Distinct Microbiomes in three Tropical seagrasses around the island of Curaçao: *Halophila stipulacea, Halodule wrightii* and *Thalassia testudinum*.

Name: Tamara Stuij
Student number: 4015991
1st supervisor: J.B.M (Jack) Middelburg, department of Earth Sciences, UU
2nd supervisor: A. (Aschwin) Engelen, MAREE Marine Ecology and Evolution department, CCMAR, Universidade do Algarve

Seagrasses represent the unique re-colonization of the marine ecosystem by angiosperms. As their terrestrial relatives, seagrasses are important habitat providers but in contrast their microbiomes are still poorly known. The microbial community associated with terrestrial plants is intensively studied and plays an important role in plant fitness.The close relation of seagrasses to terrestrial plants suggests resemblance in survival strategies, including the creation of a microbiome distinct of the surrounding environment. To obtain more knowledge regarding seagrass microbiomes and their intra- and interspecies differentiations, samples of three tropical seagrass species occurring around the island of Curaçao, the invasive *Halophila stipulacea* and the natives *Halodule wrightii* and *Thalassia testudinum,* were collected. Root and leaf associated microbes were separately analyzed using high-throughput Illumina sequencing of the region V5-V7 of the 16S rRNA gene. Sequences were aligned and clustered into Operational Taxonomic Units (OTUs). Results displayed the occurrence of a seagrass-specific microbiome, distinct from that of the surrounding seawater and sediment. The existence of a species and tissue (root/leaf) specific bacterial community and structure was detected, along with a bacterial community that was shared among the seagrasses. OTUs belonging to the shared seagrass community were mostly of the orders rhizobiales. Desulfobacterales was the most abundant order associated with the roots and Rhodobacterales with the leaves of the three seagrass species. Species specific bacteria are represented mostly by OTUs of the same orders as the common OTUs, along with a few species specific orders. The high abundant and widespread bacterial OTUs were identified to be mostly associated to sulfur and nitrogen cycling, which point towards the importance of these processes in seagrass fitness.

1 INTRODUCTION

As soon as a plant colonizes a certain habitat, a complex system of symbiotic interactions between the emerging seedling and the environment will start to form, creating a holobiont. A holobiont contains a wide variety of microbes which are associated with a certain host organism (Sánchez-Cañizares *et al*., 2017). A plants holobiont can be spatially determined as the rhizosphere (the soil influenced by the root); the rhizoplane (microbiota attached to the plant root); phyllosphere (total above ground part of the plant) and the endosphere

(microbiota inside the plant) (Sánchez-Cañizares *et al.*, 2017). Recently, many studies have been conducted on composition, activity, and the role of microbial communities associated with plants, which show microbial symbionts can confer stress tolerance (Redman, 2002; Zhang et al., 2008), increase nutrient uptake and fitness (Chaparro *et al.*, 2014; Egamberdieva, 2008), and prevent pathogen attack to a wide variety of diverse plant hosts (Bulgarelli et al., 2012) .

The structure of a plants microbiome is determined by a range of abiotic and biotic factors, like the environment (soil/air/water), the geno-/phenotype and the development stage of the plant (Chaparro *et al*., 2014; Hardoim *et al*., 2015). Terrestrial plants gain the biggest part of their rhizosphere microbial community from the soil, the upper layer of sediment in which plants grow. The soil bears a wide variety of bacterial groups, which differs due to variation in sediment characteristics. Plants supply the soil with exudates released by their roots. Thereupon, specific bacterial groups which can utilize these substances cluster in the plants rhizosphere (Bulgarelli *et al*., 2013; de Souza *et al.*, 2016; Lundberg *et al.,* 2012; Wagner *et al.,* 2016). Differences in the soil community, the root system, exudates, and mucilage between monocotyledons and eudicots lead to differences in microbiota (Sánchez-Cañizares *et al.,* 2017), by which the plant creates a microbiome that differs among plant species and from the surrounding microbial community (Edwards *et al.,* 2015; Lundberg *et al.*, 2012; Wagner *et al.,* 2016). The phyllosphere microbial community is showed to be distinct from the rhizosphere microbiome (Beckers et al., 2017). The source is unlike the rhizosphere communities less defined (Vorholt, 2012). Suggested is a community composed of partly air- and soil-borne microbes.

Until recently, the main focus of research concerning the microbiome of plants covered terrestrial plants. Much less is known about the bacteria associated with marine plants. Seagrasses represent the recolonization of the marine habitat by flowering plants (angiosperms) by three independent events over four families: *Posidoniaceae, Cymodoceae, Hydrocharitaceae* and *Zosteraceae* (Olsen *et al.*, 2016). It is one of the biggest habitat shifts known by vascular plants (Hemminga & Duarte, 2000). Seagrasses are able to survive constantly submerged, tolerating high salinity levels and anoxic conditions. They fulfill important functions in coastal marine ecosystems, as they capture sediment and function as nursery grounds for fish and invertebrates (Laffoley & Grimsditch, 2009; Nellemann *et al.*, 2009). Unfortunately, seagrass meadows are vulnerable ecosystems. Due to (anthropogenic) changes in their habitat seagrass meadows are decreasing (Chapin *et al*., 2000). Given the important function of microbiome interactions in terrestrial plants, it is interesting to determine the role of bacteria in seagrass meadows.

Sediments colonized by seagrasses are often low in oxygen and high in organic compounds, which stimulates the growth of sulfate reducing bacteria, the predominant bacteria involved in anaerobic degradation of organic matter in coastal marine sediments (Jørgensen, 1982). They use sulfate as a substrate, by which they produce high amounts of hydrogen sulfide, a strong phytotoxin which makes the sediment highly reduced. Hydrogen sulfide is toxic to eukaryotic organisms, resulting in die-back events of seagrasses worldwide (Borum *et al*., 2005; Holmer & Nielsen, 1997; Pedersen *et al*., 2004). Completion of the sulfur cycle by sulfate oxidizing bacteria will lower the hydrogen sulfide levels, detoxifying the sediment, increasing seagrass fitness. The in general anoxic seagrass sediments can locally contain oxygen released from root tips by diffusion (Holmer *et al*., 2002). This stimulates the growth of sulfate oxidizing bacteria which use oxygen as a terminal electron acceptor (Hasler-Sheetal & Holmer, 2015; Jensen *et al*., 2007; Pedersen *et al*., 2004). Accordingly, the seagrass rhizosphere of European seagrasses is shown to be dominated by bacteria involved in the sulfur cycle, with *Alpha-, Gamma-, Delta-, Epsilonproteobacteria,* and *Actinobacteria* as successful root colonizers, whereas in the bulk sediment sulfate reducing *Deltaproteobacteria* represent the dominant group (Cifuentes *et al*., 2000; Cúcio *et al.*, 2016;

Fahimipour *et al.,* 2017; Jensen *et al*., 2007). Another important process in the often oligotrophic seagrass sediments is nitrogen fixation, by which atmospheric nitrogen becomes available for the plant. High nitrogen fixation rates and abundances of potential diazotrophic microbes have been detected in seagrass meadows(Lehnen et al., 2016).

Further support of the seagrass roots ability to "select" for certain microbes is given in a comprehensive analysis of microbial communities associated with *Zostera marina*. The microbiome of the roots is stable over different individuals and different from the surrounding sediment. When different beds were compared, the microbiota of the root to the adjacent sediment was not more different then from the root to a distinct sediment. Leaves however, showed higher similarity with the surrounding water within a bed compared to other beds. Leaf bacterial assemblages are variable per individual and resemble the water microbiota (Fahimipour *et al.*, 2017). Two studies, which compared the microbiomes of different seagrass species, confirm a seagrass microbiome distinct from the environment, but found inconsistent results regarding the existence of a species specific microbiome(Cúcio et al., 2016; Martin et al., 2018). Furthermore, seagrass microbiomes from two distinct locations revealed distinct microbial communities, which suggests seagrass microbiomes are, on a long scale, shaped by environmental differences (Cúcio et al., 2016).

So far, studies regarding the microbiome of seagrasses, have mainly focused on European seagrass species and mostly species of the genus *Zostera* (Cúcio *et al.*, 2016; Jensen *et al*., 2007). Just a few studies contain information concerning the microbial diversity of tropical seagrass beds, but ignore seagrass-species-specific differences (Bagwell *et al*., 2002). Tropical waters differ from temporal waters in temperature, nutrient availability (lower in tropical waters) and sediment type. How these different environmental conditions in tropical waters influence the microbiota and how/if this relates to the seagrass microbiome has yet to be evaluated.

Here we report a detailed analysis of the bacterial communities associated with the leaf (joint phyllosphere and endosphere) and root (joint rhizoplane and endosphere) microbiomes of two native seagrass species *Thalassia testidinum, Halodule wrightii* and an invasive species *Halophila stipulacea* (Smulders *et al.*, 2017; Willette & Ambrose, 2012) collected at six different bays around the island of Curaçao. By using Next Generation Sequencing (NGS), the goal is to tackle differences in diversity, community composition and structure among species, locations and plant structures (leaves/roots) aiming to detect the possible driving forces behind microbial assemblage.

Figure 1 Studied seagrass species: Thalassia testidinum (a), Halophila stipulacea (b) and Halodule wrightii (c).

2 METHODS

2.1 SAMPLING

Samples were collected in January 2017 with an average seawater temperature varying between 28 to 32 degrees Celsius. Three seagrass species were collected: the native *Thalassia testidinum* and *Halodule wrightii* and the invasive *Halophila stipulacea.* The three species were obtained from six different locations around the island of Curaçao (Table. 1). At each location three replicates/species were collected (separating roots and leaves) along with sediment and seawater samples, compiling a total of 112 samples (Table 1). Individuals were uprooted, washed free of sediment and anything loosely attached within the seawater of their habitat. Excess seawater was shaken off and differentiated tissue samples (leaves and roots) were directly preserved in the field in 2ml eppendorfs with 0.5ml Xpedition™ Lysis/Stabilization Solution (Zymoresearch, California). In this way, the rhizoplane, phyllosphere and endosphere were conserved for further measurements. Sediments collected had a volume of roughly 1 ml and represented the sediment at the root depth of the respective seagrass. Seawater microbial communities were collected upfront of any sample by filtering 0.5L of seawater, from the water column on top of the respective meadow, over 0.2 µm filters. Filters were preserved the same way as seagrasses and sediment samples.

Table 1 Sample overview collected at each sample location per species (leaves + roots) together with seawater and sediment samples per site.

Location	*T. testudinum*		*H. wrightii*		*H. stipulacea*		Environmental	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Seawater	Sediment
Ascension 12º16'24.24N 69º03'08.53W	3	3	3	3			2	4
St. Marta 12º16'33.01N 69º07'18.21W					3	3	2	3
Piscadera 12º08'06.32N 68º37'37.75W	3	3			3	3	2	3
Jankok 12º12´15.43N 69º03'09.33W	3	3	3	3	3	3	3	3
Spanish Water 12º03'57.95N 68º51'12.92W	3	3	3	3	3	3	4	4
St. Joris 12º07'36.59N 68048'56.82W	3	3			3	3	1	3
Total	15	15	9	9	15	15	14	20

2.2 DNA EXTRACTION, BACTERIAL COMMUNITY ANALYSIS AND CHARACTERIZATION

Total genomic DNA was extracted from all samples using the Quick–gDNA kit (Zymo Research™) according to the manufacturer protocol for “Solid Tissue Samples” (page 4 of the manual).

To validate taxonomic identification, samples were barcoded using the nuclear ribosomal DNA internal transcribed spacer (ITS) covering the region between the 18S rDNA and the 26S rDNA including ITS-1, 5.8S nrDNA and ITS-2 and using the universal primers ITS1 and ITS4 (White *et al*., 1990). PCR conditions followed those of Kumar & Shukla (2005): 95 °C for 10 minutes, 30 cycles of 95 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1 minute, and a final elongation of 72 °C for 10 minutes. The 25 µl reaction mixture contained 200 µM dNTPs, 0.2 µM of each primer, 1× PCR buffer mix, 2 µl of template DNA and 0.25 µl of Taq polymerase. Sequences were then outsourced for Sanger sequencing and further visualized and edited using Geneious (http://www.geneious.com,Kearse *et al*., 2012).

For bacterial community characterization, the total 16S rRNA was amplified using the universal primers 27F and 1492r with the following changes to the original protocol (Lane, 1991): an initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 3 min. The 25 µl reaction mixture contained 250 µM dNTPs, 0.6 µM of each primer, 1× 2 PCR buffer mix, 2 µl of template DNA (with a final concentration of about 10 ng µl−1) and 0.3 µl of Taq polymerase (Advantage R2 Clontech). PCR products were cleaned using ExoFastAP enzyme following the Thermo Scientific™ protocol. Amplified DNA was sent to Molecular Research (MR DNA), Shallowater, Texas where a nested–PCR was performed prior to sequencing. The modified 8 bp key–tagged primer 799F along with the reverse primer 1193R, covering the regions V5-V7 from 16S rRNA and amplifying a fragment of ~ 400 bp, were used to avoid chloroplast cross amplification (Bodenhausen *et al*., 2013). PCR conditions were as follow: 95 °C for 3 min, 10 cycles of 95 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 3 min. Samples were pooled together in equal proportions based on their molecular weight (calculated based on the size of the amplicon) and DNA concentrations (using Qubit™ Invitrogen®) and purified using calibrated Agencourt® AMPure® XP beads. DNA libraries were prepared by following Illumina TruSeq DNA library preparation protocol and paired–end (2 x 250 bp) sequencing performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

Sequencing data was processed using QIIME version 1.8. (Caporaso *et al*., 2010) and clustered into operational taxonomic units (OTUs) at >97% similarity using open-reference OTU picking with the UCLUST algorithm against the SILVA reference database (version 128, www.arb-silva.de). After selection of one representative sequence per OTU the database was aligned and chimeras were removed. Taxonomy was assigned using SILVA database. From the created OTU table, eukaryotic sequences (e.g. chloroplasts), singletons and doubletons were removed.

2.3 STATISTICAL ANALYSIS

Statistical analyses and data visualization were based on the resulting OTU table using PRIMER6 & PERMANOVA and Rstudio version 3.4.2(Clarke & Gorley, 2006; R Development Core Team, 2016).

To determine α-diversity indices, the OTU table was rarefied to the minimum number of sequences (29021 sequences of sample PisHs2). Bacterial richness was displayed by the number of OTUs and Shannon-index (Shannon & Weaver, 1949) was used to include evenness. Data was tested for normal distribution with the Shapiro-Wilk test in R (version 3.4.2). As data was not normally distributed, PERMANOVA (Permutational multivariate ANOVA) was used to determine significant differences ($P < 0.05$) between the different sample groups using Euclidean distance (PRIMER6 & PERMANOVA, number of permutations was set to 999)(Anderson, 2001; Gower, 1982). A Monte Carlo test was done when the number of unique permutations was less than 900 (Metropolis & Ulam, 1949). Boxplots were created using the function boxplot in R (version 3.4.2), in which samples of the same seagrass species and tissue type where treated as biological replicates and were plotted together as one group. The deviation of the centroid was calculated using PERMDISP (PRIMER6 & PERMANOVA), to detect differences between variances of the sample groups.

Bacterial community composition and structure was assessed through OTUs presence/absence and square root transformations, respectively, after which a resemblance matrix was compiled using the Bray-Curtis dissimilarity measure (Bray & Curtis, 1957).

Statistical analysis were done using a three-factor PERMANOVA: species (fixed factor with 3 levels), tissue (fixed factor with 2 levels), and location (random factor with 6 levels). First a main test was carried out after which more specific differences were analyzed in pairwise tests which compared two or three factors. Monte Carlo test was done when the number of unique permutations was less than 990. Sample groups were considered significantly different if $P < 0.05$. After comparisons on OTU-level, the data was treated identically, changing the taxonomic level from OTU-level to genus level. Community composition and structure tests were conducted in PRIMER6 & PERMANOVA, community differentiations were visualized with Canonical analysis of Principal coordinates (CAP) plots based on the resemblance matrix obtained from the Bray-Curtis dissimilarity measure.

Differential abundance (Deseq function, Deseq2 package, R) and indicator species analysis (multipatt function, indicspecies package, R) were used to identify the specific genera causing the detected differences. The positive predictive value (A) and the sensitivity (B) for selecting indicators were both set to be > 0.6.

Core microbiome was determined using the non-rarefied OTU table. A microbe was labeled as part of the "core" when it occurred in at least 80% of the samples of a defined group (e.g. species or tissue). Furthermore, all microbes with a relative abundance of more than 0.01% in seawater or sediment were labelled as seawater- /sediment-like microbes, and were removed from the initial seagrass core results. Established "cores" per species-tissue were displayed in VENN diagrams to observe the similarities and dissimilarities. Barplots were created to visualize the microbial taxa occurring in the core microbiomes.

3 RESULTS

The complete dataset (all seagrass and environmental samples) resulted in 9,465,162 sequences in which a total of 560,855 OTUs were detected (after removal of one sample due to an extremely low amount of counts). After chimeric, unassigned and chloroplasts sequences removal, the OTU table consisted of 8,908,965 sequences distributed through 445,744 unique OTUs. A considerable part of these OTUs represented singletons and doubletons (42%) and were removed to avoid possible sequence artifacts. After removal of these single- and doubletons the dataset contained a total of 257,757 unique bacterial and 16 Archaea OTUs out of 8,533,147 sequences. Around 85 % percent of the detected bacterial OTUs belonged to the phyla Proteobacteria (62.91 %), Bacteriodetes (17.61 %) and Actinobacteria (4.98 %) (Fig. 2). Another 10 % is represented by Planctomycetes (2.21%), Acidobacteria (2.03%), Firmicutes (1.83%), Spirochaetae (1.36%), Fibrobacteres (1.01%), AC1 (0.71%) and Deferribacteres (0.64%). The remaining 5% is composed of a variety of 52 less abundant phyla (4.55%) and unidentified bacterial sequences (0.66%).

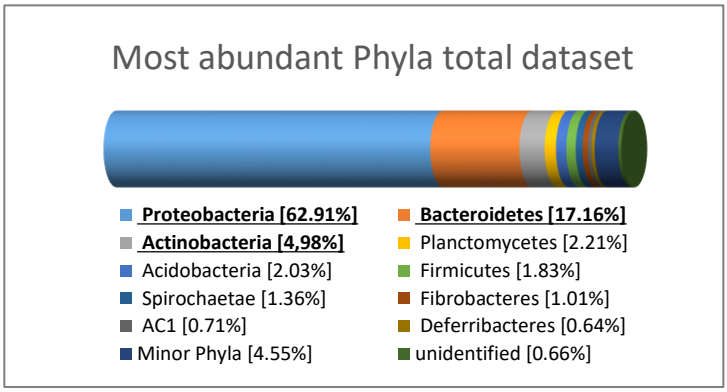

Figure 2 Bacterial Phyla composition of the complete dataset.

3.1 ALPHA DIVERSITY

Rarefaction resulted in a dataset containing a total amount of 232,238 unique OTUs. Alpha diversity estimated as OTU number and Shannon index (H') differed among species (P_{OTU}

$_{number}$ = 0.002; $P_{H'}$=0.005) and between tissue-type ($P_{OTU\ number}$ =0.001; $P_{H'}$=0.001), regardless of the location of the samples ($P_{OTU\ number}$ =0.283; $P_{H'}$=0.343). However, when factors were combined (location x species/tissue), the location of the samples did show to have an effect ($P_{OTU\ number} < 0.05$; $P_{H'} < 0.05$, Supp. Table 1.1 & 1.4). Pairwise PERMANOVA tests gave more insight, by which the location-effect occurred sporadic over different comparisons (Supp. Table 1.3 & 1.6).

Constant over both the number of OTUs and Shannon diversity, seawater and sediment had the least and most diverse communities, respectively ($P_{OTU\ number}$ = 0.001; $P_{H'}$= 0.001, Fig. 3a and 3b). Furthermore, sediment was more diverse than all seagrass microbiomes and seawater was less diverse than all but *H. stipulacea* leaves (number of OTUs and Shannon index) and *H. wrightii* leaves (Shannon index, P < 0.05, Supp.Table. 1.2 & 1.5). Inter-species diversity differences were observed as *H. stipulacea* roots display high diversity measures compared to the other species ($P_{OTU\ number} < 0.05$; $P_{H'} < 0.05$, except for *H. wrightii* roots number of OTUs: $P_{OTU\ number}$ = 0.116; and *H. wrightii* leaves Shannon diversity: $P_{H'}$=0.071, Supp.Table. 1.2 & 1.5). According to leaves, the OTU number of *H. wrightii* roots were in the same diversity range as the roots of *H. stipulacea* (Fig. 3a, Supp. Table 1.2 & 1.5), whereas based on the Shannon index they equal the roots of *H. wrightii* with the other seagrass microbiomes and showed a relative increase of *H. wrightii* leaves with respect *H. stipulacea* ($P_{H'}$=0.071).

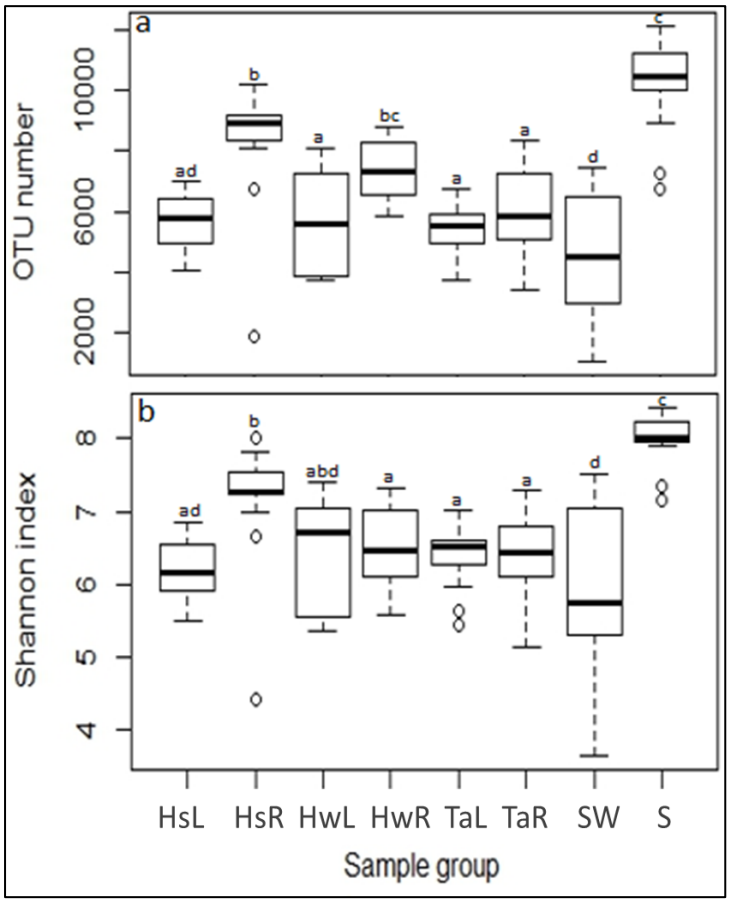

Figure 3. Alpha diversity estimates by number of OTUs (a) and Shannon index (b) of seagrass associated microbiomes from Curaçao, Netherlands Antilles. Sample groups display the three seagrass species per tissue type (Leaf and Root), seawater and sediment. HL: H. stipulacea Leafs (n=15), HR: H. stipulacea Roots (n=15), SyL: S. filiforme Leafs (n=9), SyR: S. filiforme Roots (n=8), TaL: T. testudinum Leafs (n=15), TaR: T. testudinum Roots(n=15), SW: seawater (n=14) and S: sediment (n=20). Boxplot values are given by the minimum and maximum values (lower and upper whisker), the first (25%) and the third (75%) quartile (lower and upper hinge) and the median with a 95% confidence interval (the notch). Outliers are displayed as bullets. Differences were analyzed with PERMANOVA pair-wise tests (n=999 permutations), computed using the euclidean distance between the sample groups. Letters above boxplots represent similarity/dissimilarity among groups (sign. difference: P < 0.05).

3.2 COMMUNITY COMPOSITION AND STRUCTURE

Seawater, sediment and seagrass samples displayed a unique microbial community structure and composition which differed among seagrass species, tissue-type and location (P = 0.001). Furthermore, all possible factor combinations resulted in significant interactions

(SpeciesxTissue, SpeciesxLocation, TissuexLocations & SpeciesxTissuexLocation, P = 0.001, Supp. Table 1.7 & 1.17). Pairwise tests of these interactions revealed that seagrass bacterial communities differed between tissue-type in each species (0.001 > P < 0.002, Supp. Table 1.12 & 1.22), among all seagrass' roots and leaves and from the sediment/seawater (0.001 > P < 0.015, Supp. Table 1.11 & 1.21). Although less significant, communities also differed among almost all locations in each seagrass (0.002 > P < 0.05, Supp. Table 1.14 & 1.24). The CAP analysis based on a species-tissue classification correctly clustered 92,793% of the samples (Fig. 4). Seawater clustered distinct from the seagrass microbial communities, whereas sediment samples clustered more closely with the seagrass roots (Fig. 4).

The differences between root and leaf communities were further examined by a differential abundance analyzes, which revealed 642 significantly different abundant genera in leaf and root samples at a significance level of P = 0.05 (Deseq2, R). The forty most differential abundant genera were mostly composed by members of the order Desulfobacterales, which were high abundant in root samples, whereas members of the orders Rhodobacterales and Sphingobacterales were strongly leaf-associated (Fig. 5). Additional, the indicator species analysis revealed seagrass-species-tissue and environmental (sediment/seawater) related bacteria (Table 2). Overall, there were two indicator genera for *H. stipulacea,* three for *H. stipulacea* leaves, 6 for *T. testudinum* Roots, 2 for *T. testudinum* leaves, 2 for *H. wrightii* roots, and 3 bacterial genera for *H. wrightii* leaves (Table 2). The relative abundances of these indicator bacteria were low, as all were less than 0.2%, more widespread genera had higher relative abundances (Supp. Mat. 2). Sediment and seawater contained a wider variety of indicator bacteria (20) than the seagrasses (Table 2). Sediment shared many indicator bacteria with seagrass roots (95, Supp. Table 2.2), in contrast, seawater did not show many indicator bacteria in common with leaves (5, Supp. Table 2.2).

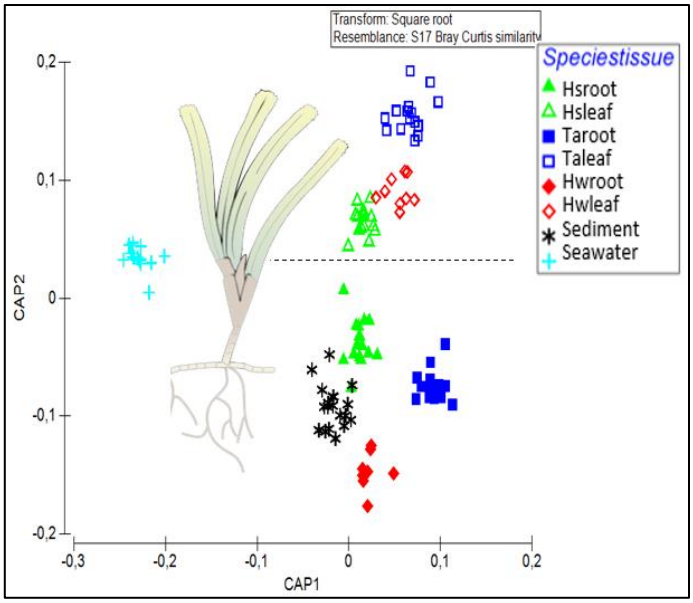

Figure 4. Canonical analysis of Principal coordinates (CAP) of microbiome communities of leaves and roots of three seagrass species and their environment (seawater and sediment) from six bays on Curaçao (Netherlands Antilles) using Bray-curtis similarity of the counts (square-root transformed) to the taxonomic level genus. Sample groups display the three seagrass species per tissue type, seawater and sediment. Hsroot: H. stipulacea roots (n=15), Hsleaf: H. stipulacea leaves (n=15), Taroot: T. testudinum roots (n=15), Taleaf: T. testudinum leaves (n=15), Hwroot: H. wrightii roots (n=8), Hwleaf: H. wrightii leaves (n=?), sediment (n=20) and seawater (n=14).

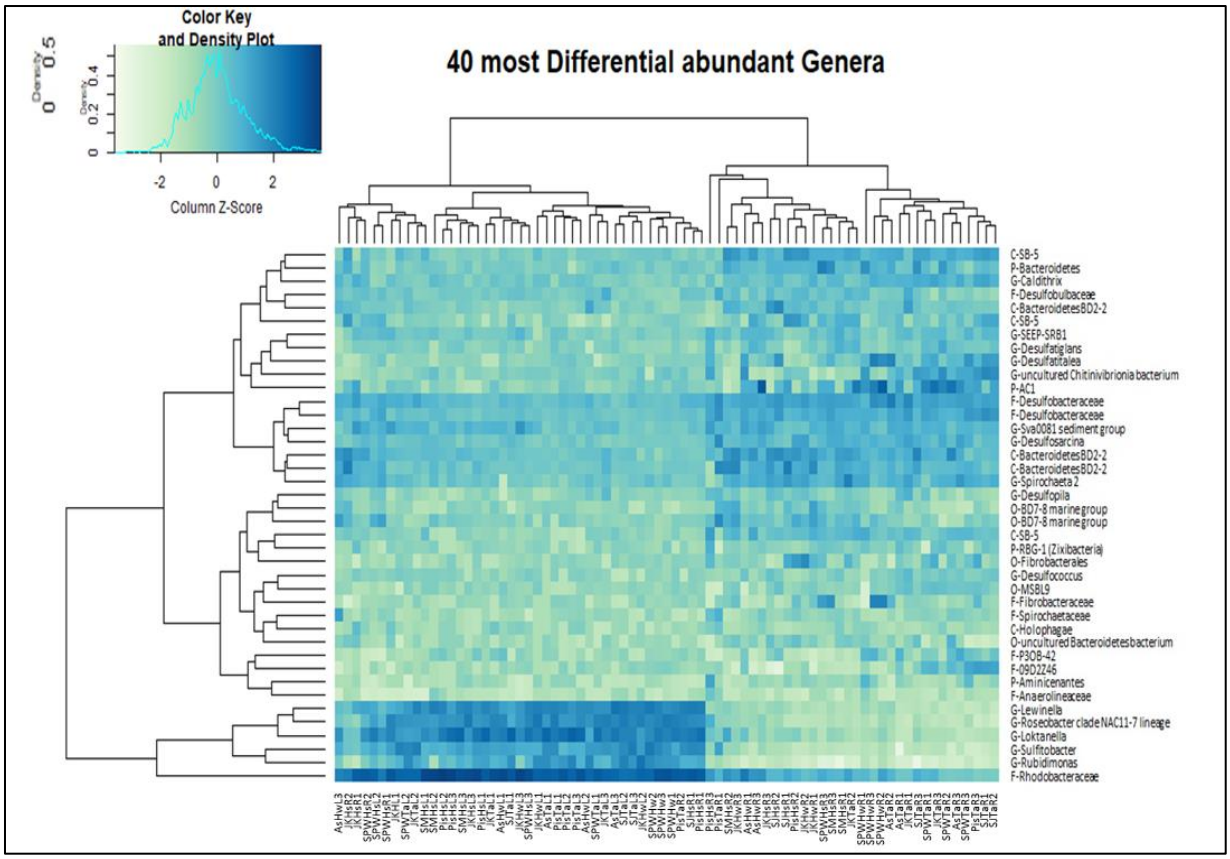

Figure 5. Heatmap of leaf and root samples (x – axis) of three seagrass species from Curaçao, Netherlands Antilles and their 40 most differential abundant genera (y – axis, Lowest adjusted p-values, DESeq2). Raw counts are transformed using a variance stability transform (vsd). OTUs are presented at their lowest known taxonomic level. Letter indicates taxonomy: G = Genus; F = Family; O = Order, C = Class and P = Phylum. The dendogram (on top) groups the samples based on their similarity and the dendogram (on the side) groups the bacteria based on their similarity. Sample names represent: location (As: Ascension, JK: JanKok, Pis: Piscadera, SJ: Sint Joris: SM: Sint Marta, SPW: Spanish Water, – species (H: H. stipulacea, Hw: H. wrightii, Ta: T. testudinum) – tissue (L: Leaf, R: root) – replicate (1,2,3).

Table 2. Indicator analysis results, giving the indicator genera associated with one specific group. The Minimum value for the positive predictive value (A) and sensitivity factor (B) was 0.6. P-value: degree of statistical significance of the associated bacteria. Relative abundance: mean relative abundance of the specific genera in the specified sample group.

Seagrass x tissue	Bacteria	A	B	p.value	rel. Abundance
***H. stipulacea* Roots**	G-Thalassolituus	0.6667	0.8333	0.001	0.000758072
	G-Rs-M59 termite group	0.6876	0.75	0.002	0.000629429
***H. stipulacea* Leaves**	F-Rhizobiales Incertae Sedis	0.855	0.8	0.001	0.000254988
	O-Rickettsiales	0.6607	0.9333	0.001	0.000652401
	G-Rhodococcus	0.7577	0.8	0.001	0.000128642
***T. testudinum* Roots**	F-uncultured sediment bacterium	0.6923	0.875	0.001	6.03012E-05
	F-uncultured soil bacterium	0.6918	0.875	0.001	0.000146446
	O-uncultured Codakia orbicularis gill symbiont	0.9078	0.625	0.001	4.30723E-05
	G-Candidatus Thiopilula	0.8884	0.625	0.001	9.90662E-05
	G-Campylobacter	0.8718	0.625	0.007	0.001262017
	G-Candidatus Riegeria	0.6546	0.75	0.010	0.001266324
***T. testudinum* Leaves**	O-Flavobacteriales	0.749	0.7778	0.010	0.001286425
	G-Dokdonia	0.6559	0.7778	0.001	0.000126345
***H. wrightii* Roots**	O-Fibrobacterales	0.8163	0.6111	0.001	0.000248096
	O-Fibrobacterales	0.6729	0.7222	0.001	0.00014702
***H. wrightii* Leaves**	G-Butomus umbellatus	0.8597	0.9333	0.001	0.000558216
	O-Rickettsiales	0.6431	1	0.001	0.000186072
	F-Caulobacteraceae	0.9377	0.6	0.001	0.000397413
Sediment	O-Xanthomonadales	0.8072	1	0.001	0.000680542
	O-Rhodospirillales	0.8758	0.9	0.001	0.000706385
	O-Acidimicrobiales	0.7823	1	0.001	0.000654698
	O-HOC36	0.8223	0.95	0.001	0.00098377
	G-Acidihalobacter	0.7874	0.95	0.001	0.000168843
	O-Gaiellales	0.7923	0.9	0.001	0.00036353
	G-Candidatus Entotheonella	0.6711	0.95	0.001	0.000659867
	F-Acidiferrobacteraceae	0.8237	0.75	0.001	0.000503084
	F-ABS-19	0.8192	0.75	0.001	0.000161952
	F-FW13	0.9411	0.65	0.001	0.000380759
	O-NB1-n	0.7282	0.8	0.001	0.000382482
	O-NB1-n	0.8284	0.7	0.001	0.000356638
	F-MSB-1E8	0.7466	0.75	0.001	0.000158506
	O-uncultured Acidobacteria bacterium	0.6453	0.85	0.001	0.000189518
	O-NB1-n	0.7828	0.7	0.001	0.00020847
	C-MACA-EFT26	0.7717	0.7	0.001	0.000163675
	G-Zeaxanthinibacter	0.7179	0.75	0.001	0.000601289
	F-C8S-102	0.7721	0.65	0.001	0.000179181
	G-Epulopiscium	0.7096	0.7	0.001	7.40843E-05
	C-BD2-11 terrestrial group	0.6138	0.6	0.002	8.95903E-05
Seawater	F-SAR86 clade	0.9615	1	0.001	0.009042713
	F-SAR86 clade	0.9492	1	0.001	0.009692489
	O-SAR11 clade	0.9452	0.9286	0.001	0.000236282
	F-SAR116 clade	0.9559	0.8571	0.001	0.000132909
	F-Surface 1	0.9537	0.8571	0.001	0.000534096
	F-Surface 1	0.9482	0.8571	0.001	0.000689156
	G-AEGEAN-169 marine group	0.9329	0.8571	0.001	0.000758072
	F-Mitochondria	0.9221	0.8571	0.027	0.006741424
	P-Marinimicrobia (SAR406 clade)	0.9202	0.8571	0.005	0.003864197
	F-Surface 1	0.9677	0.7857	0.001	0.000137831
	G-Candidatus Aquiluna	0.9415	0.7857	0.023	1.96902E-05
	F-OM182 clade	0.949	0.7143	0.001	0.003091357
	O-Bacillales	0.9599	0.6429	0.001	0.000297814
	F-Staphylococcaceae	0.8596	0.7143	0.001	0.000285508
	G-MWH-UniP1 aquatic group	0.9522	0.6429	0.001	0.001786883
	F-SAR116 clade	0.9402	0.6429	0.001	0.000162444
	O-Micrococcales	0.9396	0.6429	0.001	0.000706385
	F-Microbacteriaceae	0.9348	0.6429	0.008	0.00191487
	P-Firmicutes	0.8046	0.7143	0.003	0.000401187
	F-NS7 marine group	0.6408	0.6429	0.004	0.000147676

3.3 CORE MICROBIOME

The core microbiome of each seagrass species contained a small number of OTUs (Fig. 6). *H. stipulacea's* core microbiome was the most diverse, represented by 32 root and 75 leaf specific OTUs. *H. wrightii* contained 38 root and leaf OTUs, whereas *T. testudinum* had the least diverse core with 10 root and 8 leaf OTUs (Fig. 6). The seagrass core microbiome shared among all seagrass species consisted of 18 root specific, 48 leaf specific and 8 OTUs shared among both tissue-types. The 8 tissue-independent OTUs are Alpha- and Gammaproteobacteria of the orders Rhizobiales, Rhodobacterales (Alpha), Oceanospirillales and Thiotrichales (Gamma). Despite species specific differences in OTU core composition, the seagrass roots were characterized by Deltaproteobacteria of the family Desulfobacteraceae (33%). Furthermore, a variety of Bacteroidetes (22%) occurred along with phyla represented by one or two OTUs (Supp. Table 3.2). Seagrass leaves showed a variety of Rhizobiales (OCS116 clade and Phyllobacteriaceae) and Rhodobacteraceae (37.5%), with additional OTUs of the Saprospiraceae (12.5%), Alteromonadaceae (6.25%), Methylophilaceae (4%), and a diverse group of Gammaproteobacteria (13%) containing Alteromonadaceae; Arenicellaceae; Porticoccaceae; Ectothiorhodospiraceae (Supp. Table 3.3).

OTUs that were species specific were mostly of the same orders as the shared OTUs (Fig. 6, Supp. Table 3.1 t/m 3.9). Additionally, there were some species-specific orders. The roots of *H.* stipulacea contained an Ignavibacteriales and a Latescibacteria of unknown order, *H. wrightii's* microbial community embodies a Caldithrix (Deferribacteres) and an Ardenticatenia (Chloroflexi, unknown order). The leaves of *H. stipulacea* showed occurrence of a Rhodococcus (order Corynebacteriales) and a Kordiimonas (order Kordiimonadales), whereas *H. wrightii* harbors some Truepera (order Deinococcales) and a Bradymonadales. Sulfurimonas (order Campylobacterales) only occurred in the core bacterial root community of *T. testudinum*.

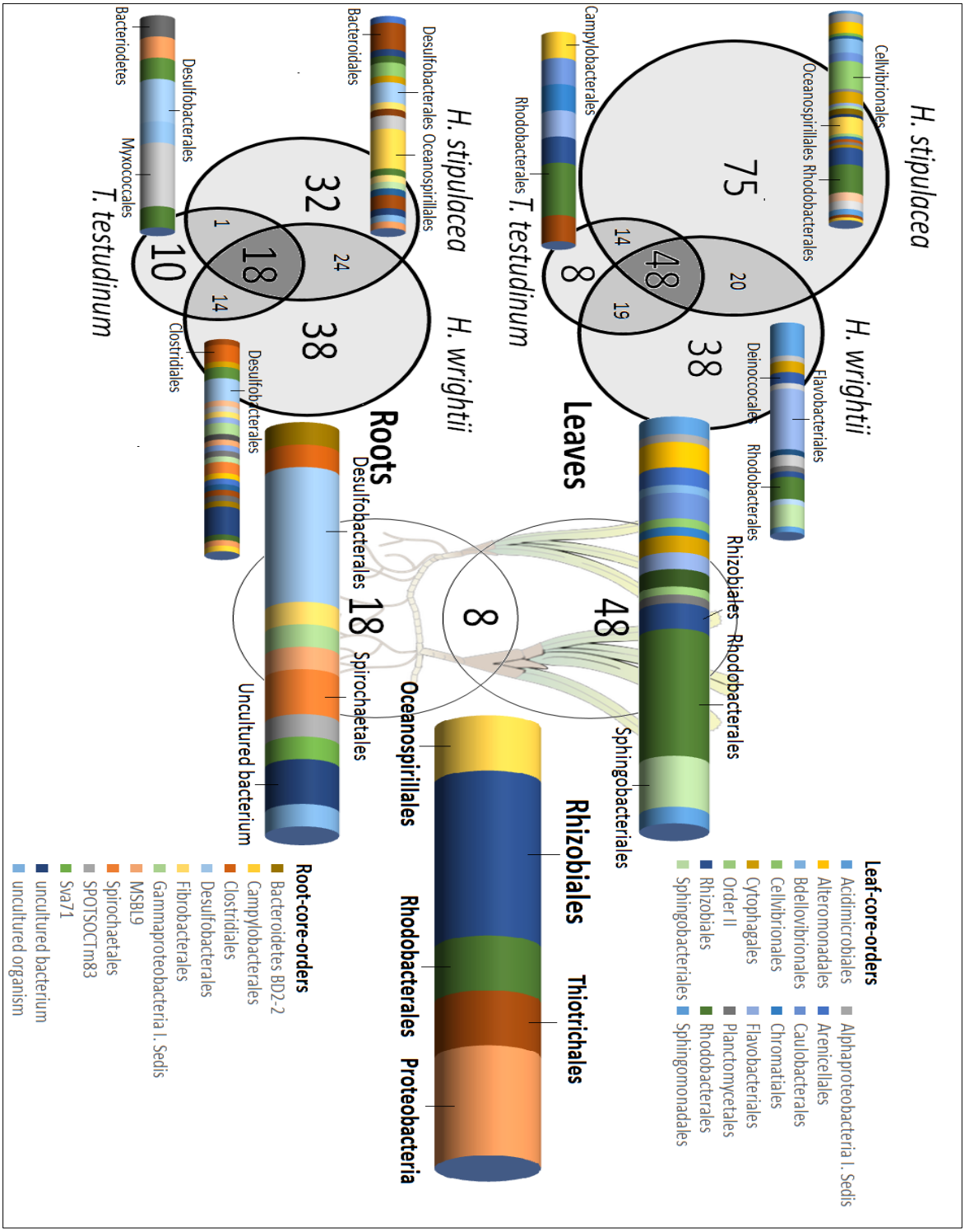

Figure 6. Venn diagrams of root and leaf core bacteria of H. stipulacea, H. wrightii and T. testudinum (Bardou *et al.*, 2014)*. Barplots represent bacterial diversity up to order level of the root and leaf species-specific core bacteria (left), the core root bacteria and the core leaf bacteria shared among seagrasses (middle), and the core bacteria shared between roots and leaves and among all seagrass species (right). Legends display the core root bacteria and the core leaf bacteria shared among seagrasses up to order level. The most abundant orders were graphically represented.*

4 DISCUSSION

This study aimed to detect the driving forces behind microbial assemblage in seagrasses, by which the bacterial communities associated with the roots and leaves of three species from six different locations were analyzed. The results revealed the existence of a tissue- and species-specific seagrass microbiome distinct from the surrounding environment. In contrast with findings regarding the microbiomes of two other seagrass species (Cúcio et al., 2016), the location of the seagrasses influenced the seagrass microbiome to a lesser extent.

4.1 THE MICROBIAL COMMUNITY ASSOCIATED WITH SEAGRASSES DIFFERS IN DIVERSITY, COMPOSITION AND COMMUNITY STRUCTURE FROM THE SURROUNDING ENVIRONMENT

The sediment contained a higher alpha diversity (Richness and Shannon index) than the seagrass microbial community, which is in accordance with the pattern observed for the roots and leaves of *Zostera marina* (Ettinger *et al*., 2017) and a recent study covering the roots of three seagrass species collected in Shark Bay, Western Australia (Martin *et al.*, 2018). Likewise, terrestrial plants have shown decreased diversity in host associated sample types compared to bulk sediment samples (Bulgarelli *et al*., 2012; Chen *et al*., 2016; Edwards *et al*., 2015; Hartman *et al.,* 2017; Rascovan *et al.,* 2016). Community structure and composition (CAP and indicator species) analyses revealed a seagrass microbiome distinct from the sediment. However, seagrass roots shared a great part of their community with the sediment. In parallel with terrestrial endophytic plant communities where soil is considered the most determining factor (Hardoim *et al.*, 2015), seagrasses likewise seem to derive a great part of their microbial community from the sediment.

Differences between seagrass species were observed as *H. stipulacea* roots resembled sediment communities more than the other seagrasses in both alpha and beta diversity. Literature mentions long root hairs as a cause of a relatively more sediment like microbiome compared to species with less pronounced root hairs (Martin *et al.,* 2018). However, as *T. testudinum* and *H. stipulacea* both grow massive root hairs(Larkum et al., 2006), this does not explain *H. stipulacea's* relatively more sediment-like microbiome in this study. Another explanation relates to niche theory, which has been shown to apply for rhizosphere communities(Lebeis, 2015). *H. stipulacea* is the smallest among all seagrasses with roots penetrating to a depth of 4 to 5 cm (Larkum et al., 2006). Recent colonization of *H. stipulacea* around Curaçao means recent colonization of this niche in the sediment, resulting in less pronounced community differences between the species and the sediment.

The in this study observed lower alpha diversity in seawater compared to the leaves is in contrast with findings in a global study regarding the microbiome of *Z. marina* (Fahimipour et al., 2017) but resembles studies done regarding terrestrial plant leaves and air (Bowers *et al.,* 2009; Redford *et al*., 2010; Vorholt, 2012; Womack *et al*., 2015). Like a recent study regarding the microbiomes of two seagrass species (Z. marina and Z. japonica) (Crump *et al.*, 2018), community structure and composition of seawater was distinct from the seagrass microbiomes. As in alpha diversity results, the study regarding the global *Z.marina* microbiome published contrasting results (Fahimipour et al., 2017). Inconsistent results could be related to differences in sampling methods. As seawater samples were collected just above each plant (Fahimipour et al., 2017), the leaf microbial community might have interfered in the seawater community. Whereas seawater samples taken a bit more distant from the seagrass leafs display the "true" marine microbial community. Important members of the marine microbial community detected in this study consisted of the SAR86 clade

(Oceanospirillales), the Surface 1 family and other SAR11 members, SAR116 (Rickettsiales), Microbacteriaceae and Firmicutes. Case studies in other marine waters displayed comparable community structures (Cram *et al*., 2015; Fuhrman & Hagström, 2008; Pommier *et al.*, 2007). SAR86 and SAR11, which represent the largest part of the seawater community found in this study, specialize in the transport and degradation of lipids and polysaccharides and assimilate dissolved DMSP (dimethylsulfoniopropionate) and amino acids, respectively *(*Dupont *et al., 2012;* Malmstrom *et al.*, 2004).

4.2 Microbial community diversity, composition and structure is seagrass' species and tissue specific

Consistent with several earlier seagrass studies(Crump *et al*., 2018; Crump & Koch, 2008; Ettinger *et al*., 2017; Fahimipour *et al*., 2017), leaves and roots contained distinct microbial communities. Differences among species were observed, as leaf and root samples of *H. stipulacea* grouped closer together compared to leaf and root samples of the other seagrasses (Fig 4). In line with these results, the different abundance analysis clustered some root samples of *H. stipulacea* with leaf samples. An explanation could be given by its morphology (Fig. 1). *H. stipulacea* has the smallest shoots among seagrasses (around 1 cm) and produce, as mentioned before, an unbranched root of 4-5 cm containing long root hairs. *H. wrightii* and *T. testudinum* penetrate much deeper into the sediment and have shoots that grow much bigger (30 up to 50 cm*)*(Larkum *et al*., 2006)*. H. stipulacea's* smaller root and leaf size makes the two tissues less distinct from each other and could explain the 'leaf-like' root microbial community.

More debated is the occurrence of a species specific microbiome *(*Crump *et al.,* 2018; Cúcio et al., 2016; Martin et al., 2018). The in this study host specific alpha diversity and community composition/structure is supported by a recent study which evaluated different seagrass species (Martin *et al.*, 2018). Interestingly, no distinguishable microbial communities were detected in the rhizobiomes of three species in the North Eastern Atlantic Ocean (Cúcio et al., 2016), as in two species of the genus *Zostera* within tidal mudflats harboring mixed beds (Crump et al., 2018). Contrasting findings could be explained by differences in sampling strategy among studies. Where in the North Eastern Atlantic they only considered rhizosphere communities(Cúcio et al., 2016), and did thereby not include endosphere and rhizoplane communities. Additional, the occurrence of a host specific microbiome might be influenced by phylogenetic distance. Species of the same genus displayed overlapping microbial communities (Crump et al., 2018), whereas species of different families or genera displayed distinct microbiomes (Fig. 4 & 6, Martin et al., 2018).

The here detected species related differences in root microbiomes could be related to differences in root exudation rates and composition. Literature confirmed variation in root exudates over different seagrass species (Holmer et al., 2001; Martin et al., 2017; Küsel et al., 2006). *H. stipulacea* was not covered in known literature, but *T. testudinum* DOC (dissolved organic carbon) excretion levels showed to be higher compared to *H. wrightii* (Wetzel & Penhale, 1979).

4.3 Seagrass enriched microbial taxa are involved in Sulfur and Nitrogen cycling

The differential abundance analyses between the roots and leaves together with the root and leaf specific cores revealed that roots contained a wide variety of Desulfobacterales. Desulfobacterales are sulfate reducing Deltaproteobacteria, commonly associated with roots on predominately anoxic, sulfide-rich sediments, such as seagrass sediments (Bahr et al., 2005; Isaksen & Finster, 1996; Klepac-Ceraj et al., 2004; Martin et al., 2018). Metatranscriptome results of a recent study revealed increased expression of genes involved

in sulfide oxidation and sulfate reduction (dissimilatory sulfite reductase genes dsrA and dsrB, and adenylyl-sulfate reductase genes aprA and aprB) in seagrass roots compared to leaves and surrounding seawater microbiomes (Crump et al., 2018). Bacteria associated with these genes (sulfate oxidizing Gammaproteobacteria and sulfate reducing deltaproteobacteria) were highly abundant in the root-associated bacterial community detected in this study (e.g. desulfobacterales, Gammaproteobacteria Incertae Sedis), which supports the importance of the sulfur cycle in the seagrass rhizo- and endosphere.

Bacteria of the order Rhizobiales occurred in all species and tissue types. Rhizobiales include a diversity of nitrogen-fixing microbes which often form symbiotic relationships with terrestrial plants (Fischer *et al.*, 2012; Krol *et al*., 2011). Further support of the importance of the nitrogen cycle in seagrass microbiomes is given by several studies which evaluate gene expression of nifH genes (genes related to nitrogenase). High nifH gene expression was found in seagrass associated microbial communites (Bagwell et al., 2002; Crump et al., 2018; Lehnen et al., 2016). The genes related to either sulfate reducing Deltaproteobacteria (Lehnen et al., 2016), sulfur-oxidizing Gammaproteobacteria and nitrogen-fixing Bacteriodetes (Crump et al., 2018). The core microbiomes detected in this study reveal Delta and Gammaproteobacteria are strongly associated with the roots as Bacteriodetes (e.g. Sphingobacteriales of the family Saprospiraceae) are widespread in leaf microbiomes. Leaves also contained significantly more Rhodobacterales (Roseobacter, Sulfitobacter, Loktanella), which are also abundant in marine waters (Dang et al., 2008; McIlroy & Nielsen, 2014). Rhodobacterales are Alphaproteobacteria capable of aerobic anoxygenic photosynthesis (AAnP), degrade algal DMSP, oxides CO and inorganic sulfur compounds (Dang et al., 2008; Wagner-Döbler & Biebl, 2006). Some Rhodobacterales of the genus Roseobacter have shown to be able to produce antibiotics (Wagner-Döbler & Biebl, 2006), by which a function in the protection against bacterial pathogens could be suggested.

Interestingly, genes associated with mentioned nutrient cycles occurred in bacterial phyla/orders which were detected in the cores of all species. Selection on function rather than taxonomy is suggested in an earlier study regarding the microbiome of a green macroalga (Burke et al., 2011), and could also explain species related differences obtained in this study.

5 CONCLUSION

This study revealed the occurrence of a seagrass microbiome, distinct from the surrounding environment. Microbial diversity, community composition and structure differed between roots and leaves and among the three species, whereas the location influenced the seagrass microbial community to a lesser extent. High abundant and widespread species turned out to be members associated with sulfur and nitrogen cycling, which point towards the importance of these metabolic processes in seagrass survival. Further insight in the detected community differences can be given by combining Next generation sequencing with metatranscriptomic studies, which couples detected bacterial taxa to functional genes. Separation of the seagrass microbiome in not only phyllo and rhizosphere, but also evaluate the endosphere separately, could show in which microenvironment the detected differences among species are apparent.

6 ACKNOWLEDGEMENTS

I wish to thank A. Engelen for data collection around the Island of Curacao. For guidance during lab work, analyses and writing I would like to thank A. Engelen and T. Aires.

7 REFERENCES

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*, 32–46. http://doi.org/10.1111/j.1442-9993.2001.tb00081.x

Bagwell, C. E., La Rocque, J. R., Smith, G. W., Polson, S. W., Friez, M. J., Longshore, J. W., & Lovell, C. R. (2002). Molecular diversity of diazotrophs in oligotrophic tropical seagrass bed communities. *FEMS Microbiology Ecology*, *39*(2), 113–119. http://doi.org/10.1016/S0168-6496(01)00204-5

Bahr, M., Crump, B. C., Klepac-Ceraj, V., Teske, A., Sogin, M. L., & Hobbie, J. E. (2005). Molecular characterization of sulfate-reducing bacteria in a New England salt marsh. *Environmental Microbiology*, *7*(8), 1175–1185. http://doi.org/10.1111/j.1462-2920.2005.00796.x

Bardou, P., Mariette, J., Escudié, F., Djemiel, C., & Klopp, C. (2014). Jvenn: An interactive Venn diagram viewer. *BMC Bioinformatics*, *15*(1), 1–7. http://doi.org/10.1186/1471-2105-15-293

Beckers, B., Op De Beeck, M., Weyens, N., Boerjan, W., & Vangronsveld, J. (2017). Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field-grown poplar trees. *Microbiome*, *5*(1), 25. http://doi.org/10.1186/s40168-017-0241-2

Bodenhausen, N., Horton, M. W., & Bergelson, J. (2013). Bacterial Communities Associated with the Leaves and the Roots of Arabidopsis thaliana. *PLoS ONE*, *8*(2). http://doi.org/10.1371/journal.pone.0056329

Borum, J., Pedersen, O., Greve, T. M., Frankovich, T. A., Zieman, J. C., Fourqurean, J. W., & Madden, C. J. (2005). The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, Thalassia testudinum. *Journal of Ecology*, *93*(1), 148–158. http://doi.org/10.1111/j.1365-2745.2004.00943.x

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349. http://doi.org/10.2307/1942268

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., … Schulze-Lefert, P. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature*, *488*(7409), 91–95. http://doi.org/10.1038/nature11336

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annual Review of Plant Biology*, *64*(1), 807–838. http://doi.org/10.1146/annurev-arplant-050312-120106

Burke, C., Steinberg, P., Rusch, D. B., Kjelleberg, S., & Thomas, T. (2011). Bacterial community assembly based on functional genes rather than species. *Proceedings of the National Academy of Sciences of the USA*, *108*(34), 14288–14293. http://doi.org/10.1073/pnas.1101591108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1101591108

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K.,

… Knight, R. (2010). correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nature Publishing Group*, *7*(5), 335–336. http://doi.org/10.1038/nmeth0510-335

Chaparro, J. M., Badri, D. V, & Vivanco, J. M. (2014). Rhizosphere microbiome assemblage is affected by plant development. *The ISME Journal*, *8*(4), 790–803. http://doi.org/10.1038/ismej.2013.196

Chapin, F. S., Zavaleta, E. S., Eviner, V. T., Naylor, R. L., Vitousek, P. M., Reynolds, H. L., … Díaz, S. (2000). Consequences of changing biodiversity. *Nature*, *405*(6783), 234–42. http://doi.org/10.1038/35012241

Chen, L., Brookes, P. C., Xu, J., Zhang, J., Zhang, C., Zhou, X., & Luo, Y. (2016). Structural and functional differentiation of the root-associated bacterial microbiomes of perennial ryegrass. *Soil Biology and Biochemistry*, *98*, 1–10. http://doi.org/10.1016/j.soilbio.2016.04.004

Cifuentes, A., Antón, J., Benlloch, S., Donnelly, A., Herbert, R. A., & Rodríguez-Valera, F. (2000). Prokaryotic Diversity in Zostera noltii -Colonized Marine Sediments. *Applied and Environmental Microbiology*, *66*(4), 1715–1719. http://doi.org/10.1128/AEM.66.4.1715-1719.2000.Updated

Clarke, KR, Gorley, R. (2006). PRIMER v6: User Manual/Tutorial. *PRIMER-E, Plymouth UK*, 192.

Cram, J. A., Chow, C.-E. T., Sachdeva, R., Needham, D. M., Parada, A. E., Steele, J. A., & Fuhrman, J. A. (2015). Seasonal and interannual variability of the marine bacterioplankton community throughout the water column over ten years. *The ISME Journal*, *9*(3), 563–580. http://doi.org/10.1038/ismej.2014.153

Crump, B. C., & Koch, E. W. (2008). Attached bacterial populations shared by four species of aquatic angiosperms. *Applied and Environmental Microbiology*, *74*(19), 5948–5957. http://doi.org/10.1128/AEM.00952-08

Crump, Byron C., Wojahn, John M., Tomas, Fiona, Mueller, R. S. (2018). Metatranscriptomics and Amplicon Sequencing Reveal Mutualisms in Seagrass Microbiomes. *Front. Microbiology*, *388*(9). http://doi.org/10.1016/S2213-8587(14)70049-X

Cúcio, C., Engelen, A. H., Costa, R., & Muyzer, G. (2016). Rhizosphere microbiomes of European + seagrasses are selected by the plant, but are not species specific. *Frontiers in Microbiology*, *7*(MAR), 1–15. http://doi.org/10.3389/fmicb.2016.00440

Dang, H., Li, T., Chen, M., & Huang, G. (2008). Cross-ocean distribution of Rhodobacterales bacteria as primary surface colonizers in temperate coastal marine waters. *Applied and Environmental Microbiology*, *74*(1), 52–60. http://doi.org/10.1128/AEM.01400-07

de Souza, R. S. C., Okura, V. K., Armanhi, J. S. L., Jorrín, B., Lozano, N., da Silva, M. J., … Arruda, P. (2016). Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Scientific Reports*, *6*(1), 28774. http://doi.org/10.1038/srep28774

Dupont, C. L., Rusch, D. B., Yooseph, S., Lombardo, M. J., Alexander Richter, R., Valas, R., … Craig Venter, J. (2012). Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. *ISME Journal*, *6*(6), 1186–1199. http://doi.org/10.1038/ismej.2011.189

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., … Sundaresan, V. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences*, *112*(8), E911–E920. http://doi.org/10.1073/pnas.1414592112

Egamberdieva, D. (2008). Plant growth promoting properties of rhizobacteria isolated from wheat and pea grown in loamy sand soil. *Turkish Journal of Biology*, *32*(1), 9–15.

Ettinger, C. L., Voerman, S. E., Lang, J. M., Stachowicz, J. J., & Eisen, J. A. (2017). Microbial communities in sediment from *Zostera marina* patches, but not the *Z. marina* leaf or root microbiomes, vary in relation to distance from patch edge. *PeerJ*, *5*, e3246. http://doi.org/10.7717/peerj.3246

Fahimipour, A. K., Kardish, M. R., Lang, J. M., Green, J. L., Eisen, J. A., & Stachowicz, J. J. (2017). Global-scale structure of the eelgrass microbiome. *Applied and Environmental Microbiology*, AEM.03391–16. http://doi.org/10.1128/AEM.03391-16

Fischer, D., Pfitzner, B., Schmid, M., Simões-Araújo, J. L., Reis, V. M., Pereira, W., … Hartmann, A. (2012). Molecular characterisation of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (Saccharum sp.). *Plant and Soil*, *356*(1-2), 83–99. http://doi.org/10.1007/s11104-011-0812-0

Fuhrman, J. A., & Hagström, Å. (2008). Bacterial and Archaeal Community Structure and its Patterns. In *Microbial Ecology of the Oceans: Second Edition* (pp. 45–90). http://doi.org/10.1002/9780470281840.ch3

Gower, J. C. (1982). Euclidean distance geometry. *Mathematical Scientist*, *7*(1), 1–14. Retrieved from http://convexoptimization.com/TOOLS/Gower2.pdf

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., … Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews*, *79*(3), 293–320. http://doi.org/10.1128/MMBR.00050-14

Hartman, K., van der Heijden, M. G. A., Roussely-Provent, V., Walser, J. C., & Schlaeppi, K. (2017). Deciphering composition and function of the root microbiome of a legume plant. *Microbiome*, *5*(1). http://doi.org/10.1186/s40168-016-0220-z

Hasler-Sheetal, H., & Holmer, M. (2015). Sulfide intrusion and detoxification in the seagrass zostera marina. *PLoS ONE*, *10*(6). http://doi.org/10.1371/journal.pone.0129136

Hemminga, M. A., & Duarte, C. M. (2000). *Seagrass Ecology*. *Cambridge University Press*. http://doi.org/10.1016/S0304-3770(01)00171-1

Holmer, M., Andersen, F. O., Nielsen, S. L., & Boschker, H. T. S. (2001). The importance of mineralization based on sulfate reduction for nutrient regeneration in tropical seagrass sediments. *Aquatic Botany*, *71*(1), 1–17. http://doi.org/10.1016/S0304-3770(01)00170-X

Holmer, M., Gribsholt, B., & Kristensen, E. (2002). Effects of sea level rise on growth of Spartina anglica and oxygen dynamics in rhizosphere and salt marsh sediments. *Marine Ecology Progress Series*, *225*, 197–204. http://doi.org/10.3354/meps225197

Holmer, M., & Nielsen, S. L. (1997). Sediment sulfur dynamics related to biomass-density patterns in Zostera marina (eelgrass) beds. *Marine Ecology Progress Series*, *146*(1-3), 163–171. http://doi.org/10.3354/meps146163

Isaksen, M. F., & Finster, K. (1996). Sulphate reduction in the root zone of the seagrass Zostera noltii on the intertidal flats of a coastal lagoon (Arcachon, France). *Marine Ecology Progress Series*, *137*(1-3), 187–194. http://doi.org/10.3354/meps137187

Jensen, S. I., Kühl, M., & Priemé, A. (2007). Different bacterial communities associated with the roots and bulk sediment of the seagrass Zostera marina. *FEMS Microbiology Ecology*, *62*(1), 108–117. http://doi.org/10.1111/j.1574-6941.2007.00373.x

Jørgensen, B. B. (1982). Mineralization of organic matter in the sea bed – the role of sulphate reduction. *Nature*. http://doi.org/10.1038/296643a0

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649. http://doi.org/10.1093/bioinformatics/bts199

Klepac-Ceraj, V., Bahr, M., Crump, B. C., Teske, A. P., Hobbie, J. E., & Polz, M. F. (2004). High overall diversity and dominance of microdiverse relationship in salt marsh sulphate-reducing bacteria. *Environmental Microbiology*, *6*(7), 686–698. http://doi.org/10.1111/j.1462-2920.2004.00600.x

Krol, E., Blom, J., Winnebald, J., Berhörster, A., Barnett, M. J., Goesmann, A., … Becker, A. (2011). RhizoRegNet-A database of rhizobial transcription factors and regulatory networks. *Journal of Biotechnology*, *155*(1), 127–134. http://doi.org/10.1016/j.jbiotec.2010.11.004

Küsel, K., Trinkwalter, T., Drake, H. L., & Devereux, R. (2006). Comparative evaluation of anaerobic bacterial communities associated with roots of submerged macrophytes growing in marine or brackish water sediments. *Journal of Experimental Marine Biology and Ecology*, *337*(1), 49–58. http://doi.org/10.1016/j.jembe.2006.06.004

Laffoley, D. d'., & Grimsditch, G. (2009). *The management of natural coastal carbon sinks. IUCN, Gland, Switzerland* (Vol. (eds)). http://doi.org/10.1007/s00114-001-0283-x

Lane, D. J. (1991). 16S/23S rRNA Sequencing. *Nucleic Acid Techniques in Bacterial Systematics*, 115–175. http://doi.org/10.1007/s00227-012-2133-0

Larkum, A. W. D., Orth, R. J., & Duarte, C. M. (2006). *Seagrasses; Biology, Ecology and Conservation*. *Springer*. http://doi.org/10.1017/CBO9781107415324.004

Lebeis, S. L. (2015). Greater than the sum of their parts: Characterizing plant microbiomes at the community-level. *Current Opinion in Plant Biology*, *24*, 82–86. http://doi.org/10.1016/j.pbi.2015.02.004

Lehnen, N., Marchant, H. K., Schwedt, A., Milucka, J., Lott, C., Weber, M., … Kuypers, M. M.

M. (2016). High rates of microbial dinitrogen fixation and sulfate reduction associated with the Mediterranean seagrass Posidonia oceanica. *Systematic and Applied Microbiology*, *39*(7), 476–483. http://doi.org/10.1016/j.syapm.2016.08.004

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., … Dangl, J. L. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature*, *488*(7409), 86–90. http://doi.org/10.1038/nature11237

Malmstrom, R. R., Kiene, R. P., Cottrell, M. T., & Kirchman, D. L. (2004). Contribution of SAR11 bacteria to dissolved dimethylsulfoniopropionate and amino acid uptake in the North Atlantic Ocean. *Applied and Environmental Microbiology*, *70*(7), 4129–4135. http://doi.org/10.1128/AEM.70.7.4129-4135.2004

Martin, B. C., Gleeson, D., Statton, J., Siebers, A. R., Grierson, P., Ryan, M. H., … Martin, B. C. (2018). Low Light Availability Alters Root Exudation and Reduces Putative Beneficial Microorganisms in Seagrass Roots, *8*(January), 1–16. http://doi.org/10.3389/fmicb.2017.02667

Martin, B. C., Statton, J., Siebers, A. R., Grierson, P. F., Ryan, M. H., & Kendrick, G. A. (2017). Colonizing tropical seagrasses increase root exudation under fluctuating and continuous low light. *Limnology and Oceanography*. http://doi.org/10.1002/lno.10746

McIlroy, S. J., & Nielsen, P. H. (2014). The Family Saprospiraceae. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea* (pp. 863–889). Berlin, Heidelberg: Springer Berlin Heidelberg. http://doi.org/10.1007/978-3-642-38954-2_138

Metropolis, N., & Ulam, S. (1949). The Monte Carlo Method. *Journal of the American Statistical Association*, *44*(247), 335–341. http://doi.org/10.1080/01621459.1949.10483310

Nellemann, C., Corcoran, E., Duarte, C. M., Valdés, L., De Young, C., Fonseca, L., & Grimsditch, G. (2009). *Blue carbon: A Rapid Response Assessment. Environment*. Retrieved from http://www.grida.no/files/publications/blue-carbon/BlueCarbon_screen.pdf

Ofek-Lalzar, M., Sela, N., Goldman-Voronov, M., Green, S. J., Hadar, Y., & Minz, D. (2014). Niche and host-associated functional signatures of the root surface microbiome. *Nature Communications*, *5*, 4950. http://doi.org/10.1038/ncomms5950

Olsen, J. L., Rouzé, P., Verhelst, B., Lin, Y.-C., Bayer, T., Collen, J., … Van de Peer, Y. (2016). The genome of the seagrass Zostera marina reveals angiosperm adaptation to the sea. *Nature*, *530*(7590), 331–335. http://doi.org/10.1038/nature16548

Pedersen, O., Binzer, T., & Borum, J. (2004). Sulphide intrusion in eelgrass (Zostera marina L.). *Plant, Cell and Environment*, *27*(5), 595–602. http://doi.org/10.1111/j.1365-3040.2004.01173.x

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, *11*(11), 789–799. http://doi.org/10.1038/nrmicro3109

Pommier, T., Canbäck, B., Riemann, L., Boström, K. H., Simu, K., Lundberg, P., … Hagström, Å. (2007). Global patterns of diversity and community structure in marine bacterioplankton. *Molecular Ecology*, *16*(4), 867–880. http://doi.org/10.1111/j.1365-294X.2006.03189.x

R Development Core Team. (2016). R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing Vienna Austria*, *0*, {ISBN} 3–900051–07–0. http://doi.org/10.1038/sj.hdy.6800737

Rascovan, N., Carbonetto, B., Perrig, D., Díaz, M., Canciani, W., Abalo, M., … Vazquez, M. P. (2016). Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. *Scientific Reports*, *6*. http://doi.org/10.1038/srep28084

Redman, R. S. (2002). Thermotolerance Generated by Plant/Fungal Symbiosis. *Science*, *298*(5598), 1581–1581. http://doi.org/10.1126/science.1072191

Sánchez-Cañizares, C., Jorrín, B., Poole, P. S., & Tkacz, A. (2017). Understanding the holobiont: the interdependence of plants and their microbiome. *Current Opinion in Microbiology*, *38*, 188–196. http://doi.org/10.1016/j.mib.2017.07.001

Shannon, C. E., & Weaver, W. (1949). The mathematical theory of communication. *University of Illinois Press*, 144p. http://doi.org/10.1145/584091.584093

Smulders, F. O. H., Vonk, J. A., Engel, M. S., & Christianen, M. J. A. (2017). Expansion and fragment settlement of the non-native seagrass Halophila stipulacea in a Caribbean bay. *Marine Biology Research*, *1000*, 1–8. http://doi.org/10.1080/17451000.2017.1333620

Timmusk, S., & Wagner, E. G. (1999). The plant-growth-promoting rhizobacterium Paenibacillus polymyxa induces changes in Arabidopsis thaliana gene expression: a possible connection between biotic and abiotic stress responses. *Molecular Plant-Microbe Interactions : MPMI*, *12*(11), 951–959. http://doi.org/10.1094/MPMI.1999.12.11.951

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nature Reviews Microbiology*. http://doi.org/10.1038/nrmicro2910

Wagner, M. R., Lundberg, D. S., del Rio, T. G., Tringe, S. G., Dangl, J. L., & Mitchell-Olds, T. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature Communications*, *7*, 12151. http://doi.org/10.1038/ncomms12151

Wagner-Döbler, I., & Biebl, H. (2006). Environmental Biology of the Marine *Roseobacter* Lineage. *Annual Review of Microbiology*, *60*(1), 255–280. http://doi.org/10.1146/annurev.micro.60.080805.142115

Wetzel, R. G., & Penhale, P. A. (1979). Transport of carbon and excretion of dissolved organic carbon by leaves and roots/rhizomes in seagrasses and their epiphytes. *Aquatic Botany*, *6*(C), 149–158. http://doi.org/10.1016/0304-3770(79)90058-5

White, T. J., Bruns, S., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phyologenetics.pdf. *PCR Protocols: A Guide to Methods and Applications*. http://doi.org/citeulike-article-id:671166

Willette, D. A., & Ambrose, R. F. (2012). Effects of the invasive seagrass Halophila stipulacea on the native seagrass, Syringodium filiforme, and associated fish and epibiota communities in the Eastern Caribbean. *Aquatic Botany*, *103*, 74–82. http://doi.org/10.1016/j.aquabot.2012.06.007

Zhang, H., Kim, M.-S., Sun, Y., Dowd, S. E., Shi, H., & Paré, P. W. (2008). Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. *Molecular Plant-Microbe Interactions : MPMI*, *21*(6), 737–744. http://doi.org/10.1094/MPMI-21-6-0737