment						Sediment					
ID	Sequence count	OTU count	Chao 1	Shannon	Simpson	ID	Sequence count	OTU count	Chao 1	Shannon	Simpson
LUC14.006.05	9713	2898	18073	7.78	0.000393	LUC14.009.05	6424	1288	11028	7.11	0.000201
LUC14.006.10	7454	4098	17470	8.18	0.000168	LUC14.009.15	7100	1660	15386	7.33	0.000253
LUC14.006.15	7034	3297	17602	7.99	0.000173	LUC14.009.20	6986	1422	12455	7.16	0.000368
LUC14.006.20	6408	2629	16249	7.78	0.000198	LUC14.009.25*	2757	475	5840	6.14	0.000284
LUC14.006.25	6061	2285	13866	7.65	0.000202	LUC14.009.30	6883	2304	16084	7.66	0.000176
LUC14.006.30	4340	1464	11735	7.20	0.000293	LUC14.009.35*	2045	306	987	4.88	0.018387
LUC14.006.35	3986	1312	10904	7.09	0.000326	LUC14.009.40*	2092	329	3359	4.82	0.049908
LUC14.006.40	8154	2733	18049	7.81	0.000215	LUC14.009.45*	3622	583	5435	5.56	0.022997
LUC14.006.45	8100	2825	17999	7.86	0.000169	LUC14.009.50*	1306	253	2151	5.18	0.008763
LUC14.006.50	10689	3507	20355	8.03	0.000209	LUC14.010.05	5503	1245	9120	7.04	0.000368
LUC14.007.05	7805	2004	13620	7.50	0.000266	LUC14.010.10	5920	1563	10689	7.28	0.000223
LUC14.007.10	9424	3007	20590	7.90	0.000202	LUC14.010.15	3715	1012	11904	6.88	0.000215
LUC14.007.15	7702	2178	15767	7.58	0.000274	LUC14.010.20	6685	1995	16723	7.54	0.000149
LUC14.007.20	5626	1455	13348	7.19	0.000335	LUC14.010.25	2590	678	9494	6.50	0.000139
LUC14.007.25	6858	1759	13774	7.39	0.000247	LUC14.010.30	5836	1351	9900	7.16	0.0002
LUC14.007.30	6427	1683	12428	7.35	0.000252	LUC14.010.35	5874	1407	11530	7.16	0.000326
LUC14.007.35	4209	974	8248	6.81	0.00035	LUC14.010.40	6104	1534	12587	7.27	0.000204
LUC14.007.40	4300	1096	13187	6.94	0.000268	LUC14.010.45	7061	1651	11649	7.34	0.000208
LUC14.007.45	8578	2397	18875	7.71	0.000158	LUC14.010.50	6361	1486	10738	7.22	0.000335
LUC14.007.50	4080	1033	11714	6.90	0.000211						
LUC14.008.05	3548	899	8288	6.71	0.000493						
LUC14.008.10	3506	792	6133	6.61	0.000428						
LUC14.008.15	6423	1444	8479	7.15	0.000453						
LUC14.008.20	5336	1217	7986	7.00	0.000463						
LUC14.008.25	7486	1801	12750	7.42	0.000203						
LUC14.008.30	8330	2077	10909	7.52	0.00032						
LUC14.008.35	3935	865	9445	6.70	0.000359						
LUC14.008.40	3989	855	8866	6.65	0.000621						
LUC14.008.45	3729	840	7709	6.67	0.000385						
LUC14.008.50	9420	2301	18557	7.67	0.000151						

Table 6. Final sequence and OTU counts after pre-processing sediment samples. Alpha diversity indices include Chao 1 estimated number of OTUs and Shannon and Simpson indices of richness/evenness.

Porewater						Gill (G) and Foot (F) Tissues					
ID	Sequence count	OTU count	Chao 1	Shannon	Simpson	ID	Sequence count	OTU count	Chao 1	Shannon	Simpson
LUC14.013	3094	1081	3475	5.86	0.0115	<i>Stewartia</i> 6-10GA	473	14	26	0.155	0.958
LUC14.014	5746	2408	7344	7.05	0.0021	<i>Stewartia</i> 6-10GB	204	24	67	0.333	0.901
LUC14.015	4309	1081	3406	5.15	0.0322	<i>Stewartia</i> 6-10FB	52	4	4	0.615	0.698
LUC14.016	2576	1091	3832	6.07	0.0091	<i>Stewartia</i> 10-10GA	409	20	174	0.186	0.950
LUC14.017	5379	2163	7212	6.78	0.0039	<i>Stewartia</i> 10-10GB	53	8	23	0.219	0.927
LUC14.018	3384	1403	4967	6.38	0.0054	<i>Stewartia</i> 10-10GC	80	15	43	0.255	0.925
LUC14.019	5340	2157	7013	6.73	0.0047	<i>Ctena</i> 10G	21	10	17	0.597	0.785
LUC14.020	5144	1697	4676	6.41	0.0062	<i>Ctena</i> 10F	388	125	179	2.171	0.434
LUC14.021	4262	941	3396	4.76	0.0486	<i>Lucinisca</i> 9G	661	40	139	0.443	0.884
LUC14.022	2128	561	2222	4.59	0.0492	<i>Lucinisca</i> 9F	308	85	108	2.609	0.274
LUC14.023	4619	1535	5506	5.64	0.0302						
LUC14.024	3773	1088	3749	5.43	0.0210						

Table 7. Relative abundance of the top twenty OTUs observed in lucinid gill tissues.

Class	Genus	<i>C. orbiculata</i> foot	<i>L. nassula</i> foot	<i>S. floridana</i> Foot 6B	<i>S. floridana</i> Gill 6A	<i>S. floridana</i> Gill 10A	<i>S. floridana</i> Gill 10B	<i>S. floridana</i> Gill 10C	<i>S. floridana</i> Gill 6B	<i>L. nassula</i> Gill	<i>C. orbiculata</i> Gill
Gammaproteobacteria	Sedimenticola	0.68	0.52	0.05	0.99	0.98	0.97	0.97	0.96	0.97	0.91
Bacilli	Enterococcus	0.00	0.00	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gammaproteobacteria	Unclassified	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified phylum	Unclassified	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.02	0.00	0.00
Alphaproteobacteria	Neorickettsia	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified phylum	Unclassified	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified Proteobacteria	Unclassified	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
Alphaproteobacteria	Unclassified	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gammaproteobacteria	Sedimenticola	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Betaproteobacteria	Ralstonia	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gammaproteobacteria	Unclassified	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gammaproteobacteria	Acinetobacter	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified Bacteroidetes	Unclassified	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gammaproteobacteria	Unclassified	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mollicutes	Mycoplasma	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Alphaproteobacteria	Unclassified	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Alphaproteobacteria	Nesiotobacter	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Unclassified phylum	Unclassified	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Clostridia	Peptoniphilus	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Unclassified phylum	Unclassified	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02

Table 8. Counts of *Sedimenticola*-like sequences recovered from the free-living environment.

Sequence counts	Sediment (n=49)	Pore fluid (n=11)
Mean total sequences per sample	5867 +/- 2171	4146 +/- 1172
Mean <i>Sedimenticola</i> sequences	1.63 +/- 3.0	8.5 +/- 13.1
Mean % <i>Sedimenticola</i> sequences	0.031 +/- 0.06	0.21 +/- 0.31

Appendix B: Figures

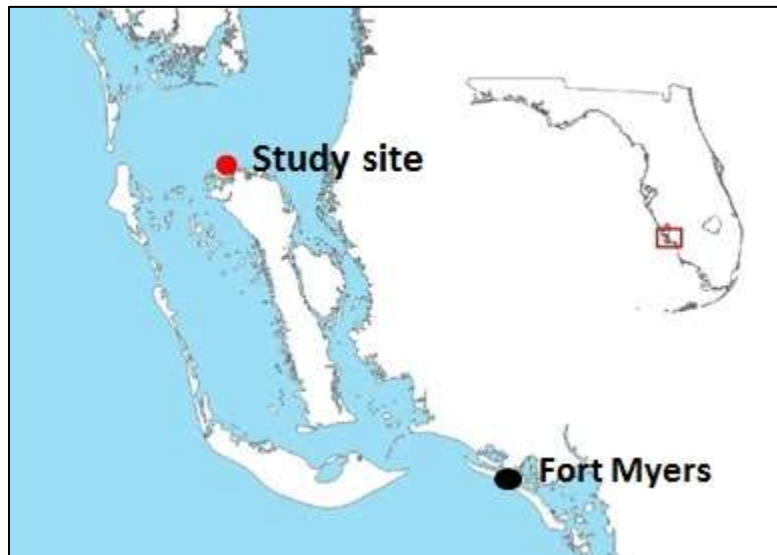


Figure 1 Pine Island, Florida, plotted in ESRI ArcMap 10.2.2



Figure 2. Sampling site at Bokeelia, Florida, on Pine Island, Lee County, Florida. Orange dots indicate sampling locations. Chemistry data were collected at purple points. Sample points are plotted on an ESRI ArcMap basemap. Transect numbers are marked in boxes.

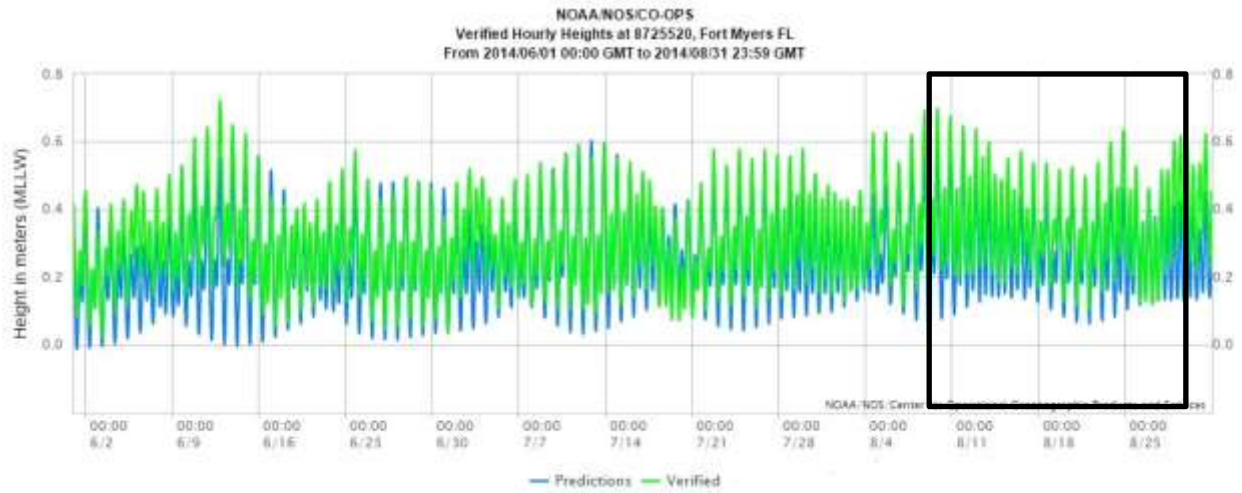


Figure 3. Water levels in the Fort Myers area preceding and during sampling. Sampling period within black box.

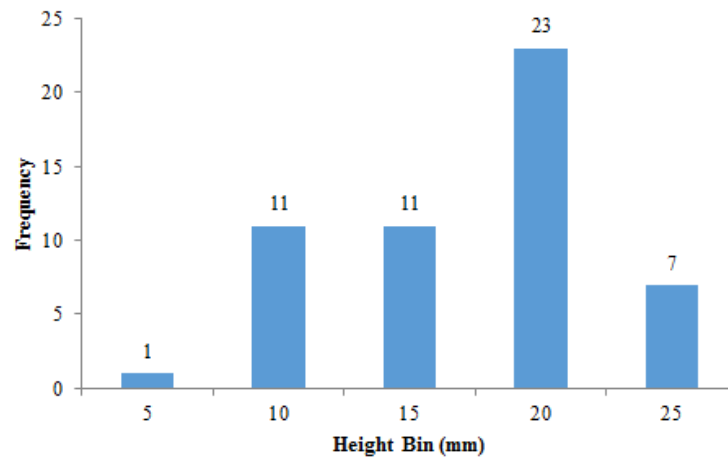


Figure 4. Size distribution of live *S. floridana*.retrieved from sediments at Bokeelia, Florida. Measurements were taken from commissure to hinge, the maximal anterior-posterior dimensions

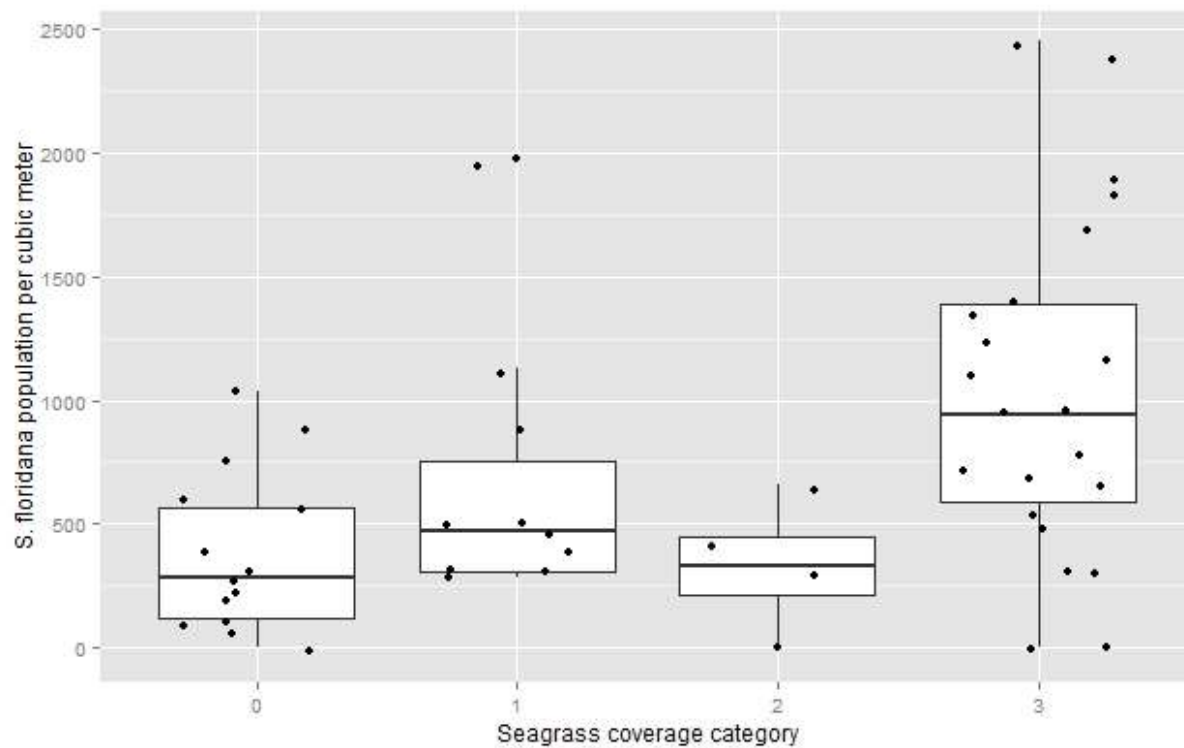


Figure 5. *S. floridana* population density per each seagrass coverage category. Significant positive correlation, $\rho = 0.41$, $p = 0.003$.

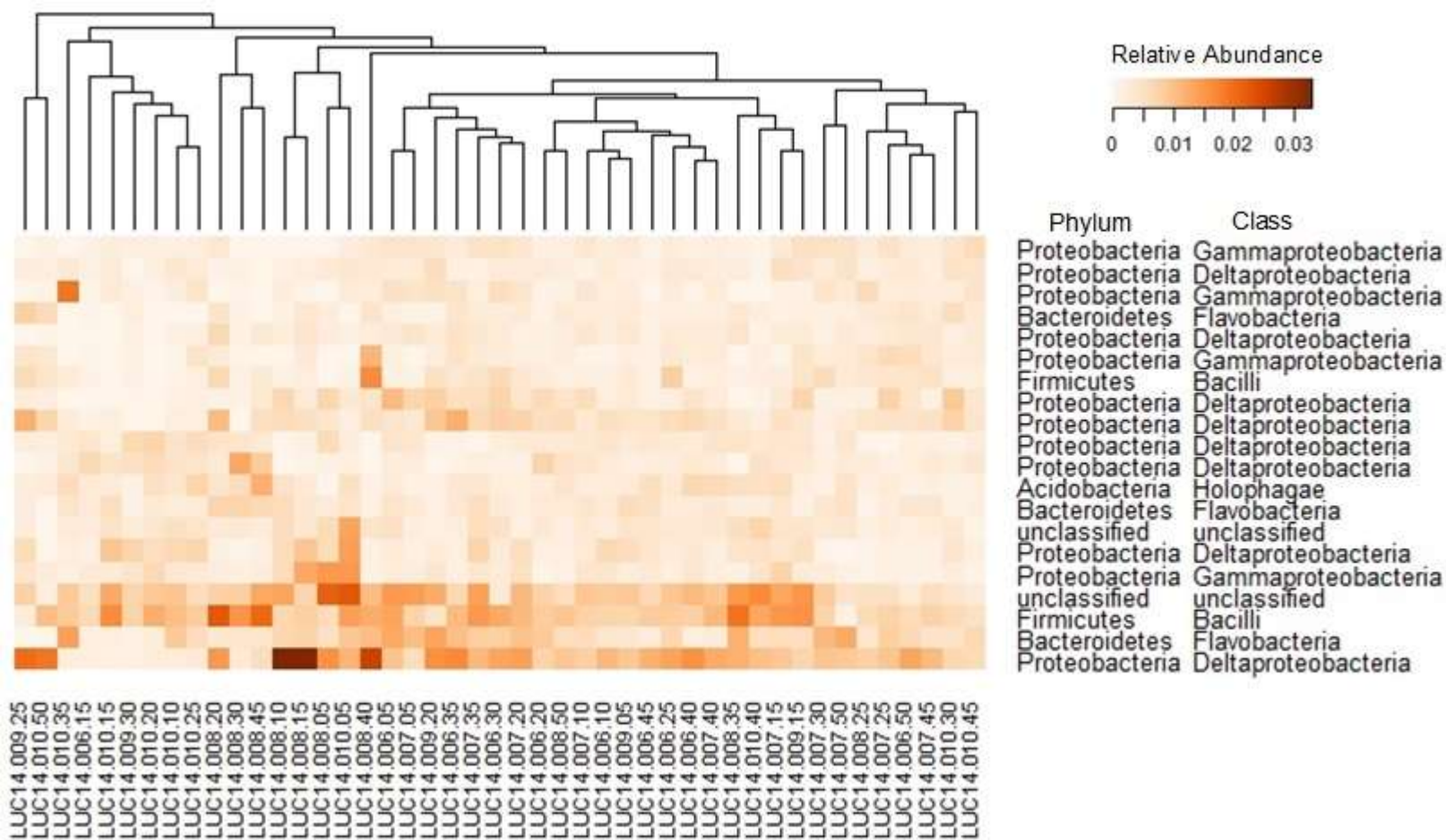


Figure 6. Heatmap of the relative abundance of the top twenty OTUs in sediment samples with Bray-Curtis dissimilarity dendrograms. Four samples removed as outliers.

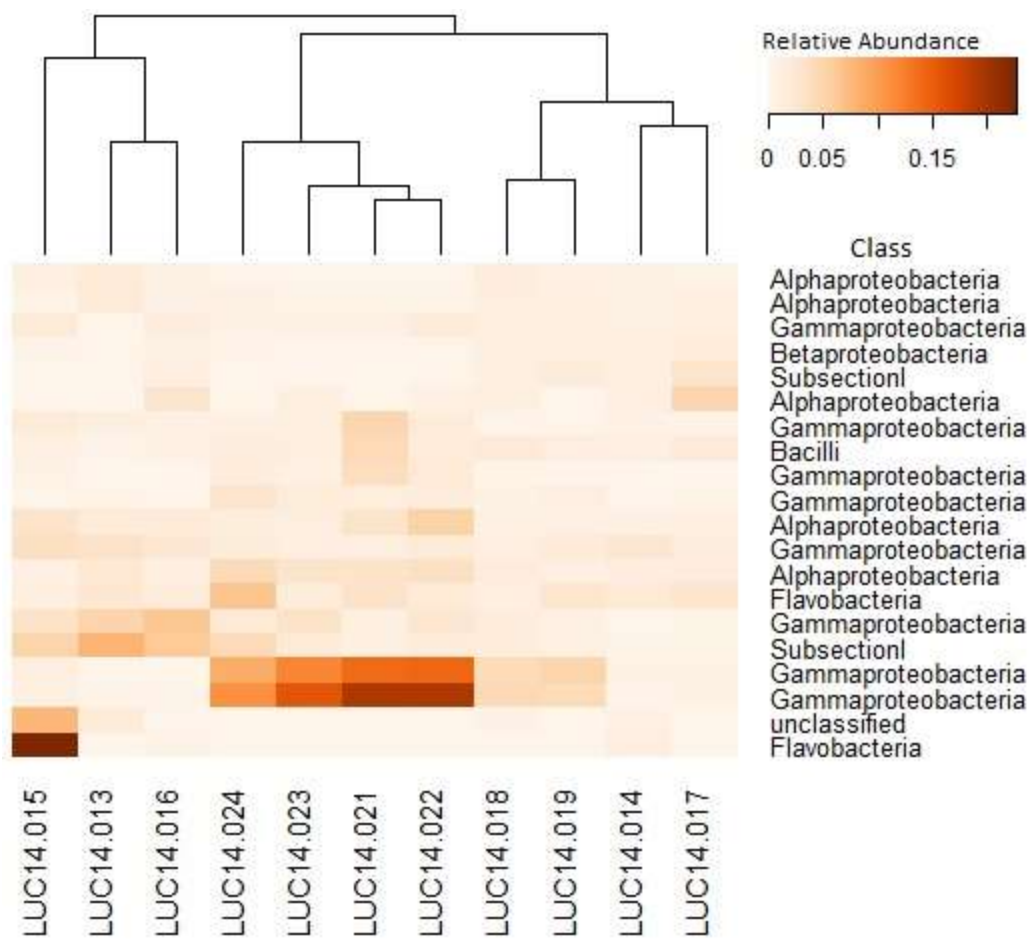


Figure 7. Heatmap of the relative abundance of the top twenty OTUs in pore water microbial samples with Bray-Curtis dissimilarity dendrograms.

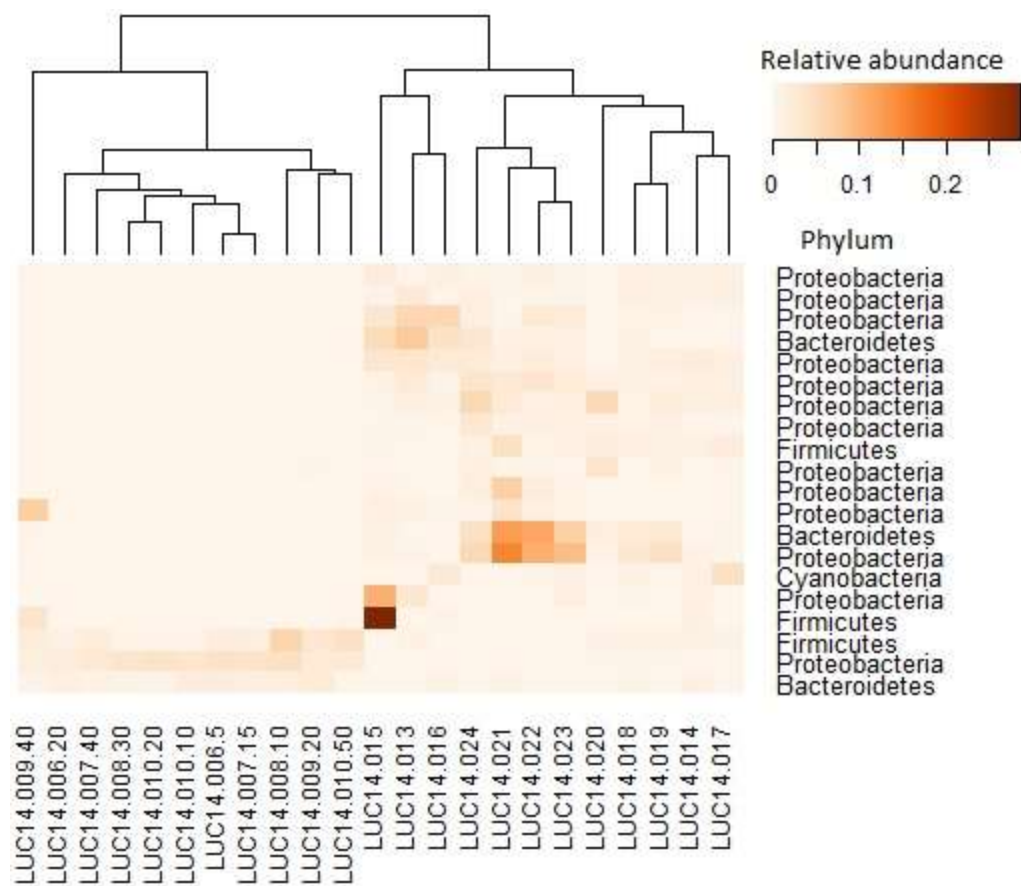


Figure 8. Heatmap of the relative abundance of the top twenty OTUs in porewater and sediment samples taken from the same sampling point with Bray-Curtis dissimilarity dendrograms.

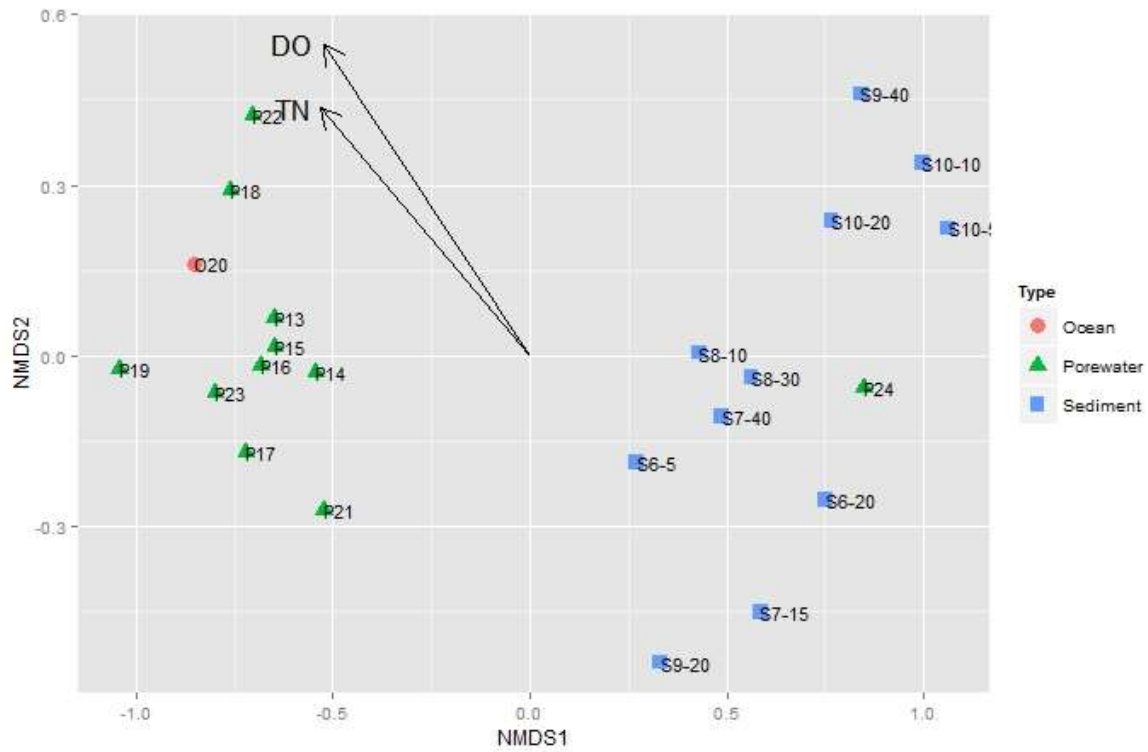


Figure 9. Sediment, porewater, and ocean microbial samples plotted in non-metric multidimensional space. Significant vectors were determined by vegan’s “envfit” function and include porewater total nitrogen and dissolved oxygen concentrations.

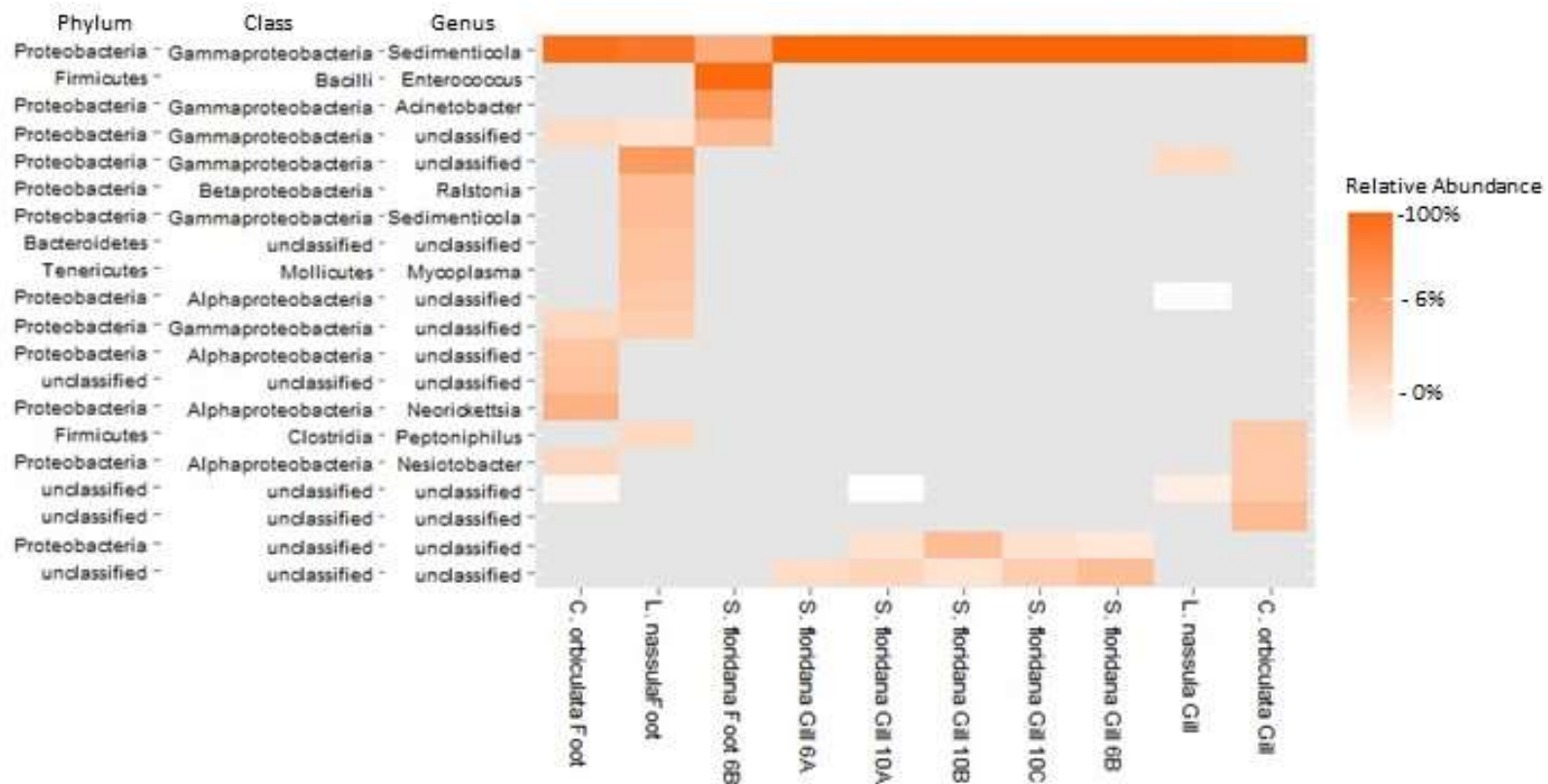


Figure 10. Relative abundance of OTUs in tissue samples at OTU sequence similarity level of 97%.

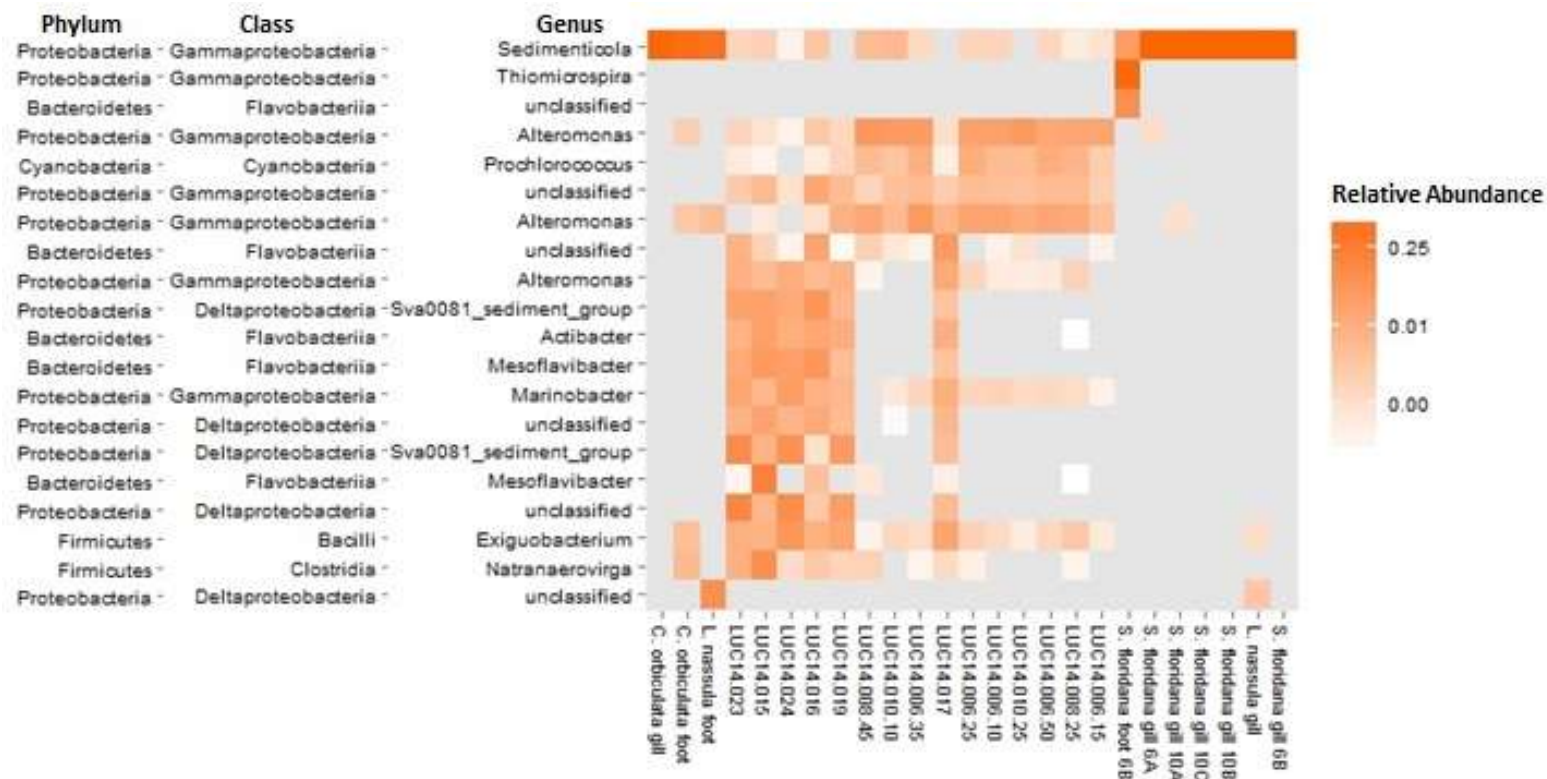


Figure 11. Top 20 OTUs shared by lucinid tissue samples, sediments, and porewaters.

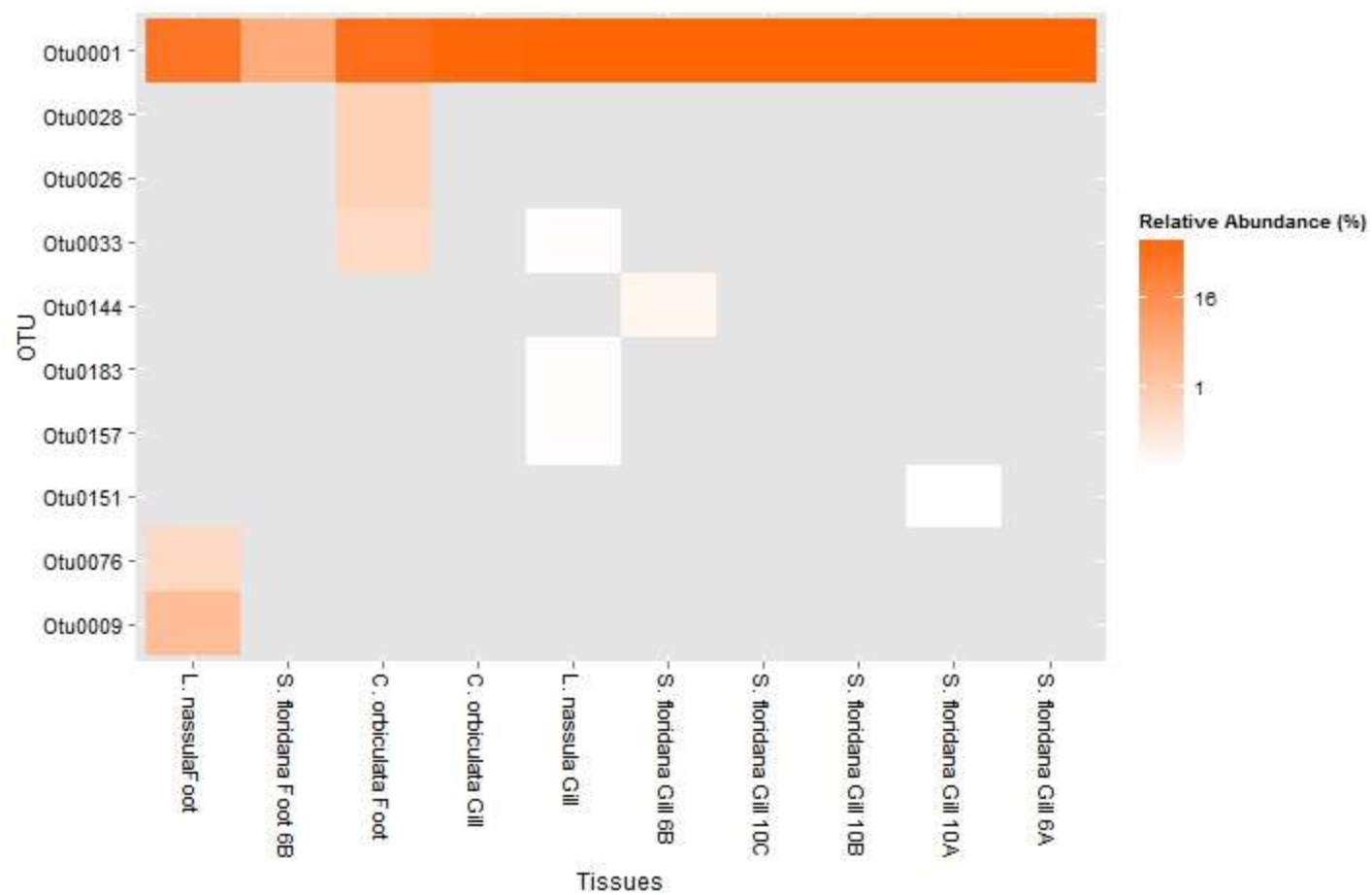


Figure 12. Relative abundance of different operational taxonomic units of the genus *Sedimenticola* within lucinid tissues. OTU sequence similarity threshold of 97%.

Appendix C: mothur pipeline

```
sffinfo(sff=111214AE27F1.sff, flow=T)
trim.flows(flow=current, oligos=LUC14_1.oligos, pdiffs=2, bdiffs=1)
shhh.flows(file=111214AE27F1.flow.files)
trim.seqs(processors=4, fasta=111214AE27F1_full.fasta, oligos=LUC14_1.oligos, maxambig=0, maxhomop=8, bdiffs=1, pdiffs=2,
minlength=200, flip=T)
align.seqs(fasta=current, reference=silva.bacteria.fasta)
screen.seqs(fasta=current, name=current, group=current, start=1044, minlength=200)
chimera.uchime(fasta=current, reference=self)
remove.seqs(fasta=current, name=current, group=current, accnos=current)
filter.seqs(fasta=current, vertical=T, trump=.)
classify.seqs(fasta=current, template=silva.bacteria.fasta, taxonomy=silva.bacteria.silva.tax, cutoff=80)
dist.seqs(fasta=current, output=lt, cutoff=0.15)
cluster(cutoff=0.15)
classify.otu(list=current, taxonomy=current)
make.shared(list=current, group=current)
```

Appendix D: SAS input

ANOVA and correlation matrix for *S. floridana* abundance and seagrass coverage

Data hold;

input TransectPosition Seagrass Halodule Syringodium Thalassia ClamCount DeadClamClamDensity SquareClam;
lines;

```
6 5 0 0 0 0 0 1 0.00.0
6 10 3 3 0 0 25 2 1473.738.4
6 15 3 3 0 0 12 8 707.4 26.6
6 20 0 0 0 0 3 2 176.8 13.3
6 25 1 1 0 0 5 5 294.7 17.2
6 30 3 0 3 0 14 2 825.2 28.7
6 35 3 0 3 0 19 0 1120.033.5
6 40 1 1 0 0 21 3 1237.935.2
6 45 1 1 0 0 5 0 294.7 17.2
6 50 1 0 1 0 3 0 176.8 13.3
7 5 0 0 0 0 6 1 353.7 18.8
7 10 0 0 0 0 3 6 176.8 13.3
7 15 3 3 0 0 26 5 589.5 24.3
7 20 3 3 0 0 10 5 176.8 13.3
7 25 1 1 0 0 3 1 648.4 25.5
7 30 0 0 0 0 11 6 766.3 27.7
7 35 3 0 3 0 13 4 294.7 17.2
7 40 3 0 3 0 5 0 0.00.0
7 45 2 0 1 2 0 5 0.00.0
7 50 3 0 2 2 0 6 58.9 7.7
8 5 0 0 0 0 1 0 353.7 18.8
8 10 3 3 0 0 6 1 235.8 15.4
8 15 2 2 0 0 4 0 176.8 13.3
8 20 3 3 0 0 3 0 707.4 26.6
8 25 1 1 0 0 12 0 176.8 13.3
8 30 2 2 0 0 3 10 235.8 15.4
8 35 0 0 0 0 4 0 0.00.0
8 40 3 0 3 0 0 0 471.6 21.7
8 45 3 1 1 3 8 3 176.8 13.3
8 50 1 1 0 0 3 26 530.5 23.0
9 5 0 0 0 0 9 0 117.9 10.9
9 10 0 0 0 0 2 3 117.9 10.9
9 15 0 0 0 0 2 0 117.9 10.9
```

```

9 20 0 0 0 0 1 0 58.9 7.7
9 25 0 0 0 0 1 0 58.9 7.7
9 30 1 1 0 0 9 0 530.5 23.0
9 35 3 0 3 0 10 3 589.5 24.3
9 40 3 2 2 1 7 2 412.6 20.3
9 45 3 1 1 0 8 1 471.6 21.7
9 50 3 3 1 0 20 1 1178.9 34.3
10 5 0 0 0 0 8 0 471.6 21.7
10 10 3 3 0 0 12 2 707.4 26.6
10 15 3 3 0 0 3 3 176.8 13.3
10 20 3 0 3 0 7 7 412.6 20.3
10 25 3 3 0 0 18 3 1061.0 32.6
10 30 0 0 0 0 6 12 353.7 18.8
10 35 2 2 0 0 7 2 412.6 20.3
10 40 3 3 0 0 15 1 884.2 29.7
10 45 1 0 1 0 4 4 235.8 15.4
10 50 1 1 0 0 5 0 294.7 17.2
;
Proc glm;
class Transect Site Seagrass;
model Clams = Transect Seagrass /P;
means Seagrass Transect / t lines;
run;
proc corr spearman;
var Clams Seagrass;
run;
Proc glm;
class Transect Site Halodule Syringodium Thalassia;
model Clams = Transect Halodule Syringodium Thalassia /P;
means Halodule Syringodium Thalassia Transect / t lines;
run;
proc corr spearman;
var Clams Halodule Syringodium Thalassia;
run;
Proc univariate normal data=hold;
var Clams;
run;

```

Appendix E: Spearman's rho correlation coefficients for relationships between *S. floridana* and seagrasses

```

data hold;
input Clam Distance pH DO Sulfide TNAlk Temp Salinity Cl SO4 Br Ca K MgNa NH4 LOI DOC;
lines;
0 5 7.5 0.13 0.09 2.04 176.168 29 35 0.45217 0.02265 0.00042 0.01027 0.00955 0.05218 0.43823 0.00102 0.94 27.57
13.29807598 20 7.75 0.2 0.03 0.84 125.172 30 34 0.52503 0.02638 0.00050 0.01086 0.01028 0.05617 0.47141 0.00075 0.71 24.14
39.14847569 40 8.31 0.21 0.01 1.07 145.668 31 34 0.44 0.02150 0.00040 0.00750 0.00704 0.03860 0.32104 0 0.43 28.37
17.16774228 15 7.75 0.2 0.1 1.52 167.872 32 34 0.53033 0.02673 0.00050 0.01015 0.00958 0.05233 0.43949 0.00072 0.8 22.26
18.80631941 10 7.8 0.09 0.05 1.03 157.136 30 33 0.51791 0.02625 0.00049 0.00536 0.00502 0.02822 0.24208 0 0.33 24.27
13.29807598 30 7.48 0.08 0.03 0.9 146.4 31 27 0.52162 0.02639 0.00049 0.00928 0.00878 0.04780 0.40076 0.00078 0.82 24.04
7.67764775 40 7 0.1 0.04 0.67 148.596 31 27 0.44817 0.02195 0.00041 0.01026 0.00970 0.05305 0.44744 0 0.27 0.24
20.3131466 20 7.73 0.08 0.09 0 143.472 30 33 0.52022 0.02630 0.00050 0.01036 0.00981 0.05354 0.45074 0.00072 0.24 24.03
26.59615197 10 7.83 0.11 0.1 1.08 163.236 31 32 0.44683 0.02183 0.00043 0.00974 0.00914 0.04961 0.41450 0.00087 NA 26.38
20.3131466 20 7.86 0.09 0.06 0.83 153.232 32 34 0.52369 0.02658 0.00053 0.00643 0.00611 0.03292 0.27471 0.00069 NA
24.84
17.16774228 50 7.78 0.13 0.08 0.94 156.163 32 35 0.54397 0.02743 0.00055 0.01087 0.01029 0.05520 0.45211 0.00144 NA 25.99
;
proc corr spearman;
var Clam Distance pH DO Sulfide TNAlk Temp Salinity Cl SO4 Br Ca K MgNa NH4 LOI DOC;
run;

```

Vita

Aaron Goemann was born and raised on his family's farm in South-Central Minnesota. His parents encouraged him to read, write, do math, and work hard from an early age. Annual family vacations broadened Goemann's awareness of the world and exposed him to the natural wonders of the North American continent. The lessons and experiences provided to Goemann by his family laid the foundation for a future scientist.

The efforts of his family encouraged Goemann to pursue higher education in science and culture at the University of Minnesota, Morris. During his time as an undergraduate student, he busied himself as a student-athlete, Community Adviser, student-government activist, research assistant and independent researcher, and enjoyed summer employment with the U.S. Fish and Wildlife Service. The varied activities Goemann was involved in challenged him, demanded a diversity of responsibilities from him, and required interactions with a diverse array of people. Those experiences, and recent experiences as a graduate student, have reinforced in him the importance of taking a positive and mindful stance when communicating and working with others and taught him the necessity of being concise and transparent about personal and professional issues at home and in the workplace.

Academic lives, activities, and interactions often cross personal and professional boundaries. In this unique setting, it is exceptionally important that faculty, staff, and graduate students at academic institutions be mindful of those boundaries and be transparent about expectations with regard to interpersonal communication and the commitment of time and labor to research, service, and teaching.

Today, we realize that mental health is an urgent and little-understood issue within populations of high school and college students, military veterans, and within society at large (Soet & Sevig 2006; Corrigan et al. 2012; Schomerus et al. 2012). College counseling services are currently dealing with student populations that exhibit a wider range of mental disorders and disorders of greater severity than in the past, putting unanticipated burdens on counseling services as well as academic faculty and administrative staff (Watkins et al. 2012). Depression and anxiety disorders rank among the most common experienced by college student populations (Soet & Sevig 2006; Cranford et al. 2009). Recent research indicates that mental health plays a significant role in the academic and economic success of students ranging from high school to doctoral (Hyun et al. 2006; Eisenberg et al. 2009). Out of a sample of over 3000 graduate students, 46-50% reported feeling frequently or chronically overwhelmed or exhausted and had experienced at least one significant emotional or stress-related problem in a period of 12 months (Hyun et al. 2006). Factors implicated in the mental health of this group of students included the nature of student-advisor relationships and the financial confidence of students, where positive student-advisor relationships were correlated to better student mental health and financial stress was correlated with poor mental health (Hyun et al. 2006).

Goemann believes that the expectations advisors and instructors have of their graduate students and the expectations graduate students have of their advisors and instructors be clearly expressed between these groups at every opportunity. A transparent understanding between advisors, instructors, and students, of preferences in interpersonal communication and application of effort to activities including research, service, and teaching would contribute greatly to reducing the stress experienced by faculty, staff, and students. Open, mindful communication can alleviate many problems that many graduate students, staff, and faculty face.