UNIVERSITA' DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI SCIENZE MATEMATICHE, FISICHE E NATURALI

DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA ED ECOLOGIA

CICLO XXVII

CHARACTERIZATION OF THE EPIPHYTIC MICROBIAL COMMUNITIES ASSOCIATED WITH SEAGRASSES IN A CHANGING ENVIRONMENT

Ph.D. Thesis

Astrid Mejia

A.A. 2013/2014

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THESIS SUMMARY

Seagrasses are benthic ecosystems that provide important services in the coastal zones, yet are declining worldwide at an alarming rate. Seagrasses provide habitat to commercially important fish and crustaceans, and contribute to coastal protection through the stabilization of sediment run-off and erosion. They play a critical role in the maintenance of biogeochemical cycles and bury approximately 12% of the global atmospheric carbon production. Rising anthropogenic and natural threats, including human-induced climate change may negatively affect seagrasses, resulting in permanent habitat loss and reduction of biodiversity. With most of seagrasses environmental monitoring based on long term responses to environmental pressures, there is a growing interest to develop alternative diagnostic tools that more effectively identify changes in seagrasses conservation status at an early stage. Microbial communities respond rapidly to changing environmental conditions and may be useful bioindicators of seagrass ecophysiology. To assess this potential, the microbial communities of two seagrass species, namely *Cymodocea nodosa* and *Halophila stipulacea* were investigated using molecular tools.

The study of the effects of climate change impacts like ocean acidification on marine epiphytic microbial communities is scant and still poorly understood. This thesis investigated the effects on the seagrass microbiome of four controlled conditions of CO_2 and nutrients in a mesocosm experiment. The CO_2 concentrations represented scenarios ranging from current to late century (2100) estimations based on predictions by the Intergovernmental Panel on Climate Change (IPCC). The epiphytic microbial communities associated with the leaves, rhizomes and roots of *Cymodocea nodosa* where characterized after one month incubation, both in

summer and winter. Plant responses were also measured to investigate the possible link between plant ecophysiology and microbial communities. Results highlighted the influence of increased CO_2 and nutrients on the microbial community structure and the differences in seasonal microbial assemblages. The plants did not show variations in response to the experimental conditions, neither on leaf biometry nor on leaf -carbon to nitrogen- ratios. The molecular tool employed, Single Strand Conformation Polymorphism (SSCP), allowed for the characterization of the microbial communities. Different and diversified microbial communities are host by plants both above (leaves) and belowground (rhizomes and roots). The dominant phylum of the microbial community composition was *Proteobacteria,* mainly classes *Alphaproteobacteria, Gammaproteobacteria* and *Deltaproteobacteria*. Shortcomings with SSCP generated bands were highlighted by the low resolution of the 16S rRNA gene sequencing which did not allow for a robust analysis of the microbial community assemblages.

Considering the initial findings on *Cymodocea nodosa*, the microbial communities associated with a second seagrass species were investigated in the field using more advanced molecular tools. Plant responses were also evaluated to explore more in depth the possible link between plant ecophysiology and their associated microbial communities.

Changes in environmental conditions due to natural causes and human pressure may cause shifts in seagrass ecophysiology and their associated microbial communities. The seagrass *Halophila stipulacea* was selected to investigate differences in microbial community assemblages and plant ecophysiology along a natural gradient of different environmental conditions and human uses. Three seagrass meadows: North Beach (NB), Tur Yam (TY) and South Beach (SB), were selected for sampling along an 11 Km

coastal stretch in the Gulf of Aqaba, Red Sea, Israel. Plant descriptors, including leaves morphometrics, photosynthetic pigments and total phenols contents were measured to assess plants' ecophysiological status. Environmental variables, as well as the geomorphological features of the meadows and the human uses were also examined. Results highlighted significant differences among meadows on both microbial community and in plants ecophysiology. Different and diversified microbial communities are host by plants both above (leaves) and belowground (rhizomes and roots). The dominant phylum of the microbial community composition was *Proteobacteria*, mainly classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*. In particular, a higher microbial diversity on the leaves was observed under conditions of low hydrodynamics, fine sediments and high water turbidity; on plants with large leaves, high photosynthetic activity and low phenol content. On the contrary, the microbial diversity belowground (rhizomes and roots) seemed to be influenced by conditions of high hydrodynamics, coarse sand and high light availability, when plants show low photosynthetic activity and surface area but high phenol content. Clear dissimilarities in the microbial community assemblages were also found between plant compartments above *vs.* belowground on the three meadows. Among the phylum *Proteobacteria* the class *Alphaproteobacteria* was dominant on the leaves; while belowground the dominance was shared with classes *Gammaproteobacteria* and *Deltaproteobacteria*. The plants of the three stations showed significant differences in leaf biometry as well as in photosynthetic pigments and total phenols contents. The findings suggest an influence of plants photosynthetic pigment contents on microbial community responses. High abundance of the class *Alphaproteobacteria* and *Cyanobacteria* were found associated with conditions of high light availability and low chlorophyll *a* contents, as it has been observed in

bacterial biofilms attached to artificial substrates in Australian coral reef areas. The synchronic response of both plants and microbial communities to local environmental conditions suggests a strong link between plant's ecophysiology and their epiphytic microbial communities.

Differences in the local environmental conditions along spatial gradients may also alter seagrasses ecophysiology and their microbial communities. To study the effect of depth, the microbial communities associated with *Halophila stipulacea* were investigated at 4, 9, 18 and 28 meters in the SB meadow. Plant descriptors and environmental conditions were also measured. Results highlighted differences among depth on both microbial community and in plants ecophysiology. Again, different and diversified microbial communities are host by plants both above (leaves) and belowground (rhizomes and roots). The dominant phylum along the vertical gradient was again *Proteobacteria*, classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*. Along the depth gradient, the plants and their microbial communities showed again synchronic responses; displaying the most dissimilarity at the greatest depth limit, 28 meters. The influence of particular environmental conditions and plants' ecophysiology was also observed. The microbial diversity was consistently higher belowground than aboveground at most of the depths examined. The findings suggest an influence of hydrodynamics and sediment granulometry on microbial diversity and richness, especially at the shallower (4 m) and deeper edges (28 m) of the meadow. Again, clear differences in the microbial community assemblages were found associated with the plant compartments. The dominance of the phylum *Proteobacteria* was confirmed along the vertical gradient. The class *Alphaproteobacteria* was the most abundant phylotype found on the leaves, while belowground the dominance was shared with classes *Gammaproteobacteria* and *Deltaproteobacteria*.

The findings highlighted the influence of depth in both plants and their microbial community assemblages.

Overall, epiphytic microbial communities associated with seagrasses are influenced by plant ecophysiology and environmental conditions. Furthermore, the seagrasses microbiome can be influenced by temporal and spatial differences. This thesis represents a stepping stone in the study of microbial communities as a marker of environmental change in seagrasses. A functional relationship has been suggested between seagrasses and their associated microbial communities. Transcriptomic studies may help explore deeper the functional relationship between seagrasses and its microbiome. Future investigations should also focus on the exploration of the rare microbial community component. Understanding their physiology and metabolic capabilities *(i.e.* through gene expression) is critical to effectively assess bacterial community shifts in a changing environment. Studies of CO_2 effects and other climate change related factors should be investigated with NGS; the incorporation of more descriptive and quantitative techniques (*i.e.* Next-Generation Sequencing, quantitative PCR) can lead to a better understanding of the seagrass microbiome. This can help monitor seagrass health and conservation under a changing environment.

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DEFINITIONS

BIOFILM: an organized aggregation or community of microbes growing on an environmental surface and frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS; for examples see [Witt *et al*, 2012]).

EPIPHYTIC BACTERIA: bacteria that are capable of living (*i.e.* multiplying) on plant surfaces (for review see [Gnanamanickam and Immanuel, 2006])

GENOMICS: is the analysis of genomic DNA from an individual organism or cell (for review [Gilbert and Dupont, 2011]).

HOLOBIONT: is the collective community of host and its symbionts (for examples see [Rosenberg, *et al.*, 2007; Singh and Reddy, 2014]).

MICROBIAL COMMUNITY: an integrated assemblage of microbes belonging to different taxon and with different metabolic capabilities.

MICROBIAL METAGENOMICS: is the isolation and analysis of the collective genome from a whole microbial community obtained from an environmental sample. The technique is usually applied to determine both the composition (diversity) and capabilities (function) of a community of microorganisms (for review [Gilbert and Dupont, 2011]).

MICROBIOME: the collective genome of microorganisms or microbial assemblage (Bacteria, Archaea) associated with any system such as a plant compartment, or the body of an animal, a water or soil sample, or an entire sea.

NEXT-GENERATION SEQUENCING: massively parallel sequencing of large sample sets (for review [Zhang *et al.*, 2011]).

RHIZOSPHERE: is the narrow area in the soil directly influenced by roots secretions around a growing plant, often colonized by microorganisms (for review see [Lugtenberg, 2015]).

1 INTRODUCTION

1.1 Seagrasses: Overview, Ecological role and Importance

Seagrasses are flowering plants that adapted to life in shallow oceanic and estuarine waters about 100 million years ago (Duarte, 2011). They spend their entire life cycle in shallow seawater and have reproductive organs (flower and seeds), roots, rhizomes, and leaves (Björk *et al.*, 2008); features that differentiate them from macroalgae (seaweeds). There are about 60 identified seagrass species, which often form prominent seagrass meadows composed of single species or highly mixed stands (Short *et al.*, 2007). They have a worldwide distribution and are commonly found along temperate and tropical coastlines (Fig. 1).

Temperate seagrasses are known to provide habitat for commercially important fish and shellfish (Green and Short, 2003) and are crucial in sediment deposition and reducing wave and current energy. In tropical ecosystems, seagrasses are an important component of a natural network of three interconnected habitats formed alongside mangroves and coral reefs (Green and Short, 2003). These habitats in conjunction exert a stabilizing effect on the environment, resulting in important physical and biological support for other communities. Mangroves serve as the primary sediment trapping habitat and natural filtration system of coastal water and sediment inputs. Coral reefs often absorb

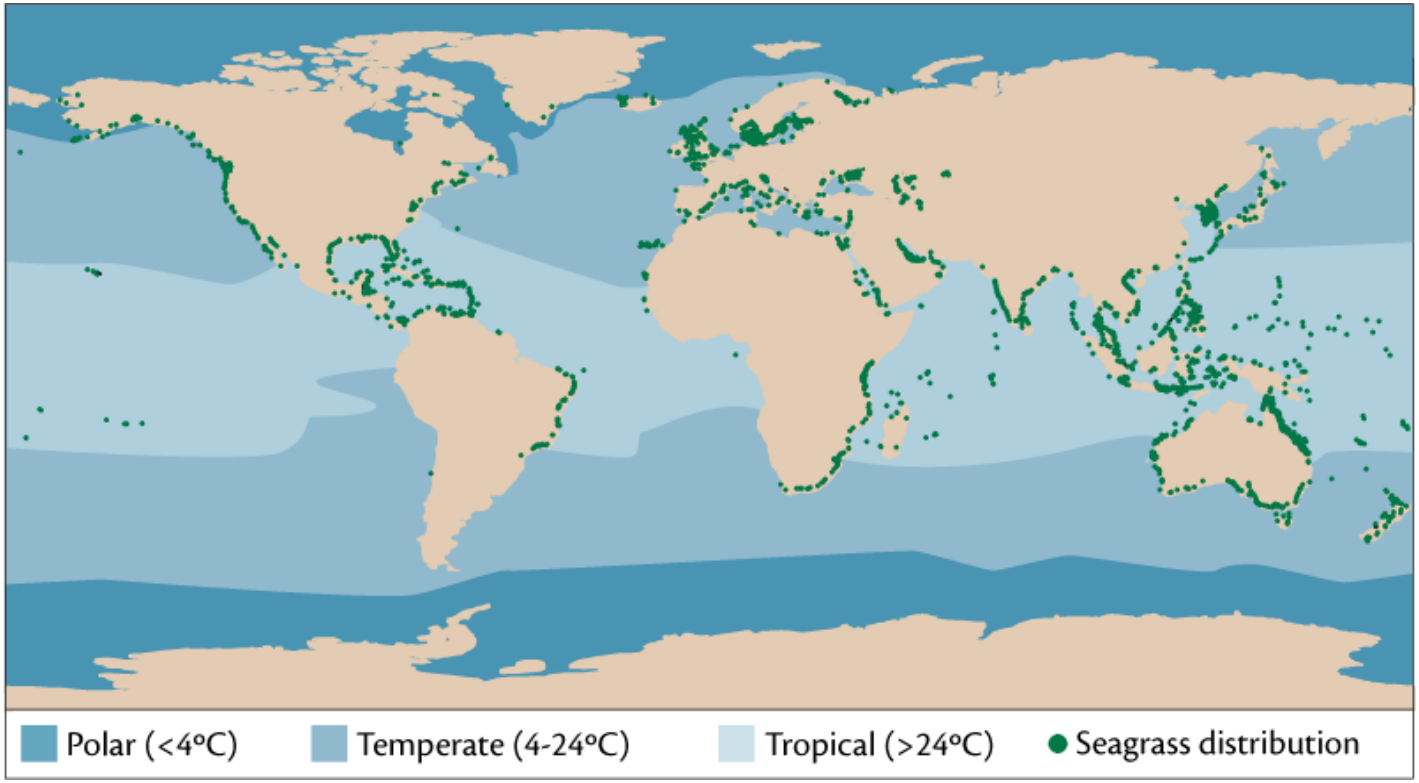


Fig. 1 Map showing the global distribution of seagrasses based on the temperature zones of the earth according to latitude. (http://www.teachoceanscience.net/)

the effect of the waves, acting as a line of natural defense and protection to coastal shorelines. Seagrasses act as nursery grounds and feeding areas for reef fish and crustaceans, in addition to the stabilization of sediments from upland runoff.

Due to their many ecosystem services, seagrasses are one of the most valuable marine habitats on earth, with an estimated value of US$2.8 10^6 $yr^{-1} \cdot km^{-2}$ (Costanza *et al.*, 2014). Given their ecological and economic importance, they have been legally recognized in the European Union (EU) Water Framework Directive (WFD, Directive 2000/60/EC) as key coastal ecosystems and are identified as bioindicators of ecosystem quality (Marbà *et al.*, 2013). However, they are experiencing worldwide declines mainly due to anthropogenic and natural threats (Björk *et al.*, 2008; Short *et al.*, 2011). Global assessments indicate that seagrasses have been disappearing at a rate of 110 $km^2\ yr^{-1}$ since 1980. Continuous global loss rates are estimated at 2-5% $year^{-1}$, compared to 0.5% $year^{-1}$ for tropical forests (Waycott *et al.*, 2009). Human-induced climate change may also impact seagrasses as sea level rises and severe storms become more frequent (Green and Short, 2003). Implementing monitoring programs has been an important step in assessing the conservation status of seagrass meadows and to identify the causes and effects of potential stressors. Large scale efforts through programs such as Seagrass-Watch (www.seagrasswatch.org; McKenzi *et al.*, 2000), and Seagrass Net (www.seagrassnet.org; Short *et al.*, 2004,

2005) have been carried out worldwide to monitor changes in the health status of seagrass meadows.

1.2 Seagrasses Descriptors

With seagrasses declining at a fast rate, the identification of suitable parameters to effectively assess seagrasses health has become more critical and necessary to prompt timely actions for their protection and conservation (Marbà *et al.*, 2013; Unsworth *et al.*, 2014). Traditionally, most seagrass monitoring approaches have been based on the follow up of changes in community based parameters such as bed composition, percent cover and biomass (Buia *et al.*, 2004). However, those descriptors represent relatively slow (plant) responses to environmental changes (Marbà *et al.*, 2013). There is a growing interest in developing new diagnostic tools to describe more effectively and rapidly the current state of seagrass meadows.

Functional and biochemical descriptors focusing on plant physiology are increasingly being applied on seagrasses to assess plant responses to environmental changes (Migliore *et al.*, 2007; Rotini *et al.*, 2011; Arnold *et al.*, 2012; Rotini *et al.*, 2013a,b; Silva *et al.*, 2013). Physiological responses such as the production of photosynthetic pigments and the synthesis of secondary metabolites (total phenols), are influenced by changes in the environment and thus represent the plants' eco-physiological status. To keep photosynthetic efficiency, plants change their photosynthetic pigments concentrations in

response to differences in water quality and/or light regimes (Campbell *et al.*, 2003; Ralph *et al.*, 2007; Beer *et al.*, 2014). Changes in phenol content have been previously observed in different seagrass species in response to several abiotic and biotic pressures, including pollution and disease (Vergeer and Develi, 1997; Ferrat *et al.*, 2003; Migliore *et al.*, 2007; Rotini *et al.*, 2011; Rotini et al, 2013a,b), ocean acidification (Arnold *et al.*, 2012; Migliore *et al.*, 2012; Arnold *et al.*, 2014), competition (Pergent *et al.*, 2008), herbivory (Arnold *et al.*, 2008; Vergés *et al.*, 2008; Darnell and Heck, 2013) and light reduction (Silva *et al.*, 2013).

Microbial community shifts are being increasingly studied in the marine environment, particularly when associated with eukaryotes to better understand the impacts of changing environmental conditions, for example in corals (Rosenberg *et al.*, 2007; Ainsworth *et al.*, 2009), macroalgae (Dubilier *et al.*, 2008; Crawford and Clardy, 2011; Wahl *et al.*, 2012; Egan *et al.*, 2012; Singh and Reddy, 2014) and sponges (Hentschel *et al.*, 2012; Webster *et al.*, 2012). Differences in epiphytic microbial communities have been associated with deteriorating health of corals and sponges in response to different gradients of CO_2 concentrations (Meron *et al.*, 2011) and temperature conditions (Webster *et al.*, 2008). Marine biofilm- forming- microbial taxa (attached to artificial substrates) have been associated with particular environmental conditions, for example the phyla *Alphaproteobacteria* and *Cyanobacteria* have been correlated with oligotrophic conditions (low impacted sites with high light, low nutrients and low Chlorophyll

a concentrations), while *Gammaproteobacteria* and *Bacteroidetes* have been correlated to sites with eutrophication (Witt *et al.*, 2012).

The functional relationship between seagrasses and microbial communities has not yet properly been established. It is possible that the epiphytic microbial communities shift in response to environmental changes; and both plants and microbes may respond in synchrony to changes in environmental conditions. The exploration of microbial community responses to changing environmental conditions along with plants ecophysiology may help reveal aspect of their interaction as well as the potential of this microbiome to be a bioindicators of ecosystem health in seagrass meadows.

1.3 Epiphytic Microbial Communities Associated with Seagrasses

Complex and highly diverse biofilms (Fig. 2) are present on the surfaces of seagrass plants (reviewed by Michael *et al.,* 2008), such as they are on macroalgae. Epiphytic microbial communities are found attached to the aboveground plant parts or compartment including the shoots, leaves, seeds, and flowers. They can be also found on the belowground compartment which involves the rhizomes and root. An important habitat for microbes is constituted by the rhizosphere, which is the area in the soil compartment influenced by the roots of growing plants (Lugtenberg, 2015). Interestingly, a significant amount of knowledge about the seagrass microbiome has originated from the study of the rhizosphere. Table 1 shows a general evolution of

seagrass microbiome studies until present and the techniques employed. The study of microbial communities has significantly progressed over the years and benefited from the development of culture independent techniques. Findings have highlighted the suitability of plant surfaces for biofilm attachment and provided the first descriptions of seagrasses associated microbial community assemblages.

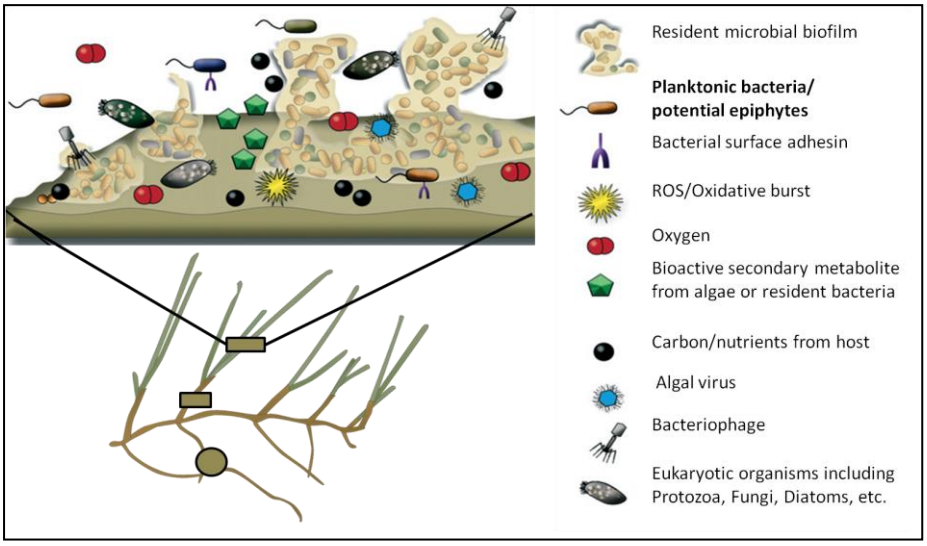


Fig. 2 Illustration of 'the predicted biofilm structure on seagrasses', which may be attached to leaves, rhizomes and roots. Adapted from Singh and Reddy, (2014); original image related to the 'Seaweed holobiont and the factors influencing bacterial colonization on macroalgal hosts'.

Different microbial communities have been found associated with seagrasses leaves, *vs.* roots, vegetated sediments, non vegetated sediments and water column on meadows of different species, including *Halophila stipulacea* (Weidner *et al.*, 2000), *Thalassodendron ciliatum*, *Thalassia hemprichii*, and *Cymodocea*

rotundata (Uku *et al.*, 2007), *Zostera noltii* (Nielsen *et al.*, 2001), *Zostera marina* (Crump and Koch, 2008), *Thalassia testudinum* and *Syringodium filiforme* (Bagwell *et al.*, 2002*).*

The introduction of molecular tools has improved bacterial detection and taxonomic identification of the microbial communities, allowing a more detailed exploration of their structure and composition. Diverse communities are associated with the oxic and anoxic layers of seagrasses sediments as well as with the different plant parts. Segrasses leaves have been found to host phototrophic *Alphaproteobacteria* (Crump and Koch, 2008) and nitrogen-fixing *Cyanobacteria* (Hamisi *et al.*, 2009), which contribute to the bulk nitrogen that seagrasses require for their productivity. On the rhizomes and roots, nitrogen-fixing and sulfide-oxidizing bacteria are commonly found (Cifuentes *et al.*, 2000; Bagwell *et al.*, 2002), which a play a crucial role in the detoxification of sulfide and reactive nitrogen in the root and sediment interface (reviewed by Welsh, 2000; Lee and Dunton, 2000). Similarly, sulfate-reducing bacteria are commonly found associated with the anoxic sediments surrounding seagrasses roots (Blaabjerg and Finster, 1998, García-Martínez *et al.*, 2009).

The presence of distinct assemblages attached to the surfaces of seagrass plants in comparison with the surrounding environment suggests complexity in this relationship. Understanding these

interactions could help reveal possible causes of seagrass decline and enhance efforts in restoration.

Tab. 1 List of studies pertaining to bacterial communities associated with different seagrasses.
Techniques: **CUD**, culture-dependent methods. Microscopic methods: **EM**, electron microscopy; **SEM**, scanning electron microscopy; **TEM**, transmission electron microscopy; **EPF**, epifluorescence microscopy. *Molecular techniques*: **ARDRA**, Amplified rDNA restriction analysis; **FISH**, fluorescence *in situ* hybridization; **PCR-DGGE**, denaturing gradient gel electrophoresis; **RFLP**, restriction fragment length polymorphism; **ARISA**, automated ribosomal intergenic spacer analysis; **SSCP**, single strand conformation polymorphism; **NGS**, next-generation sequencing.

Seagrass species	Plant part or compartment	Microbial community estimation	Technique employed	Reference
Cymodocea nodosa	Leaves, rhizomes and roots	Bacterial diversity and composition	SSCP and 16S rRNA gene Sanger sequencing	Mejia *et al.*, 2015*****
Cymodocea rotundata	Leaves	Bacterial enumeration, diversity and abundance	Light microscopy;16S rRNA genes and *nif*H gene sequencing	Hamisi *et al.*, 2013
Cymodocea rotundata, Thalassodendrum ciliatum, Thalassia hemprichii	Leaves	Bacterial density, biomass, abundance	PCR-DGGE; 16S rRNA gene sequencing	Uku *et al.*, 2007
Enhalus acroides	Leaves	Bacterial diversity and composition	ARISA and 16S rRNA gene-Illumina NGS.	Hassenrück *et al.*, 2015
***Enhalus* sp.**	Whole plant tissues (epiphytes and endophytes)	Bacterial enumeration and antifouling activity	CUD	Marhaeni *et al.*, 2010
Halodule wrightii, Thalassia testudinum, Syringodium filiforme	Vegetated sediments	Bacterial diversity and composition	PCR-DGGE; 16S rRNA gene sequencing	Guevara *et al.*, 2014
Halodule wrightii Thalassia testudinum	Vegetated and non-vegetated sediments	Bacterial diversity	Double gradient DGGE	James *et al.*, 2006
Halodule wrightii, Thalassia testudinum	Roots (anaerobes, acetogenic and sulfate-reducing bacteria (SRB)	Bacteria enumeration, microbial colonization patterns	Microscopy, FISH, phospholipid fatty acid analysis	Küsel *et al.*, 1999

*****Studies presented in this thesis.

Tab. 1 Continued

Seagrass species	Plant part or compartment	Microbial community estimation	Technique employed	Reference
Halophila stipulacea	Leaves, rhizomes and roots	Bacterial diversity and composition	16S rRNA gene 454-pyrosequencing (NGS)	Mejia *et al.*, 2015*
Halophila stipulacea	Leaves	Bacterial diversity and abundance	ARDRA and 16s rRNA gene sequencing	Weidner *et al.*, 2000
Halophila stipulacea	Leaves	Bacterial diversity	ARDRA	Weidner *et al.*, 1996
Posidonia oceanica	Leaves, rhizomes, roots (endophytes)	Bacterial diversity and abundance	PCR-DGGE; 16S rRNA gene sequencing	Garcias-Bonet *et al.*, 2012
Posidonia oceanica	Vegetated sediments (Sulfate-reducing *Deltaproteobacteria*)	Bacterial diversity and abundance	FISH and 16S rRNA gene sequencing	García-Martínez *et al.*, 2009
Posidonia australis, Posidonia oceanica, Posidonia sinuosa	Roots	Bacterial colonization patterns and abundance	SEM	García-Martínez *et al.*, 2004
Posidonia oceanica	Water-sediment interface	Bacterial enumeration (density, biomass, abundance)	EPF	Danovaro *et al.*, 1998
Posidonia oceanica	Vegetated sediments	Bacterial enumeration (density, biomass, abundance)	EPF	Danovaro *et al.*, 1994
Posidonia oceanica	Vegetated sediments	Bacterial activity	Exoenzymatic activity, ammonification rates, exoglucosidase activity	López *et al.*, 1998; 1995
Posidonia oceanica	Vegetated sediments	Bacterial enumeration (density, biomass, abundance)	EPF	Danovaro *et al.*, 1996
Posidonia oceanica	Vegetated sediments	Bacterial enumeration (density, biomass, abundance)	EPF	Danovaro and Fabiano, 1995

*****Studies presented in this thesis.

Tab. 1 Continued

Seagrass species	Plant part or compartment	Microbial community estimation	Technique employed	Reference
Thalassia testudinum	Vegetated and non vegetated sediments	Bacterial activity, abundance and composition	RFLP	Smith *et al.*, 2004
Thalassia testudinum, Syringodium filiforme	Vegetated and non-vegetated sediments	Diazotroph diversity and composition	PCR-DGGE; 16S rRNA gene sequencing	Bagwell *et al.*, 2002
Thalassodendrum ciliatum	Leaves (epiphytes and endophytes)	Photographic account	TEM and SEM	Barnabas, 1992
Thalassia testudinum Zostera marina	Rhizome detritus	Bacterial distribution, biomass and acetylene production	EPF and SEM	Kenworthy *et al.*, 1987
Zostera marina	Leaves and roots	Bacterial diversity and composition	PCR-DGGE; 16S rRNA genes sequencing	Crump and Koch, 2008
Zostera marina	Vegetated and non-vegetated sediments	Bacterial density, biomass, abundance	RFLP and 16S rRNA gene sequencing	Jensen *et al.*, 2007
Zostera marina Zostera japonica	Vegetated sediments	Bacterial enumeration and abundance	EPF and sole source carbon usage	Hahn, 2003
Zostera marina	Rhizomes and roots	O_2 sensitivity and sulfate reducing activity	O_2 incubations and Hypochlorite treatments	Blaabjerg and Finster, 1998
Zostera noltii	Vegetated sediments	Bacteria and Achaea abundance and diversity	16S rRNA gene sequencing	Cifuentes *et al.*, 2000
Zostera capricorni	Roots and rhizomes	Bacterial and *Alteromonas* sp. counts and abundance	Immunofluorescence and EPF	Glazebrook *et al.*, 1996
Zostera capricorni	Vegetated sediments	Gram-negative and Gram-positive bacteria composition	TEM	Moriarty and Hayward, 1982

1.4 Seagrasses-Microbes Interactions

Seagrasses enrich benthic bacterial abundances by the release of oxygen from its roots during photosynthesis. Benthic bacterial abundance differs seasonally influenced by plants productivity and has shown higher activity during summer than winter (reviewed by Duarte *et al.,* 2005). Microbial communities participate in multiple metabolic processes including the oxidation of organic carbon, nitrogen fixation, iron reduction, sulfate reduction and sulfide oxidation, some of which may be beneficial to the plants. The relationship between seagrasses and its microbiome can be considered mutually beneficial (Duarte *et al.,* 2005), encompassing a delicate balance in which seagrasses maintain bacterial activity through photosynthesis while microbial communities provide nutrients to the plants through remineralisation processes. Changes in abiotic and biotic conditions can disrupt this relationship (i.e. changes in plant photosynthesis may cause shifts in bacterial production of toxic sulfide which may be threatening to seagrasses habitats).

Different environmental and sediment conditions have been correlated to benthic bacterial activity, including temperature (Danovaro and Fabiano, 1995; García *et al.,* 2009), inorganic and organic nutrient contents (Danovaro, 1994, López *et al.,* 1995; López *et al.,* 1998), biochemical composition (Danovaro, 1994, Danovaro and Fabiano, 1995) and meiofauna distributions (Danovaro, 1996). Similarly, a three-stage symbiosis between seagrasses, lucinids, and

sulfide-oxidizing bacteria has been reported to be crucial in reducing sulfide stress in seagrass meadows (Van der Heide *et al*., 2012). This highlights the importance of examining different trophic interactions in seagrass meadows.

The study of the microbiome in terrestrial plants has revealed complex and dynamic interactions in which microbes play a crucial role in plant's growth, health and adaptation to the prevailing environmental conditions (Rout and Southworth, 2013). In this context, microbes represent a reservoir of 'different metabolic activities', which plants can use to respond to changes in the environment. By shifting their (resident) community structure and/or metabolic function, microbes may confer plants the capability to rapidly cope with changes in environmental conditions, securing their survival.

Similar interactions have also been observed with some marine organisms, *i.e.*, in macroalgae, microbes (from the *Roseobacter* clade) are known to be involved in their morphogenesis and growth (Singh and Reddy, 2014); in corals, symbiotic and/or pathogenic bacteria shifts have been observed in response to changes in water quality and pH (*i.e.* increase of pathogenic *Gammaproteobacteria Vibrio* spp.: Rosenberg *et al.*, 2007; Meron *et al*., 2011); likewise, in marine sponges, microbial shifts have been observed in response to changes in temperature and water quality (*Alphaproteobacteria* abundance reduces with high temperature, while *Gammaproteobacteria* increases

with increased concentrations of dissolved organic carbon (Teira *et al.*, 2010; Hentschel *et al.*, 2012).

The physical and chemical conditions to which the plant parts are exposed may influence the patterns of microbial assemblages on the plant surfaces as well as their chemically mediated interactions which in turn may impact plant's health (Michael *et al.*, 2008; Egan *et al.*, 2008). In seagrasses, differences in the microbial communities have been observed in response to local nutrient gradients (Nitrogen and Phosphorus) in three seagrass species, *Thalassia testudinum, Syringodium filiforme* and *Halodule wrightii,* in the Florida Keys (Guevara *et al.*, 2014). However, the research is still scant, particularly about the effects of increasing CO_2 causing ocean acidification (Hassenrück *et al.,* 2015), changes in temperature, and about the influence of spatial or temporal differences.

In the last decades the CO_2 absorbed by the ocean is changing the chemistry of seawater through a process known as ocean acidification (OA). OA poses a risk to the biogeochemical processes in the ocean and the ecological functions and associations of marine organisms. Seagrasses play a critical role in sequestering atmospheric CO_2, yet they continue to decline at a fast rate. Microbial shifts have been observed in the associated communities on corals and the seagrass *Enhalus acroides* (Hassenrück *et al.,* 2015) under increased CO_2 conditions, suggesting the need to explore further the resilience of plants and the (host-associated) microbial communities in a changing climate.

The existing literature highlights some important aspects about the microbial communities associated with seagrasses, despite the intrinsic limitations related to PCR, cloning and fingerprinting techniques. However, more in depth analyses are required to adequately assess differences in microbial community assemblages. The use of more advance methodologies such as Next Generation Sequencing (NGS) represents a great advantage to deepen the study and analysis of microbial communities.

To address some of the gaps in seagrass microbes' research, studies should focus on the effects of changing environmental conditions on microbial community structure, composition and diversity. Also to be considered is the evaluation of different temporal and spatial scales on different seagrass species. These can increase our understanding of the interaction between microbes and seagrasses, and of these with the surrounding environment.

2 AIMS OF THE STUDY

This study aims to explore the structure, diversity and composition of epiphytic microbial communities associated with seagrasses under different environmental conditions.

To reach this aim, the following objectives were undertaken:

1. Characterize and investigate the microbial communities associated with *Cymodocea nodosa* under the effects of experimental seawater acidification and eutrophication conditions.

2. Characterize and investigate the microbial communities associated with *Halophila stipulacea* along a gradient of different human pressure.

3. Characterize and investigate the microbial communities associated with *Halophila stipulacea* along a depth gradient (4 – 28 meters).

4. Infer about possible responses of seagrass plants and their microbial communities under different environmental conditions.

This work hopes to contribute to the long term goal of employing seagrass microbial community assemblages as descriptors of environmental changes to help ensure seagrasses conservation.

CHARACTERIZATION OF THE EPIPHYTIC MICROBIAL COMMUNITIES ASSOCIATED WITH SEAGRASSES

The following sections provide a description of all the steps carried out to characterize the epiphytic microbial communities associated with the seagrasses *Cymodocea nodosa* and *Halophila stipulacea*.

First, the methodological steps undertaken for selecting the seagrasses species and study sites are described, followed by a description about the sampling, processing and analysis of the microbial community samples. Then, the results of the microbial community analyses are presented. Next, the findings are discussed and the overall conclusions presented. Finally, common patterns about the seagrasses associated microbial communities are exposed along with perspectives for future studies.

The descriptions referred above will be presented separately by seagrass species.

3 THE MICROBIAL COMMUNITIES ASSOCIATED WITH CYMODOCEA NODOSA

3.1 Study site and experiment set up

Cymodocea nodosa (Ucria) Ascherson is a monocotyledon belonging to the order Alismatales, family *Cymodoceaceae*. This temperate seagrass is known to occur extensively in coastal lagoons and estuaries, and where harsh hydrodynamic conditions persist (Short *et al.,* 2007). This species is a dominant resident in the very dynamic Ria Formosa lagoon, Portugal, where it forms extensive monospecific and mixed stands with *Zostera marina*. The Ria Formosa is a coastal lagoon in which at least three seagrass species can be found, *Zostera noltii*, *Zostera marina* and *Cymodocea nodosa*. This lagoon is characterized by an important tidal cycle which subjects the seagrasses to periods of desiccation and constant renewal of water from the sea. The seagrasses are thus exposed to great variations of environmental conditions due to the wide tidal range within the lagoon ecosystem, oscillating from 1.5 meters during neap tides to 3.5 meters during spring tides (Cabaço *et al.,* 2009).

During the experimental set up, *C. nodosa* plants were collected in the Ria Formosa coastal lagoon and transplanted to a mesocosm facility (Fig. 3), established at the Ramalhete Marine Station in Faro (PT). This mesocosm facility was designed and managed by the Marine Plant Ecology Research group (ALGAE group) based at the Centre of Marine Sciences (CCMAR), University of Algarve. This

experimental set up was supported by three grants provided by the European Community - Research Infrastructure Action under the FP7 "Capacities" Specific Programme, ASSEMBLE grant agreement no. 227799. The project “Qualitative assessment of the microbial component in the structure of the seaweed community” was funded by the 3rd (set up of the method), 4th (1st sampling) and 5th (2nd sampling) calls of the programme.

In the mesocosm, the plants were exposed for 1 month to four experimental conditions, in which two levels of CO_2 -current (360 ppm) and future (700 ppm)- and two levels of nutrients -ambient (2 µM NH^{4+}-NO^3; 0.5 µM PO^{4-}) and enriched (8 µM NH^{4+}-NO^3, 5 µM PO^{4-})- were combined as follows:

- Current CO_2 and ambient nutrient (C-N-);
- Current CO_2 and enriched nutrient (C-N+);
- Future CO_2 and ambient nutrient (C+N-); and
- Future CO_2 and enriched nutrient (C+N+).

The ALGAE group collected plant samples and performed analysis on plant growth rates and Carbon to Nitrogen ratios on the leaves to monitor the effects of the experimental conditions on the plant’s physiology.

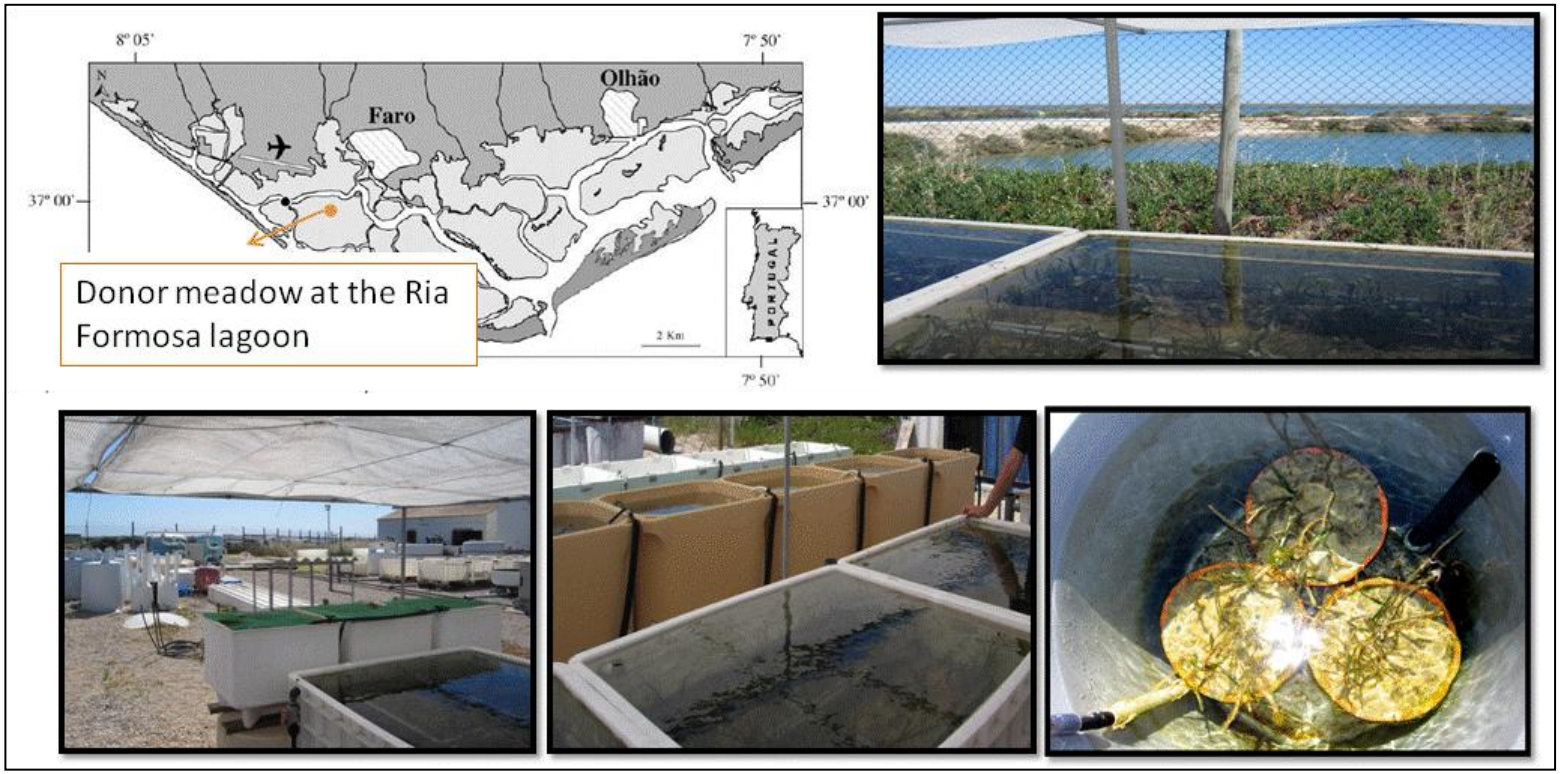
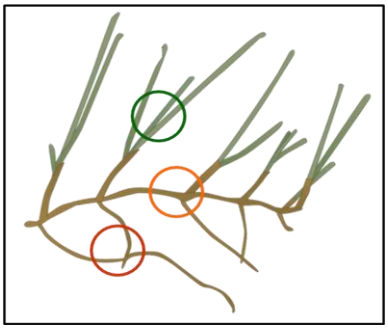


Fig. 3 Map of the Ria Formosa lagoon, southern Portugal showing the Cymodocea nodosa meadow chosen to transfer plants to a mesocosm set up in the Ramalhete marine station for the characterization of their epiphytic microbial community.

3.2 Bacterial sampling and processing



In two separate seasons, summer 2010 and winter 2011, plants were collected for the characterization of the epiphytic microbial community. Three shoots, each composed of 3 leaves and their accompanying rhizomes and roots were collected from the mesocosm tanks described earlier to obtain 3 replicates per plant part and experimental condition. The replicates were then processed for their bacterial pellets.

In addition, plants from the field were also collected to also establish a baseline of the microbial communities associated with the plants in the Ria Formosa Lagoon.

3.2.1 Bacterial pellet

The bacterial pellets were collected from leaves, rhizomes and roots. To get the bacterial pellet, each plant part was placed into 50 ml tubes, soaked and washed with 10 ml of washing solution (200 mM Tris-HCl pH8, 10 mM EDTA, and 0.24% Triton X-100; Kadivar and Stapleton, 2003). Then they were gently rubbed against the tubes walls and vortexed (3 x 30s) to detach and collect the bacteria. After removing the plant parts from the tubes the bacterial suspension was centrifuged

at 15000 *g* for 20 min and pelleted. The pellet was then processed for DNA extraction. The bacterial pellets were standardized to the amount of plant materials collected.

Since the interest of this work was to collect only the epiphytic bacteria attached to the roots, at the point of collecting the plants these were carefully shaken while submerged in the tanks to get rid of sediment particles. Unavoidably, a very small fraction of sediment particles usually remained attached to the root hairs. Consequently, some of the rhizosphere bacteria were also collected. It is also important to note that the leaves of *Cymodocea nodosa* were gently scraped with a sterile scalpel to help detach the bacterial epiphytes during the washing step. This was avoided in *Halophila stipulacea* to reduce potential contaminations (extraction of chloroplasts and discharge of contaminants due to mechanical damage of the plant parts) that could interfere with further DNA processing.

3.2.2 DNA Extraction, Amplification and Sequencing

A phenol-chloroform extraction method was used to extract the bacterial DNA associated with *Cymodocea nodosa*. The DNA extraction protocol employed has been described in Evangelisti *et al.*, (2014) and it originated from adapting the protocol described in Rossolini *et al.*, (1993) and Zhou *et al.*, (1996). To conduct the DNA extraction, the bacterial pellets (samples) were mixed with 1 ml of extraction buffer Solution 1 (50 mM Tris-HCl pH 8, 20% sucrose, 50 mM EDTA, 10 mg/ml lysozyme) and kept for 30 min a 37°C. Then,

the samples were treated with 4 ml of extraction buffer Solution 2 (50 mM NaCl, 1% CTAB, 35 µl of 10 mg/ml proteinase K solution) and kept at 37°C for 30 min. The samples were shaken by inversion every 10 min during the warm incubation. Next, 0.5 ml of 20% Sarkosyl (Sigma-Aldrich, USA) was added to the samples and these were incubated at 65°C for two hours under gentle inversion. After centrifugation at 6000 g (10 min) at room temperature, the supernatants were collected and mixed with an equal volume of preheated (to 60°C) phenol-chloroform- isoamyl alcohol (25:24:1; Applichem, Germany). The aqueous phase was recovered by centrifugation (4000 g) at 4°C and precipitated with sodium acetate 3 M pH 5.2 (1/10 volume) and ethanol (2x volume). The pellet obtained after centrifugation at 15,000 g was rinsed in 70% ethanol (Sigma-Aldrich USA) and stored in 100 µl TE buffer at −20°C until PCR amplification.

The bacterial DNA extracts were used for DNA amplification targeting the 16S rRNA gene by Polymerase Chain Reaction (PCR). Next, the amplified products were prepared for DNA fingerprinting and sequencing. The procedures of DNA amplification and sample preparation for Single Strand Conformation Polymorphism (SSCP) were conducted according to the descriptions in Dohrmann and Tebbe, (2010). This technique was chosen to obtain the bacterial DNA profiles of *Cymodocea nodosa* (Fig. 4) because it allows for the evaluation of similarities of different samples through the quantitative analysis of their ‘genetic profiles’ or ‘fingerprints’. The DNA bands

on the profiles can be cut off the gels and prepare for sequencing to investigate microbial community composition.

To obtain the bacterial profiles, the following steps were carried out:

1. The bacterial metagenome was amplified by PCR with universal primers Com1 and phosphorylated primer Com2-Ph (Tab. 2) to obtain DNA amplicons of approximately 400 bp. Amplification reactions were carried out in a volume of 100 µl with 100 pmol of each primer in 2X My Taq Red Mix buffer (Bioline) and 2 µl of the DNA template. Amplification conditions for 16S rRNA genes included an initial denaturation step of 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C for 70 sec and a final extension step of 72°C for 5 min. Reaction products were checked for size and purity on a 1% agarose gel. The PCR products were purified using E.Z.N.A. Cycle-Pure Kit (OMEGA bio-tek, USA).

Tab. 2 DNA amplification primers used in this study.

Target:	**Amplification of the 16S rRNA gene by PCR**
Primers:	Universal for bacteria: **Com1** (5'-CAGCAGCCGCGGTAATAC-3'); positions 519-536 **Com2-Ph**,(5'-CCGTCAATTCCTTTGAGTTT-3'); positions907-926 Fragment length: 400 bp; Regions: V4 and V5. (Schwieger and Tebbe, 1998; Schmalenberger *et al.*, 2001).

2. The double strand DNA was digested with a lambda exonuclease enzyme to obtain single strand DNA.

3. The single strand DNA was loaded onto a polyacrylamide gel. The gels (16 cm in length, 0.4 mm in thickness) were run at 250 V, 8 mA for 17 h at 25°C in an adjustable slab gel kit (C.B.S. Scientific Co., USA). The DNA fingerprints were then stained with silver nitrate to visualize and analyze the profiles.

4. The DNA profiles were photographed and the images analyzed with the software Phoretix 1D Totallab to conduct the bacterial community analysis.

5. The DNA bands with densitometric values higher than 500 pixels in the fingerprinting profiles were selected for Sanger sequencing. The selected bands were excised and processed by PCR re-amplification prior to sequencing (Dohrmann and Tebbe, 2010).

3.2.3 Microbial Community Analysis

3.2.3.1 SSCP profiles

The densitometric values calculated by Phoretix 1D Totallab software for each sample, corresponding to a plant part and experimental condition were used as raw data to perform microbial community analyses. Individual gels (Fig. S1) pertaining to the four experimental conditions and two seasons were prepared and analyzed. These gels contained two or three replicates per plant compartment and season. The relative abundance of the bands in the profiles (or lanes) were used as an input matrix to measure the Shannon-Weaver diversity index (H') in PAST software v1.89b. Non metric Multi Dimensional

Scaling (n-MDS; PAST software, v1.89b) was used to visualize the different fingerprints.

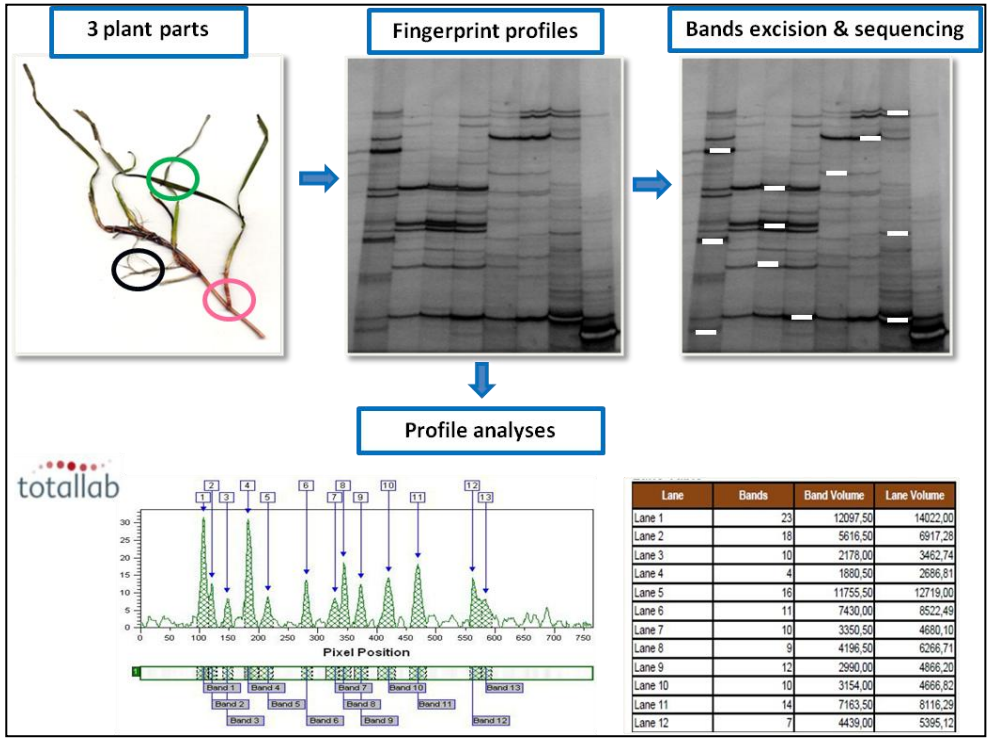
3.2.3.2 Operational Taxonomic Units (OTUs) based analysis

To identify the closest taxon represented by the bands in the fingerprinting profiles, SSCP band sequences were compared with the sequences available in the Ribosomal Database Project (RDP; Maidak *et al.*, 1999) and then subjected to BLAST analysis using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/; Altschul *et al.*, 1990). To visualize similarities of the bacterial composition, phylogenetic trees of the SSCP sequences were built in the ClustalW website (www.genome.jp/tools/clustalw/; Larkin *et al.*, 2007). The sequences were inputted in a randomized order and trees were built using the neighbor-joining method. Sequences of strains belonging to the class *Flavobacteria* were taken from the public RDP database and used as outgroups. The classifier function (Wang *et al.*, 2007) on the Ribosomal Database Project website was used to determine the taxonomy of the SSCP sequences obtained; using a cutoff of 80%. All sequences determined in this study have been deposited in GenBank under accession no. KR133495 to KR133579.

3.2.3.3 Statistical Analysis

Analysis of Variance (ANOVA, Two factor with replication) was used to determine significant differences in diversity of the different SSCP

fingerprinting profiles corresponding to the four experimental conditions. Significant effects were considered at $p < 0.05$.



Lane	Bands	Band Volume	Lane Volume
Lane 1	23	12097,50	14022,00
Lane 2	18	5616,50	6917,28
Lane 3	10	2178,00	3462,74
Lane 4	4	1880,50	2686,81
Lane 5	16	11755,50	12719,00
Lane 6	11	7430,00	8522,49
Lane 7	10	3350,50	4680,10
Lane 8	9	4196,50	6266,71
Lane 9	12	2990,00	4866,20
Lane 10	10	3154,00	4666,82
Lane 11	14	7163,50	8116,29
Lane 12	7	4439,00	5395,12

Fig. 4 Exemplification of the steps followed to separate the genomic bacterial DNA of *Cymodocea nodosa* on a polyacrylamide gel by electrophoresis, the selection of bands for sequencing and of the analyses of the DNA profiles with Phoretix 1D Totallab software.

3.3 Results

3.3.1 SSCP profiles analysis

The analysis of the bacterial profiles, corresponding to different plant parts and experimental conditions, revealed a higher Shannon diversity (H') on leaves than on rhizomes and roots, in both summer and winter seasons (Tab. 3). Overall, diversity values among samples were lower in winter than summer. In general, significant differences in microbial diversity were highlighted between summer and winter independently, by plant part and experimental condition (ANOVA, $p<0.05$; Tab. S1 [1, 2]). Differences in diversity influenced by the interaction of experimental conditions and plant parts were statistically significant in summer but not in winter (ANOVA, $p<0.05$; Tab. S1 [3]). In summer, the microbial diversity of leaves was significantly different than that from both rhizomes and roots (ANOVA, $p<0.05$; Tab. S1 [4, 5]). On the contrary, diversity between rhizomes and roots was not significantly different (ANOVA *n.s.*, Tab. S1 [6]). In summer, nutrient additions influenced diversity of all plant parts, (see C+N- and C+N+, Tab.3), although differences were not statistically significant (t- test *n.s.*). In winter, the experimental conditions C-N+ and C+N- reduced the microbial diversity in the different plant parts (Tab 3), although differences were not statistically significant (t- test *n.s.*). The condition C+N+ showed the highest values of all conditions and comparable values to the control (C-N-). The Shannon diversity in the plants from the field, also showed the

highest diversity values on leaves during summer (2.83±0.28) and winter (2.62±0.09). Microbial diversity of rhizomes (2.29±0.28) was slightly higher than on roots (2.09±0.08) during winter; while in summer diversity of roots was 2.39±0.28). The replicates of rhizomes showed no bands in summer.

Tab. 3 Shannon Diversity Index (H') of the microbial communities associated with the different plant parts (leaves, roots and rhizomes) of *Cymodocea nodosa* under different experimental conditions (C-N- control; C+N- high CO_2; C-N+ high Nutrients; C+N+ high CO_2 and Nutrients). Data represents the mean of two replicates.

SHANNON DIVERSITY INDEX (H')			
Experimental condition	**Leaves**	**Rhizomes**	**Roots**
SUMMER			
C-N-	2.47±0.11	1.69±0.007	1.53±0.39
C-N+	2.52±0.03	1.97±0.109	2.060±0.02
C+N-	2.03±0.02	1.79±0.03	2.32±0.05
C+N+	2.89±0.33	1.97±0.02	1.97±0.007
WINTER			
C-N-	2.68±0.06	1.87±0.26	1.77±0.05
C-N+	1.8615±0.11	0.759±1.07	1.43±0.17
C+N-	0.78±0.24	1.23±0.16	1.10±0.05
C+N+	2.43±0.22	1.31±0.22	2.09±0.16

The n-MDS analyses performed for each experimental condition (Fig. 5), based on the relative abundances of the bands in the SSCP profiles show some similarity between the microbial communities of the

experimental conditions C+N+ and C+N-; while the samples exposed to C-N+ appear more dispersed. Stress values are reported on the legend of Fig. 5. See Fig. S2 in supporting information for n-MDS results by season.

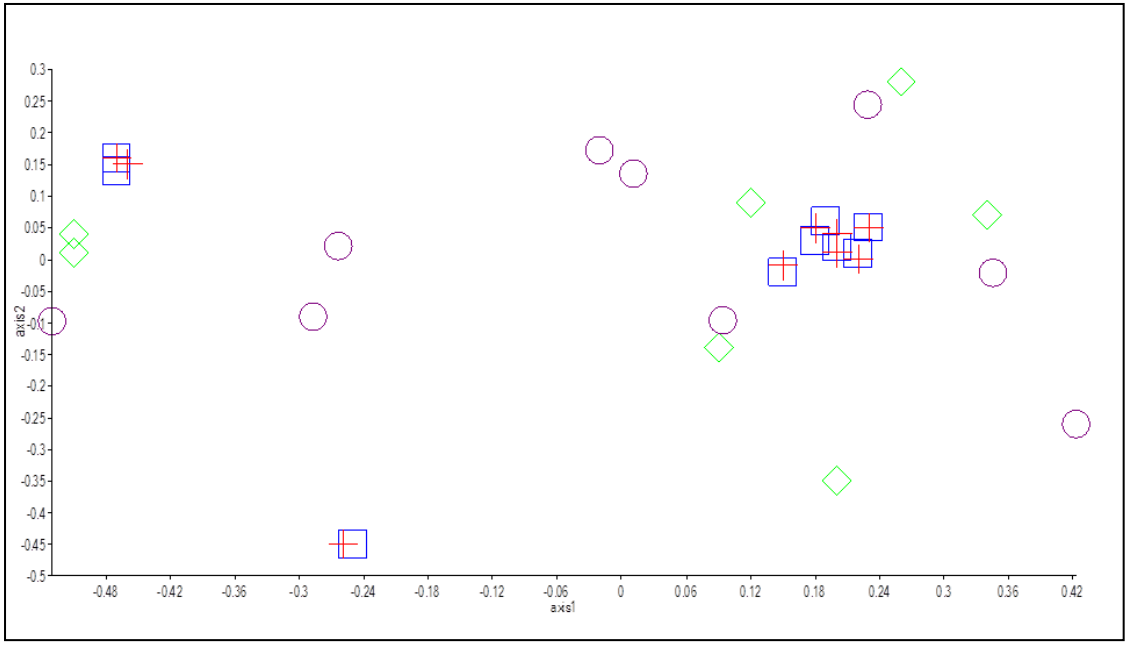


Fig. 5 Plot with n-MDS axis coordinates showing the microbial fingerprints associated with different plant parts (leaves, roots and rhizomes) of *Cymodocea nodosa* plants under different experimental conditions (C-N- control; C-N+ high Nutrients; C+N- high CO_2; C+N+ high CO_2 and Nutrients). The analysis was performed using Bray-Curtis similarity indices. Data represents two and three replicates per plant part for each experimental condition. Symbols: C-N- = green diamond; Stress value= 0.073; C-N+ = purple circle, Stress value= 0.088; C+N- = blue square. Stress value=0.157; C+N+ = red cross, Stress value=: 0.154.

3.3.2 OTUs based analysis

Major constrains were experienced with the approach used to reamplify the SSCP generated bands since many resulted in poor sequencing products. Given this, no statistical comparison can be performed with this data. Nonetheless, this limitation, the findings depict interesting aspects of the bacterial assemblages that merit discussion. The sequencing of the most intense SSCP bands on the bacterial fingerprints resulted in a total of 149 bacterial sequences that clustered into 75 different OTUs. Venn diagrams show the distribution of unique *vs.* shared OTUs by plant parts (Fig. 6 a, b). In general, unique OTUs were always higher than shared OTUs. Leaves and roots had a comparable number of total OTUs and higher than the rhizomes. The unique OTUs on each plant part accounted for about 40-50% of the total OTUs observed (rhizomes: 13/32; leaves: 19/38; roots: 21/42). Only 17% of the OTUs were shared among all plant parts. The samples from the lagoon also showed a higher number of unique *vs.* shared OTUs in all plant parts. No OTUs were shared among all three plant parts, but between leaves and rhizomes and rhizomes and roots.

Fig. 6 Venn diagram showing the unique and shared OTUs of the microbial communities associated with *Cymodocea nodosa* plants: (a) in an experimental mesocosm by plant part and (b) from the field, Ria Formosa Lagoon.

The taxonomic identification of the sequences revealed that the phylum *Proteobacteria*, classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* were the most abundant and dominant phylotypes found, representing ~80% of the total community composition (Fig. 7). The less abundant phylotypes comprising the remaining 20% of the community included the phyla *Firmicutes, Bacteroidetes, Spirochaetes, Verrucomicrobia, Planctomycetes*, as well as *Epsilonproteobacteria and Betaproteobacteria*. In addition, 18 sequences were classified as *Cyanobacteria/Bacillariophyta* chloroplasts.

The microbial taxa show differences in abundance among plant parts (Fig. 7): the class *Alphaproteobacteria* ranged from 17% on leaves to 12% on both rhizomes and roots; *Gammaproteobacteria* from 36% on leaves to 25% on rhizomes and 35% on roots; while *Deltaproteobacteria* increased from 25% on leaves to 37% on

rhizomes and roots. In each plant part, the rare taxa accounted for a total of 22% on leaves, 25% on rhizomes and 17% on roots (shown on the top of the bars, Fig. 7). Among these, *Verrumicrobia* and *Planctomycetes* were found only associated with the leaves of the plants. On the plants from the Ria Formosa lagoon, the bacterial composition was dominated by *Gammaproteobacteria* (59%), followed by *Deltaproteobacteria* (24%), *Bacteroidetes* (10%) and *Spirochaetes* (7%). At least two sequences were identified as *Cyanobacteria/Bacillariophyta* chloroplasts and these were present in all plant parts. No *Alphaproteobacteria* was observed in these samples.

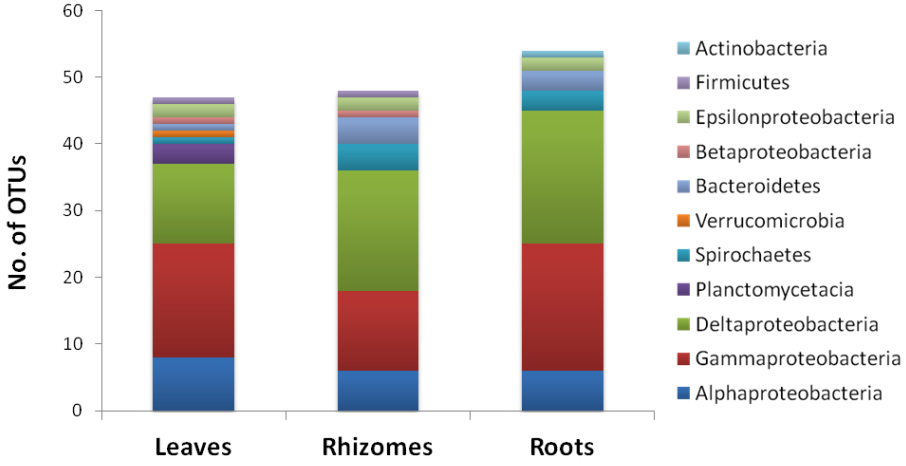


Fig. 7 Distribution of Operational Taxonomic Units (OTUs) of the microbial taxa found on the plant parts (leaves, rhizomes and roots) of *Cymodocea nodosa* when exposed to experimental condition of CO_2 and nutrient concentrations in a mesocosm.

Phylogenetic trees with the most abundant phylotypes (*Alphaproteobacteria, Gammaproteobacteria,* and *Deltaproteobacteria*) were constructed to further explore differences within and among taxonomic groups. (Fig. 8a-c).

The *Alphaproteobacteria* assemblages on the three plant parts (in blue, Fig. 8a-c) show two clear-cut clusters formed by OTUs assigned to the orders *Rhizobiales* (A1) and *Rhodobacterales* (A2), which include nitrogen-fixing and phototrophic bacteria commonly found on seagrasses.

The *Gammaproteobacteria* assemblage on leaves (in red, Fig. 8a) was mainly composed of one large cluster (L-G2 formed by OTUs assigned to the genus *Reinekea* (4 OTUs), three of which were closely related to the species *Reinekea blandensis* (Pinhassi *et al.*, 2007), and the last one had a high homology (83%) to the halophillic *Halochromatium* sp. On rhizomes and roots the OTUs formed two clusters (Fig. 8b, c); one, formed by OTUs mainly assigned to the *Vibrionaceae* family (RZ-G1 and RT-G1), and the other, mainly assigned to uncultured *Gammaproteobacterium* strains (RZ-G2 and RT-G2). The cluster RT-G2 was larger on roots than on rhizomes and at least 1 OTU was assigned to the Order *Chromatiales*.

The *Deltaproteobacteria* assemblages (in green, Fig. 8a-c) form two clusters in all plant parts assigned to *Desulfobacteraceae* (D1) and *Desulfovibrionaceae* (D2). The cluster D1 is well represented on all plant parts, with some OTUs showing similarity to the genus

Desulfonema, *Desulfomonile* and *Desulforsacina* and it is the largest cluster on the roots. The cluster D2 is formed mainly by OTUs assigned to the genus *Desulfovibrio*, and it is better represented on leaves and rhizomes, than on roots (1 OTU).

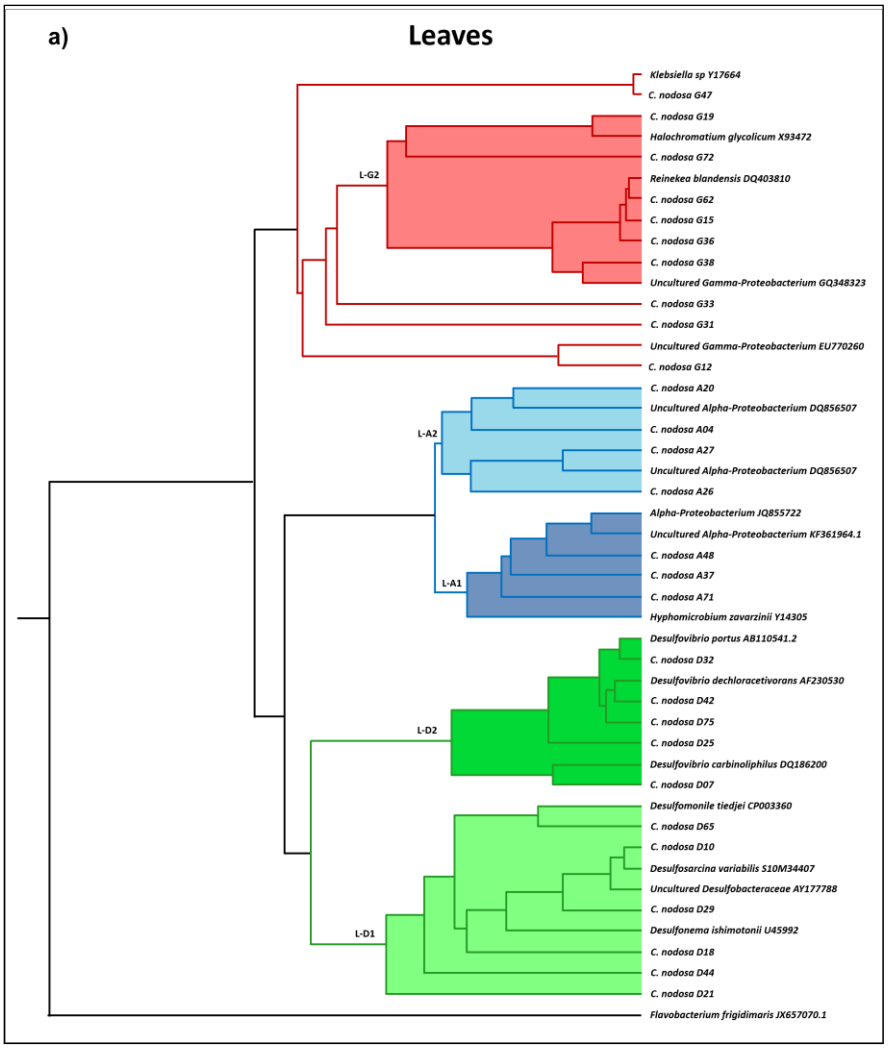
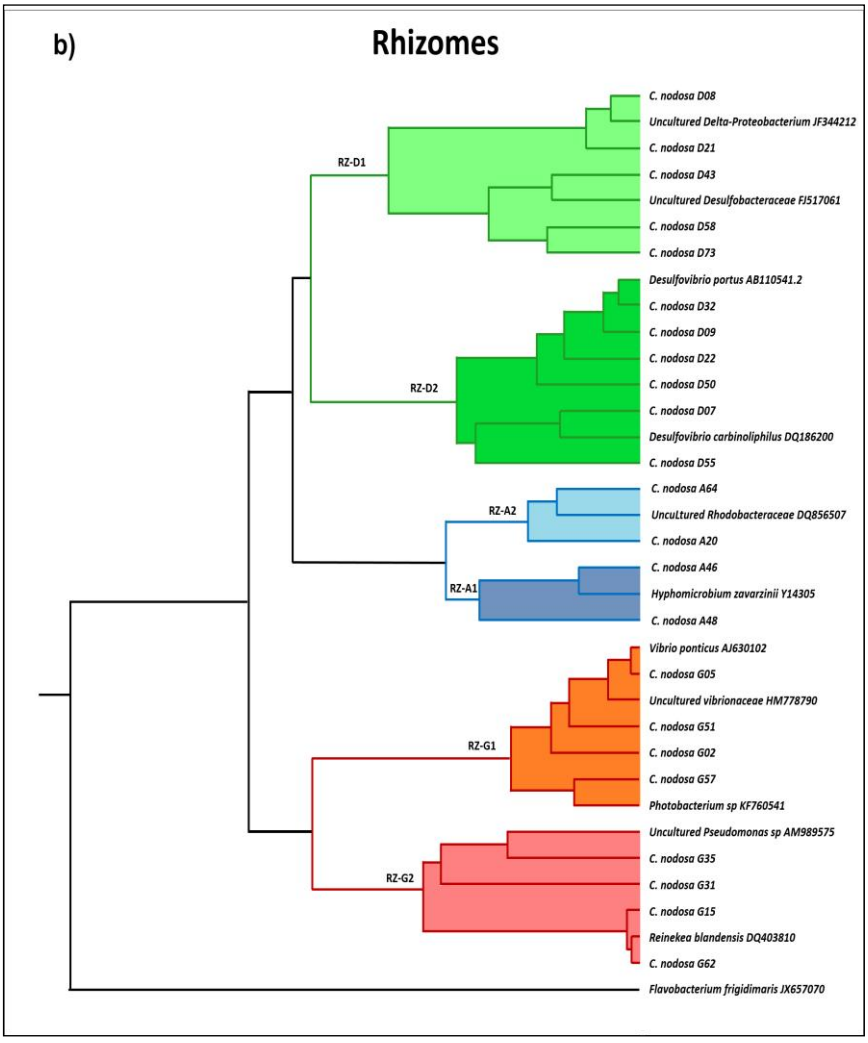


Fig. 8 Phylogenetic affiliations of the dominant OTUs collected by plant parts: (a) leaves (L), (b) rhizomes (RZ), (c) roots (RT). The dominant microbial taxa are identified by different colours, in blue *Alphaproteobacteria* (A), in green *Deltaproteobacteria* (D), in red *Gammaproteobacteria* (G); sub-groups correspond to A1 and A2, D1 and D2, G1 and G2). The OTUs of this study are referred to as *C. nodosa* plus main group initial and identification number (A#, D#, G#).


b)
Rhizomes
C. nodosa D08
Uncultured Delta-Proteobacterium JF344212
C. nodosa D21
C. nodosa D43
Uncultured Desulfobacteraceae FJ517061
C. nodosa D58
C. nodosa D73
Desulfovibrio portus AB110541.2
C. nodosa D32
C. nodosa D09
C. nodosa D22
C. nodosa D50
C. nodosa D07
Desulfovibrio carbinoliphilus DQ186200
C. nodosa D55
C. nodosa A64
UncuLtured Rhodobacteraceae DQ856507
C. nodosa A20
C. nodosa A46
Hyphomicrobium zavarzinii Y14305
C. nodosa A48
Vibrio ponticus AJ630102
C. nodosa G05
Uncultured vibrionaceae HM778790
C. nodosa G51
C. nodosa G02
C. nodosa G57
Photobacterium sp KF760541
Uncultured Pseudomonas sp AM989575
C. nodosa G35
C. nodosa G31
C. nodosa G15
Reinekea blandensis DQ403810
C. nodosa G62
Flavobacterium frigidimaris JX657070
RZ-D1
RZ-D2
RZ-A2
RZ-A1
RZ-G1
RZ-G2


c)
Roots
RT-A1
RT-A2
RT-D1
RT-D2
RT-G1
RT-G2
C. nodosa A46
Hyphomicrobium zavarzinii Y14305
C. nodosa A52
Alpha-Proteobacterium JQ855722
C. nodosa A48
C. nodosa A71
Uncultured Rhodobacteraceae DQ856507
C. nodosa A20
C. nodosa A49
C. nodosa D28
C. nodosa D18
C. nodosa D39
C. nodosa D11
Desulfonema ishimotonii U45992
Uncultured Desulfobacteraceae FJ517061
C. nodosa D30
Desulfosarcina variabilis M34407
C. nodosa D01
C. nodosa D40
Desulfomonile tiedjei CP003360
C. nodosa D34
C. nodosa D61
C. nodosa D63
C. nodosa D17
Uncultured Delta-Proteobacterium JF344212
C. nodosa D24
Uncultured bacterium KF268898
Desulfovibrio piezophilus C1TLV30
Desulfovibrio portus AB110541.2
C. nodosa D14
Desulfovibrio carbinoliphilus DQ186200
Klebsiella sp Y17664
C. nodosa G56
C. nodosa G05
Uncultured Vibrionaceae HM778790
C. nodosa G51
C. nodosa G57
Uncultured Pseudomonas AM989575
C. nodosa G35
C. nodosa G23
C. nodosa G72
C. nodosa G31
C. nodosa G19
Uncultured Gamma-Proteobacteria incertae sedis X84979
C. nodosa G15
C. nodosa G62
Uncultured Xhantomonadales EU770260
Flavobacterium frigidimaris JX657070

3.4 Discussion

This study aimed to characterize the microbial community associated with different plant parts of the seagrass *Cymodocea nodosa* in a mesocosm to assess the possible influences of different CO_2 and nutrient regimes on the diversity and composition of these communities.

The analysis of the bacterial fingerprinting profiles obtained with SSCP highlighted significant differences among plant parts and experimental conditions. Clear differences among plant parts and the effect of experimental conditions were highlighted by ANOVA. The Shannon diversity Index (H') showed that leaves, rather than rhizomes and roots, harboured the most diverse bacterial communities both in summer and winter, and this was also observed on the plants from the lagoon. Similar results have been reported for other seagrasses, like *Halophila stipulacea* (Weidner *et al.,* 2000) and *Zostera marina* (Crump and Koch, 2008).

This constitutes one of the few studies looking at the potential effects of acidification in seagrass habitats focusing on the bacterial communities. The findings showed shifts in the microbial communities in response to manipulation of nutrient and CO_2 concentrations. By shifting their (resident) community structure and/or metabolic function, microbes may confer plants the capability to rapidly cope with changes in environmental conditions, securing their

survival. The response of epiphytic microbial communities to changing environmental conditions provides evidence of their potential use as a descriptor of plant conservation status. Microbes represent a reservoir of 'different metabolic activities', which plants can use to respond to changes in the environment. The plants in the mesocosm did not show significant changes in growth rates and C:N ratios. A possible explanation for this could be related to a lack of response to the experimental conditions tested, but it is also important to note that *C. nodosa* plants are exposed to highly variable conditions in the field which can also influence the lack of response observed in the mesocosm. Large variations in environmental conditions are imposed daily by the wide tidal range that takes place in the Ria Formosa lagoon. The tidal cycle ranges from 1.5 m during neap tides and 3.5 m during spring tides (Cabaço *et al.*, 2009), this represent a constant input of nutrients and resources for the plants, and variable conditions, including periods of desiccation during the low tides.

A total of 149 SSCP bands were successfully isolated from the different parts of *C. nodosa* plants, and these clustered into 75 different OTUs. The OTUs distribution depicted in Venn diagrams showed a higher number of unique than shared OTUs associated with the different plant parts in all the experimental conditions and also on the plants from the field, suggesting the presence of microbial assemblages with distinct community compositions.

Overall, across all three plant parts, the microbial composition was dominated by the phylum *Proteobacteria*, followed by *Spirochaetes* and *Bacteroidetes.* The classes *Alphaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* were the most abundant phylotypes, showing differences among plants parts and phylogeny.

The class *Alphaproteobacteria* was mainly found associated with leaves. The phylogenetic analysis assigned all the *Alphaproteobacteria* OTUs to the orders *Rhizobiales* and *Rhodobacterales*. *Rhizobiales* comprise nitrogen-fixing bacteria and *Rhodobacterales* include chemoorganotrophs and photoheterotrophic bacteria. The latter are dominant and ubiquitous primary surface colonizers in coastal waters (Elifantz *et al.,* 2013) which may deter the attachment of other bacteria through the production of antibacterial compounds. These features may enable them to both persist and adapt rapidly to changing environmental conditions and be dominant in the epiphytic microbiota (Dang *et al.,* 2008). Members of both of these orders have been found associated with seagrass leaves (Weidner *et al.*, 2000), roots (Crump and Koch, 2008) and coastal water biofilms (Taylor *et al.*, 2014).

The *Deltaproteobacteria* was the most abundant phylotype found on rhizomes and roots. The phylogenetic analysis assigned all the *Deltaproteobacteria* OTUs to the families *Desulfobacteraceae* and *Desulfovibrionaceae*. These *Deltaproteobacteria* families include

obligate anaerobic, sulphate reducing bacteria, which have been commonly found associated with the roots of seagrasses (García-Martínez *et al.*, 2009) and in seagrass marine sediments (Boer *et al.*, 2007). The two families were differently represented on the plant parts: on the rhizomes, *Desulfobacteraceae* and *Desulfovibrionaceae* displayed a comparable number of different OTUs and the same pattern was observed on the leaves. On the roots, *Desulfobacteraceae* was dominant and only one OTU of *Desulfovibrionaceae* was found. This dominance cannot be easily explained and could be determined by several factors such as the kind and/or amount of available nutrients, the sulphide concentration, or the biotic component. Some *Desulfovibrionaceae* could be more oxygen-tolerant; hence, they would be able to persist on rhizomes and leaves.

The *Gammaproteobacteria* was the most represented class on leaves. The phylogenetic analysis assigned the great majority of *Gammaproteobacteria* OTUs to the families *Vibrionaceae* and *Oceanospirillaceae*. These include halotolerant/halophilic nitrate reducing aerobic marine bacteria, known to be able to withstand changes in nutrient conditions. On the leaves, the genus *Reinekea* (*Oceanospirillales*) was dominant, while in the below ground parts the OTUs were mainly *Vibrionaceae* (*Vibrio* and *Photobacterium*). The differences in dominance probably depend on the well documented ability of the *Vibrionaceae* to associate with benthic fauna, clams and zooplankton (Mansson *et al.*, 2011), while *Reinekea* has been isolated from coastal waters and tidal flats (Pinhassi *et al.*, 2007; Choi and

Cho, 2010). *Gammaproteobacteria* sulfur oxidizing bacteria have been found associated with the roots of seagrasses (Crump and Koch, 2008) as well as in marine sediment exposed to high concentrations of CO_2 (Kerfahi *et al.*, 2014), as it was found here on the roots and also on the leaves of *Cymodocea nodosa*.

The preliminary characterization of the bacterial community associated with the plants collected in the Ria Formosa lagoon showed patterns and composition comparable to those found on the plants in the mesocosm. Clear differences among plant parts were found, with the highest diversity on leaves. The OTUs distribution showed the presence of a higher number of unique than shared OTUs, further confirming different microbial community structure on each plant part. The plants from the lagoon showed comparable bacterial composition with that of the plants in the experimental conditions. Nonetheless, a more discriminatory molecular technique should be used to better evaluate and quantify bacterial composition. This could also help elucidate the functional interaction between microbes and seagrasses.

4 THE MICROBIAL COMMUNITIES ASSOCIATED WITH *HALOPHILA STIPULACEA*

4.1 Study site and field sampling

H. stipulacea is a monocotyledon that belongs to the order Alismatales, family Hydrocharitaceae. The genus *Halophila* comprises widely distributed species in the tropical indo-pacific bioregion (Short *et al.*, 2007). *H. stipulacea* is the dominant species found in the northern most tip of the Red Sea, Gulf of Aqaba (GOA), along the Israeli coastline, which stretches about 11 km long. This species forms discontinuous meadows along the coast and extend from 5 to over 70 meters deep (Winters, personal communication).

In the GOA (Fig. 9), the microbial communities associated with plants of three different seagrass meadows were selected for sampling: North Beach (NB), Tur Yam (TY) and South Beach (SB) at 9 m depth. These meadows were chosen based on a gradient of geomorphological features and human use (Tab. 4).

Seagrass plants were also collected to measure physiological responses. Plants descriptors measured included leaves morphometrics (length, width and area, leaf number/plant, the percentage of leaves with lost apex), photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) and total phenols contents. In addition, environmental variables (water column and pore water nutrient concentrations, granulometry and total organic carbon content of local

sediments) and geomorphological features (meadows surface area, bottom slope, Secchi depth, the diffuse attenuation coefficient-Kd_{PAR}) were also evaluated.

The sampling campaigns and processing of microbes and plant samples was partly financed by the Dead Sea-Arava Science Center, Central Arava Branch. This work was also supported by a Short Term Scientific Mission grant from the COST Action scientific programme (ES0906) on Seagrass productivity: from genes to ecosystem management (COST-STSM-ES0906-06445 and ES0906-15001. Financial support was also provided by the European Community - Research Infrastructure Action under the FP7 "Capacities" Specific Programme, ASSEMBLE 8th call, project: “Free-living or coral associated *vibrio spp*. Identification through molecular and bioinformatics tools. Trial on Eilat *vibrio* isolates”, ASSEMBLE grant agreement no. 227799.

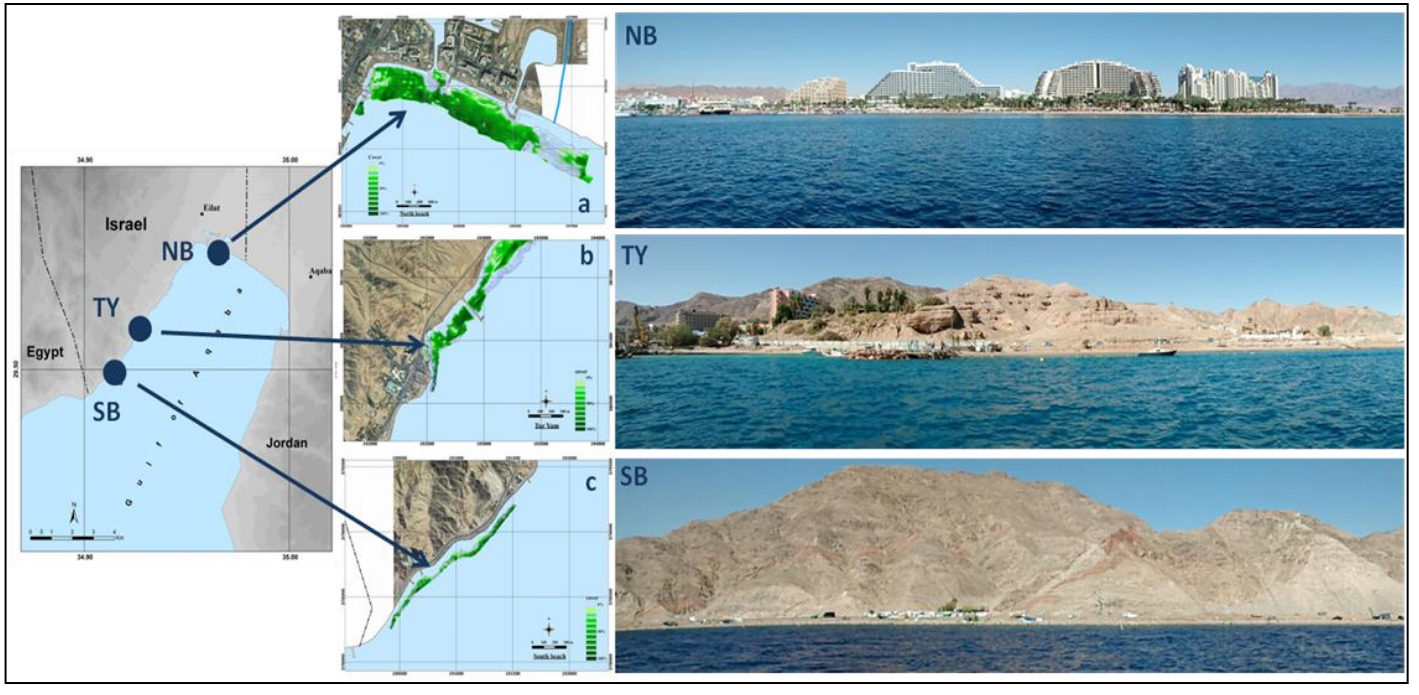


Fig. 9 The three *Halophila stipulacea* beds in the northern Gulf of Aqaba (Red Sea, Israel) selected for sampling: North Beach (NB), Tur Yam (TY) and South Beach (SB), with maps showing percent cover of *Halophila stipulacea* (a-c) and images showing the different human uses.

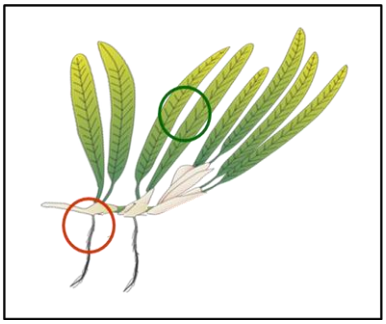
Tab. 4 Main geomorphological features and human uses of the three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB), in the Gulf of Aqaba (Red Sea, Israel).

Sampling station	Location	Meadow Area[1] (m^2)	Bottom Slope[1]	Secchi Depth[2] (m)	Kd $_{PAR}$ [3]	Corals present	Human use
North **B**each (NB)	29.546150°N 34.964819°E	343,032	2.55°	23.13±2.69	0.196	No	High
Tur **Y**am (TY)	29.516527°N 34.927205°E	67,365	5.26°	25.63±1.65	0.102	Partial	Medium
South **B**each (SB)	29.497664°N 34.912737°E	61,900	17.92°	26.38±2.16	0.155	Yes	Low

[1]Bottom slopes and surface areas of meadows were obtained from ongoing efforts to map seagrasses along the Israeli coast of the northern Gulf of Aqaba (Winters, personal comm.). [2]Data are average values of Secchi depths from October 2006-2013 (NMP, 2013); NMP's monitoring sites differ slightly from the sites in this study, the reference NMP sites used are: Taba (200 m from SB), Navy, (some 400 m from NB) and water control station (some 300 m from TY). [3]PAR measurements were taken in each sampling station at noon from 0-9 m and the corresponding diffuse attenuation coefficient (Kd) based on PAR reading at 2 and 9 m depth; using the 2π-quantum sensor of the Diving-PAM (pulse amplitude modulated) fluorometer (Walz, Germany). Human uses: High = intense tourism shoreline infrastructure, popular sandy beaches, city marina, fish farms 200 m away were operative until 2008; Medium= Low-frequented beach, a small marina, diving activities, coastal infrastructures related to a local oil terminal, rocky beach; Low= Pristine rocky shoreline with no coastal infrastructures, but busy diving activities.

4.2 Bacterial sampling and processing

For *Halophila stipulacea* 8 shoots, each composed of 8 leaves (excluding the apical), ~ 4 cm of rhizomes and accompanying roots, were collected in the field to obtain two replicates per plant part and sampling station. In this case, rhizomes and roots were pooled before processing to get sufficient amount of material for the bacterial pellets.



4.2.1 Bacterial pellets

The bacterial pellets were obtained following the procedure described above for *Cymodocea nodosa* (see section 3.2.1).

4.2.2 DNA Extraction, Amplification and Sequencing

The metagenomic bacterial DNA associated with *Halophila stipulacea* was extracted with the Power Soil® DNA isolation kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions.

The pure bacterial DNA extracts were sent to the Molecular Research LP in Texas, USA (MR DNA, http://www.mrdnalab.com/, Dowd *et al.*, 2008) for PCR amplification and 454 pyrosequencing of the 16S

rRNA gene. The universal primers, Com1 and Com2-non phosphorilated (Tab. 2) were used for DNA amplification. The samples were sequenced with Roche 454 FLX titanium instruments and reagents following the manufacturer's instructions.

4.2.3 454 Sequencing Output Analysis

The raw sequence data was processed with the open source software MOTHUR (http://www.mothur.org/), according to the 454 Standard Operating Procedure pipeline (Schloss et al., 2009) available online at the software's website. Briefly, in the pipeline, quality trimming was performed, sequences were depleted of their barcodes and those <200bp and with ambiguous base calls were removed. These sequences were then dereplicated, aligned against the greengenes core-set template alignment, and screened to make them overlap in the same region. Putative chimeras were identified with the "uchime" algorithm and removed. Finally, high quality sequences were classified with naïve Bayesian classifier in MOTHUR using the RDP taxonomy reference database-trainset 9_032012. Sequences classified at phylum level as unknown, archaea and chloroplast were removed. Operational Taxonomic Units (OTUs) were defined by clustering of sequences at 3% divergence (97% similarity). A final matrix of the OTUs assigned to each sample with their taxonomic identification was build in MOTHUR and used in downstream analyses. To describe the bacterial composition at different taxonomic levels, the final matrix was filtered to include only OTUs with taxonomic identities of ≥80%

confidence at the phylum and class levels and that had at least 10 sequence reads per sample. The complete set of raw sequences obtained in this study has been deposited in GenBank at the Sequence Read Archive (SRA) under the study accession no. SRP057388, BioProject no. PRJNA281491.

4.2.3.1 Microbial Community Analysis

4.2.3.1.1 Operational Taxonomic Units (OTUs) based analysis

Microbial community structure and composition were analyzed in MOTHUR, as follows: i) rarefaction curves were built to evaluate differences in sampling effort; ii) dendrograms were built using the UPGMA algorithm to evaluate differences in community composition; iii) Venn diagrams were built to state the relationship between unique and shared OTUs; iv) the Shannon index (H') was measured to evaluate bacterial diversity, v) histograms were built to visualize the bacterial community composition and their relative abundances. In addition, the SIMilarity PERcentage (SIMPER) test was used to identify the taxonomic groups (phylotypes) contributing most to the dissimilarity between the samples.

4.2.3.1.2 Statistical Analysis

Non-metric Multi-Dimensional Scaling (n-MDS) was used to visualize differences in microbial community composition based on OTU's abundance. The n-MDS ordination was conducted on a Bray-Curtis distance matrix calculated between sampling plots with log

(x+1)-transformed abundance data. A one-way non-parametric analysis of similarities (ANOSIM, n = 999 randomizations; Clarke and Warwick, 2001) was employed to test for significant differences among sampling stations. Ordination analyses and ANOSIM were performed in MOTHUR.

The Unifrac weighted algorithm in MOTHUR was used to determine if the microbial community structure of the samples differed significantly based on their phylogenetic relationships and the abundances of the taxonomic groups present in the samples. The samples were clustered in dendrograms using Bray-Curtis distance matrix. The significance of pairwise comparisons were defined at $p<0.01$.

The SIMPER analysis was performed in PAST software (version 3.1).

4.3 Results

4.3.1 454 Sequencing output

The raw output of the 454 pyrosequencing resulted in a total of 109,622 bacterial sequences, comprising all samples and stations. The cleaning of raw sequences resulted in a total of 75,028 sequences, assigned to a total of 5,752 OTUs. On average, each individual sample was represented by 6,252 sequences, ranging from 740 to 9,146 sequences.

The rarefaction curves analysis (Fig. 10) showed that some samples did not reach the asymptote in OTU richness; in those cases, more sequences would be required to recover the full taxonomic diversity. Nevertheless, the shortcomings of these samples did not affect the final output of the study. For standardization, the dataset was normalized to 726 sequences reads across samples.

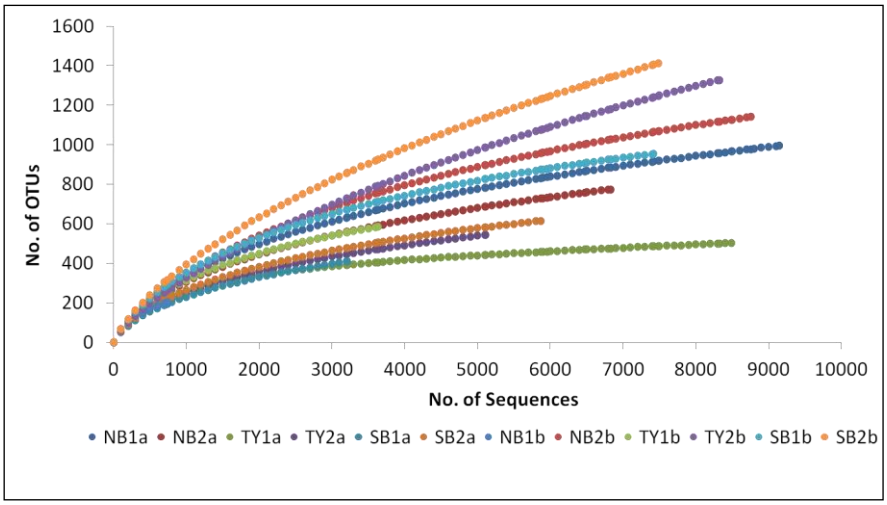


Fig. 10 Rarefaction curves of the microbial communities associated with the different plant compartments, aboveground (a=leaves) and belowground (b=rhizomes and roots) of *Halophila stipulacea* in the three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB). Two replicates per sampling station are shown. No. of OTUs=Number of Operational Taxonomic Units.

4.3.2 OTUs based analysis

The Non-metric Multi Dimensional Scaling (n-MDS; Fig. 11) showed a clear separation between aboveground (leaves) and belowground (rhizomes with roots) compartments. In addition, a separation by

stations was evident for SB belowground. Significant differences were found between the plant compartments (ANOSIM Pairwise Test R:0.74; $p<0.01$).

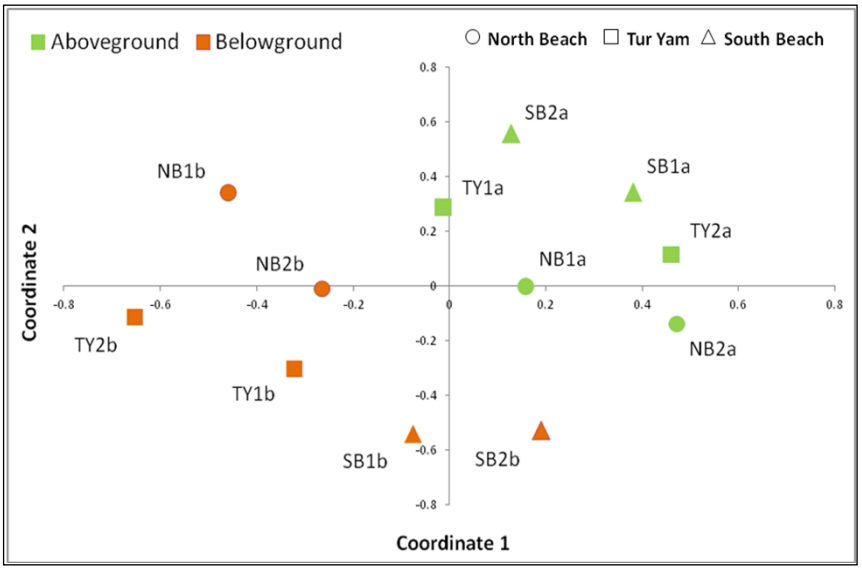


Fig. 11 n-MDS analysis of the microbial community associated with different plant compartments, a=aboveground (leaves) and b=belowground (rhizomes and roots) of *Halophila stipulacea* in the three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB). The analysis was performed using Bray-Curtis similarity indices (Stress: 0.29), of the normalized dataset. Two replicates per station are shown.

The Unifrac weighted analysis highlighted significant differences in the microbial community structure and composition among stations. The pairwise comparisons showed significant differences among the three stations aboveground, NB *vs.* TY *vs.* SB (Unifrac weighted $p<0.001$; Tab. S2 in supporting information), while belowground,

differences were significant only between NB *vs.* TY (Unifrac weighted, $p<0.001$; Tab. S2 in supporting information).

Venn diagrams (Fig. 12) showed a lower number of observed OTUs in the stations aboveground (2,635) than belowground (3,923). A total of 1,260 OTUs were shared among all the samples in the study and of these 68% were common to both plant compartments. In all the samples, the number of unique OTUs was higher than the shared ones, with only 7% of the OTUs being shared among the stations aboveground and 8% belowground.

The Shannon diversity index (H') values of the stations aboveground showed a decreasing trend from NB to SB. On the stations belowground, SB showed the highest diversity, with no clear trend among stations (Tab. 5).

The taxonomic identification of the bacterial OTUs revealed that the phylum *Proteobacteria* accounted for at least 70% of bacterial composition of each sample (Fig. 13, in blue). Within this Phylum, *Alphaproteobacteria, Gammaproteobacteria* and *Deltaproteobacteria* were the most abundant classes observed, and these showed differences in abundance in the above and belowground compartments (Fig. 14, pie charts). Aboveground, *Alphaproteobacteria* accounted for 68% of the total community composition and was thus the sole dominant class among stations. Belowground, the dominant groups were classes *Gammaproteobacteria, Alphaproteobacteria,* and

Deltaproteobacteria, representing 32%, 31% and 21% of the whole community composition, respectively.

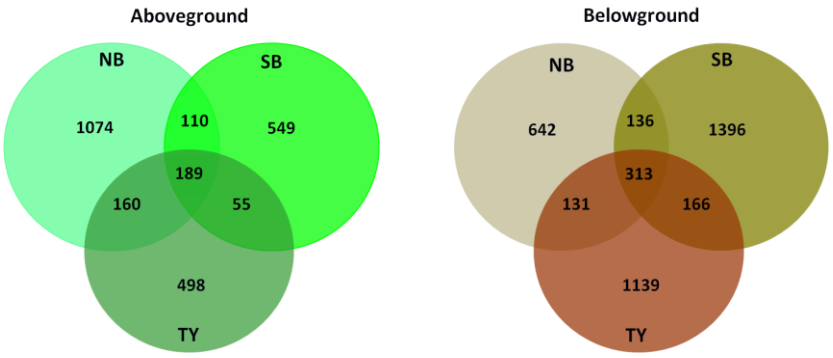


Fig. 12 Venn diagrams of the bacterial OTUs, specific and shared, associated with *Halophila stipulacea*, above and belowground, in the three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB). Venns were built using all the OTUs (full dataset) found in two replicates per station.

Tab. 5 Shannon diversity index (H') of the microbial communities associated with *Halophila stipulacea* above and belowground compartments in the three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB). Values are means ±SD of two replicates, using the normalized dataset.

Station	Shannon Diversity (H')± SD	
	Aboveground	Belowground
NB	4.85±0.05	4.83±0.09
TY	4.49±0.44	4.49±0.02
SB	4.05±0.02	4.91±0.04

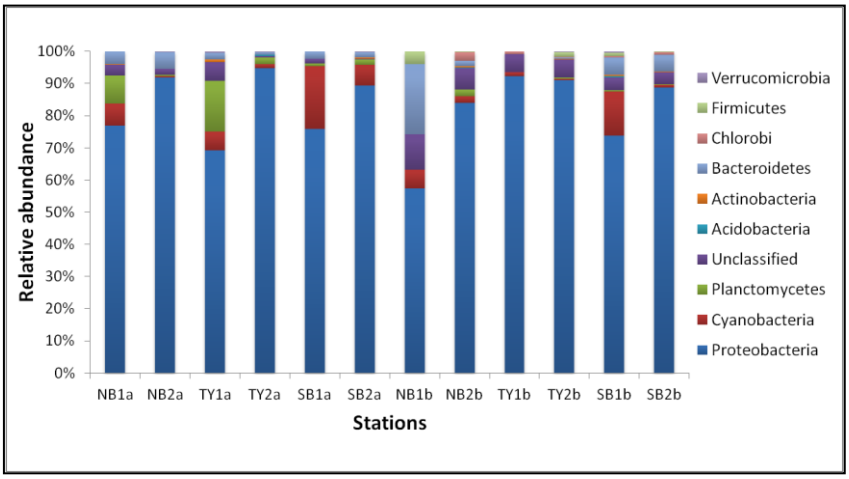


Fig. 13 The bacterial community composition at Phylum level associated with *Halophila stipulacea*, aboveground (a) and belowground (b), in the three sampling stations (NB=North Beach, TY=Tur Yam, SB=South Beach). Data corresponds to two replicates per sampling station.

Several phyla made up the less represented (rare) bacterial community components (Fig. 14, top of the bars), including *Planctomycetes* (4.0%), *Bacteroidetes* (2.8%), *Chlorobi* (0.5%), *Firmicutes* (0.3%), *Actinobacteria* (0.3%), *Verrucomicrobia* (0.2%) and *Acidobacteria* (0.1%). *Cyanobacteria* were assigned to 4.8% of the sequences, while unclassified bacteria accounted for 3.8% of the sequences. This rare component included many classes which combined represented 9% aboveground and 7% belowground (Fig. 14, bar plot). Specifically, classes *Planctomycetacia, Flavobacteria, Actinobacteria* and *Cyanobacteria* were found in all the stations, showing different relative abundance above and belowground. Classes *Epsilonproteobacteria, Ignavibacteria, Clostridia* and *Bacilli* were only present belowground.

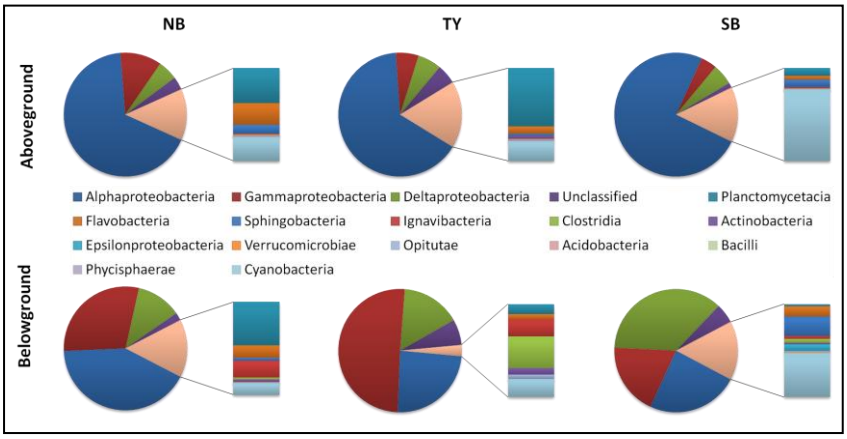


Fig. 14 The bacterial community composition at Class level associated with *Halophila stipulacea*, aboveground and belowground, in the three sampling stations (NB=North Beach, TY=Tur Yam, SB=South Beach). The pies represent the dominant bacterial component while the bars the rare components. Values are means of two replicates from each sampling station.

The sampling stations showed further differences in the presence; absence and relative abundance of microbial phylotypes at lower taxonomic levels (see Tab. S3 in supporting information). Aboveground, NB showed the highest number of different phylotypes (28) followed by TY (26) and SB (23). Belowground, SB had the highest number of different phylotypes, 33, followed by NB (28) and TY (26). Some phylotypes were only found in SB, including *Alteromonadaceae*, *Cytophagaceae*, *Bacilli* and *Epsilonproteobacteria.* Similarly, the family *Sphingomonadales* (0.4%) was found only in NB and the class *Clostridales* was only found in TY.

A SIMPER test analysis (See Tab. S4 in supporting information) showed a high dissimilarity between plant compartments (55.95%) with five phylotypes contributing to >70% of the differences: *Rhodobacteraceae, Gammaproteobacteria*, *Alphaproteobacteria*, *Desulfobulbaceae* and *Planctomycetaceae*. The general comparison among stations showed the greatest dissimilarity between TY and SB (47.86%), followed by NB *vs.* SB (44.44%) and TY *vs.* NB (38.98%). The analysis by plant compartment also showed that the greatest differences aboveground were between SB and both, NB and TY stations, with average dissimilarity values of 33.71% and 31.82%, respectively. On the contrary, the dissimilarity between NB *vs.* TY was considerably low (14.61%). The main phylotypes contributing to differences with SB included: *Cyanobacteria* and *Flavobacteracea*, in addition to the phylotypes already mentioned above for differences between compartments. Belowground, the dissimilarity values among stations was comparable, with the highest value found between SB *vs.* NB (36.76%), followed by SB *vs.* TY (36.45%) and NB *vs.* TY (32.12%). The phylotypes contributing to >70% of the differences included: *Desulfobulbaceae, Gammaproteobacteria, Cyanobacteria*, *Myxococcales, Sphingobacteriales, Rhodobacteraceae* and *Desulfobacteraceae.* The taxonomic identification of the shared OTUs revealed the 'core microbiome' composition of both plant compartments (Tab. 6). The phyla *Proteobacteria and Planctomycetes* represented >70% of the shared OTUs on both plant compartments.

Tab. 6 The core microbiome associated with the above and belowground compartments of *Halophila stipulacea* in the Gulf of Aqaba, Red Sea. No. OTUs=Number of Operational Taxonomic Units. Tables were built using all OTUs found in two replicates per station.

Aboveground				
Phylum	**No.OTUs**	**Class**	**No.OTUs**	**No. Sequences**
Actinobacteria	4	*Actinobacteria*	4	92
Bacteroidetes	7	*Sphingobacteria*	3	233
		Flavobacteria	4	423
Cyanobacteria	24	*Cyanobacteria*	24	2068
Planctomycetes	25	*Planctomycetacia*	24	1558
		Phycisphaerae	1	22
Proteobacteria	117	*Alphaproteobacteria*	83	19280
		Deltaproteobacteria	7	1600
		Gammaproteobacteria	24	1365
		*Proteobacteria*_unclassified	3	62
Verrucomicrobia	2	*Verrucomicrobiae*	2	38
Unclassified	10	Unclassified	10	305
Total	**189**		**189**	**27046**

Tab 6. ***Continues***

Belowground				
Phylum	**No. OTUs**	**Class**	**No. OTUs**	**No. Sequences**
Acidobacteria	3	*Acidobacteria*	3	40
Actinobacteria	10	*Actinobacteria*	10	192
Bacteroidetes	11	*Sphingobacteria*	4	189
		*Bacteriodetes*_unclassified	1	14
		Flavobacteria	6	320
Chlorobi	1	*Ignavibacteria*	1	272
Chloroflexi	3	*Anaerolineae*	2	13
		*Chlorofexi*_unclassified	1	15
Cyanobacteria	21	*Cyanobacteria*	21	1125
Firmicutes	1	*Clostridia*	1	98
Planctomycetes	25	*Planctomycetacia*	23	440
		Phycisphaerae	2	11
Proteobacteria	194	*Alphaproteobacteria*	77	7322
		Betaproteobacteria	1	7
		Deltaproteobacteria	46	5608
		Epsilonproteobacteria	1	94
		Gammaproteobacteria	63	7699
		*Proteobacteria*_unclassified	6	67
Unclassified	43	Unclassified	43	1392
WS3	1	WS3_incertae_sedis	1	5
Total	**313**		**313**	**24923**

4.4 Discussion

Our study revealed that on *Halophila stipulacea* the microbial community composition is significantly different between plant compartments, leaves *vs.* rhizomes and roots, as it has been observed also on other seagrasses (*Cymodocea nodosa*, Mejia *et al.*, 2014; *Zostera marina*, Crump and Koch, 2008). The class *Alphaproteobacteria* was particularly dominant on the leaves of the plants in all three stations; particularly of the family *Rhodobacteraceae*, which are dominant in marine biofilms, found attached to different artificial substrates in oligotrophic waters (Elifantz *et al.*, 2013; Dang *et al.*, 2008). Belowground the bacterial community dominance was distributed among classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*. The class *Gammaproteobacteria* includes halotolerant-halophilic aerobic bacteria involved in nitrogen cycling, as well as sulfur oxidizing bacteria commonly found associated with the rhizosphere of seagrasses (Mejia *et al.*, 2014; Crump and Koch, 2008). The order *Rhodobacterales* was again the most abundant of the *Alphaproteobacteria*. The family *Desulfobulbaceae* of the class *Deltaproteobacteria*, represented 15% of the whole community belowground which include strict anaerobes involved in the reduction of sulfate to sulfide and nitrate to ammonia (Minz *et al.*, 1999). Member of the class *Deltaproteobacteria* has been found associated with the roots of *Posidonia oceanica* (García-Martínez *et al.*, 2009),

Zostera marina (Crump and Koch, 2008), and *Cymodocea nodosa* (Mejia *et al.*, 2014).

The Shannon diversity index (H') showed little discrimination between compartments, however differences among stations were highlighted by a higher number of different phylotypes found belowground than aboveground, 29 *vs.* 25. Similarly, the distribution of unique and shared OTUs, assigned a higher total number of OTUs to the belowground compartment than aboveground, suggesting differences in the bacterial richness between the compartments. The OTUs distribution also revealed the 'core microbiome' of *H. stipulacea* along short spatial scales. The phyla *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Planctomycetes* and *Proteobacteria* were shared among both plant compartments and consequently represent the 'essential bacterial composition' of *H. stipulacea* microbial epiphytes in the Red Sea. Despite the evidently high abundance of the phylotype *Rhodobacteraceae* in all the samples, the SIMPER test analysis showed that many other phylotypes contributed to differences among the plants compartments and station, including: *Gammaproteobacteria, Desulfobulbaceae, Cyanobacteria*, *Myxococcales, Sphingobacteriales, Flavobacteracea*, *Rhodobacteraceae, Planctomycetaceae, Verrumicrobia* and *Alphaproteobacteria.*

Microbial communities linked to plant's ecophysiological descriptors

The three seagrass meadows showed differences based on all the plant descriptors measured: differences in the plants' morphometrics (leaf area, width and length, number of leaves/shoot and % leaves with lost apex), plant eco-physiology (total phenols and photosynthetic pigments as Chlorophyll *a* and *b*, carotenoids) and microbial communities were found, despite the relatively short geographical distances between the meadows. In general, differences between the stations NB and SB, at the edges of the gradient, were more evident than with TY, the middle station.

The microbial community in NB showed the highest microbial diversity on the leaves, which may be associated with the high levels of Chlorophyll *a* and the availability of resources in the turbid water column. As noted before, the highest incidence of the opportunistic phyla, *Gammaproteobacteria* and *Bacteroidetes* (*Flavobacteria*), were found on leaves from this station, and these are known to thrive under high Chlorophyll *a* levels (Witt *et al.*, 2012) and with the increase influx of organic nutrients (Teira *et al.*, 2014).

The microbial community composition was also comparable to NB, both aboveground (14.61%) and belowground (32.12%). Fitting its midway geographical position, TY displayed intermediate responses in most of the parameters measured, although overall, this site showed greater similarity to NB than SB. The microbial communities in this station showed the most dissimilarities when compared to NB and TY

both aboveground and belowground. In this station, the aboveground plant compartment microbial community had the highest incidence of *Alphaproteobacteria* and *Cyanobacteria* which can be related to the high light availability and low Chlorophyll *a* (Witt *et al.*, 2012). Belowground, this station had the highest incidence of *Deltaproteobacteria* (and thus, sulfur-reducing bacteria), which are known to increase with high nutrients; as well as *Cyanobacteria* (possibly attached to the rhizomes) which are known to increase with high light availability and water clarity (Witt *et al.*, 2012).

The results on the microbial communities are part of an integrated approach that was used to study the meadows. Both the morphological (leaves morphometrics, number of leaves/shoot and % leaves with lost apex) and the biochemical descriptors (foliar photosynthetic pigments and total phenol content), showed differences among stations, demonstrating how morphological plasticity and physiological adaptation of plants can occur under different environmental conditions, as the plants cope with environmental pressures. Significantly different microbial communities were also found among stations, in association with the plant's eco-physiological status and the local environmental conditions. This suggests a functional relationship between seagrasses and their associated microbial communities; which need to be investigated further to be properly established.

5 MICROBIAL COMMUNITIES ASSOCIATED WITH HALOPHILA STIPULACEA ALONG A DEPTH GRADIENT (4 – 28 M)

The meadow of South Beach (SB) in the Gulf of Aqaba (Fig. 9) was selected to evaluate the microbial communities along a vertical gradient. As it was described before, SB is a very dynamic meadow, characterized by high hydrodynamics and where plant host a different microbial community in comparison with the other two meadows, North Beach and Tur Yam, found in the same location (see section 4, Tab. 4). Four depths were selected for sampling, the shallow edge of the meadow at 4 meters, the mid- shallow depth at 9 meters, the mid-deep edge at 18 meters and the deep edge at 28 meters.

In this study, the plants and their bacterial pellets were collected, processed and analyzed as described before in sections (4.1 and 4.2). The sections that follow will present the results and a brief discussion of the analyses conducted. A detailed analysis of the bacterial community diversity and composition was performed. Plants descriptors were again evaluated and then the bacterial community analyses were compared with plants ecophysiology and the environmental conditions of the meadow.

5.1 Results

5.1.1 454 Sequencing output

The raw output of the 454 pyrosequencing resulted in a total of 107,782 bacterial sequences, comprising all samples. The cleaning of sequences with the MOTHUR software resulted in a total of 84,892 sequences, assigned to a total of 6,793 OTUs. The average number of sequences of all samples was 5,306 sequences, ranging from 2,764 to 9,001 sequences. For standardization, the dataset was normalized to 2,764 sequences reads per sample.

The rarefaction curves analysis (Fig. 15) showed that some samples did not reach the asymptote in OTU richness, in those cases, more sequences would be required to recover their full taxonomic diversity.

5.1.2 OTUs based analysis

The Non metric Multi Dimensional Scaling (n-MDS; Fig. 16) shows a greater separation of the samples by plant compartment than by depth, although some clustering by depth can be observed, especially belowground at depths of 4m and 28m. ANOSIM highlighted significant differences between plant compartment but not by depth (ANOSIM Pairwise Test R: 0.14; $p<0.05$).

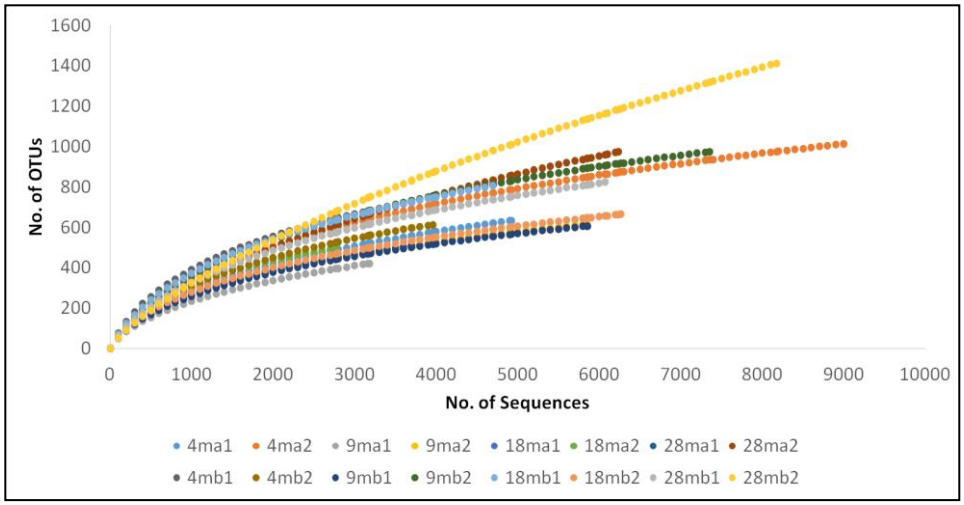


Fig. 15 Rarefaction curves of the microbial communities associated with the different plant compartments, above and belowground, of *Halophila stipulacea* along a depth gradient: 4m, 9m, 18m, 28m. Two replicates per sample are shown (1) and (2). Aboveground (leaves)=a, Belowground (rhizomes and roots)=b.

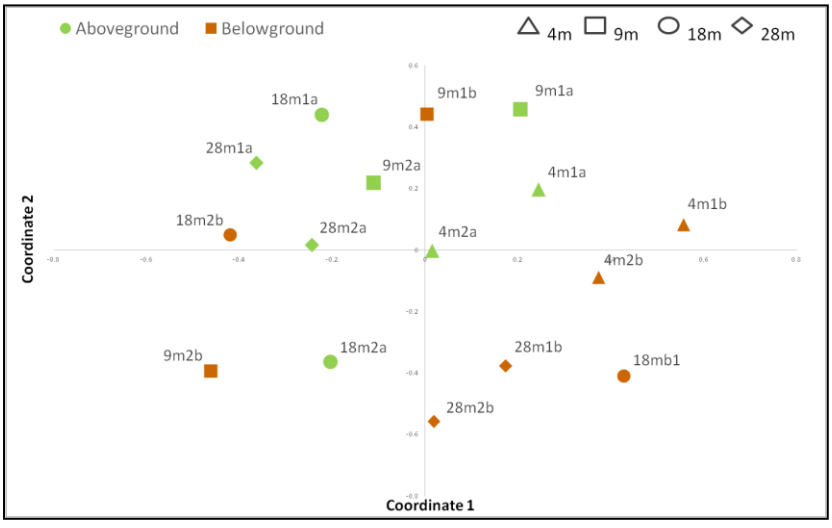


Fig. 16 n-MDS analysis of the microbial community associated with *Halophila stipulacea* along a depth gradient: 4m, 9m, 18m, and 28m. m=meters; 1 and 2=replicate number; a=Aboveground (leaves), b=Belowground (rhizomes and roots). The analysis was performed using Bray-Curtis similarity indices (Stress: 0.31) of the normalized dataset. Two replicates per depth are shown.

The unifrac weighted analysis highlighted significant differences in of the microbial communities by depth. Aboveground, the pairwise comparisons showed significant differences (Tab. S5; Unifrac weighted $p<0.05$) between the communities at 4m *vs.* those at 18m and 28m, while at 28m communities were different from all other depths. Belowground, differences were significant between communities at 28m with those at all other depths.

Venn diagrams (Fig.16) shows that all the samples had a higher number of unique than shared OTUs. A total of 1,482 OTUs were shared among all the samples. A lower number of OTUs was observed on the samples aboveground (3,378) than belowground (4,573). The number of shared OTUs between compartments was 1,158. The highest total number of OTUs was observed at 4m aboveground (1,447 OTUs) and 28m belowground (1,984 OTUs). In both plant compartments the number of shared OTUs among samples was about 4.4% and 3.4 % of the total OTUs observed.

The Shannon diversity index (H') values were slightly lower aboveground at all depths than belowground, except at 28m (Tab. 7). The highest diversity in both plant compartments (above and belowground) was found at 4m.

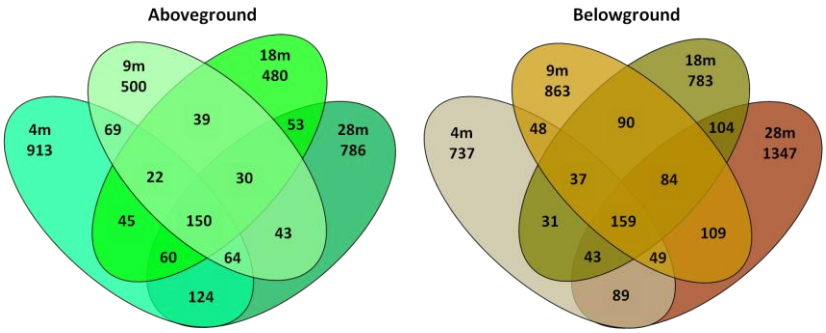


Fig.16 Venn diagrams of the bacterial OTUs (specific and shared) associated with the different plant compartments, above (leaves) and belowground (rhizomes and roots), of *Halophila stipulacea* along a depth gradient: 4m, 9m, 18m, and 28m. Data represent two replicates per station, using the full dataset.

Tab. 7 Shannon diversity index (H') of the microbial communities associated with the different plant compartments, above and belowground, of *Halophila stipulacea* along a depth gradient: 4m, 9m, 18m, and 28m. Data represents two replicates per station.

Depth	Shannon Diversity (H')± SD	
	Aboveground	Belowground
4m	5.04±0.15	5.30±0.046
9m	4.37±0.25	4.85±0.55
18m	4.98±0.33	5.05±0.37
28m	4.67±0.07	4.51±0.56

The bacterial community composition of *Halophila stipulacea* along the depth gradient was dominated by the phylum *Proteobacteria* (Figure 17, in blue). This phylum accounted for 81% of the bacterial composition found in all samples. The less represented or rare bacterial component (Fig 17, top of bars) included the Phyla:

Cyanobacteria (7%), *Planctomycetes* (4%), *Bacteroidetes* (3%), *Actinobacteria* (0.5%) and *Verrucomicrobia* (0.1%), which were present in all samples. *Firmicutes* (0.5%) and *Chlorobi* (0.1%) were present only on the belowground samples. *Acidobacteria* and *Chlorofexi* had the lowest abundance (<1%) and were present only at some depths (18m above- and 9m belowground, respectively). The unclassified bacteria accounted for 3% of the whole community composition.

The most abundant class within the *Proteobacteria* was *Alphaproteobacteria* that represented 61% of the whole community composition in the study (Figure 18, pie charts). In general, the abundance of this class was higher above (71%) than belowground (63%). The second and third most abundant classes were *Deltaproteobacteria* (8%) and *Gammaproteobacteria* (7%); and both were slightly more abundant belowground than aboveground.

Many classes composed the rare component above and belowground (Figure 18-barplot); the classes *Planctomycetes*, *Flavobacteria*, *Sphingobacteria*, *Actinobacteria*, *Epsilonproteobacteria*, *Verrucomicrobia* and *Acidobacteria* were present on both plant compartments. A number of classes were only present belowground, these included: *Clostridia*, *Ignavibacteria*, *Bacilli*, *Opitutae*, *Betaproteobacteria*, *Caldilineae* and *Phycisphaerae*.

A SIMPER test (See Tab. S6 in supporting information) revealed dissimilarities among depths. Aboveground, the highest dissimilarities were found between 4m and 18m (44.91%).

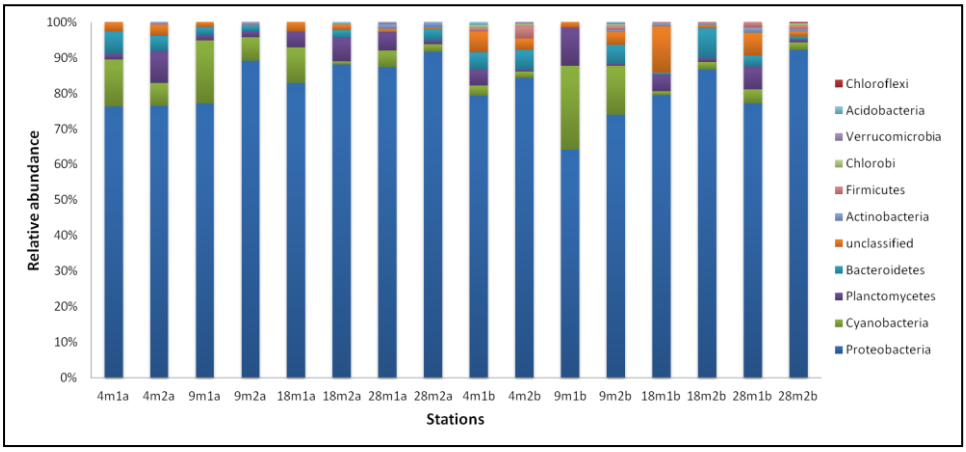


Fig. 17 The bacterial community composition at Phylum level associated with *Halophila stipulacea*, along a depth gradient: 4m, 9m, 18m, 28m. m=meters; 1 and 2=replicate number; a=Aboveground (leaves), b=Belowground (rhizomes and roots). Data corresponds to two replicates per sampling station.

The phylotypes contributing most to the dissimilarity included *Cyanobacteria*, *Rhodobacteraceae* and *Alphaproteobacteria*. Belowground, the highest dissimilarity was found between 18m and 28m (45.03%). The phylotypes contributing most to the dissimilarity included *Roseibium*, *Desulfopila*, *Planctomycetacea*, *Rhodobacteracea* and *Cyanobacteria.*

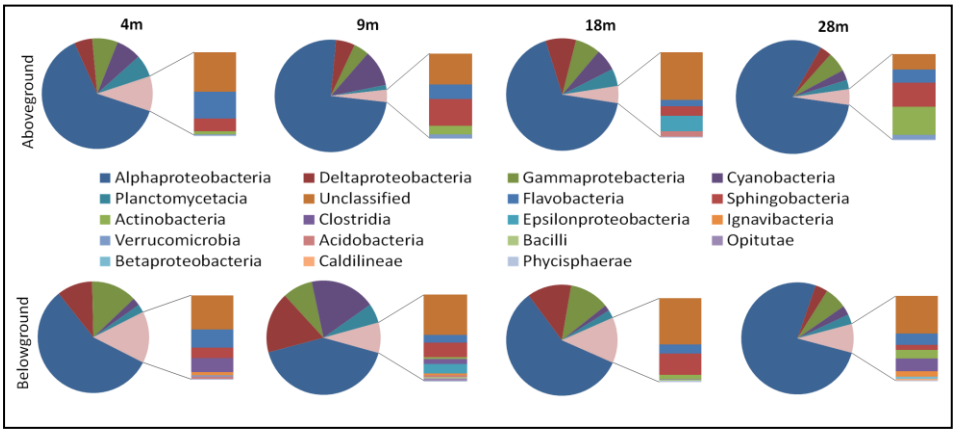


Fig. 18 The bacterial community composition at Class level associated with *Halophila stipulacea*, aboveground (leaves) and belowground (rhizomes and roots) compartments, along a depth gradient: 4m, 9m, 18m, 28m; m=meters. The pies represent the dominant bacterial component while the bars the rare components. Values are means of two replicates from each sampling station.

5.2 Discussion

Different and diverse microbial communities were found associated with the SB meadow at the four different depths. Plant descriptors also showed variations with depth in leaf morphology and physiological responses.

The microbial communities showed significant differences in microbial diversity and composition at the edges of the meadow, namely 4m and 28 m, while more constant communities at the mid-shallow (9m) and mid deep (18m) depths. The microbial diversity was consistently higher belowground than aboveground at most of the depths examined. The findings suggest an influence of hydrodynamics and sediment granulometry on microbial diversity and richness, especially at the

shallower (4 m) and deeper edges (28 m) of the meadow. The highest diversity was observed at 4m both above and belowground. This may be related to its vicinity to the shoreline and thus availability to higher amount of nutrients from coastal sediments runoff and deposition. The leaves at 4m displayed the highest community richness suggesting suitable environmental conditions for a diverse and rich community to thrive. Plant physiological responses showed high phenol content on leaves at this depth and this can be indicative of as higher environmental dynamics which can lead to stress in ecophysiology. Belowground, the highest richness was found at 28m and this may also be linked to the environmental conditions present there. This depth displayed the largest leaves; the most different granulometric composition and the highest organic matter content found among depths (See more details in Annex-Depth Gradient). Leaf surface area increases with depth according to the reduction of light availability. The biggest leaves were found at 28 m where only 5% of total surface PAR reaches the bottom. Reductions in pigment contents were observed with depth, with the lowest levels displayed at 28m.

Again, clear differences in the microbial community assemblages were found associated with the plant compartments. The dominance of the phylum *Proteobacteria* was confirmed along the vertical gradient. The class *Alphaproteobacteria* was the most abundant phylotype found on the leaves, while belowground the dominance was shared with classes *Gammaproteobacteria* and

Deltaproteobacteria. A similar composition was reported by Weidner *et al.,* (2000) for *H. stipulacea* in this region. A highly diverse rare component is also associated with *H. stipulacea* plants and many more phylotypes were found belowground than aboveground. The findings highlighted the influence of depth in both plants and their microbial community assemblages. The SIMPER analysis revealed that the microbial communities at 18m were dissimilar from all other depths. Belowground, dissimilarities were found progressively with depth, microbial communities show dissimilarity between 4m *vs.* 9m, 9m *vs.* 18m and 18m *vs.* 28m. The phylotypes contributing to dissimilarities aboveground included *Cyanobacteria*, *Rhodobacteraceae* and *Alphaproteobacteria*. Belowground, the major taxa influencing dissimilarity included *Roseibium, Desulfopila, Planctomycetacea, Rhodobacteracea* and *Cyanobacteria*.

The results indicated that in *H. stipulacea* foliar pigment and total phenol content are capable of detecting variations in plant physiological status (Migliore *et al.,* 2007; Rotini *et al.,* 2011; Rotini *et al.,* 2013a,b; Campbell *et al.,* 2003; Silva *et al,* 2013). This study also found significantly different microbial communities influenced by depth and linked to the plant's eco-physiological status and the differences in environmental conditions. These findings provide additional evidence about the functional relationship that may exist between seagrasses and their associated microbial communities.

6 OVERALL CONCLUSIONS

6.1 Cymodocea nodosa

The study on *Cymodocea nodosa* was the first attempt to investigate the microbiome associated with a seagrass when exposed to different concentrations of CO_2 and nutrients. Results showed a high bacterial diversification among the three plant parts, which significantly changed under varying conditions of CO_2 and nutrients. Microbial communities also showed differences influences by seasonality. More diverse communities were found in summer than in winter.

This work revealed that the microbial community associated with *Cymodocea nodosa* is composed by a dominant and a rare component; the dominant Phylum was *Proteobacteria*, mainly classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria.* The rare component included: *Bacteroidetes, Betaproteobacteria, Clostridia, Firmicutes, Spirochaetes, Verrumicrobia* and *Actinobacteria.*

Clear differences among plant parts were found, with the highest diversity on leaves. The OTUs distribution showed the presence of a higher number of unique than shared OTUs, further confirming different microbial structure on each plant part. The dominant bacterial phylotypes found on the plants from the lagoon showed slight differences in community composition with that of the mesocosm possibly due to the simplified and strictly controlled

conditions of the mesocosm. This was shown in particular by a high incidence of *Gammaproteobacteria* and the absence of *Alphaproteobacteria* in the lagoon.

In general, other detection techniques, especially Next Generation sequence could improve the level and quantification of the microbial shifts and should be considered for similar experiments and analyses.

6.2 Halophila stipulacea

The study on *Halophila stipulacea* was the first attempt to investigate the microbiome associated with a seagrass alongside the plant's eco-physiology in an integrated approach. This study successfully detected differences in the plant eco-physiology (as plant morphometrics, photosynthetic pigments and phenol content) and in the associated microbial communities in response to the local environmental conditions. Differences were highlighted among the three seagrass meadows studied: North Beach (NB), Tur Yam (TY) and South Beach (SB). The influence of depth was also highlighted in the microbial communities associated with plants in the SB meadow.

Based on the plant's responses to the parameters measured, a decreasing trend in the plants' eco-physiological status was unexpectedly suggested from NB to SB. The environmental conditions of the meadows and their geomorphological features seem to have a greater influence on plant physiology and the microbial communities than the anthropogenic pressure, present mainly in NB

(due to high tourism infrastructure on the coastline). The approach used revealed that *Halophila stipulacea* has much more favorable conditions to grow and thrive in conditions of fine sediments, high turbidity, lower nutrient levels (NB) than in SB, characterized by coarse sediment, low turbidity and high nutrients; being the latter more favorable for corals.

Differences in the microbial communities in SB further highlighted the link between microbial communities, plant ecophysiology and environmental conditions. The microbial communities at the shallowest depth studied (4m) were the most diverse and rich possibly influenced by input nutrients from the shoreline. Microbial communities at 28m were the most different along the gradient and this may be related to the differences in light conditions, granulometry and organic matter content found at this depth.

The studies in *Halophila stipulacea* showed how morphological plasticity and physiological adaptation of plants can occur under different environmental conditions, as the plants cope with environmental pressures. Similarly, microbial diversity and richness display changes in the above and belowground compartments influenced by the local environmental conditions and plant ecophysiology. These studies suggest a functional relationship between seagrasses and their associated microbial communities; which needs more exploration.

6.3 Common patterns of the microbial communities associated with seagrasses

The analyses of the microbial communities associated with the two seagrasses selected in this thesis work, highlighted common patterns (See Tab. 8).

Tab. 8 Common patterns of the microbial communities associated with the two seagrasses analyzed in this study, *Cymodocea nodosa* and *Halophila stipulacea*.

Pattern	***Cymodocea nodosa***	***Halophila stipulacea***
Differences between plant parts	Yes	Yes
Overall diversity (H'): Leaves *vs.* Rhizomes/Roots	Higher on leaves	Comparable between parts
Overall richness: Unique *vs.* Shared OTUs	Unique OTUs higher	Unique OTUs higher
Microbial shifts observed and linked to environmental conditions	Yes, significant	Yes, significant
Influence of temporal and/or spatial differences	Yes (temporal)	Yes (spatial)
Synchrony with the plant's physiological response to environmental conditions	Observed	Observed

In general, different microbial communities were found associated with the above and belowground compartments of both seagrass species and with different environmental conditions, including seawater acidification. The bacterial diversity differed between plant

compartments. On both species, consistently, the dominant microbial taxa belonged to the phylum *Proteobacteria*, classes, *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, despite the fact that both species were exposed to different environmental conditions and analyzed with different techniques.

On both species the total number of unique OTUs was higher than the shared OTUs suggesting unique microbial communities are associated with seagrasses. In addition, a synchrony between plant responses and their associated microbial communities was also highlighted.

The methodological upgrade resulting in the employment of 454 pyrosequencing, allowed for an in-depth analysis of the microbial communities associated with *Halophila stipulacea*. Consequently, it was possible to highlight links between microbial assemblages, the plant's eco-physiological status and specific environmental conditions:

- Under low hydrodynamics, fine sediments and high water turbidity, when plants showed the highest photosynthetic activity and surface and low phenols, the microbial community showed the highest diversity values aboveground. The dominant microbial community was mainly composed by *Alphaproteobacteria* and *Gammaproteobacteria* and the rare component by *Planctomycetes* and *Flavobacteria*.

- Conversely, under high hydrodynamics, coarse sand, high light availability, when plants showed the lowest photosynthetic activity and surface area but high phenol content, the microbial community showed the highest diversity belowground. The dominant microbial community was mainly composed by *Deltaproteobacteria* and *Gammaproteobacteria* and the rare component by *Flavobacteria, Sphingobacteria* and *Cyanobacteria*.

Overall, epiphytic microbial communities associated with seagrasses are influenced by plant ecophysiology and environmental conditions. Furthermore, the seagrasses microbiome can be influenced by temporal and spatial differences. Microbial descriptors can increase the effectiveness to identify the conservation status of seagrasses (at an early stage) and should therefore be incorporated in seagrasses monitoring programs. This thesis represents a stepping stone in the study of microbial communities as a marker of environmental change in seagrasses. A functional relationship has been suggested between seagrasses and their associated microbial communities. Transcriptomic studies may help explore deeper the functional relationship between seagrasses and its microbiome. Future investigations should also focus on the exploration of the rare microbial community component. Understanding their physiology and metabolic capabilities *(i.e.* through gene expression) is critical to effectively assess bacterial community shifts in a changing environment. Studies of CO_2 effects and other climate change related

factors should be investigated with NGS; the incorporation of more descriptive and quantitative techniques (*i.e.* Next-Generation Sequencing, quantitative PCR) can lead to a better understanding of the seagrass microbiome. This can consequently improve monitoring seagrass health and conservation under a changing environment.

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7 ANNEX

I. Manuscript: *Cymodocea nodosa* microbiome shifts at varying CO_2 and nutrients conditions.

II. Manuscript: Assessing the ecological status of seagrasses using morphology, biochemical descriptors and microbial community analyses. A study in *Halophila stipulacea* meadows in the northern Red Sea.

III. Supporting information

Includes Tables and Figures.

ANNEX I

Cymodocea nodosa microbiome shifts at varying CO_2 and nutrients conditions.

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Abstract

Microbial epiphytes play an important role in seagrass habitats, yet they remain poorly studied. Diversified microbial communities may confer seagrasses the capacity to adapt rapidly to changes in environmental conditions and help secure their health and conservation. In here, we characterized the microbial communities associated with leaves, rhizomes and roots of *Cymodocea nodosa* exposed to ambient and enriched concentrations of CO_2 (360-700 ppm) and nutrients (2-8 µM NH_4+ and NO_3-; 0.5-5 µM PO_4-), using the PCR-based technique Single Strand Conformation Polymorphism (SSCP). The microbial community varied with different CO_2 and nutrient concentrations. A high bacterial diversification among the three plant parts was also highlighted. Microbial communities also showed differences influenced by seasonality. More diverse communities were found in summer than in winter. The microbial Shannon diversity (H') highlighted leaves as the most colonized compartment than rhizomes and roots. The phylum *Proteobacteria*, classes *Alphaproteobacteria, Gammaproteobacteria and Deltaproteobacteria* were dominant in all plant parts. On leaves, members of the *Oceanospirillaceae* family, class *Gammaproteobacteria* comprising halotolerant-halophilic aerobic bacteria involved in the nitrogen cycling were dominant. On rhizomes and roots, the families *Desulfobacteraceae* and *Desulfovibrionaceae*, class *Deltaproteobacteria*, which play a major role in the sulfur cycle were dominant. This constitutes one of the few studies looking at the potential effects of acidification in seagrass habitats focusing on the bacterial communities.

ANNEX II

Assessing the ecological status of seagrasses using morphology, biochemical descriptors and microbial community analyses. A study in *Halophila stipulacea* meadows in the northern Red Sea.

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Abstract

Seagrasses are one of the most valuable marine ecosystems on earth, yet they are declining worldwide at an alarming rate. With most of seagrass monitoring based on long term responses to environmental pressures, there is a growing interest to develop alternative diagnostic tools that more effectively identify changes in seagrasses conservation status at an early stage. Besides morphological indicators, widely used in seagrass monitoring, functional and biochemical descriptors focusing on plant physiology can provide a good understanding of the plant responses to environmental changes. Moreover, the epiphytic microbial communities of seagrasses may shift in synchrony with plants as a response to changes in environmental conditions. However, until now they have been seldom used as a descriptor of environmental change. In this study three *Halophila stipulacea* (Forsk.) Aschers meadows (North Beach, NB, Tur Yam, TY, South Beach, SB) found along a gradient of different human uses and geomorphological features in the Gulf of Aqaba (northern Red Sea), were characterized using an integrated approach to highlight possible differences in their ecological status. For this, plant descriptors, namely leaves morphometrics (length, width and area, leaf number/plant, the percentage of leaves with lost apex), photosynthetic pigments (Chlorophyll *a*, Chlorophyll *b* and carotenoids) and total phenols contents were coupled with the plants' epiphytic microbial community structure and composition, studied using 454 pyrosequencing. These plant descriptors were then linked to the environmental variables (water column and pore water nutrient concentrations, granulometry and total organic carbon content of local

sediments), geomorphological features (meadows surface area, bottom slope, Secchi depth, the diffuse attenuation coefficient-KdPAR) and human uses of each meadow. The entire suite of descriptors depicted differences among the meadows plants eco-physiology and their microbial communities in response to their different physico-chemical and geo-morphological features. The mutual response of the seagrass plants and the microbial communities provided evidence of their functional relationship, which undoubtedly needs further investigation. To the best of our knowledge, this is the first time that such descriptors have been used in an integrated approach. We provide evidence to their effectiveness in discriminating seagrasses conservation status, even at small spatial scales. This work constitutes a new approach to the assessment of seagrasses and a stepping stone in the application of microbial communities as a putative marker in a changing environment.

8 SUPPORTING INFORMATION

Tab. S1 Results of ANOVA: Two Factor analyses with replication performed in PAST software for the analysis of the microbial community associated with *Cymodocea nodosa* in a mesocosm experiment. Shannon Diversity indices were log transformed and used as input. Two replicates per sample were used. Statistical significance *p*=0.05; n.s = not significant.

ANOVA: Two-Factor With Replication				
Source of Variation				
Summer *vs*. Winter samples	***F***	***P-value***		
1. Exp. Conditions (summer *vs*. winter)	4.135125	0.004107		
2. Plant Part (summer *vs*. winter)	7.844502	0.002391		
Exp. Conditions (summer *vs*. winter) x plant part	1.669845	0.130471 n.s		
	SUMMER		**WINTER**	
Source of Variation	***F***	***P-value***	***F***	***P-value***
Experimental conditions	5.659109	0.007311	4.677669	0.021856
Leaves *vs.* Rhizomes *vs.* Roots	24.03958	9.36E-06	2.579993	0.116946 n.s.
3. Interaction (Exp. Condition x plant part)	5.006416	0.0025	1.563377	0.239921 n.s.
Experimental conditions	15.29379	0.001123	2.799656	0.108614 n.s.
Leaves *vs.* Rhizomes	129.7358	3.19E-06	3.343564	0.104871 n.s.
4. Interaction (Exp. Condition x plant part)	6.233065	0.017288	1.798929	0.225235 n.s.
Experimental conditions	3.068519	0.090995 n.s.	52.92825	1.28E-05
Leaves *vs.* Roots	20.15311	0.002031	6.50877	0.034109
5. Interaction (Exp. Condition x plant part)	6.85407	0.013336	8.31219	0.007686
Experimental conditions	5.621375	0.022713	1.75774	0.23271 n.s.
Rhizomes *vs.* Roots	0.970101	0.353502 n.s.	1.573587	0.245096 n.s.
6. Interaction (Exp. Condition x plant part)	2.545229	0.129313 n.s.	0.944227	0.463595 n.s.

Tab. S2 Results of Unifrac Weighted analysis performed in MOTHUR software for the analysis of the microbial community associated with *Halophila stipulacea* in three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB).. Two replicates per sample were used. Statistical significance *p*=0.05; n.s=not significant. Bray Curtis matrix was build using the normalized dataset of 726 sequences per sample.

UNIFRAC WEIGHTED		
Bray Curtis distances		
PAIRWISE COMPARISONS		
Aboveground	WScore	WSig
North Beach *vs.* South Beach	0.936209	**<0.001**
North Beach *vs.* Tur Yam	0.885509	**<0.001**
South Beach vs.Tur Yam	0.870773	**<0.001**
Belowground		
North Beach *vs.* South Beach	1	n.s
North Beach *vs.* Tur Yam	0.88972	**<0.001**
South Beach *vs.* Tur Yam	1	n.s
By station		
North Beach *vs.* South Beach	0.866127	**<0.001**
North Beach *vs.* Tur Yam	0.74914	**<0.001**
South Beach *vs.* Tur Yam	0.849188	**<0.001**

Tab. S3 The microbial community composition associated with *Halophila stipulacea* at family level on three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB).

Phylum	Class	Order	Family	NB	TY	SB	Total sequences
Acidobacteria	Acidobacteria	Acidobacteria incertae sedis	Acidobacteria incertae sedis	0	13	0	13
Actinobacteria	Actinobacteria	Unclassified_Actinobacteria	Unclassified_Actinobacteria	13	31	13	57
		Acidimicrobiales	Acidimicrobiaceae	0	32	0	32
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	40	11	20	71
			Unclassified_Sphingobacteriales	109	25	54	188
	Flavobacteria	Flavobacteriales	Flavobacteriaceae	382	158	40	580
	Unclassified_Bacteroidetes	Unclassified_Bacteroidetes	Unclassified_Bacteroidetes	0	0	12	12
Cyanobacteria	Cyanobacteria	Cyanobacteria incertae sedis	Cyanobacteria incertae sedis	279	312	627	1218
		Unclassified_Cyanobacteria	Unclassified_Cyanobacteria	207	165	114	486
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	5985	5425	4244	15654
		Unclassified_Alphaproteobacteria	Unclassified_Alphaproteobacteria	1332	1169	530	3031
		Rhizobiales	Hyphomicrobiaceae	0	20	0	20
			Unclassified_Rhizobiales	46	87	33	166
			Rhodobiaceae	63	12	0	75
			Cohaesibacteraceae	0	0	16	16
			Phyllobacteriaceae	214	97	73	384
		Rhodospirillales	Rhodospirillaceae	198	85	28	311
		Sphingomonadales	Erythrobacteraceae	180	278	36	494

Tab. S3. Continued

		Alphaproteobacteria incertae sedis	**Alphaproteobacteria incertae sedis**	**11**	**26**	**14**	**51**
		Caulobacterales	Hyphomonadaceae	11	0	0	11
	Deltaproteobacteria	Unclassified_Deltaproteobacteria	Unclassified_Deltaproteobacteria	0	0	34	34
		Desulfobacterales	Desulfobacteraceae	35	16	0	51
			Desulfobulbaceae	325	481	19	825
		Myxococcales	Unclassified_Myxococcales	256	192	311	759
		Desulfuromonadales	Desulfuromonadaceae	10	0	0	10
	Gammproteobacteria	Unclassified_Gammaproteobacteria	Unclassified_Gammaproteobacteria	892	453	188	1533
		Gammaproteobacteria incertae sedis	Gammaproteobacteria incertae sedis	320	207	24	551
		Alteromonadales	Alteromonadaceae	70	0	67	137
		Thiotrichales	Thiotrichales incertae sedis	12	0	0	12
			Piscirickettsiaceae	10	0	0	10
	Unclassified_Proteobacteria	Unclassified_Proteobacteria	Unclassified_Proteobacteria	39	71	19	129
Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	613	1210	79	1902
	Phycisphaerae	Phycisphaerales	Phycisphaeraceae	10	0	0	10
	Unclassified_Planctomycetes	Unclassified_Planctomycetes	Unclassified_Planctomycetes	0	0	10	10
Verrucomicrobiae	Opitutae	Puniceicoccales	Puniceicoccaceae	0	26	0	26
	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	27	11	14	52
Unclassified	Unclassified	Unclassified	Unclassified	311	436	45	792
Total no. of sequences				**12000**	**11049**	**6664**	**29713**

Tab. S4 Results of SIMPER analysis conducted in PAST v.3 software for the analysis of the microbial community associated with *Halophila stipulacea* in three sampling stations: North Beach (NB), Tur Yam (TY) and South Beach (SB).

SIMPER ANALYSIS ABOVEGROUND			
Overall dissimilarity between stations	14.61%	**33.71%**	31.82%
Taxon contribution >60% dissimilarity	**NB vs. TY**	**NB vs. SB**	**SB vs. TY**
Planctomycetaceae	17.7	8.5	20.1
Rhodobacteraceae	16.6	27.7	21.0
Gammaproteobacteria	13.0	11.2	4.7
Cyanobacteria	1.3	5.5	5.6
Alphaproteobacteria	4.9	12.8	11.3
Desulfobulbaceae	4.6	4.9	8.2
Unclassified	3.7	4.2	6.9
Total	61.8%	70.6%	77.8%

SIMPER ANALYSIS BELOWGROUND			
Overall dissimilarity between stations	32.12%	**36.76%**	**36.45**%
Taxon contribution >60% dissimilarity	**NB vs. TY**	**NB vs. SB**	**SB vs. TY**
Desulfobulbaceae	9.6	38.0	27.0
Cyanobacteria	1.9	9.9	10.2
Rhodobacteraceae	6.6	4.8	0.5
Desulfobacteraceae	2.0	4.1	2.3
Myxococcales	1.9	4.0	4.9
Gammaproteobacteria	47.0	2.9	36.0
Total	68.92%	63.71%	80.92%

Tab. S5 Results of Unifrac Weighted analysis performed in MOTHUR software for the analysis of the microbial community associated with *Halophila stipulacea* along a depth gradient: 4m, 9m, 18m, 28m. Two replicates per sample were used. Statistical significance *p*=0.05; n.s=not significant. Bray Curtis matrix was build using the normalized dataset of 2,764 sequences per sample

UNIFRAC WEIGHTED		
Bray Curtis distances		
PAIRWISE COMPARISONS		
Aboveground	WScore	WSig
4m *vs.* 18m	0.882919	<0.001
4m vs. 28m	0.962379	0.005
9m *vs.* 28m	0.907865	<0.001
18m *vs.* 28m	0.818577	<0.001
Belowground		
4m vs. 28m	1	<0.001
9m *vs.* 28m	0.980158	<0.001
18m *vs.* 28m	0.980158	<0.001

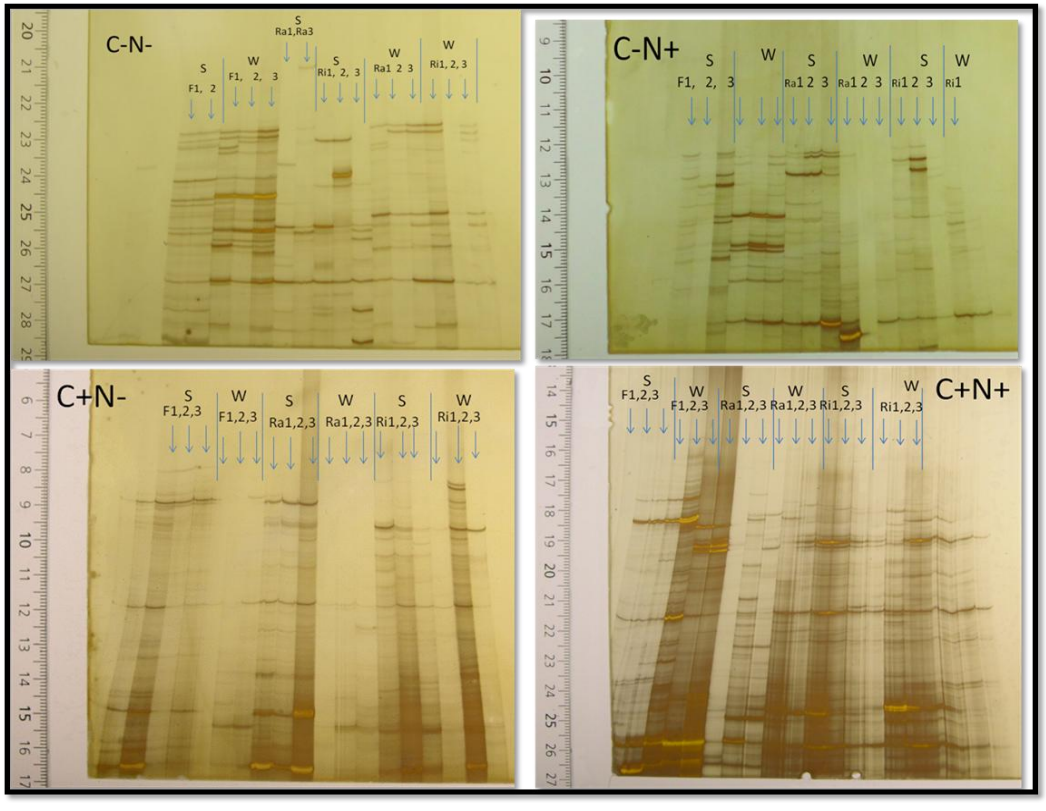
Tab. S6 Results of SIMPER analysis conducted in PAST v.3 software for the analysis of the microbial community associated with *Halophila stipulacea* along a depth gradient: 4m, 9m, 18m, 28m. Table shows the top 10 phylotypes contributing the most to the dissimilarity between samples.

ABOVEGROUND

	4m *vs.* 9m		4m *vs.* 18m		4m *vs.* 28m		9m *vs.* 18m		9m *vs.* 28m		18m *vs.* 28m	
Cumulative dissimilarity %	31.09		44.91		33.8		39.13		30.12		36.83	
	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.
1.	Rhodobacteraceae	6.476	Rhodobacteraceae	8.891	Haliea	5.454	Rhodobacteraceae	10.25	Rhodobacteraceae	7.477	Rhodobacteraceae	12.89
2.	Planctomycetaceae	5.804	Cyanobacteria	5.619	Planctomycetaceae	5.166	Cyanobacteria	6.57	Cyanobacteria	7.443	Alphaproteobacteria	6.45
3.	Desulfopila	4.75	Haliea	4.46	Cyanobacteria	5	Roseibium	5.24	Alphaproteobacteria	6.61	Acidimicrobiales	5.77
4.	Alphaproteobacteria	4.74	Flavobacteriaceae	4.08	Cyanobacteria	4.92	Desulfopila	5.16	Myxococcales	5.95	Desulfopila	5.71
5.	Unclassfied	4.72	Cyanobacteria	4	Rhodobacteraceae	4.65	Myxococcales	4.5	Acidimicrobiales	4.83	Gammaproteobacteria	4.91
6.	Muricauda	4.61	Planctomycetaceae	3.96	Unclassfied	4.52	Sphingobacteriales	4.07	Sphingobacteriales	4.21	Cyanobacteria	4.56
7.	Myxococcales	4.29	Alphaproteobacteria	3.93	Desulfopila	4.53	Alphaproteobacteria	4	Roseibium	3.94	Myxococcales	3.63
8.	Cyanobacteria	3.99	Desulfopila	3.82	Flavobacteriaceae	3.92	Cyanobacteria	3.68	Gammaproteobacteria	3.81	Phyllobacteriaceae	3.47
9.	Flavobacteriaceae	3.77	Sphingobacteriales	3.59	GammaIncertae	3.86	Gammaproteobacteria	3.51	Haliea	3.61	Sphingobacteriales	3.44
10.	Haliea	2.47	Muricauda	3.55	Myxococcales	3.44	Haliea	3.05	Desulfopila	3.33	Cyanobacteria	2.6

Tab. S6 continued

BELOWGROUND												
4m *vs.* 9m		4m *vs.* 18m		4m *vs.* 28m		9m *vs.* 18m		9m *vs.* 28m		18m *vs.* 28m	
Cumulative dissimilarity %	42.93		39.16		36.29		42.33		40.67		45.03
Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.	Taxon	% Contrib.
Cyanobacteria	11.95	Rhodobacteraceae	6.336	Roseibium	19.17	Rhodobacteraceae	6.6	Roseibium	5.408	Roseibium	17.16
Planctomycetaceae	5.13	Sphingobacteriales	4.184	Acidimicrobiales	3.67	Cyanobacteria	5.16	Rhodobacteraceae	2.601	Clostridiales	4.07
Desulfopila	4.99	Desulfopila	3.82	Planctomycetaceae	3.33	Desulfopila	5.12	Cyanobacteria	1.932	Desulfopila	3.83
Rhodobacteraceae	4.57	Unclassfied	3.62	Flammeovirgaceae	2.56	Unclassfied	4.44	Clostridiales	1.636	Acidimicrobiales	3.36
Desulfobulbaceae	4.25	Myxococcales	3.22	Alphaproteobacteria	2.45	Sphingobacteriales	3.95	Unclassfied	1.549	Rhodobacteraceae	3.26
Alphaproteobacteria	4.08	Pelagibius	3.19	Nannocystaceae	2.41	Gammaproteobacteria	3.44	Myxococcales	1.367	Unclassfied	3.08
Gammaproteobacteria	3.89	Haliea	3.13	Desulfosarcina	2.35	Myxococcales	3.27	Desulfopila	1.152	Cyanobacteria	3
Desulfobacteraceae	2.99	Rhizobiales	2.92	Proteobacteria	2.25	Haliea	3.07	Hyphomicrobiaceae	1.032	Alphaproteobacteria	2.83
Sphingobacteriales	2.72	Cohaesibacter	2.92	Desulfobacteraceae	2.21	Loktanella	2.89	Planctomycetaceae	1.018	Cyanobacteria	2.71
Rhizobiales	2.51	Clostridiales	2.82	Muricauda	2.12	Cohaesibacter	2.79	Acidimicrobiales	1.016	Gammaproteobacteria	2.59



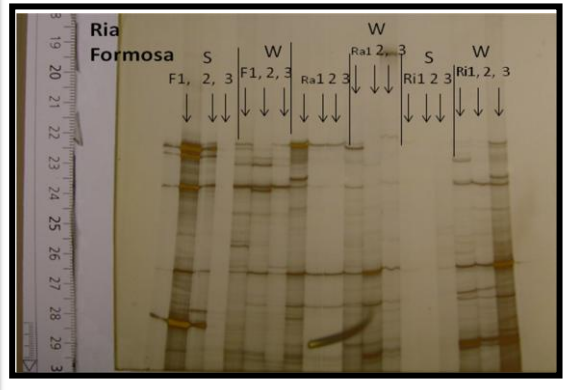


Fig. S1 SSCP gels of the microbial communities associated with the different plant parts (leaves, roots and rhizomes) of *Cymodocea nodosa* plants under different experimental conditions (C-N- control; C-N+ high Nutrients; C+N- high CO_2; C+N+ high CO_2 and Nutrients) and in plants from the field (Ria Formosa Lagoon).

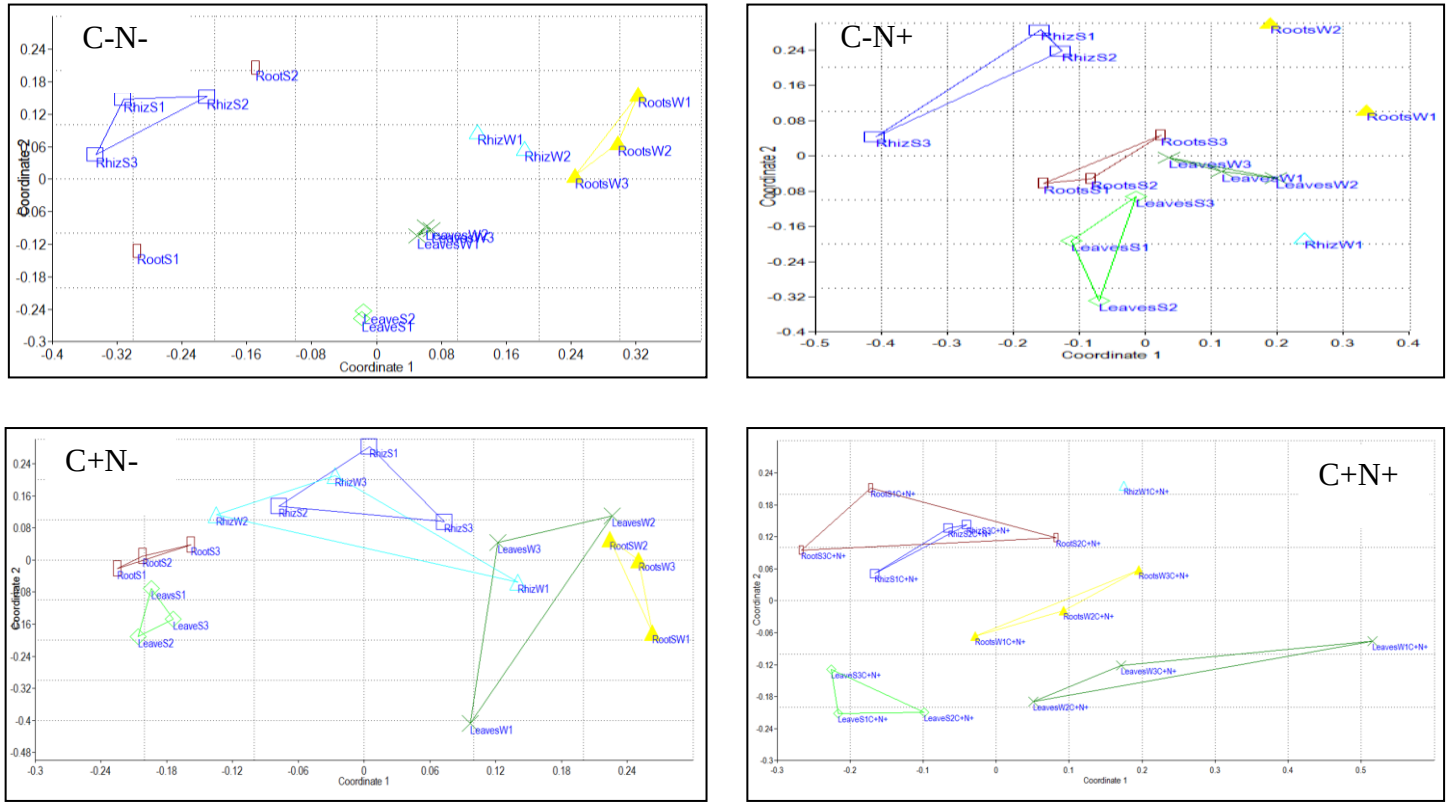


Fig. S 2 n-MDS charts of the microbial communities associated with *Cymodocea nodosa* plants during summer and winter when exposed to different experimental conditions (C-N- control; C-N+ high Nutrients; C+N- high CO_2; C+N+ high CO_2 and Nutrients). The analysis was performed using Bray-Curtis similarity indices. Charts show all replicates from three different plant parts (leaves, roots and rhizomes). Symbols: Leaves summer = Green diamond; rhizomes summer = Blue square; roots summer = Red box; Leaves winter = Green X; rhizomes winter = Blue triangle; roots winter = Yellow filled triangle: Stress value= C-N- = 0.1788; C-N+ = 0.3039; C+N- = 0.1641; C+N+ = 0.3208.