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Abstract: Bacterial communities inhabiting non-calcified gorgonian corals (Octocorallia, Gorgoniidae) are apparently unique in taxonomic composition and may benefit their host by several means, including chemical defense and nutrient provision. However, in spite of their distinct phylogenetic diversity, the gorgonian symbiotic consortium has thus far been scarcely addressed, and thus the microbiota of several gorgonian hosts remains uncharted.

This thesis describes the bacterial diversity found in the gorgonian host *Eunicella labiata*, determines its degree of uniqueness in comparison with the structure of the surrounding bacterioplankton and reveals the readily cultivatable fraction within this symbiotic consortium. To this end, a custom methodological approach was employed allowing direct comparison between the "total" and "cultivatable" bacterial community inhabiting *E. labiata* using massive Illumina sequencing of 16S rRNA gene amplicons. The 16S rRNA gene Operational Taxonomic Units (OTUs) were established at a cut-off of 97% gene similarity. Biodiversity indices such as the Shannon-Wiener were highest in the bacterial community obtained from seawater. A Tukey Honest Post Hoc test determined significant differences between biodiversity indices and sample categories. A closer look into taxonomic profiles and the relative abundances of dominant genera, clearly showed differences between microhabitats, with *Candidatus Actinomarina* dominating the seawater samples whereas within *Ruegeria* and *Endozoicomonas* represented the dominant fraction within *E. labiata*. The cultivatable community retrieved from *E. labiata* was close to the total community (62%) and speaks for a successful cultivation attempt of the *E. labiata* bacteriome. Unique OTUs within the gorgonian coral sum up to 4% in contrast to the unique fraction of OTUs found in the seawater, accounting to 31.1%. Finally, a Principle Coordinate Analysis and bootstrap values obtained from a hierarchical Cluster Analysis support the clear separation of the taxonomic community between the seawater and the gorgonian coral.

Resumo: Embora os recifes de coral ocupem apenas até 2% do ambiente marinho, eles abrigam um terço de todas as espécies marinhas descritas. Através de sua estrutura tridimensional, eles suportam vários nichos e são freqüentemente usados como habitats

de reprodução e exploração por várias espécies. Além disso, os invertebrados marinhos ganham cada vez mais interesse na indústria, abrigando um reservatório de enorme biodiversidade, promovendo interdependências de espécies complexas e um terreno fértil para compostos medicamente ativos. A informação ecológica sobre os corais moles de ambientes temperados é limitada quando comparada aos seus correspondentes tropicais calcificados, especialmente informações sobre diversidade bacteriana e cultivabilidade de seus microbiomas. As gorgonias representam um componente característico das comunidades bentônicas do fundo duro e são espécies engenherias de alta longevidade. A sua conservação é crucial para manter a biodiversidade das comunidades marinhas. As comunidades bacterianas que habitam corais gorgonianos não calcificados (Octocorallia, Gorgoniidae) são aparentemente únicas na composição taxonômica e podem beneficiar seu hospedeiro por vários meios, incluindo proteção química e provisão de nutrientes. No entanto, apesar da sua diversidade filogenética distinta, o consórcio simbiótico das gorgônias até agora não foi completamente abordado e, portanto, a microbiota de vários hospedeiros gorgonianos permanece desconhecida. Um coral é um holobionte complexo composto pelo animal e sua microbiota associada, interna e externa, composta por procariotas, eucariotas microbianos, fungos, protozoários e vírus. Muitas incertezas permanecem sobre os mecanismos de como a população microbiana associada aos corais nativos interfere com a nutrição dos corais, metamorfose larval e resistência a agentes patogênicos.

Esta tese descreve a diversidade bacteriana encontrada no hospedeiro gorgônio *Eunicella labiata* (amostrado em Faro, Portugal, 2015), determina seu grau de singularidade em comparação com a estrutura do bacterioplâncton envolvente e revela a fração prontamente cultivável dentro desse consórcio simbiótico. Para este fim, empregou-se uma abordagem metodológica específica, permitindo uma comparação direta entre a comunidade bacteriana "total" e "cultivável" que habita *E. labiata* usando sequenciação maciça Illumina de amplicões de genes de ARNr 16S. As unidades taxonômicas operacionais (OTUs) do gene rRNA 16S foram estabelecidas em um corte de 97% de similaridade de genes. Uma curva de rarefação foi criada para investigar a integridade da comunidade bacteriana obtida a partir de cada categoria de amostra, nomeadamente: (i) tecido de *E. labiata* recolhido in situ, (2) a comunidade de bactérias cultiváveis de *E.*

labiata obtida em meio Agar Marinho ("lavagem de placas") e (3) água do mar . Considerando que as amostras de água do mar e de lavagem de placas quase atingiram um plateau, indicando que, com maior esforço de amostragem, não serão adicionadas muitas espécies, o aumento do esforço de amostragem em *E. labiata* ainda pode aumentar consideravelmente as espécies detectadas. As OTUs bacterianas dominaram o conjunto de sequências do rRNA 16S obtidas por sequenciação, enquanto as arqueias nunca ultrapassaram 4% das sequências totais em cada categoria de amostra. O gênero arqueano *Nitrosopumilus* foi detectado em *E. labiata* (30 OTUs) e sabidamente contêm genes de oxidação de amônia utilizados para a nitrificação de amônia para nitrito, um mecanismo utilizado para a remoção de resíduos nitrogenados no holobiontecoralíneo. A riqueza procariótica (OTUS) observada, bem como a estimativa da riqueza procariótica total (índices Chao1 e ACE) e o índice de diversidade de Shannon foram calculados para descrever a biodiversidae bacteriana em cada categoria de amostra. Os índices de biodiversidade, como o índice Shannon-Wiener, apresentaram valores médios de 4,2 na água do mar, 3,4 em *E. labiata* e 2,8 nas amostras cultivadas. Para identificar se houve diferença significativa entre os valores obtidos para cada categoria de amostra, foi realizado um teste Tukey Post Hoc, após a obtenção de valores p significativos estatísticos de uma ANOVA. A água do mar é estatisticamente diferente nos quatro parâmetros de biodiversidade medidos quando comparada às OTUs obtidas de *E. labiata* tanto pelo método independente (in situ) como pelo método dependente (lavagem de placas) de cultivo, excepto para o índice de Shannon-Wiener onde diferenças significativas só puderam ser encontradas entre a categoria de água do mar e a lavagem de placas. A maior diversidade dentro de cada índice foi exibida na água do mar. O gráfico de barras empilhadas mostra a composição taxonômica em quatro níveis taxonômicos, a saber Classe, Ordem, Família e Gênero. Para uma ilustração clara dos gêneros dominantes em cada categoria de amostra, as OTUs com uma abundância relativa inferior a 2% foram filtradas e resumidas dentro de uma barra de pilha. Um olhar mais atento sobre os perfis taxonômicos e a abundância relativa de gêneros dominantes mostrou claramente diferenças entre microhábitats, com *Candidatus Actinomarina* dominando as amostras de água do mar enquanto *Ruegeria* e *Endozoicomonas* representavam a fração dominante em *E. labiata*. *Ruegeria*, *Vibrio* e *Sphingorhabdus*

dominaram as amostras de lavagem da placa. O maior número de táxons raros (OTUs com abundâncias mais baixas do que 2%) foi encontrado nas amostras in situ de *E. labiata*. Cerca de 47,1% das 1092 OTUs obtidas neste estudo foram cultivadas com sucesso em Agar marinho semi-sólido (1/2) a baixas temperaturas ao longo de várias semanas. A comunidade cultivável recuperada de *E. labiata* foi próxima da comunidade total (62%) evidenciando uma tentativa de cultivo bem sucedida da comuidade bacteriana em *E. labiata*. As OTUs únicas dentro do coral gorgônio somam até 4% em contraste com a fração única de OTUs encontrada na água do mar, representando 31,1%. Uma Análise de Coordenadas Principais (PCoA) usando uma matriz de distância euclidiana, obtida para todas as amostras com base na sua composição de OTUs, foi realizada ilustrando as 20 OTUs mais abundantes do conjunto de dados no diagrama de ordenação de acordo com suas mudanças em abundância nas categorias de amostra estudadas. A estreita associação entre o gênero *Endozoicomonas* e as amostras de gorgônias in situ foi evidente pela sua proximidade no diagrama de ordenação e seu consequente distanciamento das amostras de água do mar.. *Ruegeria* foi posicionada entre *E. labiata* in situ e as amostras de lavagem de placas, refletindo a recuperação considerável de ambas as fontes. *Shewanella*, *Sphingorhabdus* e *Vibrio* também estavam claramente distantes das amostras de água do mar e mais próximas das amostras de lavagem das placas. Vária OTUs não classificáveis a nível de espécies ou gênero (p.ex. “non classified *Surface1”*, *SAR86*, *Candidatus Actinomarina*, ”non classified *Marinimicrobia”*, ”non classified *Rhodobacteraceae”* e “non classified *Planktomarina”*) foram agrupadas em conjunto com as amostras de água do mar. Finalmente, os valores de bootstrap obtidos a partir de uma Análise de Clusters hierárquica suportam a clara separação da comunidade taxonômica entre a água do mar e o coral gorgônico. O gênero *Endozoicomonas* não estava realmente presente na água do mar em torno do nosso coral. Vários estudos identificaram *Endozoicomonas* como uma bactéria dominante na comunidade procariótica associada aos corais. As descobertas deste estudo motivam investigações adicionais com a gorgônia *E. labiata*, especialmente devido aos resultados promissores relativamente ao cultivo de seu microbioma bacteriano, uma vez que a informação ecológica sobre a biodiversidade microbiana, obtida apenas a partir de estudos independentes de cultivo, é difícil de interpretar.

Keywords: Metagenomics, Octocorallia, Holobiont, Microbiology, *Eunicella labiata*

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Acronym list

-ACE: Abundance-based coverage estimator

-AU: Approximately unbiased p-value

BP: Bootstrap probability

-bp: Base pair

-BBD: Black band disease

-$CaCO_3$: Calcium carbonate

-CO_2: Carbon dioxide

-DCM: Deep-chlorophyll maximum

-DMSP: Dimethylsulfoniopropionate

-DNA: Deoxyribonucleic acid

-ml: Milliliter

-NCBI: National center for biotechnology information

-OTU: Operational taxonomic unit

-PCoA: Principle coordinate analysis

-PCR: Polymerase chain reaction

-Psu: Practical salinity unit

-Rca: *Roseobacter* group affiliated

-QS: Quorum sensing

-rDNA: Ribosomal deoxyribonucleic acid

-RDP-II: The ribosomal database project

-Rel. abundance: Relative abundance

-RNA: Ribonucleic acid

1. Introduction

Coral reefs represent one of the most diverse ecosystems found in our oceans. Occupying only up to 2% of the marine environment, coral reefs harbor around one third of all described marine species (Veron et al. 2009). The three-dimensional landscapes created by corals, harbor a reservoir of enormous biodiversity promoting complex species interdependencies and a fertile ground for medically active compounds. Coral reefs are however increasingly threatened by direct human activities, indirect human activities and indirect global climate shifts (Kakonikos et al. 1999; Bellwood et al. 2004).
Nowadays one-third of all coral species are at risk of extinction, while they support food production, tourism and emerging biotechnology development, providing coastal protection from natural disasters. The coral crisis is often related to global change and coastal urbanization, which is expanding disproportionally to human population growth and coastal coral reefs, like other marine coastal ecosystems are increasingly exposed to growing loads of nutrients, sediment and pollutants discharged from the land (Bourne et al. 2009; Wood et al. 2012; Erftemeijer et al. 2012; Sunagawa et al. 2009; Fabricius & Sea 2005).

Recent studies support the importance of some types of bacteria that may exclude undesirable microorganisms through the production of secondary metabolites and antibiotics, highlighting one important component of coral microbe symbiosis (Littman et al. 2009). The bacterial community associated with the black band disease of the scleractinian corals *Diploria strigose, Montastrea annularis* and *Colpophyllia natans* support previous studies indicating the low bacterial similarity in respect to abundance and diversity between healthy and diseased individuals. *Bacteria* from the class *Delta-* and *Epsilonproteobacteria* were exclusively present within the diseased individuals, whereas *Beta-* and *Gammaproteobacteria* were identified in both, healthy and diseased individuals. *Alphaproteobacteria* dominated both samples, healthy and diseased corals (Frias-lopez et al. 2002; Cooney et al. 2002). A strain affiliated with the *Roseobacter* (*Alphaproteobacteria)* clade produces an antibiotic called tropodithietic adic, a

compound showing strong inhibiting properties in respect to marine bacteria of various taxa and marine algae (Brinkhoff et al. 2004).

However, there are still many uncertainties about the mechanisms how the native coral-associated microbial population influence coral nutrition, larval metamorphosis and resistance to pathogens. The first process to investigate bacteria typically starts with culturing them. Unfortunately, uncultured microorganisms still represent the majority of the microbial diversity and therefore culture-independent methods are crucial to understand genetic diversity, population structure and ecological roles (Riesenfeld et al. 2004).

Using DNA directly from an environment was initially suggested by Pace (1997) and first implemented by Schmidt et al. (1997), who described the phylogenetic diversity of an oligotrophic marine picoplankton community by analyzing the sequences of cloned ribosomal genes in the north central Pacific (Riesenfeld et al. 2004). Mixed population (i.e., "metagenomic") DNA was cloned into the bacteriophage lambda and portions of the ribosomal RNA (rRNA) gene were amplified via polymerase chain reaction (PCR) and sequenced. The resulting sequences were compared with an established data base of rRNA sequences obtaining 15 unique eubacterial sequences including four from *Cyanobacteria* and eleven from *Proteobacteria* (Schmidt et al. 1991).

Whereas associations between bacteria and eukaryotic hosts are common in the marine environment, known symbioses involving archaea are limited. Archaea have been generally characterized as microorganisms that inhabit relatively circumscribed niches, largely high-temperature anaerobic environments but molecular phylogenetic surveys indicate that the evolutionary and physiological diversity of archaea is far greater than previously supposed (Preston et al. 1996).

Since sponges excrete ammonium, like many other marine invertebrates, as a waste product, microbial nitrifies are believed to use this substrate and play an important role in waste treatment (Bayer et al. 2008). The ammonia-oxidizing marine archaeon *Cenarchaeum symbiosum* for example, has been found in association with several

different sponge species and is regarded as a typical sponge associate (Preston et al. 1996; Amils & Puentes 2002). Findings of Holmes & Blanch 2007 support that archaea have evolved closely with their sponge hosts when analyzing 23 poriferan species, where 19 of them showed evidence of harboring archaeal communities. Most of the genes found in *C. symbiosum* from previously sequenced genomes are shared with its planktonic relatives. However, a considerable number of unique genes were found that might potentially be involved in archaeal-sponge symbiotic associations (Hallam et al. 2006). Therefore, management and prevention of coral disease will be difficult unless we can generate the data that allow us to understand the interaction between causative agents, corals and their environment combining culture dependent and culture independent techniques (Bourne et al. 2009).

Corals

Anatomy

Corals belong to the phylum Cnidaria, which further includes the hydroids, jellyfishes, sea anemones and sea fans. Along with the Placozoa and Porifera the Cnidaria represent the most ancient form of multicellular organization. Cnidarian polyps and medusae are radial symmetric and their body architecture is diploblastic consisting of two germ layers, the ectoderm and the endoderm. The diagnostic feature within this group is the organelle-like capsules with eversible tubules called Cnidae (Fig 1).
These stinging cells mainly serve in prey capture and defense. Mechanical stimulation of these cell's cnidocyil apparatus by prey or an offender leads to the explosive discharge of

the cnidocyst via bioelectrical signal transduction (Tardent 1995).

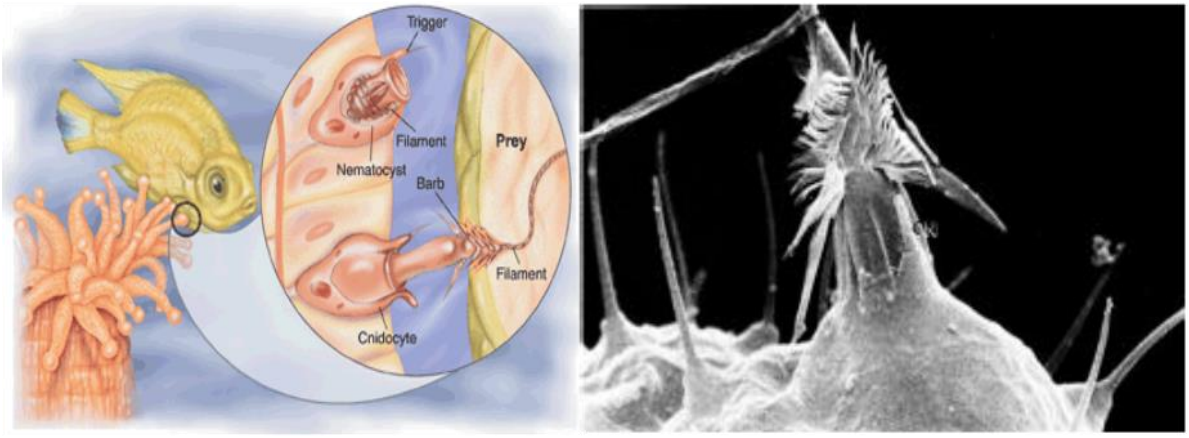

Figure 1- Schematic and microscopic illustration of a cnidocyte. ("http://www.jayreimer.com/TEXTBOOK/iText/products/0-13-115516-4/ch26/ch26_s3_1.html," https://en.wikipedia.org/wiki/Cnidocyte#/media/File:Nematocyst-discharged.png")

Corals are found within the class of the Anthozoa. All members of this class are exclusively polypoid and may be colonial, clonal or solitary, skeleton-less or with a mineralic and/or proteinaceous skeleton (Daly et al. 2003).

Corals have only a limited degree of organ development and each polyp consists of three basic tissue layers namely an outer epidermis, an inner layer of cells lining the gastrovascular cavity which acts as an internal space for digestion and a layer called the mesoglea in between these layers (Fig.2). In general, all polyps share two structural features with other members of their phylum. The first one a gastrovascular cavity that opens at only one end and is often referred as the mouth, where food is consumed and waste products are expelled. Secondly, all corals possess a circle of tentacles, which are extensions of the body wall that surround the mouth. Corals and all other members of the Cnidaria are carnivores using the cnidocyle equipped tentacles to catch prey within the water column and transport it to the mouth and into its stomach for final digestion. The tentacles are furthermore used to clear away debris from the mouth and act as the animal's primary means of defense.

The polyp of a hard coral is sitting within a cup-like structure. The walls surrounding this cup are called “theca” and the floor is referred to as the basal plate. Calcareous septa provide integrity and protection and an increased surface area for the polyp’s soft tissues, extend upward from the basal plate and radiate outward from its center. Additionally, each polyp is connected to its neighbor through a thin horizontal sheet of tissue called the coenosarc.

The soft corals are anatomically similar to their hard coral counterparts, with a few exceptions such as they do not secrete calcium carbonate skeletons and are therefore missing the calyx, theca, tabulae, septae or a basal plate (Fig.2B) (Barnes 1987; National Oceanic and Atmosperic Administration 2014).

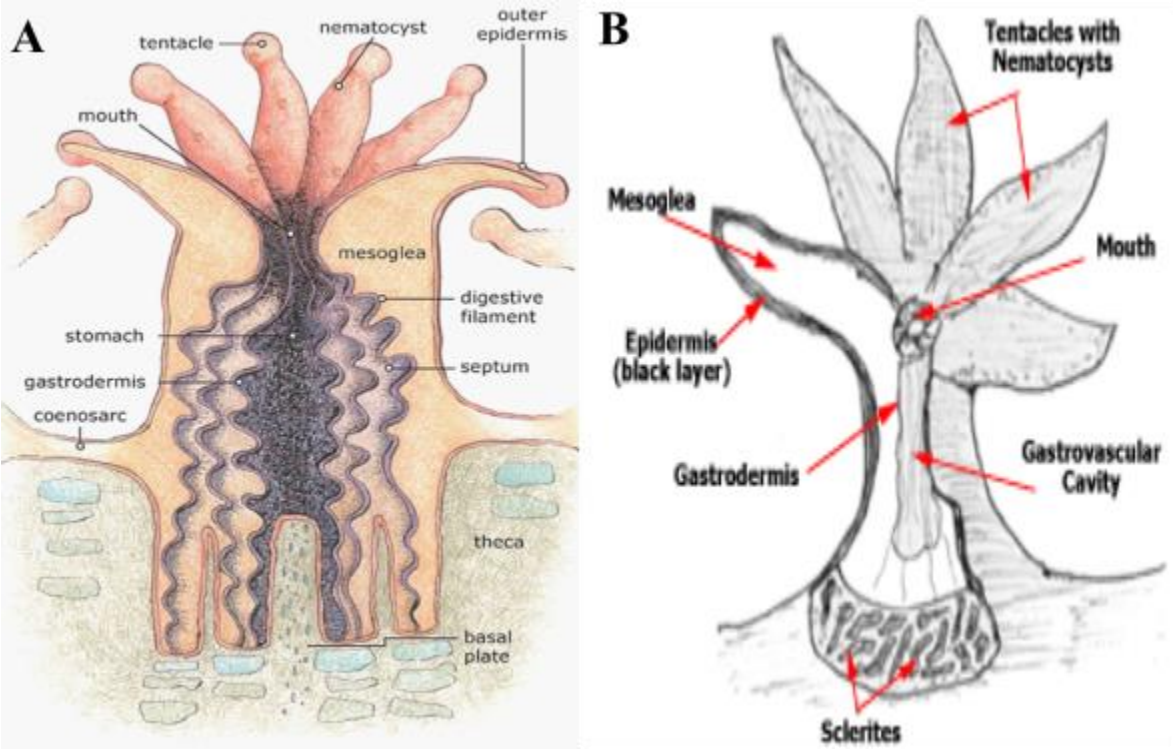

Figure 2- A schematic illustration of a hard (A) - and soft coral (B) polyp. Cnidae equipped tentacles are used to catch organisms within the water column, transport it into the stomach, where extracellular enzymes digest the prey. (“http://www.peteducation.com/article.cfm?c=0&aid=2987“, ”https://coralreef.noaa.gov/aboutcorals/coral101/anatomy/ “)

Modes of Reproduction

Corals can reproduce in both ways, sexually and asexually. Within the asexual reproduction, new clonal polyps separate from the parent polyp and start forming new colonies. It is a process that continues throughout the animal's life and appears once the parent polyp has reached a certain size (National Oceanic and Atmosperic Administration 2014).

Within the sexual reproduction, the phenomenon of broadcast spawners, where male and female individuals release massive numbers of eggs and sperm into the water to distribute their offspring's over a broad geographic area is in many reefs a mass synchronized event, where all the coral species in an area release their eggs and sperm at about the same time. Eggs and sperm become free-floating, or planktonic larvae called planulae. Because male and female individuals cannot move into reproductive contact with each other, the timing of this event is very important and is triggered in response to multiple environmental cues (Veron 2000; National Oceanic and Atmosperic Administration 2014).

Planulae swim upwards toward the light being transported by the current and will return to the bottom again afterwards, where, if conditions are favorable, they will settle. After settling they metamorphose into polyps and form colonies (National Oceanic and Atmosperic Administration 2014).

Taxonomy

Our current taxonomic understanding of corals on a species level is still a challenge. As debates concerning taxonomic uncertainty refer to that coral species are broad and fuzzy entities, capable of interbreeding with distantly related forms and exhibiting wide morphological variability and large ecological and geographical ranges (Knowlton 2001). However, recent studies suggest, that corals are well defined reproductively and more

narrowly distributed and less variable than conventionally assumed. *Montastraea annularis* was thought to show a wide variability in colony morphology. This phenotypic plasticity has therefore been viewed as a largely phenotypic response to variation in environmental conditions. Weil & Knowlton (1989) redescribed this species through a multi-character analysis due to the coexistence of discrete colony morphologies at the same site, often with little evidence of intermediate forms, resulting in defining three broadly sympatric shallow-water species. This study indicates that at present, it might not be impossible to say how many coral "species" are in fact species complexes, since literature reports numerous forms or morphotypes that remain to be analyzed (Knowlton 2001; Weil & Knowlton 1994).

The approximately 7.500 extant species within the class Anthozoa are comprised of two monophyletic lineages, the Octocorallia and the Hexacorallia.
The subclass Hexacorallia currently contains six orders: Actiniaria (sea anemones), Antipatharia (black corals), Ceriantharia (tube anemones), Scleractinia (stony corals), Corallimorpharia (corallimorpharians) and Zoanthidae (zoanthids) (Daly et al. 2003). Typical tropical coral reefs are formed from stony corals when each polyp secretes a skeleton of calcium carbonate ($CaCO_3$) As mentioned before, not all corals are reef builders (hermatypic) as some do not produce enough $CaCO_3$ or some being solitary and have single polyps (ahermatypic) (National Oceanic and Atmosperic Administration 2014; Barnes 1987).

Whereas most Hexacorallia own a hexamerous symmetry, the Octocorallia own eight tentacles and eight mesenteries of octocoral polyps that are invariant within the clade. The subclass Octocorallia is subdivided into three orders namely the Alcyonacea (soft corals and sea fans), Pennatulacea (sea pen) and the Helioporacea (blue corals) (Fig.3), whereas the Alcyonacea (soft corals and sea fans) is further divided into six sub-ordinal groups (Table 1) (Mcfadden et al. 2006).

Table 1 - Current higher taxonomic classification to the anthozoan subclass Octocorallia. The order is represented with an "O." and "N" represents the described numbers of families (from Williams, 1992).
**One described family might not be valid.*

Taxonomic group	N	Defining characteristics
O. Pennatulacea [sea pens]	14	Axial polyp differentiated into basal peduncle and distal rachis
O. Helioporacea [blue coral]	2	Massive aragonite skeleton
O. Alcyonacea		
[soft corals - no skeletal axis]		
Protoalcyonaria	2 *	Solitary polyps
Stolonifera	5	Polyps united basally by simple stolons which may fuse to form ribbons
Alcyoniina	5	Polyps united within fleshy mass of coenenchyme
[sea fans - with skeletal axis]		
Scleraxonia	7	Inner axis (or axial-like layer) consisting predominantly of sclerites
Holaxonia	4	Axis of scleroproteinous gorgonin, commonly with small amounts of embedded non-scleritic calcium carbonate; axis with hollow cross-chambered central core
Calcaxonia	5	Axis of scleroproteinous gorgonin with large amounts of non-scleritic calcium carbonate as internodes or embedded in the gorgonin; axis without hollow cross-chambered central core

A molecular phylogenetic analysis of the Octocorallia based on mitochondrial protein-coding sequences supports previous results that divide the subclass Octocorallia into two or three distinct clades, where one clade includes all members of the sea fan sub-order Holaxonia, a majority of the taxa belonging to the soft coral group Alcyoniina and most of the taxa in groups Scleraxonia and Stolonifera. The Second large clade includes all of the sea pens (Pennatulacea), blue corals (Helioporacea) and the sea fan sub-order Calcaxonia. The molecular data further do not support the phylogenetic distinction of the three sub-ordinal groups of the Alcyonacea namely the Alcyoniina, Scleraxonia and Stolonifera. These groups might represent grades of morphological construction that have likely evolved repeatedly during the history of Octocorallia. For instance, although the taxa included within the group Scleraxonia share a skeletal axis or axial-like layer containing sclerites, the details of skeletal construction differ substantially with multiple independent derivations of this type of axis (Mcfadden et al. 2006). Taxonomy and phylogenetic relationships within the Octocorallia still remain poorly understood and need further research effort to clarify phylogenetic concordance. At present the subclass Octocorallia includes approximately 3.000 extant species (Daly et al. 2007; Bellwood et al. 2004).

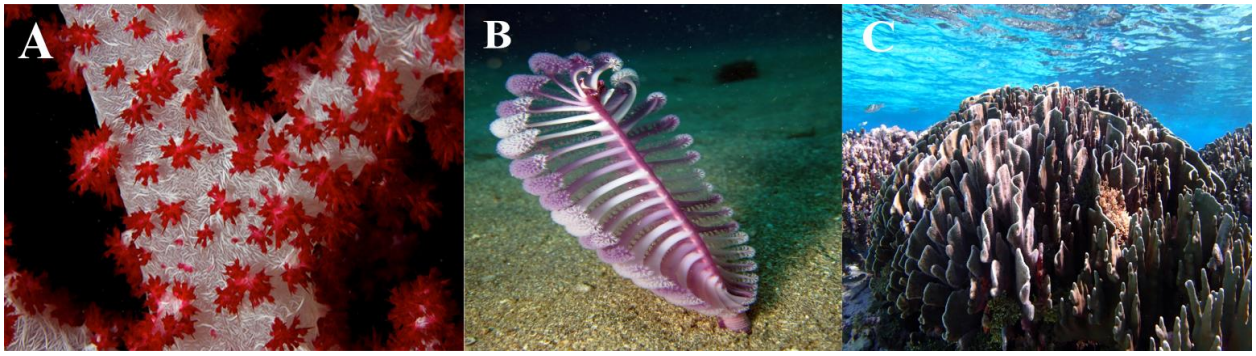

Figure 3 – Illustration of one representative of each order of soft corals. A,Alyonacea; B, Pennatulacea; C, Helioporacea ("http://coral.org/coral-reefs-101/coral-reef-ecology/soft-corals/","http://mesosyn.com/unusualAnimals.html#SeaPen"," http://ulithimarineconservation.ucsc.edu/blue-coral-helioporacea/")

Gorgonian corals

Despite their ahermatypic character gorgonians, (Alcyonacea) still make up an important part of the coral community composition within coral reefs. Analyzing three parallel submarine terraces along the southeast coast of Florida, 27 species of scleractinian corals and 39 species of gorgonians defined a typical coral-reef community, with gorgonians reaching maximum diversity at a depth of 15-20 meters, while scleractinians were most diverse within in shallower waters (Goldberg 1973).

Besides their presence in coral reefs and deep waters, gorgonians represent a characteristic component of hard bottom benthic communities and contribute significantly to the biodiversity of these ecosystems (Ballesteros 2006). Gorgonians are long-lived engineering species and their conservation is crucial to maintain the biodiversity of marine communities. The slow dynamics displayed by these species make them especially vulnerable when faced with increasing disturbances (Benayahu & Loya 1986). During the summers of 1999 and 2003, two mass mortality events affected the population of the slow-growing and long-lived Mediterranean gorgonian *Paramuricea clavata* living in the Gulf of La Spezia (Italy). Monitoring the population for three years after the mortality events, only a small increase in density of recruits and of older undamaged colonies was recorded, suggesting that the population recovers slowly (Cupido et al. 2008). Taylor et.al. (2011) reviewed mass mortalities involving

Mediterranean shallow water benthic communities caused by diseases attributed to factors such as toxic microalgae, mucilage, pathogenic agents, thermal stress and oxygen depletion. Although several species and groups of sessile organisms have been hit by mass mortalities in the Mediterranean Sea, diseased sea fans played a crucial part in altering the seascape of rocky bottoms, with the first recorded episode of gorgonian mass mortality described in 1984 mainly regarding *Eunicella singularis* and *Corallium rubrum* (Cerrano & Bavestrello 2008).

Although gorgonians are ecologically similar to scleractinians, they possess a comparatively limited number of reproductive strategies, where the most common is gonochorism combined with brooding (Brazeau & Lasker 1990).
Fertilization might occur internally or externally depending on the species. *Sarcophyton glaucum* is a dioecious species, where females attain maturity at a much larger colony size of at least 61 cm^3 when compared to male colonies bearing testes at a colony size of 11 cm^3. *S. glaucum* has a brief annual spawning period which occurs in the majority of the population during a single night. Fertilization is external and larvae swim actively promoting a wide dispersal of the species (Benayahu & Loya 1986).
In a reproductive study of a common Caribbean gorgonian coral, *Briareum asbestinum*, from the San Blas Islands (Panama), all colonies examined were either male or female during the height of the reproductive season since males could only be identified from April to August when spermaries were present. Spawning in males is synchronous and occurs following the full moons of June and July, whereas in this species, fertilization is internal. Embryos are released from polyps very early in development and remain attached to the outside of the colony for three to five days (Brazeau & Lasker 1990). As with many long-lived marine species, detailed illustrations of life-histories are rare since data of those marine species are difficult to acquire over the large temporal scales of their long life spans (Hughes & Connell 1999).

The family Gorgoniidae is found within the order Alcyonacea, which includes 30 families of soft corals and gorgonians (octocorals with a supporting skeletal axis of scleroproteinous gorgonin and/or calcite). Seventeen genera and approximately 260 species of gorgonians with retractile polyps and an axis of gorgonin surrounding a

narrow, hollow, cross-chambered central core are known within the family Gorgoniidae (Daly et al. 2007). A study in 2009/2010 investigated the distribution patterns of gorgonians in south Portugal, where the occurrence and abundance of gorgonian species in rocky bottoms were quantified over more than 25 km of coast down to 30 m depth. Most abundant gorgonian species along this study area were *Eunicella labiata*, *Eunicella gazella*, *Eunicella verrucosa* and *Leptogorgia sarmentosa* (Fig.4) (Curdia et al. 2013). In this study, one of the common and most abundant gorgonians present in the southern coast of Portugal was used, *E. labiata*. Figure five shows an overview of the global distribution of the genus *Eunicella*.

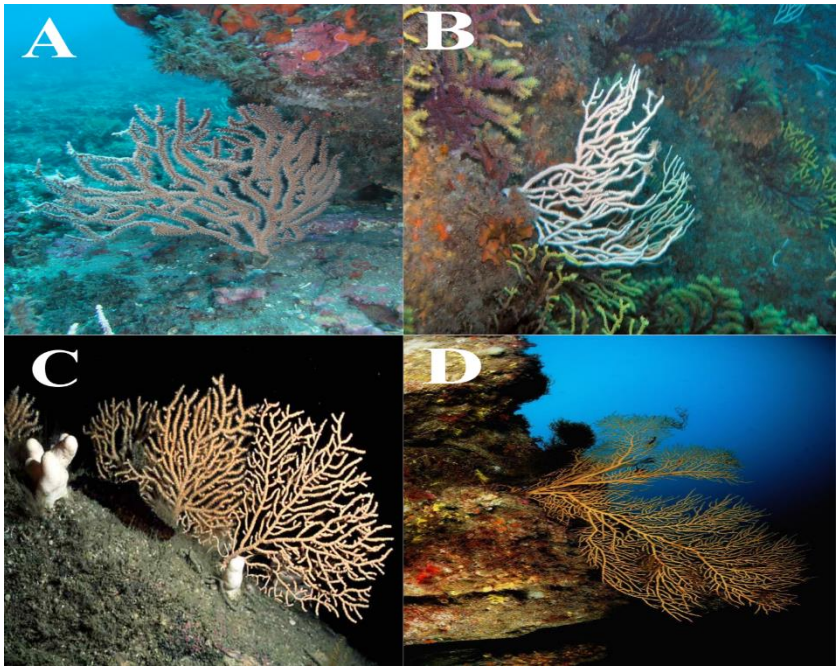

Figure 4- Abundant gorgonians from the southern coast of Portugal. A, E. labiata; B, E. gazella; C, E. verrucosa; D, L. Sarmentosa (http://www.granadasubmarina.org/art.php?id=Eunicella%20labiata", " http://www.marlin.ac.uk/species/detail/1121", " http://www.marlin.ac.uk/species/detail/1121", " https://www.asturnatura.com/fotografia/submarina-fotosub/gorgonia/7688.html")

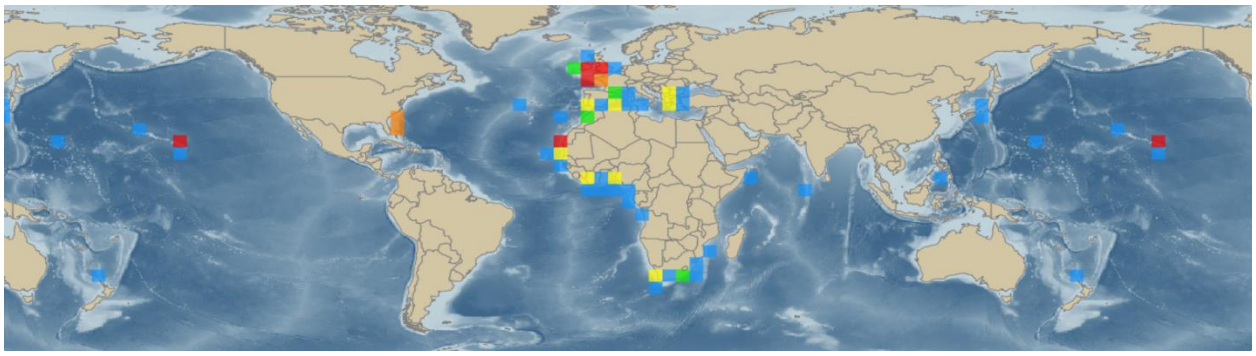

Figure 5- Global geographic distribution of the genus Eunicella. Warm colors represent high numbers of abundance whereas cold colors represent low levels of abundance (http://www.marinespecies.org/aphia.php?p=taxdetails&id=125301=

Prokaryotic life within our oceans

Studying the different life forms on our planet, microbiology was the last field to be established (Amann et al. 1995). Even after the first discovery of the microbial realm about 300 years ago, the right knowledge and biotechnological capacities were missing to fully investigate and understand prokaryotic life. Marine microbiology started gaining interest after the second half of the nineteenth century, when scientists were able to isolate ammonia- and nitrite-oxidizing bacteria from the Atlantic Ocean. Microorganisms are defined by their size (<150 µm) and include all three domains of life whereas prokaryotes include two domains, the bacteria and the archaea (Lutz-Arend 2005).

For the continental shelf and the upper 200 meters of the open oceans, the microbial density is about 5 x 10^5 cells/ml, whereas a big portion of these cells are represented by the marine cyanobacteria and *Prochlorococcus* spp. reaching an average cellular density of 4 x 10^4 cells/ml. Additionally the world's oceans comprise a rich diversity of microbial life with current estimates reaching over a million different species. From the 15 different phyla within the bacterial domain, the *Proteobacteria*, gram-positive bacteria (mostly *Actinobacteria*) and *Cyanobacteria* are of particular importance in our oceans (Whitman et al. 1998).

Generally marine bacteria are more psychrophilic than terrestrial species. Many marine bacteria have special requirements for inorganic ions such as Na^+ for growth as seen in

some species for transporting substrates into the cells. Furthermore their combination of Mg^{++} and Ca^{++} exceeds that of most terrestrial species (Macleod 1965).

Compared to *Bacteria*, *Archaea* show differences within cell walls, cytoplasmic membranes, transcription and translation mechanisms, structure of coenzymes and prosthetic groups as in the mechanisms regarding the autotrophic CO_2 – fixation (Lutz-Arend 2005). In the marine environment, archaeal habitats were believed to be limited to shallow or deep-sea anaerobic sediments, hot springs or deep-sea hydrothermal vents and highly saline land-locked seas. As reported by Delong (1992) archaea are not restricted to only anaerobic and harsh environments but also appear in oxygenated coastal surface waters in the east and west coasts of North America. Studying the abundances and distributions of prokaryotes in the western waters of the Antarctic Peninsula, during austral summer surface abundances of archaea were generally low accounting for about 1% of the total picoplankton assemblage. The abundance of *Archaea* increase significantly with depth, comprising 9-39% of the total picoplankton abundance in meso- and bathypelagic circumpolar deep waters. During winter, archaea and bacteria were more evenly distributed throughout the water column, with archaeal abundances averaging 10% in surface waters and 13% in the circumpolar deep waters (Church et al. 2003). Further studies suggest, that *Archaea* play a crucial role in the oceanic carbon cycle. Herndl et al. (2005) estimates that archaeal production in the mesopelagic and bathypelagic North Atlantic contributes between 13-27% to the total prokaryotic production in the oxygen minimum layer and 41-84% in the Labrador Sea Water, declining to 10-20% in the North Atlantic Deep Water.

Microbial Culturing and Metagenomics

Cultured species of *Bacteria* and *Archaea* represent only a minor fraction of the existing diversity. In several types of environments, more than 99% of organisms seen microscopically are not cultivated by routine techniques (Taga 1979). There are known species for which the applied cultivation conditions are just not suitable or which have entered a nonculturable state and unknown species that have never been cultured before where appropriate methods of cultivation are missing. *Vibrio cholerae* enters a

nonculturable state upon exposure to salt water, freshwater or low temperatures very quickly (Amann et al. 1995; Pace 1997). Ecological information on microbial biodiversity obtained from culture independent studies (such as metagenomics) is hard to interpret using only culture independent approaches (Fierer et al. 2007). Cultures of microbial organisms are crucial to dissect the functions and interactions of the prokaryotes with their environment (Smith & Bidochka 1998). Out of plenty of possibilities of information which can be retained from culturing, it can be used to investigate metabolic interactions between community members (Møller et al. 1998) or the effect of antibiotics on the bacterial colony. A further important example of culturing is shown by Beaty et al. 1994 who investigated the pathogenesis caused by the bacterium *Chlamydia trachomatis* using a variety of cell culture model systems. Therefore, it is crucial to further improve culturing methods in order to isolate single bacterial colonies for physiological and ecological studies. Since the majority of bacteria are uncultivable (Li 2009), culture-independent methods can be used to complete the knowledge of microbial diversity found within an ecosystem in order have an idea, on how much of the complete prokaryotic assemblage is still not cultivated (Goodman et al. 2011).

Metagenomic approaches analyze microbial communities in any given environment. Metagenomics describes the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Riesenfeld et al. 2004). The use of ribosomal RNA genes obtained from DNA isolated directly from the environment opened up new insights into microbial biodiversity. The highly-conserved nature of ribosomal RNA allows the use of universal PCR primers that can anneal to sequences conserved in the ribosomal RNA genes from all three phylogenetic domains, since each organism in an environment has its unique set of genes stored within its genome. Ribosomal RNA genes gathered from the environment are snapshots of organisms, which can be targeted for further characterization if they seem interesting and useful (Pace 1997). Because most of the coral-associated bacteria are novel at the genus and species level (Rohwer et al. 2002; Garcia et al. 2013; Wegley et al. 2007; Thurber et al. 2009; Littman et al. 2011) this task is especially challenging.

Rohwer and colleagues (2002), identified 430 novel bacterial ribotypes associated with three coral species. Many of these bacteria appear to have symbiotic relationships with the animal suggesting that corals may harbor specific microbial communities that positively contribute to host fitness. *Bacteria* seem to form species-specific associations with corals that are maintained over space and time, reflecting phylogenetic relationships among coral species (Bourne & Munn 2005).

Coral Holobiont

As first described by Heinrich Anton de Bary in 1879 the expression symbiosis was referred as "the living together of unlike organisms". Nowadays symbiosis commonly describes close and long-term interactions, including mutualistic, parasitic and commensal relationships, between different biological species (Li 2009). Growing evidence indicate that the microbial associates play essential roles in coral physiology and health. Several coral-associated bacteria have been shown to possess antibacterial activity, antibiofilm and anti-pathogenic properties (Porporato et al. 2013). Culture-dependent and independent studies have shown remarkable microbial diversity of microbial communities associated with living marine sessile organisms (Egan et al. 2008). The coral is a complex holobiont consisting of the animal and its associated suite of internal and external microbiota such as prokaryotes, microbial eukaryotes, fungi, protozoa and viruses (Wegley et al. 2007).

Carbon fixing zooxanthellae are essential symbionts in most hermatypic corals and provide the animal with energy reserves that are used for constructing the skeleton and producing the mucus sheet. Loosing those symbionts as seen in coral bleaching events is fatal for coral communities and coral bleaching events represent one of the major threats in respect to global warming. This drastic example shows the direct need between host and microbes (Wegley et al. 2007). Besides the well-known symbiotic relationship between zooxanthellae and coral, other members of the holobiont include bacteria, archaea, viruses, fungi and endolithic algae, which provide mutualistic benefits. Disturbance or shifts in any of these partners can compromise the health of the whole

animal (Bourne et al. 2009). Affecting the holobiont fitness has been shown to influence the response of bacterial communities to thermal stress, which leads to susceptibility of the holobiont to bleaching, disease and colonization by opportunistic potential pathogens (Bourne et al. 2013).

Typical prokaryotic groups present within a corals holobiont are *Alphaproteobacteria, Gammaproteobacteria* and *Bacteroidetes,* whereas *Alphaproteobacteria* are overrepresented (Pantos et al. 2015). Besides the well-studied members of the Hexacorallia, gorgonian corals are able to develop dense communities forming crucial three dimensional structures that act as nursing stations, shelter and creates microhabitats inhabited by several other species It is suggested that several members of gorgonians have a distinct prokaryotic profile that separates itself from the surrounding seawater (Bayer et al. 2013; Roberts et al. 2006; Gray et al. 2017). Roumagnac et al. (2013) highlighted that the bacterial community associated with the gorgonian coral *Paramuricea clavata* in the Mediterranean Sea is very different in its composition compared to the seawater. surrounding the coral. Shifts within the prokaryotic structure of those corals lead to higher susceptibility to pathogens and viruses (Vezzulli et al. 2010). Healthy members of the cold-water gorgonian coral *Eunicella verrucosa* seem to affiliate with the class *Gammaproteobacteria* (Ransome et al. 2017). Especially, one member of this class, the genus *Endozoicomonas,* showed high relative abundances (rel. abundances) within most healthy soft corals sometimes exceeding 50% of the total community make-up. Further, but less abundant genera found within the gorgonian coral *Eunicella cavolini* are *Ruegeria, Aquamarina, Haliea, Roseovarius, Sphingopyxis* and *Methylobacterium* (Ransome et al. 2017; Apprill et al. 2016; Woo et al. 2017; Bayer et al. 2013). Because the genus *Endozoicomonas* can only be found in very low concentrations in the seawater, this taxon appears to be intimately associated with gorgonians (Bleijswijk et al. 2015; Roumagnac et al. 2013). A more comprehensive study confirms the presence and dominance of the genus *Endozoicomonas* within five sympatric gorgonians namely *Eunicella singularis, Eunicella cavolini, Eunicella verrucosa, Leptogorgia sarmentosa* and *Paramuricea clavata.* Therefore, the concept of a core microbiome (operational taxonomic units consistently present in a species) is established.

Whereas species-specific prokaryotes assemble with certain taxa, the concept also takes in account locally variant members that might influence these local associations. For the three gorgonians *E. cavolini*, *E. singularis* and *L. sarmentosa* a core microbiome was established reaching from four to five core OTUs (Water et al. 2017).

Aims

Literature about the microbial taxonomic composition associated with *Eunicella labiata* is scarce if even not established until now. Until recently, research focused on the discovery of novel structures with potentially useful biological activity such as diterpenoids extracted from *E. labiata* (Berrue & Kerr 2009; Kakonikos et al. 1999). Long-lived species such as gorgonians, displaying slow growth, late maturity and low fecundity are among the most affected species considering anthropogenic threats and strong disturbances. Additionally, their presence is crucial to maintain the organization and diversity of the communities in which they live (Linares et al. 2008).

This Master thesis will describe the bacterial diversity found in the gorgonian host *E. labiata*, determine its degree of uniqueness in comparison with the structure of the surrounding bacterioplankton, and reveal the readily cultivatable fraction within this symbiotic consortium. To this end, a custom methodological approach will be employed allowing direct comparison between the "total" and "cultivatable" bacterial community inhabiting *E. labiata* using massive Illumina sequencing of 16S rRNA gene amplicon.

Materials and Methods

Sampling and sample processing

Sampling took place at Faro Island (Fig.6) in Southern Portugal ("Pedra da Greta": Lat. 36º 58' 47.2N, Long. 7º 59' 20.8W) on the 15th of March 2015. Sample processing, bacterial culturing and DNA extraction for sequencing analyses were performed by colleges and will hereby be discussed shortly. The water temperature on that day was

14°C, with a pH of 8.13 and a salinity of 36.30 psu (practical salinity units). In this study, we used the gorgonian coral *E. labiata* (Fig.4A).

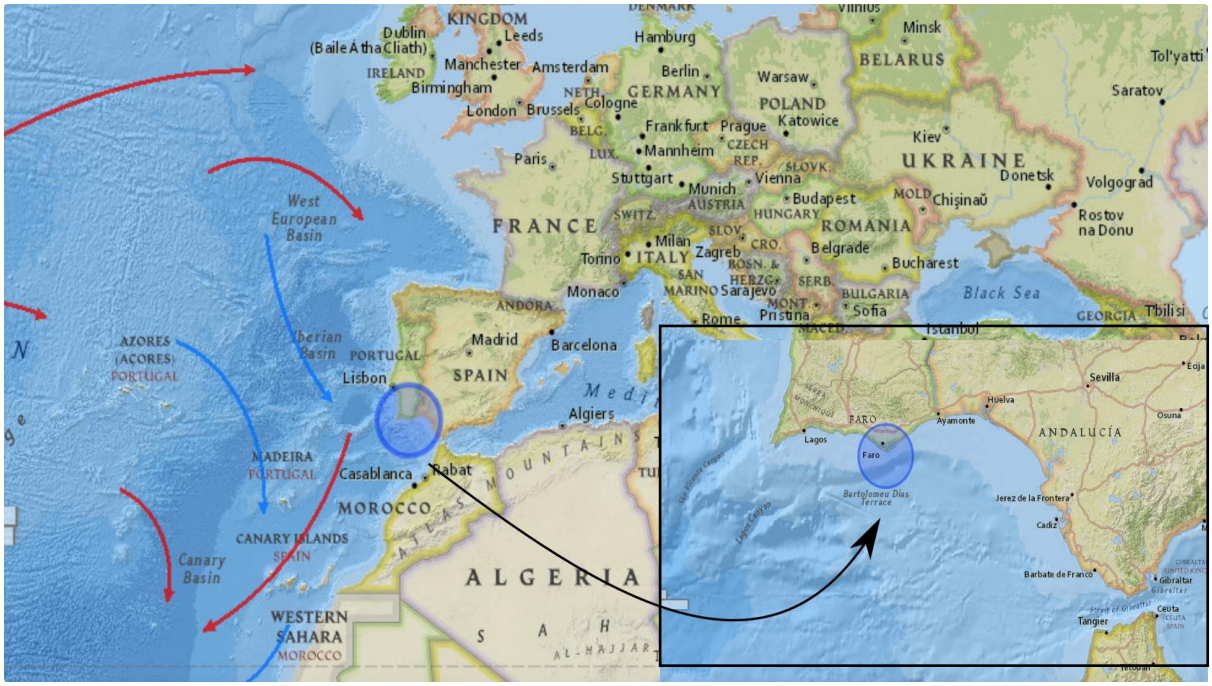

Figure 6- Location of the sampling area Faro, Portugal ("https://mapmaker.nationalgeographic.org/").

Three samples were taken from the coral *E. labiata* (EL01; EL02; EL 03) in 18 meters depth and four samples were taken from the surrounding sea water (SW05; SW06; SW07, SW08). Furthermore, semi-solid Marine Agar (1/2) was used for incubating and culturing bacterial symbionts at 18ºC over 4 weeks. The cultivated prokaryotic community was used as plate washes to be further analyzed. "Plate washing" involves extracting the total community DNA from washes of Marine Agar culture plates and is therefore a culture-dependent methodology for assessing the coral-associated microbiome without having to purify and singularize colonies (Hardoim et al. 2014).

The DNA-extraction method and plate washing follows the protocol from Hardoim and colleges 2014 and will be reviewed shortly. The coral tissue was ground with a sterile mortar and pestle in seawater. The suspension was well mixed to reach a coral-derived homogenate, which was vortexed in a sterile polypropylene tube, centrifuged to decant

coral cells and debris. After removing the supernatant into new polypropylene tubes, it was centrifuged a second time, resulting in the final microbial pellet, which was used for DNA extraction.

For the plate washing method, aliquots of homogenates prepared as above, were serially diluted and plated onto Marine Agar. After four weeks of incubation at 18ºC, all grown colonies on a given plate were re-suspended in sterile artificial seawater and transferred into a sterile polypropylene tube. The suspension was thoroughly mixed and centrifuged, the supernatant was discarded and DNA extraction was performed. This cell pellet was then used as starting material resulting in three in situ plate wash samples (Pl.W.01, Pl.W.02, Pl.W.03).

Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq apparatus following the manufacturer's guidelines, enabling high-throughput microbial ecology at the greatest coverage yet possible (Caporaso et al. 2012).

Because the 16S rRNA gene is universally present across bacteria, highly conserved and can be easily amplified using universal primers, microbial analyses are often performed using 16S rRNA amplicon sequencing. The starting point of the typical pipeline for 16S amplicon analysis, the use of primers designed to amplify the hypervariable regions of the 16S rRNA gene. The derived sequences are then clustered to "Operational Taxonomic Units" (OTU) based on similarity. The similarity between a pair of sequences is computed as the percentage of sites that agree in a pairwise sequence alignment, where a common similarity threshold used is 97%. From each OTU, a single sequence is selected as a representative. This representative sequence is annotated and that annotation is applied to all remaining sequences within that OTU. Since 16S amplicon analysis might have millions of reads, the clustering into OTUs is beneficial since it may result in only thousands of OTUs, making downstream analysis become more tractable (Nguyen et al. 2016).

In this study, the 16S rRNA gene V4 variable region PCR primers 515/806 (Apprill et al. 2015) were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions:

- 94°C for 3 minutes
- Followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed.

After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were then purified using calibrated Ampure XP beads. Then the pooled and purified PCR products were used to prepare a DNA library following Illumina TruSeq DNA library preparation protocol.

Libraries were created out of each of the individual amplicons. MR DNA does not use long concatemer primers as part of illumina data to keep amplification bias to a minimum. Additionally, MrDNA started processing the received sequence data, which will be summarized next.

After a q25 trimming of the ends, the reads were joined together. The inserted barcodes for identification were localized in the joined reads. The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline. Sequences were depleted of barcodes and primers; then short sequences <200bp were removed, sequences with ambiguous base calls were removed and sequences with homopolymer runs exceeding 6 bp were also removed. The sequences were than denoised and Operational Taxonomic Units were defined clustering at 3% divergence (97% similarity) followed by the removal of singleton sequences and chimeras (Capone et al. 2011).The final OTUs were taxonomically classified using BLASTn against a curated database derived from Green Genes, RDPII and NCBI (www.ncbi.nlm.nih.gov, Desantis et al.,

2006, http://rdp.cme.msu.edu) and compiled into each taxonomic level into both "counts" and "percentage".

Finally, 1911 OTUs were created from MR DNA and from this point on my part of the analysis started. The 1911 OTUs were used by MR DNA to classify the bacterial diversity blasting it against the Greengene database. We decided to re-do the classification using a more up-to date database, SILVA (https://www.arb-silva.de/aligner/). This database is a comprehensive web resource for up to date, quality-controlled databases of aligned ribosomal RNA gene sequences from bacteria, archaea and eukaryotes. We used SINA to compute the alignment. SINA is a reference-based alignment tool, designed to maintain high alignment accuracy while allowing for volume sequence processing (Quast et al. 2012). The file containing the representative sequence of each of the 1911 OTUs was split in two separate files, uploaded and aligned. The new classification replaced the classification done by MR DNA. Sequences corresponding to chloroplasts, mitochondria, eukaryotes, unclassified organisms and singletons were removed (Table 2).

After cleaning procedure, the dataset shrinked from 1,911 OTUs and 362,384 reads down to 1,092 OTUs and 256,715 reads.

Table 2- Number of operational taxonomic units (OTUs) and reads removed during the cleaning process.

Data	**Nº Reads**	**Nº Otus**
Raw Data	362.384	1911
Unclassified	55.089	516
Eukaryotes	6999	82
Chloroplasts	43.324	195
Mitochondria	237	6
Singletons	20	20
Cleaned-Data	256.715	1092

Alpha diversity

Biodiversity aims to quantify the ecological status of different biotopes by known abundances of species. The term biological diversity dates back to the early 1980's and was referred to as the number of species present (Lovejoy 1980).

In this study, rarefaction curves were created using the package vegan within R with the function "rarecurve", to compute rarefied species richness. The function draws a rarefaction curve for each row. Within rarefaction curves each sample category is randomly subsampled. The species accumulation point after each new sampling effort is saved and finally a smooth curve is drawn through the points. The system is perfectly sampled if the curve reaches an asymptote and no more new species are added.

So-called biodiversity indices are based on species differences that are insensitive to abundance conditions (Izsak & Papp 2000). To describe alpha diversity in the samples, four parameters were used: the observed richness, Chao1, abundance-based coverage estimator (ACE) and the Shannon Wiener index.
The observed species richness is the total of species observed in a sample or a set of samples.

As a popular diversity index, the Shannon-Wiener Index intuitively depends on the number of species occurring and the evenness of the distribution of individuals among the species, and follows the formula

Equation 1- Shannon Wiener Index

$$-\sum_{i=1}^{s} p_i \, log \, p_i$$

(Hughes et al. 2001)

Where *s* stands for the number of species and p_i *(i = 1,…, s)* denotes the probability of an individual belonging to the ith species (Izsak & Papp 2000).

The Chao1 (Chao 1984) estimator calculates the estimated true species diversity of a sample following the formula

Equation 2- Chao1

$$S_1 = S_{obs} + \frac{F_1^2}{2F_2}$$

(Hughes et al. 2001)

Where S_{obs} is the number of species in the sample, F_1 is the number of singletons and F_2 is the number of doubletons. The estimator assumes that if rare species are still found, it might be likely, that not all species have been discovered.

Compared to Chao1, the ACE estimator (Chao & Leen 1992) included data from all species with fewer than 10 individuals, when Chao1 just uses singletons and doubletons found within the dataset. The ACE-Estimator follows the formula

Equation 3- ACE

$$S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{F_1}{C_{ACE}} \gamma_{ACE}^2$$

(Hughes et al. 2001)

S_{rare} represents the number of rare samples (abundances equal or lower than 10) and S_{abund} is the number of abundant species (abundances higher than 10).

Equation 4- ACE – C_{ACE}

$$C_{ACE} = 1 - \frac{F_1}{N_{rare}}$$

(Hughes et al. 2001)

C_{ACE} estimates the sample coverage, F_1 being the number of species with i individuals and

Equation 5- ACE – N_{rare}

$$N_{rare} = \sum_{i=1}^{10} iF_i$$

(Hughes et al. 2001)

γ^2_{ACE} is estimating the coefficient of variation of the $F_{i's}$ with the formula

Equation 6- ACE -γ^2_{ACE}

$$\gamma^2_{ACE} = \max\left[\frac{S_{rare}\ \sum_{i=1}^{10} i(i-1)F_i}{C_{ACE}(N_{rare})(N_{rare}-1)} - 1\right]$$

(Hughes et al. 2001)

To even out the differences between the amount of reads in our samples, we decided to rarefy our dataset. The dataset with the lowest number of reads found between the sample categories was within the *E. labiata* in situ sample number one with 10,162 reads. The highest number of reads were found in the seawater sample number two (42,768). Using the function rarefy_even_depth in R, the program resamples an OTU table such that all samples have the same library size, which in our case referred to the smallest library size containing 10,162 reads. Rarefying is used to normalize microbiome counts that have resulted from libraries of widely-differing sizes and was first proposed by Howard L. Sanders, who recognized the problem that sample size increases and individuals are added at a constant arithmetic rate but species accumulate at a decreasing logarithmic rate when comparing samples of different sizes (Sanders 1968).

Taxonomic stacked bar charts were completed using R in addition with the R-packages GGPlot2, dplyr and Phyloseq. For the stacked bar chart, we continued using the rarefied dataset. For a clear illustration, OTUs lower in their abundance than 2% were removed and placed into one stacked bar. The taxonomic composition was assessed for four taxonomic levels namely Class, Order, Family and Genus level.

Statistical Analysis

To investigate if there are any significant differences between the biodiversity indices obtained across the sample categories, four One-way ANOVA's (Analysis of Variance)

were performed and assumptions of normality and homogeneity of variances have been tested.

Normality over each independent variable (sample categories) in respect to the dependent variables (biodiversity indices) was tested performing the Shapiro and Wilk's test for normality (Shapiro & Wilk 1965). The test uses a W-statistic and refers to the ratio of two estimates of the variance of a normal distribution based on a random sample of n observations. The numerator of W is proportional to the square of the best (minimum variance, unbiased) linear estimator of the standard deviation and the denominator is the sum of squares of the observations about the sample mean. W has a simple interpretation as an approximate measure of the straightness of the normal quantile-quantile probability plot (Royston 2013). The null hypothesis assumes that the values are normally distributed. Assuming a significance level of $\alpha = 0.05$, a p-Value below this threshold will lead to a rejection of the null hypothesis.

The Levene's test was used to check the assumption that the variances in the populations are equal. To verify whether the populations have the same shapes, means, variances and that the dependent variable is indeed equal across all groups (seawater, *E. labiata* in situ samples, plate wash samples) the null hypothesis was tested in respect to a significance level of $\alpha = 0.05$.

Null-Hypothesis- Levene's Test:

Observed richness: $H_0: \sigma^2_{Observed.Seawater} = \sigma^2_{Observed.E.labiata.in.Situ} = \sigma^2_{Observed.Plate.Wash}$

Chao1: $H_0\ \sigma^2_{Chao1.Seawater} = \sigma^2_{Chao1.E.labiata.in.Situ} = \sigma^2_{Chao1.Plate.Wash}$

ACE: $H_0: \sigma^2_{ACE.Seawater} = \sigma^2_{ACE.E.labiata.in.Situ} = \sigma^2_{ACE.Plate.Wash}$

Shannon-Wiener: $H_0: \sigma^2_{Shannon.Seawater} = \sigma^2_{Shannon.E.labiata.in.Situ} = \sigma^2_{Shannon.Plate.Wash}$

For the ANOVA the r-function "aov" of the package "car" was used, to test the null hypothesis which assumes that there are no significant differences between the means.

Null-Hypothesis-One-way-ANOVA:

Observed richness: $H_0: \mu_{Observed.Seawater} = \mu_{Observed.E.labiata.in.Situ} = \mu_{Observed.Plate.Wash}$

Chao1: $H_0: \mu_{Chao1.Seawater} = \mu_{Chao1.E.labiata.in.Situ} = \mu_{Chao1.Plate.Wash}$

ACE: $H_0: \mu_{ACE.Seawater} = \mu_{ACE.E.labiata.in.Situ} = \mu_{ACE.Plate.Wash}$

Shannon-Wiener: $H_0: \mu_{Shannon.Seawater} = \mu_{Shannon.E.labiata.in.Situ} = \mu_{Shannon.Plate.Wash}$

The null hypothesis will be rejected, when the p-Value is lower than the significance level $\alpha = 0.05$.

Because ANOVA only gives information if there is a difference between one or more pairs of groups, a Post Hoc Test was carried out to distinguish which sample categories differ from each other in respect to the biodiversity indices.

Beta diversity

Venn diagrams were created with the full dataset using the Venni 2.1 online tool (http://bioinfogp.cnb.csic.es/tools/venny/). The sum of all OTUs in each sample category was built and used to create the diagram. The diagram illustrates all shared and unique OTUs between the sample categories.

To investigate further differences between the sample categories a Principal Coordinates Analysis (PCoA) was performed (Gower 2015). Unconstrained ordinations are generally extremely useful for visualizing broad patterns across the entire data cloud, and allow the visualization of potential patterns of difference in the location or relative dispersion among groups (Zealand & Zealand 2003). The principal coordinate analysis was done with the R-packages “phyloseq” and “vegan”. We used the Hellinger transformation (Legendre & Gallagher 2001) to even out high differences between values of abundance. The transformation is well suited to species abundance data with low counts and many zeros. It is dividing each value in a data matrix by its row/column sums and taking the square root of the quotient. It is defined as

Equation 7- Hellinger Transformation

$$y'_{ij} = \sqrt{\frac{y_{ij}}{y_i}}$$

(Legendre & Gallagher 2001)

To see which OTUs play important roles in the PCoA, we included the 20 most abundant OTUs in the graph. Because our taxonomic classification was performed to the genus level, points belonging to the same genus were joined within the graph and therefore less than 20 OTUs are visible.

To test if there is a significant difference between the samples and the different OTUs found within each sample category a permutation test was done. Within the R-package vegan the function “adonis” was used. Adonis is a function for the analysis and partitioning sums of squares using dissimilarities and is directly based on the algorithm from Anderson (2001).

Finally, we performed a cluster analysis. This analysis is a statistical method, which aims to classify several objects into groups (clusters) according to similarities between them (Suzuki & Shimodaira 2006). To create a hierarchical clustering the R-package “pvclust” was used. P-values for the hierarchical clustering were calculated with the function “pvclust”. The function calculates p-values for hierarchical clustering via multiscale bootstrap re-sampling (1000 bootstrap replications were used). Two types of p-values are calculated: The approximately unbiased (AU) p-value and the bootstrap probability (BP). To calculate the AU-p-value, which has superiority in bias over BP value calculated by the ordinary bootstrap re-sampling, the multiscale bootstrap resampling is used (Shimodaira 2002).

Results

Alpha Diversity

Figure seven shows the rarefaction curve drawn for each sample category. The vertical line marks the smallest library with 10,162 reads. The rarefied species richness can be found at the crossing point of each curve with the vertical line. The plate wash samples had the slowest slope, since not many species were added with each sample effort at an early stage. A higher slope was found within our *E. labiata* in situ samples indicating a less complete sampling effort compared to the plate washes. The *E. labiata* in situ sample

number one had the highest species richness within the accumulation curve. Together with the discrepancy of *E. labiata* sample number one having higher number of species than number two and three, this might indicate that the number of species still will increase significantly when increasing the sampling effort. Highest rarefied species richness is found for seawater samples reaching species richness values between 500 and 650 after randomly subsampling the categories 10,162 times. Whereas seawater samples one to three were similar in terms of alpha diversity, sample four separated itself with higher species richness. However, it seems that the seawater samples did not differ a lot in their final plateau, resulting in the assumption that the sampling effort might already represent the bacterial community very well with only a few further species (OTUs) required to be sampled for complete coverage.

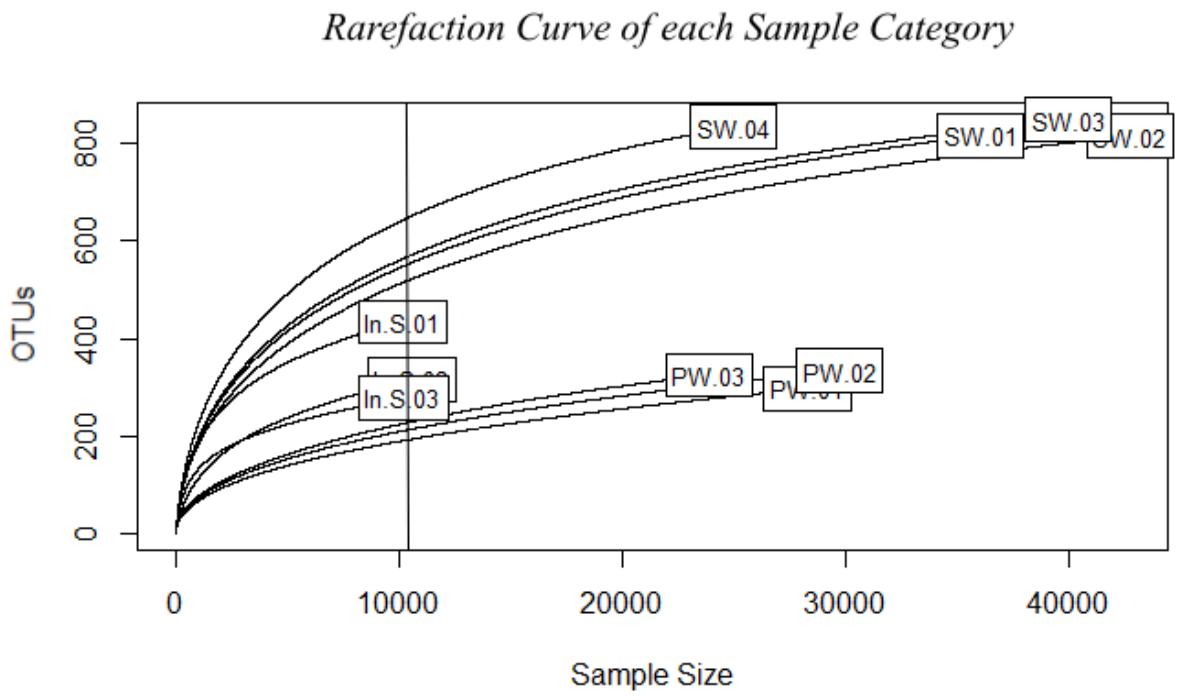

Figure 7- Rarefaction curve of each sample category. The vertical line marks the smallest library size of 10.162 reads found in the E. labiata in situ sample number two.

The ACE values were highest in the seawater and lowest in the plate wash sample categories (Fig.8). Within the *E. labiata* in situ sample category the values were slightly higher compared to the plate wash category. Abundance-based coverage values decreased more than 50% when comparing the seawater categories with the two latter ones. Because of the removal of singletons, differences between the estimators Chao1 and ACE might have gone as the estimations of both equations are very similar. Observed richness was again highest in the seawater sample and decreased approximately 47% in the *E. labiata* in situ category. The lowest richness mean value again was found within the plate wash category. The Shannon Wiener Index continued with the trend of seawater category representing the highest value (4,2), followed by the *E. labiata* in situ category (3,4) and finishes with the plate wash category (2,7) with a mean value less than three.

After retrieving significant p-values discriminating between sample categories for every biodiversity index, table three shows the significant groupings, obtained from the Tukey Honest Post Hoc test. Significant differences regarding the observed richness were found between the seawater and the *E. labiata* in situ sample category and the seawater and plate wash category. There was no significant difference between the plate wash and the *E. labiata* in situ sample. The same result was found for the comparisons of the Chao1 and ACE biodiversity indices across sample categories. Referring to the Shannon Wiener Index, the only statistical significant result was found between the seawater and the plate wash sample categories. The alpha diversity measured with the Shannon Wiener Index was not significantly different between the coral itself and its surrounding seawater.

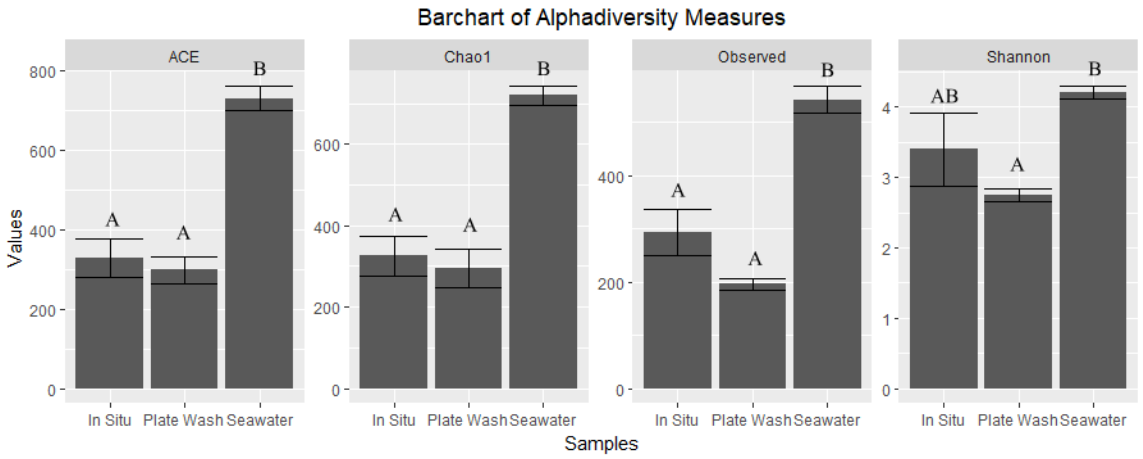

Figure 8- Alpha diversity indices used. The bar charts represent the mean of each sample category of each diversity parameter. The whiskers represent the standard errors and different letters indicate significant differences.

Table 3- The p-values of each grouping received from the Tukey Honest Post Hoc Test with a 95% confidence interval. Statistical significant p-values are highlighted in red.

Statistical significance tests for alpha diversity measures obtained from each sample category

Tukey multiple comparisons of means - Observed Richness
95% family-wise confidence level

Groupings	p-Value
Plate Wash - E.labiata.in.Situ	0.1365083
Seawater - E.labiata.in.Situ	0.0012285
Seawater - Plate Wash	0.0001607

Tukey multiple comparisons of means - ACE
95% family-wise confidence level

Groupings	p-Value
Plate Wash - E.labiata.in.Situ	0.8489365
Seawater - E.labiata.in.Situ	0.0002844
Seawater - Plate Wash	0.0001783

Tukey multiple comparisons of means - Chao1
95% family-wise confidence level

Plate Wash - E.labiata.in.Situ	0.8682799
Seawater - E.labiata.in.Situ	0.0003858
Seawater - Plate Wash	0.0002464

Tukey multiple comparisons of means - Shannon Wiener
95% family-wise confidence level

Plate Wash - E.labiata.in.Situ	0.3085377
Seawater - E.labiata.in.Situ	0.1559569
Seawater - Plate Wash	0.0157201

Bacteria dominated the 16S rRNA gene dataset whereas archaea never exceeded 4% of the total reads within each sample category. Eighteen different OTUs were found within the archaeal domain *Euryarchaeota* and two distinct OTUs within the *Thaumarchaeota* (Table 5). From the twenty OTUs assigned as *Archaea* only one was taxonomically distinct, *Halococcus* belonging to *Euryarchaeota*. All other OTUs in this phylum were assigned as unclassified *Marine Group II*.

The highest diversity within the archaea was found in the seawater samples and decreased in the *E. labiata* in situ samples, being lowest in the plate wash samples.

In the seawater sample category (total sum of all 4 seawater samples), highest counts of reads in the bacterial domain were found within the *Proteobacteria*, with 71,833 reads, followed by *Actinobacteria* (37,336 reads) and *Bacteroidetes* (23,415 reads) (Table 4). The 37,336 reads assigned to the phylum *Actinobacteria* corresponded to 60 different OTUs whereas the 23,415 reads assigned to the phylum Bacteroidetes corresponded to 197 different OTUs. Highest number of OTUs were detected in Proteobacteria (633). Joining the three samples of the *E. labiata* in situ samples, highest number of reads within the bacterial domain was still represented by the *Proteobacteria* (26.339 reads), followed by *Actinobacteria* (2.064 reads) and *Bacteroidetes* (979 reads).

Considering the sum of the plate wash samples a slight difference occurred. While *Proteobacteria* dominated the culturable community from *E. labiata* (81.123 reads), the phylum *Firmicutes* (337 reads) was the second most abundant, followed by *Actinobacteria* (273 reads) and *Bacteroidetes* (139 reads).

Table 4- Total bacterial OTUs and reads found within each sample category at Phylum level.

	Seawater		E.L. In Situ		E.L. Plate Wash	
Phyla	**Otus**	**Sequences**	**Otus**	**Sequences**	**Otus**	**Sequences**
Acidobacteria	6	15	7	66	1	1
Actinobacteria	60	37336	30	2064	29	273
Bacteroidetes	197	23415	71	979	44	139
Chlamydiae	0	0	1	8	0	0
Chlorobi	1	9	1	1	1	1
Chloroflexi	1	5	4	23	0	0
Cyanobacteria	9	1120	7	129	2	8
Euryarchaeota	18	4590	6	142	3	8
Fibrobacteres	2	6	0	0	0	0
Firmicutes	5	108	8	352	6	337
Fusobacteria	2	14	1	1	0	0
Gemmatimonadetes	7	31	2	8	3	3
Lentisphaerae	2	21	0	0	1	1
Marinimicrobia (SAR406 clade)	18	3850	11	273	7	28
Nitrospirae	0	0	1	13	0	0
Parcubacteria	3	10	2	18	0	0
Planctomycetes	24	886	11	146	6	10
Proteobacteria	633	71833	427	26339	403	81123
SBR1093	1	25	0	0	1	1
Thaumarchaeota	2	255	1	30	1	1
TM6 (Dependentiae)	1	3	0	0	0	0
Unclassified (Bacteria)	1	18	0	0	1	1
Verrucomicrobia	23	307	12	325	6	6
Total	1016	143.857	603	30.917	515	81.941

Table 5- Taxonomic and abundance overview of all archaeal OTUs found in all sample categories. Number within sample category columns represent the sum of the number of reads joined for each sample category.

OTU	Domain	Phyla	Class	Order	Family	Genus	Seawater	E.L.In.S.	Pl.W.In.S
OTU_1267	Archaea	Euryarchaeota	Halobacteria	Halobacteriales	Halobacteriaceae	Halococcus	5	0	0
OTU_904	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	7	0	0
OTU_757	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	9	0	0
OTU_916	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	10	0	0
OTU_1327	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	11	0	0
OTU_808	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	12	0	0
OTU_973	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	12	0	0
OTU_758	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	14	0	0
OTU_1875	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	14	0	0
OTU_987	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	14	2	0
OTU_375	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	29	0	0
OTU_1137	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	61	0	0
OTU_1394	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	122	0	0
OTU_927	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	147	12	0
OTU_95	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	184	35	0
OTU_69	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	405	31	1
OTU_300	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	663	5	1
OTU_12	Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine Group II	Uncl. Marine Group II	2871	57	6
OTU_407	Archaea	Thaumarchaeota	Marine Group I	Uncl. Marine Group I	Uncl. Marine Group I	Candidatus Nitrosopelagicus	55	0	1
OTU_102	Archaea	Thaumarchaeota	Marine Group I	Uncl. Marine Group I	Uncl. Marine Group I	Candidatus Nitrosopumilus	200	30	0
						Total	4845	172	9

Figure nine shows the relative abundance (rel. abundance) distribution of bacterial classes across all samples, where OTUs with a rel. abundance lower than 2% were filtered and joined within a single stack, represented by an olive color. Whereas the relative taxonomic distribution remained similar over all four seawater samples, there was higher variability within each of the other two sampling environments (*E. labiata* in situ and *E. labiata* plate wash) especially in the within-sample variability in the in-situ sample. *Alphaproteobacteria*, *Acidimicrobia* and *Gammaproteobacteria* were the classes with the highest relative abundances within the four seawater samples. Moving on to the *E. labiata* in situ samples, sample "In.S.02" showed a clear difference regarding the abundance of *Gammaproteobacteria*, which dominated the sample. In all three *E. labiata* in situ samples the relative abundances of the *Flavobacteria* and of the *Gammaproteobacteria* decreased and increased, respectively, revealing one obvious difference in the taxonomic compositions of the seawater and *E. labiata* in situ samples. The first plate wash sample (PW.01) was dominated by *Alphaproteobacteria*. As seen for the *E. labiata* in situ samples, there was considerable degree of variability in taxonomic composition between replicates of the plate wash sample category, as in the second

(PW.02) and the third sample (PW.03). Reads assigned to *Gammaproteobacteria* were well represented and sharing dominance with *Alphaproteobacteria* reads.

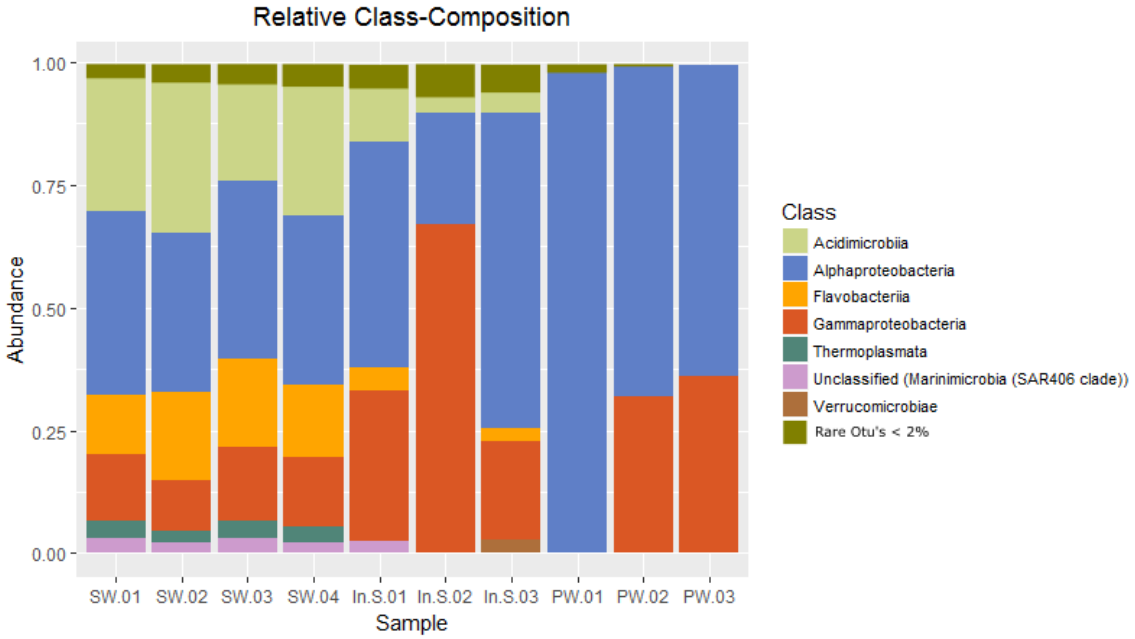

Figure 9- Relative Taxonomic composition (Taxonoic level: Class) of each sample. OTUs less than 2% in their abundance were removed and placed within the olive colored stacked bar.

Moving down the taxonomic hierarchy from the class to the order level, the overall similarity between each seawater sample remained high and constant (Fig. 10). No large differences were seen between each of the seawater samples. The highest relative abundance was found for the *Acidimicrobiales*, followed by *SAR11* clade, *Flavobacteria*, *Rhodobacteriales* and *Oceanospirillales*. Lowest rel. abundances were shared between the *Cellvibrionales*, *Thermoplasmatales*, *Sphingomonadales* and *Rickettsiales*. The in-situ coral samples showed increased abundances of the order of *Rhodobacterales*, *Oceanospirillales*, *Sphingomonadales* and *Alteromonadales* when compared with seawater. However, variability between replicates could also be depicted. For instance, while the order *Rhodobacterales* was dominant in one sample, *Oceanospirillales* was the prevalent order in the remaining samples. Additionally, in situ sample one "In.S.01" showed a higher abundance of *Vibrionales* when compared to "In.S.02" and "In.S.03". *Flavobacteriales*, *Cellvibrionales* and *SAR11* clade showed decreased abundances in the coral in situ samples compared to the seawater samples. In all three plate wash samples

Rhodobacterales represented the dominant order, followed by *Sphingomonadales* in sample one. In plate wash sample number two, the second most abundant OTUs found corresponded to *Vibrionales* followed by *Sphingomonadales* in sample one. In contrast, *Sphingomonadales* represented the second most abundant order before the *Vibrionales* in our third sample. Furthermore, the third sample harbored a higher number of *Alteromonadales* than plate wash samples number one and two.

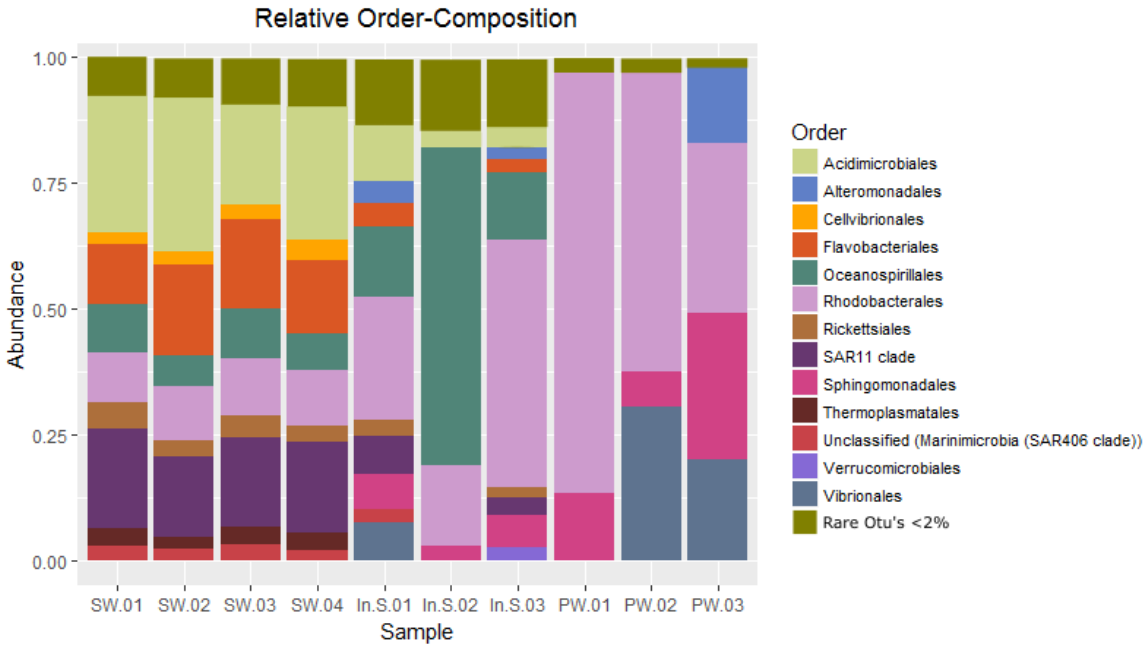

Figure 10- Relative Taxonomic composition (Taxonoic level: Order) of each sample. OTUs less than 2% in their abundance were removed and placed within the olive colored stacked bar.

At the family level, the similarity between each seawater sample decreased in comparison with those observed for higher taxonomic ranks (Fig. 11). While the *OM1* clade dominated all four samples, sample two, three and four differed from the first sample considering the relative abundance of *Hahellaceae*, which was lower in the seawater sample number one. After the *OM1* clade, *Surface1* is second, followed by *Rhodobacteraceae*, *SAR86* clade, *SAR116* clade and *Flavobacteriaceae*. The lowest relative abundances within the seawater samples before reaching the 2% threshold of filtered OTUs were shared between the *Cryomorphaceae*, *Marine Group II*, *NS9 Marine Group*, and two unclassified OTUs belonging to the *Oceanospirillales* and

Marinimicrobia. Within the *E. labiata* samples the rel. abundance of *Rhodobacteraceae*, *Hahellaceae* and *Marine Group II* increased in comparison with seawater, whereas the families *Flavobacteriaceae*, *NS9 Marine Group*, *OM1* clade, *SAR86* clade, *SAR116* clade and *Surface1* decreased in abundance. Coral sample number two was dominated by *Hahellaceae* and differed in family composition from the in-situ *E. labiata* samples one and three. In the first coral sample, there was a higher relative abundance of *Vibrionaceae* compared to the *E. labiata* samples number two and three. The second coral sample also differed in the relative abundance of the *Surface1* family which was higher in number one and three. Finally, *E. labiata* in situ sample number three differed from the coral samples with a higher rel. abundance in *Rubritaleacaea*. In all three plate wash samples *Rhodobacteraceae* were dominant (Fig. 11). The second most abundant family in the plate wash sample number one was *Sphingomonadaceae*, while plate wash samples number two and three both had higher relative abundances of *Vibrionaceae* than *Sphingomonadaceae* (Fig. 11). A further difference between this sampling environment was the high relative abundance of *Shewanellaceae* found within the third plate wash sample. Comparing all three sample categories (seawater, *E. labiata* in situ, plate wash in situ) the highest relative abundance of "filtered OTUs" (OTUs with rel. abundance lower than 2%) was found in the coral samples, decreasing in the seawater samples and being lowest in the plate wash samples.

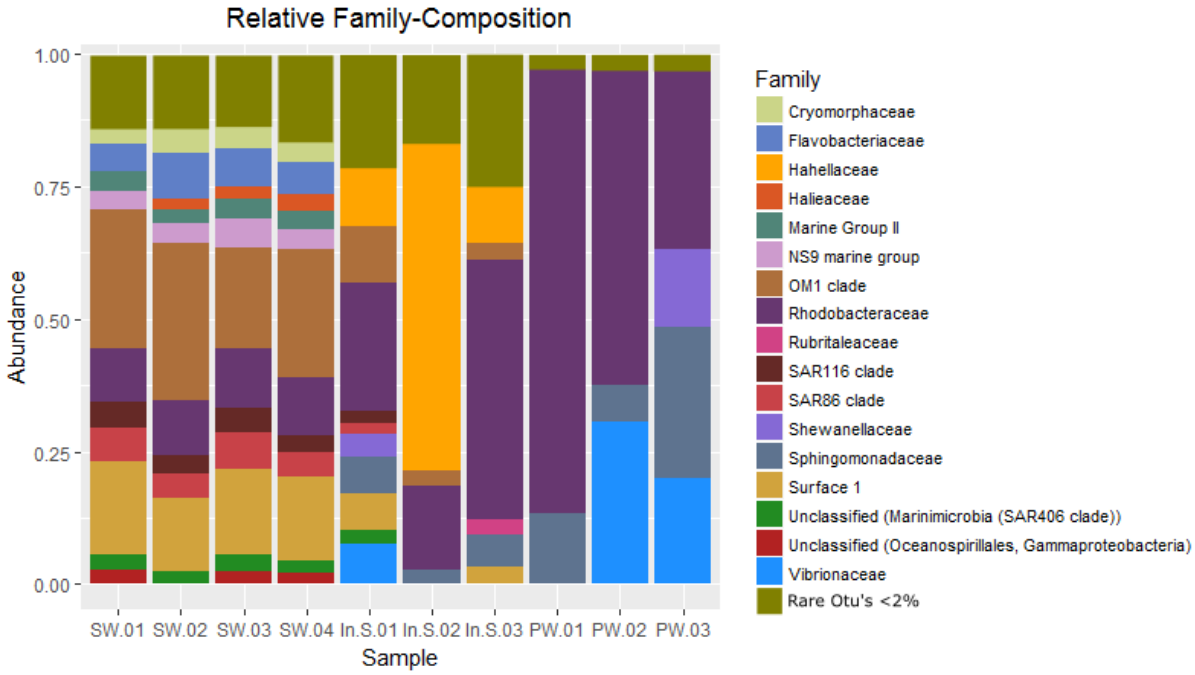

Figure 11- Relative Taxonomic composition (Taxonoic level: Family) of each sample. OTUs less than 2% in their abundance were removed and placed within the olive colored stacked bar.

Taxonomic composition at the genus level revealed large difference between every sample category and also slight variations within each sample category (Fig. 12). The two dominant genera within the seawater samples were *Canidatus Actinomarina* and the unclassified *Surface1* (*Alphaproteobacteria*). Lower and more equally relative abundances were shared between unclassified *SAR86* (*Gammaproteobacteria*), *SAR116* (*Alphaproteobacteria*), unclassified *Rhodobacteracea*e, unclassified *NS7 Marine Group*, and unclassified *Marine Group 8* (*Archaea*). Seawater samples number two, three and four had higher relative abundances of *Fluviicola*, whereas number four, three and one had higher rel. abundances of unclassified *Oceanospirillales* compared to the seawater sample number two. Moving on to the *E.labiata* in situ samples the dominant genus in sample number one and three was *Ruegeria*, whereas sample number two was dominated by *Endozoicomonas*. Besides *Ruegeria* and *Endozoicomonas*, high relative abundances were also observed for the genus *Vibrio*, unclassified *Surface1* (Alphaproteobacteria) and *Sphingorhabdus*. *E. labiata* sample number three differed from other replicates in its higher rel. abundance of *Labrenzia* and *Rubritalea*. The rel. abundance of *Candidatus Actinomarina* decreased in corals in comparison with the seawater samples. Further

differences between sample categories were evident from the analysis of the plate wash samples. While *Ruegeria* dominated plate wash samples number one and two, *Sphingorhabdus* was nearly as abundant as *Ruegeria* in the plate wash sample number three. In sample one, the second most abundant "genus" was an unclassified *Rhodobacteraceae* taxon, followed by *Sphingorhabdus* and *Labrenzia*. The relative abundance of the genus *Vibrio* was highest in the plate wash sample number two, followed by *Sphingorhabdus* and *Shewanella*. The third plate wash sample showed a more even distribution. As mentioned, *Ruegeria* and *Sphingorhabdus* were the most dominant ones, followed by *Vibrio*, *Shewanella*, *Roseovarius* and unclassified *Rhodobacteriaceae*.

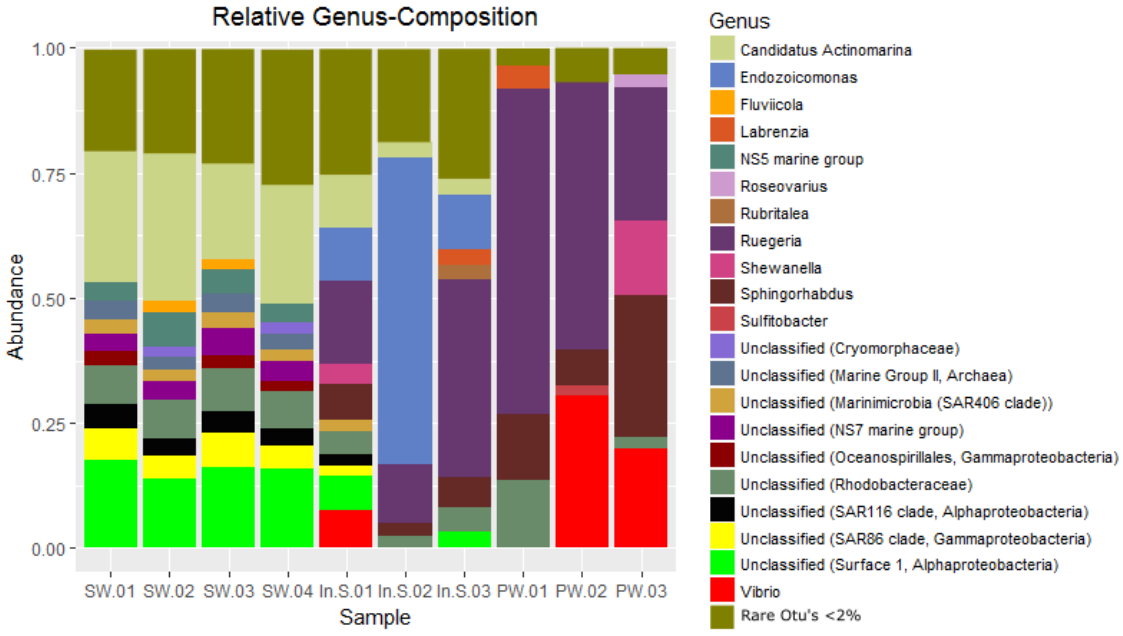

Figure 12- Relative Taxonomic composition (Taxonoic level: Genus) of each sample. OTUs less than 2% in their abundance were removed and placed within the olive colored stacked bar.

The two most dominant orders of the *E. labiata* coral microbiome (*Oceanospirillales* and *Rhodobacterales*) were subjected to more detailed investigation of their genus-level and approximate "species" (that is OUT)-level, taxonomic composition and abundance (total

number of reads) using heatmaps (Figs. 13 and 14). Concerning to the order *Oceanospirillales* (Fig. 13), after joining OTUs belonging to the same genus, six different genera could be identified and six more categories represented unclassified candidates. Differences in the abundances of the identified genera across sample categories were highest for the genus *Endozoicomonas*, and the unclassified groups (and putative candidate genera) *ZD0405*, candidates of *Oceanospirillales* (consisting of several genera) and *SAR86*. *Endozoicomonas* showed the highest abundances in the *E. labiata* in situ samples, being very dominant in sample "In.S.02". The genus displayed lowest abundance in the plate wash samples. Reads of the unclassified OTUs in the *SAR86* clade were most and least abundant in the seawater and the plate wash samples, respectively. Genera detected only in seawater were unclassified *OM182 clade*, *Litoricola* and *Neptuniibacter*. Abundant *Oceanospirillales* genera present in both between the *E.labiata* and the cultivated plate wash samples were *Kangialla*, *Endozoicomonas*, unclassified *ZD0405*, unclassified *Oceanospirillales* and unclassified *SAR86* clade.

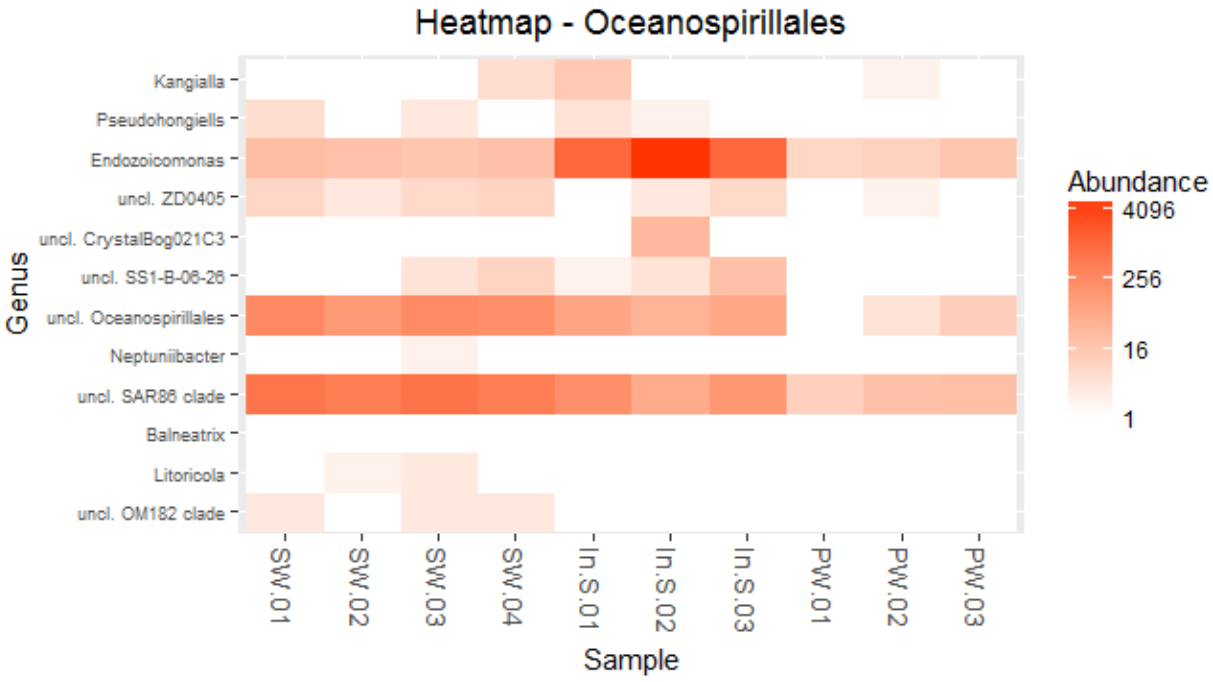

Figure 13- Heatmap of the rarefied dataset from every sample only including the order Oceanospirillales.

A closer look into the genus-level abundances and taxonomic composition of the order *Rhodobacterales* is shown in Fig. 14. Compared to *Oceanospirillales*, most of the OTUs could be assigned to a certain genus. Only one of the heatmap represents the category unclassified *Rhodobacteraceae*. *Ruegeria* showed high abundances in the *E. labiata* in situ, and the plate wash samples, displaying intermediate abundance in the seawater samples. *Roseovarius* displayed a similar profile with the highest abundance in total number of reads within the *E. labiata* in situ samples, a slight decrease in abundance in the plate wash samples and lowest abundance in the seawater samples. *Sedimentitalea* displayed low abundance in the seawater and was found to be enriched in two out of three *E. labiata* in situ samples. *Shimia* was present in very low abundances in all three sample categories and only reached noticeable number of reads in one seawater ("SW.04") and one *E. labiata* in situ sample ("In.S.03"). *Planktomarina* was detected in all ten samples, showing highest abundance in seawater and lower, but equivalent, abundances in the *E.labiata*-derived samples (in situ and plate wash). One genus showing increased abundance in the coral compared to seawater was *Labrenzia*. Its abundance was indeed lowest in seawater but increased in the gorgonian coral, showing similar numbers in all plate wash samples. A genus reaching higher abundances within our seawater and plate wash samples but a decrease in the *E. labiata* in situ samples was *Ascidiaceihabitans*. Whereas *Ascidiaceihabitans* was abundant in the seawater samples, it displayed low abundances in two *in situ* coral samples, whole it was consistently found in higher abundances in the plate wash samples. Unclassified *Rhodobacteraceae* OTUs were well presented in all samples.

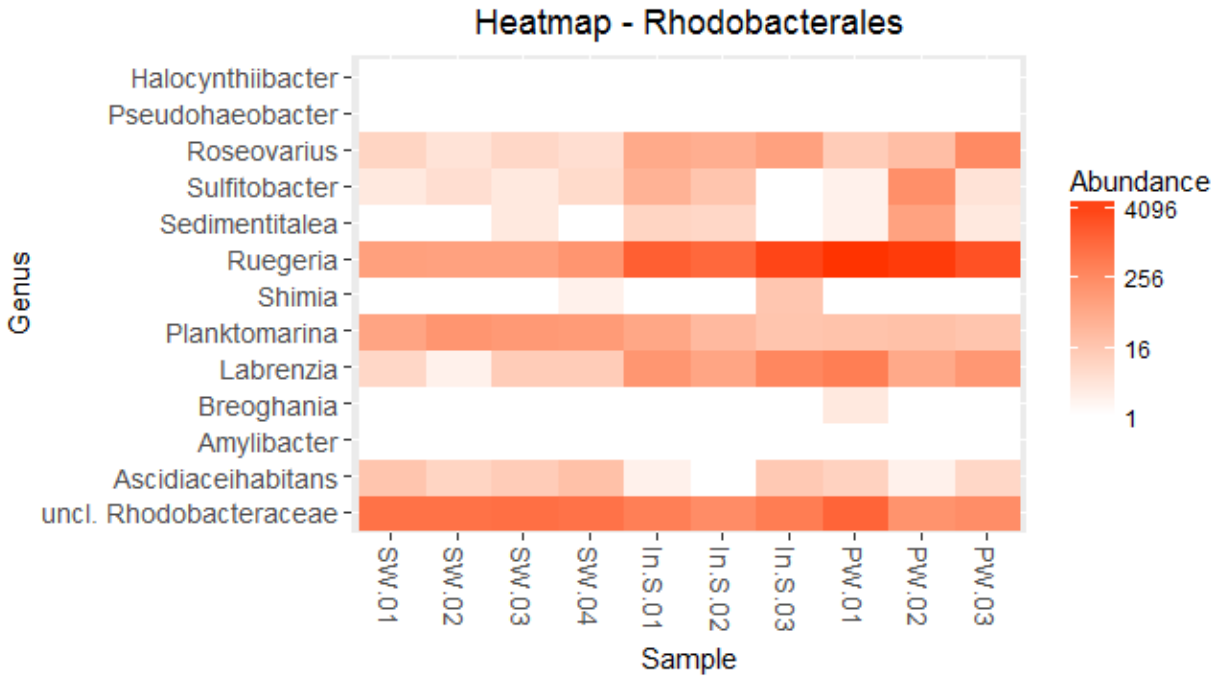

Figure 14- Heatmap of the rarefied dataset from every sample only including the order Rhodobacterales.

Beta Diversity

Figure 15 shows the uniqueness of each sample category through a Venn diagram. Four percent of the *E. labiata* OTUs were unique to this category being only found within the in situ coral samples. OTUs shared between the coral and the seawater represented 49% of the total OTU diversity uncovered in this study. From all the 1092 OTUs present in our dataset, 47.1% were rediscovered in our plate wash samples. Operational Taxonomic Units shared between the culture dependent and culture independent approach (*E. labiata in situ* vs. plate wash samples), accounted for remarkable 62% including genera such as *Ruegeria, Sphingorhabdus, Labrenzia*, other unclassified *Rhodobacteraceae.* The highest unique diversity in terms of OTUs was found for the seawater sample category, with 350 unique OTUs accounting for 32% of the total bacterial diversity observed across the data.

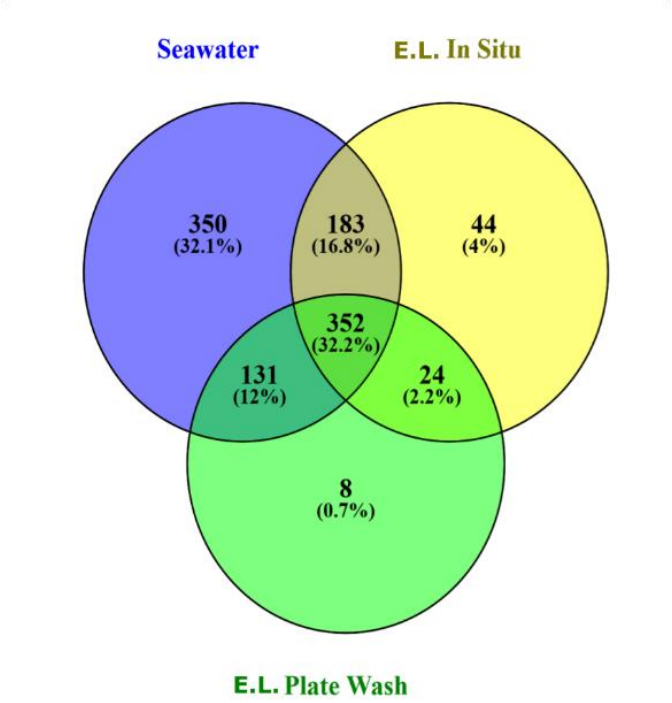

Figure 15- Venn-Diagram representing total number of OTUs found in each sample category using the non-rarefied dataset. Blue represents the seawater, yellow the E. labiata in-situ samples and green the plate wash in-situ samples.

Fig 17 shows a Principle Correspondence analysis (PCoA) using an Euclidean distance matrix obtained for all samples based on their OTU composition, and plots the 20 most abundant OTUs (representing 12 bacterial genera) in the dataset in the ordination diagram according to their shifts in abundance across the studied sample categories. Thus, the graph represents the approximate contribution of the displayed OTUs in differentiating the taxonomic profiles of the sample categories analyzed. For instance, the genus *Endozoicomonas* clearly depicts its tight association with (and higher abundance in) the in situ gorgonian samples, being placed far apart from the seawater samples in the ordination diagram. *Ruegeria* was positioned between the *E. labiata* in situ and the plate wash samples, reflecting its considerable recovery from both sources. *Shewanella*, *Spingorhabdus* and *Vibrio* were also clearly distant from the seawater samples, being much closer to the plate wash samples. Clustered all close together with the seawater samples were unclassified *Surface1*, unclassified *SAR86*, *Candidatus Actinomarina*, unclassified *Marinimicrobia*, unclassified *Rhodobacteraceae* and *Planktomarina OTUs*. Table 6 shows the result of the permutational Analysis of Variance run for the distance

matrix with 999 permutations, supporting statistical differences between the samples with a p-value of 0.001.

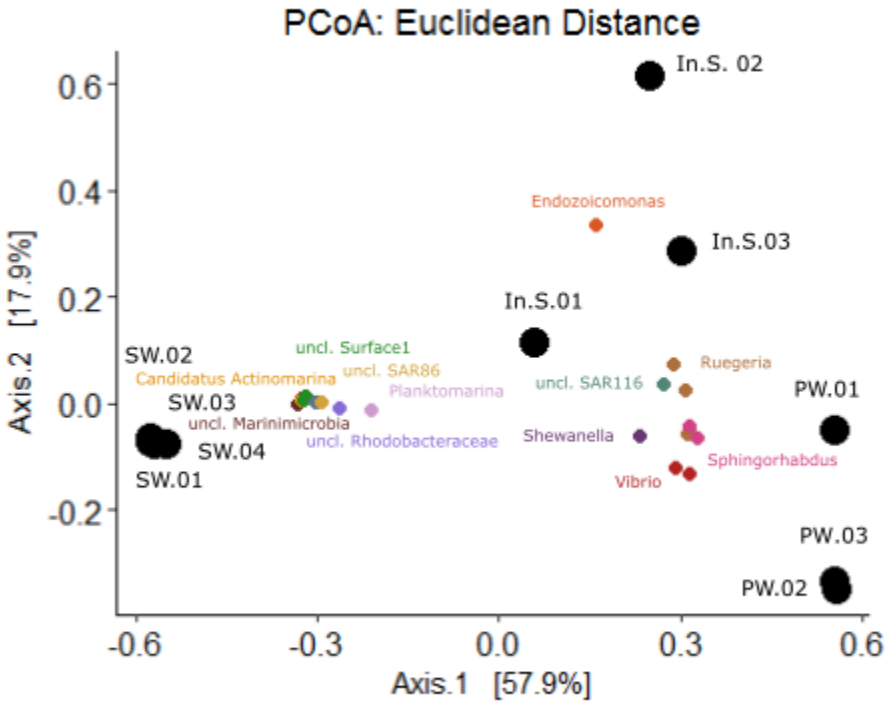

Figure 16 - Principal Coordinates Analysis (PCoA) using Hellinger transformed data on Euclidean distances calculated for each pair of samples based on their OTU community profiles. The 20 most abundant OTUs in the dataset are displayed in the ordination diagram reflecting the relative abundances across the three sample categories.

Table 6 - Results of the Permutation test, testing the three different sample categories.

Permutational Multivariate Analysis of Variance Using Distance Matrices

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Environment	3	3.15809672	1.052698907	6.832096019	0.773553	0.001
Residuals	6	0.924488389	0.154081398		0.226447	
Total	9	4.082585109			1	

Cluster analysis (Fig. 18) revealed that the seawater samples clearly separated from the *E. labiata* in situ and plate wash samples and the plate wash samples, with 100% bootstrap

support. Comparing the *E. labiata* in situ samples with the plate wash samples, bootstrap values decreased considerably not clearly supporting the separation of these sample categories from each other, revealing that the plate wash cultured community, which originated from the *E. labiata* in situ community, represents to some extent the original symbiotic consortium. The first branching indicates that the microbial community of "In.S.01" and "In.S.02" separates from the other plate wash samples and the in situ "In.S.03".

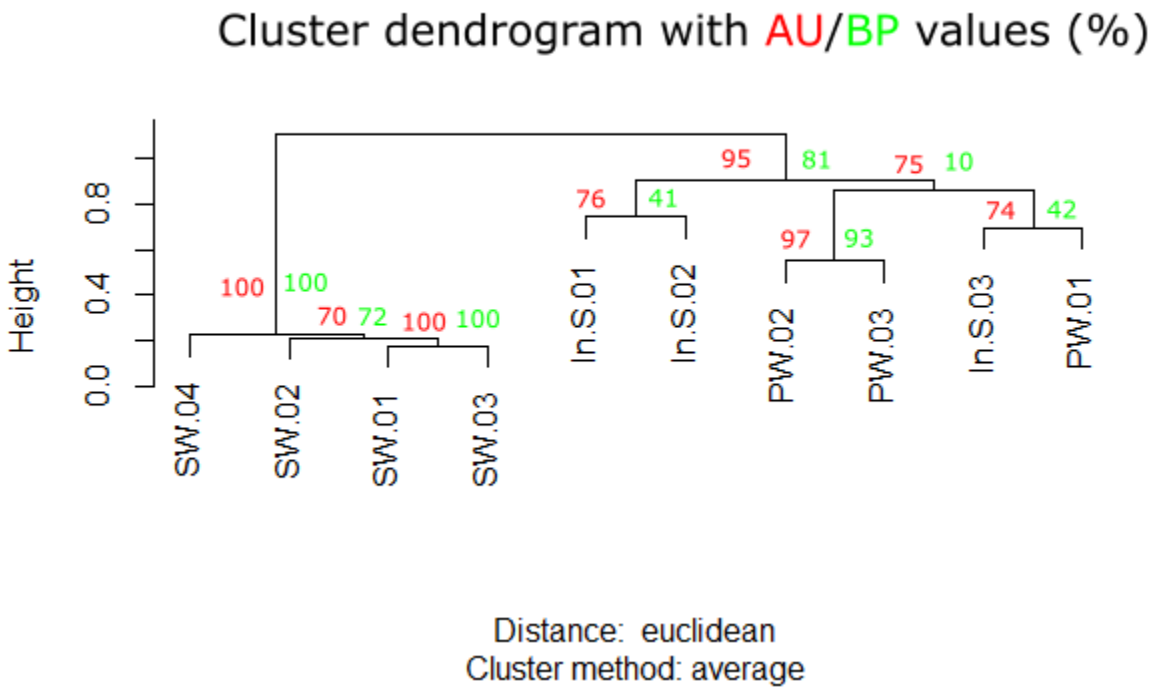

Figure 17- Hierarchical cluster analysis of each sample using the Hellinger transformed dataset. Red values represent the approximately unbiased values (AU) and green ones represent the bootstrap probabilities (BP).

Discussion

Alpha Diversity

The archaeal contribution to the total number of OTUs was about 1.8%, and most of the archaeal OTUs belonged to the unclassified *Marine Group II*. Highest diversity within the archaeal genera were found in the seawater. Studies on archaeal associations with

gorgonians are scarce. It was only recently that some studies shed light on the roles of archaea in association with sessile marine invertebrates. A study on the microbial assemblages of a cold-water coral mount in the North Atlantic, identified archaeal communities on dead coral skeleton and in freshly produced mucus of living corals. Skeleton and mucus contained a substantial amount of *Thaumarchaeota Marine Group 1* of which the majority was unclassified at the genus level. The genus *Nitrosopumilus* made up 3% and *Cenarchaeum* 0.4% of the total prokaryotic community in the skeleton and 0.1% in the mucus (Bleijswijk et al. 2015). Within the sponge *Axinella sp.* 65% of the symbiotic community associated with the sponge was represented by a single archaeal 16S rRNA gene sequence leading to conclusions of a true symbiotic association between archaeon and sponge (Riesenfeld et al. 2004). In the marine sponge *Sarcotragus spinosulus*, *Nitrosopumilus* (phylum *Thaumarchaeota*), known for its ability to oxidize ammonium into nitrite, was found to be the most dominant archaeal symbiont (Hardoim & Costa 2014). Archaea, such as *Nitrosopumilus* which were found within our coral samples (30 OTUs), have been found to contain ammonia oxidation genes used for nitrification of ammonia to nitrite, a hypothesized mechanism for removal of nitrogenous waste in the coral holobiont (Thurber et al. 2009). As proposed by Erwin et al. (2014), complex microbiota found within Octocorallia maintain dynamic microenvironments offering optimal conditions for different metabolic pathways such as chemical substrates (ammonia-rich host waste) and physical habitat for nitrification. Further investigations on archaeal relationships might reveal first symbiotic relationships between corals and archaea. Ammonia-oxidizing archaea have been consistently reported in associations with marine sponges in recent years (Steger et al. 2008; López-legentil et al. 2010). They are believed to contribute to host fitness by processing the highly toxic ammonium which is excreted by the animal host as metabolic waste, and similar processes may be mediated by coral-associated archaea. Symbionts of marine sponges have been in the nitrification step of the nitrogen cycle (conversion of ammonia to nitrite, and further from nitrite to nitrate) is well documented for marine sponges, and is likely that similar processes may be mediated by coral-associated archaea.

Several papers point out the importance of archaea in deeper water layers, where the

archaeal community shifts from an *Euryarchaeota*-dominated surface population to a *Crearchaeota*-dominated deep-water population (Acinas et al. 1997).

Within our dataset the seawater represented the highest diversity when referring to the Shannon-Wiener Index with an average value of 4.2. Although the observed and estimated richness decreased nearly 50% from the seawater to the *E. labiata* in samples, the Shannon-Wiener Index still corresponded to an average value of 3.4 for in situ *E. labiata,* still reflecting high bacterial diversity in these samples. Similar Shannon diversity values have been found in the gorgonian *E. cavolini* (Bayer et al. 2013). However, comparing the Shannon-Index between our samples and those retrieved from the gorgonian hosts *E. singularis* and *Leptogorgia sarmentosa* (Water et al. 2017), the *E. labiata* prokaryotic consortium depicted here showed higher biodiversity.
From our three *E. labiata* in situ samples number one displays the highest diverse bacterial community including a *Vibrio spp.* Studies investigating corals and their bacterial diversity indicate, that non-diseased samples tend to host less diverse bacterial communities (Cooney et al. 2002; Moura et al. 2009). Comparisons between the bacterial diversity within the same species might be able to detect early infection stages of corals, when colonized by a microbial pathogen. *Vibrio spp.* are Gram-negative, curved, rod-shaped bacteria belonging to the class *Gammaproteobacteria* and are regarded as the dominant culturable bacteria in the ocean (Vezzulli et al. 2012). The genus was not very well represented within our seawater samples, corroborating observations from another study where the bacterial diversity of the coral *Mussismilia braziliensis* was investigated (Moura et al. 2009). Members of the family *Vibrionaceae* are well known pathogenic agents infecting several marine organisms including gorgonians. Successful infections seem to be temperature-related since individuals from *Eunicella verrucosa* diseased at temperatures of about 20ºC whereas no infection has been observed in temperatures around 15ºC. *E. labiata* sample number one might not have been showing any morphological clues indicating a diseased state but might could still be on an early state of infection, since it is showing a higher rel. abundance of the genus *Vibrio* within its taxonomic profile. However, it is still a problem detecting origins, symptoms and reservoirs for marine diseases caused from bacteria. Sources of potentially infectious pathogens might be aquaculture, ballast waters, gut microbiota from several seabirds or

marine organisms etc. and due to the lack of barriers to dispersal within some parts of our oceans, the potential for long-term survival of pathogens outside the host is supported (Harvell et al. 2004). In terms of infections and global warming, rising temperatures might cause higher mortality rates in the future due to higher successful infection rates achieved by several bacterial pathogens such as *Vibrio* spp.. Sea et al. (2000) reported an extensive mass mortality of gorgonians related to a sudden increase in seawater temperature along the Ligurian coast in 1999.

It was shown that within the coral *Oculina patagonica* in the Mediterranean Sea, *Vibrio shiloi* seems to act as a causative agent of bleaching. Its unique survival strategy was described by Sussman et al. (2003). *V. shiloi* is not present in the coral during the winter. The fireworm *Hermodice carunculata* serves as a winter reservoir for *V. shiloi* and spring-summer vector for the coral-bleaching pathogen. After successful infection bacterial abundances increase, where Quorum Sensing (QS), a form of bacterial population density-dependent cell-cell communication, might play an important role. Several findings indicate that Quorum Sensing signals might be produced in free-living planktonic bacteria as well as in bacteria associated with marine organisms. During this process bacteria produce and excrete signals that accumulate to a threshold level within a diffusion limited environment as in corals and other organisms or environmentally available and more or less isolated areas. First QS signals from the marine environment were found in the symbiont *Vibrio fischeri* where the autoinducer synthesized by the luminous bacteria needs to reach a threshold to activate the synthesis of the bacterial luciferase. The bacteria are colonizing the light organ of the sepiolid squid *Euprymna scolopes*. High population densities of *V. fischeri* produce visible light in the light organ of the squid (Dobretsov et al. 2009; Dew et al. 1981). *Vibrio sp*. associated with the sponge *Cymbastela concentrica* produce Quorum sensing compounds, as well as isolated α- and γ-Proteobacteria from the sponges *Mycale laxissima* and *Ircinia strobilina*. Besides the coral sample number one, *Vibrio* spp. were well represented within plate wash samples number two and three (Pl.W.02, Pl.W.03). Previous studies indicate, that *Vibrio spp.* and their occurrence at a seasonal scale might be related to temperature since abundances dropped with temperatures below 18ºC increased when temperatures were equal or greater than 22ºC. Culturing at low temperatures as seen within this study shows

might prevent *Vibrio spp.* from dominating cultured samples (Vezzulli et al. 2010) but their enrichment on culture plates, in comparison with the in situ samples suggests, that these opportunistic species are already present at very low abundances in healthy coral tissue, and their proliferation in the coral-associated consortium might take place quickly once conditions favour. *Vibrio spp.* did not show high rel. abundances present within our seawater samples, which might be related to the low seawater temperatures of 14ºC in May, 2015.

The stacked bar chart in figure 12 displays the differences in the rel. abundance between the three sample categories. With the exception of *Sphingobacteria*, the prokaryotic community found within our seawater samples was similar to those reposted in other studies, where mainly *Alphaproteobacteria, Gammaproteobacteria, Sphingobacteria* and *Flavobacteria* constitute the heterotrophic bacterioplankton communities (Giebel et al. 2010), whereas in seawater *Acidimicrobiia* showed highest relative abundances. Within all the seawater samples, in terms of relative abundance of each genus, the single stacked bars showed a quite similar distribution of each genus. This indicates that the sampled seawater from 18 m depth is having a relatively constant relative bacterial composition. Acinas et al. (1997) also points out, that the homogeneity of picoplankton communities along a transect in different depth (above and below the deep-chlorophyll maximum), remains relatively constant and suggests that these assemblages were distributed fairly similar within the seawater. *Candidatus Actinomarina* is dominating within the seawater. Only a few groups of planktonic marine *Actinobacteria* have been described until today. Genomic analysis indicate that their photoheterotrophic lifestyle support a planktonic, free-living lifestyle (Mizuno et al. 2015). However, bacteria and phytoplankton dynamics are thought to be closely linked in coastal marine environments. It has been shown that the diversity, abundance and percentage of diatoms, for example, in the phytoplankton community account for a significant amount of the variability in the attached bacterial community composition (Giewat et al. 2005). Therefore, the microbial taxonomic diversity might change during the year in temperate marine ecosystems and our profile does not represent a fixed picture of diversity, when referred to the seawater surrounding corals or other marine sessile invertebrates. Future research shall shed light on the long-term fluctuations in the prokaryotic composition inhabiting gorgonians.

Hardoim & Costa Rodrigo (2014) showed in their model host, the sponge *Sarcotragus spinosulus*, that the prokaryotic symbionts displayed a state of dynamic stability over three successive years, where dominant and less dominant genera fluctuate over longer time periods without affecting the function of the sponge holobiont. *Candidatus Actinomarina* is dominating within the seawater. Only a few groups of planktonic marine *Actinobacteria* have been described until today. Genomic analysis indicate that their photoheterotrophic lifestyle support a planktonic, free-living lifestyle (Mizuno et al. 2015).

Referring to our *E. labiata* samples, the total number of dominant phyla compared to other studies of corals and their prokaryotic profile, result in similar findings with *Actinobacteria, Bacteroidetes* and *Proteobacteria* representing most dominant phyla (Harder et al. 2003; Yokouchi et al. 2006). However, contrasting findings were also observed as dominant bacteria found by Littman *et. al* (2011) in the coral *Acropora millepora* were *Cyanobacteria*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Chlorobi*. When comparing these results with other marine invertebrates inhabiting similar habitats, differences occur within the bacterial profile and the rel. abundance of genera. The sponge *Scarcotragus spinosulus* and its bacterial community composition includes *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Poribateria*, *Chloroflexi*, *Bacteroidetes*. (Hardoim & Costa 2014) already indicating host-specific differences on a very rough taxonomic scale.

Gammaproteobacteria represent the most abundant phylum in *E. labiata.* This group was also found to be dominant in other corals, such as in *Porites astreoides* (Wegley et al. 2007). The presence of photosynthetic symbionts seem to influence the two most dominant classes found within the prokaryotic community of the coral holobiont. *Gammaproteobacteria* seem to dominate when photosynthetic symbionts are presented whereas if no photosynthetic symbionts are present the dominating class is *Alphaproteobacteria* (Bourne et al. 2013).

Compared to the surrounding seawater, *Alphaproteobacteria* do not represent the dominate fraction of bacteria in our *E. labiata* in situ samples. As a sessile marine invertebrate, corals have a direct contact with the surrounding water column, but due to

the differences within the taxonomic profiling, corals might take up, exchange or stay in contact with their surrounding sediments as well since they are attached to a substrate close to the sea floor. Depending on living depth of a coral and turbulences within the water column, sediments might constantly get resuspended releasing prokaryotes inhabiting the upper layers of the sediments. Li et al. (2009) showed within a study using 16S ribosomal DNA clone library analysis that within the surface sediment γ-*Proteobacteria* represented nearly 50% and were dominating surface sediments from the Pacific Arctic Ocean.

From our cultured samples *Alphaproteobacteria* clearly dominate the rel. abundance, which is especially seen in the plate wash sample number one. Members of marine *Alphaproteobacteria* have been cultured readily on low-nutrient marine agar from coastal seawaters, showing similar dominant results in rel. abundance, using 16S rDNA sequences, accounting for 40% of the colonies isolated (Gonza & Moran 1997). Acinas et al. (1999) indicate that most bacterial cells living in the ocean cannot be cultivated on standard marine media. Nevertheless, they point out that particular cultured strains of marine bacteria may represent significant fractions of the bacterial biomass in seawater.

Coral Holobiont

Oceanospirillales & Rhodobacterales

The order *Oceanospirillales* belongs the class *Gammaproteobacteria*. Within the order *Oceanospirillales,* the genus *Endozoicomonas* is a widely distributed member of the bacterial communities associated with gorgonians (Bleijswijk et al. 2015; Roumagnac et al. 2013; Bayer et al. 2013; Ransome et al. 2017). *Endozoicomonas* contain aerobic and halophilic members reported to be associated with corals and studies show that both the mucus tissue of corals and the skeleton represent habitats for *Endozoicomonas* (Bleijswijk et al. 2015). In the Mediterranean coral *Eunicella cavolini, Endozoicomonas* account for 10 to 60% of the sequence reads (Bayer et al. 2013; Yang et al. 2010). While *Endozoicomonas* are known to be parasites in *Bathymodiolus* mussels, they seem to form symbiotic associations in all other documented cases (Bayer et al. 2013; Woo et al.

2017). The genera *Endozoicomonas* showed clear presence only within the *E. labiata* in situ samples, especially dominating sample number two, and was not abundant in seawater nor in cultivated samples. The genus was shown to be capable of metabolizing dimethylsulfoniopropionate (DMSP) that is produced by photosynthetic algae (Bayer et al. 2013; Raina et al. 2009). To explain associations of *Endozoicomonas* with corals that are not inhabited by photosynthetic symbionts, it has been suggested that members of the *Oceanospirillales clade* are able to degrade various organic substrates. This might indicate a symbiotic potential in terms of providing nutrients to the host (Roumagnac et al. 2013), as *Gammaproteobacteria* were associated with the gastrodermis tissue layer in the corals *Acropora aspera* and *Stylophora pistillata* (Ainsworth 2009). Besides *Endozoicomonas*, two unclassified members of the class *Oceanospirillales* were well represented in seawater and have been successfully cultured via the plate washing approach.

Members of the order Rhodobacterales (Garrity et al. 2015) are within the most dominant and ubiquitous primary surface colonizers in temperate coastal waters of the world, and estimates suggest that this order, especially members of the *Roseobacter* clade (Luo & Moran 2014), can comprise 25% of total marine bacterioplankton (Dang et al. 2008; Buchan & Moran 2005). *Rhodobacterales* spp. are found in diverse marine systems and a showed remarkable diversity in the subarctic waters of the North Atlantic, suggesting that different bacterial species in this group are able to adapt to cold environments (Fu et al. 2010). A further aspect of their distributional success is due to a tremendous diversity of metabolic capabilities (Luo & Moran 2014). Exposing the scleractinian coral *Fungia granulosa* to a hypersaline environment for a short term, the coral samples were dominated by bacteria from the family *Rhodobacteraceae* (Till et al. 2016)..

Several individuals within this order already have been shown to be related to coral diseases such as the white-plague disease. In the Caribbean coral *Montastraea faveolata* significant differences were found in the orders *Rhodobacterales*, *Campylobacterales*, *Planctomycetales* and *Clostridiales* when investigating healthy and white-plague diseased

individuals. The orders of *Rhodobacterales* and *Clostridiales* were significantly more abundant in diseased samples (Bourne et al. 2009).

The order consists of one family, namely *Rhodobacteraceae* (Garrity et al. 2015), that is generally present in aquatic ecosystems and comprise mainly aerobic photo- and chemoheterotrophs but also purple non-sulfur bacteria which perform photosynthesis in anaerobic environments. Currently 100 hundred genera are recognized as members of the family, although the *Stappia* group, *Ahrensia*, *Agaricicola* and *Rhodothalassium* do not belong phylogenetically to the family (Pujalte & Lucena 2014).

Members of the marine *Roseobacter* clade are major participants in global carbon and sulfur cycles. Members of this lineage encode metabolic potentials such as quorum sensing and type VI secretion systems enabling them to effectively interact with host and other bacteria (Zhang et al. 2016). A study with the largest *Roseobacter* group, the RCA (*Roseobacter* clade affiliated), showed positive correlation with phaeopigments, chlorophyll, dissolved and particulate organic carbon, turnover rates of dissolved free amino acids, temperature, and negative correlation with salinity (Giebel et al. 2010).

Ruegeria, a member of the *Roseobacter* clade, was well documented in the *E. labiata* and the plate wash samples. Moura et al. (2009) also finds *Ruegeria* as one of the dominant bacterial clusters in the coral mucus of *Mussismilia braziliensis* and its surrounding seawater.

Recent research congruently suggests that shifts in bacterial community structure, results in a lower coral host fitness and higher susceptibility to pathogens, highlighting the importance of the coral associated microbiome to holobiont health and functioning (Ben-haim et al. 2003; Bourne et al. 2009). Current research focuses on the microbiota of healthy corals, to aid our understanding of this multispecies mutualisms and will help to identify which species play a key role in maintaining coral health.

An example of bacterial community changes within the coral holobiont and their relationship with host disease was portrayed by Cooney et al. (2002) who investigated the bacterial consortium associated with the black band disease (BBD) within the scleractinian corals *Diplora strigose, Montastrea annularis* and *Colpophyllia natans*.

Whereas *Beta-* and *Gammaproteobacteria* were only found within non diseased corals, *Delta-* and *Epsilonproteobacteria* were only present in diseased samples. Because many pathogens have a wide host range coral disease outbreaks for example by generalist pathogens can infect a high number of different marine invertebrates. (Harvell et al. 2004; Green & Bruckner 2000).

Several members from the coral holobiont (in particular *Symbiodinium dinoflagellates*) influence microbial structure through the release of complex carbon-containing exudates including dimethylsulfoniopropionate (DMSP), which can be degraded to demethylsulphide, a central molecule in the global sulfur cycle which diffuses from the ocean into the atmosphere where it influences cloud formation, with consequences for atmospheric chemistry, local climate and water temperature. DMSP has been the focus of considerable attention because of its fundamental role as carbon and sulfur sources for bacteria. Coral reefs are one of the largest producers of DMSP with the source thought to be derived from marine invertebrates harboring symbiotic dinoflagellates. In the marine environment, the concentrations of DMSP are highest in reef-building coral reefs indicating one example on how coral reefs can effectively alter microbial structure and even influence environmental parameters. *Endozoicomonas* and several other members of the *Gammaproteobacteria* are able to metabolize DMSP and might form symbiotic relationships with DMSP-producing corals. Besides DMSP, photo symbionts seem to structure microbial communities in their hosts through the release of many other organic exudates (Bourne et al., 2013, Bourne et al., 2009).

A change in the microbiome of the zooxanthellae inhabiting coral *Acropora millepora* represented shifts from a system driven by autotrophy to heterotrophy after a bleaching event in 2001/2002 in the Great Barrier Reef (Littman et al. 2011). Carbohydrate, sulfur, phosphorous and fatty acid metabolism increased by two- to six-fold in the bleached libraries. Since 20-40% of the daily net photosynthetates are released as exudates from their symbionts in the coral mucus that is used as a substrate by many of the microbial associates, microbial communities potentially shifted towards other forms of nutrients acquisition. Within the *Proteobacteria phylum* the proportions of *Gammaproteobacteria* increased by 60% and *Alphaproteobacteria* decreased by 30%. *Bacterial communities*

switched from predominately Pseudomonas species to *Vibrio* species. It was proposed that pathogenic *Vibrio* spp. were capable of hydrolyzing the phospholipids within the membrane of the coral itself during heat stress. Additional studies indicated possible central roles for *Vibrios* associated with coral diseases in a variety of coral groups *(Littman, Willis, & Bourne, 2011, Burdett et al., 2010,* Sunagawa et al., 2009).

Thurber *et. al.* (2009) propose that stressed corals contain more disease-associated *Fungi* and *Bacteria* and therefore shifts from a mutualistic and/or commensal community to one that is pathogenic and opportunistic take place under stress. Furthermore, he suggests that environmental stressors caused the coral *P. compressa* to incorporate more genes encoding virulence pathways, affecting the communities of microorganisms present in the coral holobiont. Differences in the microbial communities have also been shown in white-plague affected corals. A study on *Mussismilia braziliensis* suggests that white plague in those corals is a polymicrobial disease. Diseased corals show elevated abundances of *Alphaproteobacteria* in the order, *Rickettsiales*, which seems to be involved in the acroporid white band disease (Garcia et al. 2013).

Beta Diversity

Figure 15 displays the uniqueness of OTUs found within each sample category. Four percent of the OTUs found in the in situ *E. labiata* samples were exclusive to this sample category. We point out, that in our sample the bacterial community of *E. labiata* is less diverse and distinct. April et al. (2013) investigated the bacterial diversity associated with the Mediterranean gorgonian coral *Eunicella cavolini*. To figure out if the coral provides a stable environment to the bacteria, they took three samples from three different depths. The bacterial community did not vary according to depth, suggesting that depth is not a driving force in structuring bacterial community composition in gorgonian corals. Comparing the culture dependent and culture independent method, 62% of the total *E. labiata* microbiome was including the genera *Ruegeria, Vibrio, Shewanella,* unclassified *Oceanospirillales,* unclassified *Rhodobacteraceae, Roseovarius* and *Sulfitobacter* (excluding OTUs with rel. abundances lower than 2%). The low medium agar, low temperatures and slightly longer cultivation period we accomplished a good deal for the

bacterial microbiome of *E. labiata.* In comparison with bacteria retrieved from terrestrial samples such as soil, growth rate tend to be higher, when compared with marine prokaryotes. Longer cultivation periods therefore might enhance the chance of detecting more diversity. Low temperatures also prevent some bacteria from dominating the cultivation samples, such as *Vibrio spp.* are positively correlated with temperature. A low nutrient medium might favor prokaryotes, that are used to live in nutrient poor conditions and are more efficient in nutrient uptake. Besides the remarkable culturing effort, we observe some within variability in our *E. labiata* in situ samples. *Endozoicomonas* and *Ruegeria* seem to be a member of the core microbiome in *E. labiata.* In what extent genera such as *Shewanella, Labrenzia* or *Rubritalea* vary between individuals, is still a matter of future investigation. When comparing the microbial communities of the sponge *Callyspongia* sp. with samples taken from Hong Kong and the sponge *Callyspongia plicifera* from the Bahamas, the two congeneric sponge species from different biogeographic regions were shown to have different bacterial associates. The diversity was higher in sponge samples from Hong Kong, indicating that besides species specific microbial associations the biogeographic distribution and its regional abiotic and biotic parameters further influence the prokaryotic communities associated with these species (Li 2009). Similar findings were reported by R. A. Littman et.Al. (2009), who investigated bacterial communities associated with three species of *Acropora* that were compared at two locations of the Great Barrier Reef. All three species microbiomes revealed specific microbiota that were also conserved among all three species of *Acropora* within each location, leading to the conclusion that related corals of the same genus harbor similar bacterial types. However, when profiling the prokaryotic community from different and further distanced members of *Acropora,* taxonomic community composition was different reinforcing the assumptions suggestions by Li, 2009. Taxonomic profiles grouped according to location rather than coral species indicate that certain bacterial groups associated specifically with corals, but the dominant bacterial genera still differ between geographically-spaced corals (Littman et al. 2009). Pantos et al. (2015) came to similar conclusions stating that habitat is the overall controlling factor of the coral microbiome, and not other members of the holobiont. It might make more sense in defining the core microbiome not necessarily in terms of taxonomic diversity but

rather in terms of functions and metabolic pathways within the microbiome (Krediet et al. 2013). Water et al. (2017) rather delineated the core microbiome of five gorgonian species (*Eunicella singularis, Eunicella cavolini, Eunicella verrucosa, Leptogorgia sarmentosa* and *Paramuricea clavata*) as resulting from few species-specific associations between coral and prokaryotes, while several other microbial genera followed a less strict relationship. Locally stable OTUs differed in abundance between different locations, leading to differences within the prokaryotic assemblages due to different geographical distributions, but with *Endozoicomonas* being dominant in all five gorgonian species (Water et al. 2017). A further study performed on *Pocillopora damicornis* supports the hypothesis of conserved coral-microbial consortia. Analyzing the coral mucus and the coral tissue, the coral mucus resembled a bacterial fingerprint of the water column rather than the coral tissue and was thus more closely associated with the water. A rarefaction analysis indicated higher levels of total diversity assessed for the coral tissue libraries, reaching clear asymptotes, as opposed to both the mucus and water libraries. Therefore, the mucus reflected a different coral microenvironment in comparison with the coral tissue (Bourne & Munn 2005).

Future perspective

Nowadays most of the symbiotic microorganisms remain unidentified and the potential roles of coral-associated bacteria are still unknown (Li 2009). A single coral colony clearly harbors much higher prokaryotic diversity than of zooxanthellae. Taxa occurring in multiple colonies offer the best hints to elucidate metabolic importance. Many coral-associated bacterial ribotypes are most closely related to known nitrogen fixers and antibiotic producers (Rohwer et al. 2002). Besides the insights into the diversity and biogeography of coral associated microbes, one next challenge is to discover metabolic contributions of these microbes to the functioning of the coral holobiont (Wegley et al. 2007). To fully assess a prokaryotic species, culture independent methods are not enough. For physiological studies and manipulations, it is still necessary to culture and isolate the microorganisms. To more successfully culture symbiotic microorganisms from corals or sponges, media might be enriched with sponge or coral extracts. Enriching cultures with minimal amounts of host tissue, co-occurring symbionts or long-term incubation

experiments would be valuable for characterizing symbionts (Dubilier et al. 2008). As several bacteria metabolize DMSP, as *Endozoicomonas* which was not cultivated in this work, studies indicate that growth of laboratory cultures could be enhanced with methionine and DMSP. Due to recent recognition in terms of the importance of the coral holobiont and its effects on coral well-being, Sunagawa et al. showed in 2009 that they could distinguish between healthy and diseased corals using the PhyloChip application. This application is suitable to detect the presence of sequenced 16S rDNA clones. Based on hybridization signal profiles and enriched 16S rDNA sequences, they were able to detect the presence of sequenced 16S rDNA clones categorizing more than 70% of the clones.

Also mentioning a more commercial and industrial approach of research, many host-associated microorganisms are relevant from a biotechnological perspective through the production of toxins, signaling molecules and other secondary metabolites for effective competition and defense strategies. Thus they represent a reservoir for the discovery of new drugs, therapeutic agents and bioactive molecules, with applications across medical, industrial and environmental settings (Egan et al. 2008). Porporato et al. (2013) points out, that soft corals, rather than hard corals, can produce antibiotic substances as prevention against fouling organisms or defense against microbes, which are often the causes of coral diseases.

Furthermore, little is known about symbiont transmission, which describes how hosts acquire their symbionts. Two strategies for symbiont transmission are typical among marine invertebrates. In vertical transmission, the symbionts are passed from one generation to the next through direct transmission of symbionts from the parent to the egg or embryo, whereas in the horizontal transmission, the symbionts are either taken up from the environment or from the co-occurring hosts (Dubilier et al. 2008). The genus *Endozoicomonas* shows only very low abundances within the seawater. Nevertheless, this genus is highly associated with gorgonian corals and yet it is not known how the coral transmits the bacteria from one individual or generation to another.

Conclusion

Gorgonian corals are found over a wide range of marine ecosystems starting from coastal regions neighboring hermatypic corals in tropical coral reefs all the way down to the dark deep sea. They play essential roles in structuring hard bottom habitats and dense communities harbor a high diversity of species (Ballesteros 2006). Recently publications investigating soft corals and their unique microbial associates indicate that these symbionts play essential roles in coral physiology and health (Porporato et al. 2013).

Eunicella labiata, one of the most abundant gorgonians found in the Southern Portuguese coast is clearly separating itself in its prokaryotic profile compared to its surrounding seawater. With our study, a first step was given, revealing the bacterial diversity and taxonomic composition hosted by *E. labiata.* Supporting previous studies, the genus *Endozoicomonas* seems to play and essential role in the *E. labiata* holobiont as reported for other corals. Members of this genus are usually not present in the water column or sediments surrounding the coral. It is suggested that *Endozoicomonas* metabolizes DMSP and supports coral health (Ransome et al. 2017). Culturing efforts at low temperatures over several weeks resulted in successful representatives of unclassified *Rhodobacteraceae* within our plate wash samples. The remarkable findings of the successful cultivation attempt in this study, accounting to a high cultivability of the *E. labiata* microbiome, fosters future research experiments to investigate microbial assemblages with gorgonian corals. Several unclassified candidates (unclassified *Rhodobacteraceae,* unclassified *Oceanospirillales*) were cultured and can be addressed in future research for isolation and taxonomic descriptions. Ecological studies can give further information on symbiotic importance in manipulation studies and the detection of new molecules such as antibiotics will enhance industrial evolution.

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Annex – General R script for the ecological and statistical analysis

##install packages##

install.packages('jsonlite', dependencies=TRUE, repos='http://cran.rstudio.com/')

detach("package:phyloseq", unload=TRUE)

install.packages("vegan")

install.packages("GUniFrac")

install.packages("RAM")

install.packages("genefilter")

install.packages("FSA")

```
install.packages("asbio")
install.packages("BiodiversityR")
install.packages(ReacTr)

library("phyloseq", lib.loc="~/R/win-library/3.3")
library(vegan)
library(GUniFrac)
library(RAM)
library(ggplot2)
library(dplyr)
library(scales)
library(grid)
library(reshape2)
library(FSA)
library(asbio)
library (BiodiversityR)
library(car)



##Import OTU table and taxa table and edit into the correct matrix###############

Phyla_OTU <- read.csv("~/University/Master Thesis/E labiata 16S Amplicon
Sequencing Data/R - Programming/Phylo_OTU.csv")

Phyla_OTU_Matrix<-as.matrix(Phyla_OTU[,2:11])

Phyla_OTU_row_names<-Phyla_OTU[,1]

rownames(Phyla_OTU_Matrix, do.NULL = TRUE, prefix = "row")
```

```
rownames(Phyla_OTU_Matrix) <- Phyla_OTU_row_names


Phyla_Taxa <- read.csv("~/University/Master Thesis/E labiata 16S Amplicon Sequencing Data/R - Programming/Phylo_Taxa.csv")

Phyla_Taxa_Matrix<-as.matrix(Phyla_Taxa[,2:7])

Phyla_Taxa_row_names<-Phyla_Taxa[,1]

rownames(Phyla_Taxa_Matrix, do.NULL = TRUE, prefix = "row")

rownames(Phyla_Taxa_Matrix)<-Phyla_Taxa_row_names

##########################################################################
####

### Now we tell phyloseq to include both matrixes into a phyloseq-object####

OTU = otu_table(Phyla_OTU_Matrix, taxa_are_rows = TRUE)

TAX = tax_table(Phyla_Taxa_Matrix)

Physeq<-phyloseq(OTU,TAX)

Physeq


##################Rarefy samples to equal depth#############################

Number_Reads<-sample_sums(Physeq) ###Check lowest number of Reads

Physeq.rare = rarefy_even_depth(Physeq, sample.size = 10162, rngseed = TRUE)
##Rarefy
```

```
####################Extract the OTU - Talbe from Phyloseq Object###########


Rare.OTU.Table = as(otu_table(Physeq.rare), "matrix")

write.csv(Rare.OTU.Table, "Rare.OTU.Table")


Rare.Tax.Table = as(tax_table(Physeq.rare), "matrix")

write.csv(Rare.Tax.Table, "Rare.Tax.Table")

#### Rarefaction curve #######

Data_Rarecurve<-as.data.frame(t(Phyla_OTU_Matrix))   ##### Change rows to column in OTU table ###

Rare_curve<-rarecurve(Data_Rarecurve, MARGIN = 1)


############### Alpha Diversity ###############

### calculate richness parameters#####

Alpha_Div_Values<-estimate_richness(Physeq.rare, split = TRUE, measures = c("Observed", "Chao1", "ACE", "Shannon"))

### import newly organized richness estimators into R again#####

Boxplot_Alpha_Div <- read_excel("~/University/Master Thesis/E labiata 16S Amplicon Sequencing Data/R - Programming/Boxplot_Alpha_Div.xlsx")

#### calculate counts, standard deviation, standard error of the mean and confidence interval ##

Summary_Alpha_Div<-summarySE(Boxplot_Alpha_Div, measurevar = "Values", groupvars = c("Samples", "Variable"))

####Plotting the alpha diversity values with standarderrors########

Barchart_Alpha<-ggplot(data = Summary_Alpha_Div, aes(x=Samples, y=Values)) +

  geom_col() + facet_wrap(~Variable,ncol = 4, scales = "free")


Barchart_Alpha<-ggplot(data = Summary_Alpha_Div, aes(x=Samples, y=Values)) +

  geom_col(position = position_dodge()) + facet_wrap(~Variable,ncol = 4, scales = "free") +
```

```
  geom_errorbar(aes(ymin=Values-se, ymax=Values+se))


Barchart_Alpha + ggtitle("Barchart of Alphadiversity Measures")+theme(plot.title =
element_text(hjust = 0.5))


#### ANOVA for each biodiversity index sperately comparing the three means of
seawater, E. labiata in situ & Plate wash against each sample category########

#### Test for normality – Shapiro wilk #######


View(Alpha_Div_Values)


### for seawater ###


Observed_SW<-Alpha_Div_Values[1:4,1]

Chao1_SW<-Alpha_Div_Values[1:4,2]

ACE_SW<-Alpha_Div_Values[1:4,4]

Shannon_SW<-Alpha_Div_Values[1:4,6]


Shapiro_Observed_SW<-shapiro.test(Observed_SW)

Shapiro_Observed_SW

Shapiro_Chao1_SW<-shapiro.test(Chao1_SW)

Shapiro_Chao1_SW

Shapiro_ACE_SW<-shapiro.test(ACE_SW)

Shapiro_ACE_SW

Shapiro_Shannon_SW<-shapiro.test(Shannon_SW)

Shapiro_ACE_SW


### for Eunicella labiata in situ####
```

```
Observed_E_l<-Alpha_Div_Values[5:7,1]
Chao1_E_l<-Alpha_Div_Values[5:7,2]
ACE_E_l<-Alpha_Div_Values[5:7,4]
Shannon_E_l<-Alpha_Div_Values[5:7,6]

Shapiro_Observed_E_l<-shapiro.test(Observed_E_l)
Shapiro_Observed_E_l
Shapiro_Chao1_E_l<-shapiro.test(Chao1_E_l)
Shapiro_Chao1_E_l
Shapiro_ACE_E_l<-shapiro.test(ACE_E_l)
Shapiro_ACE_E_l
Shapiro_Shannon_E_l<-shapiro.test(Shannon_E_l)
Shapiro_Shannon_E_l

#### for in situ plate wash####

Observed_P_W<-Alpha_Div_Values[8:10,1]
Chao1_P_W<-Alpha_Div_Values[8:10,2]
ACE_P_W<-Alpha_Div_Values[8:10,4]
Shannon_P_W<-Alpha_Div_Values[8:10,6]

Shapiro_Observed_P_W<-shapiro.test(Observed_P_W)
Shapiro_Observed_P_W
Shapiro_Chao1_P_W<-shapiro.test(Chao1_P_W)
Shapiro_Chao1_P_W
Shapiro_ACE_P_W<-shapiro.test(ACE_P_W)
Shapiro_ACE_P_W
Shapiro_Shannon_P_W<-shapiro.test(Shannon_P_W)
```

```
Shapiro_Shannon_P_W

### Normality test overall sample categories referring to each index #####

Observed_All<-Alpha_Div_Values[,1]
Chao1_All<-Alpha_Div_Values[,2]
ACE_All<-Alpha_Div_Values[,4]
Shannon_All<-Alpha_Div_Values[,6]



Shapiro_Observed_All<-shapiro.test(Observed_All)
Shapiro_Observed_All
Shapiro_Chao1_All<-shapiro.test(Chao1_All)
Shapiro_Chao1_All
Shapiro_ACE_All<-shapiro.test(ACE_All)
Shapiro_ACE_All
Shapiro_Shannon_All<-shapiro.test(Shannon_All)
Shapiro_Shannon_All

#### ANOVA and homogeneity of variances #####
####Observed#####
#### check homogeneity of variances####
Values_observed_anova<-Boxplot_Alpha_Div[1:10,-2]
ANOVA_observed<-aov(Values~as.factor(Samples), data = Values_observed_anova)

### plot residuals #####
res_observed<-ANOVA_observed$residuals
hist(res_observed,main = "Histogram of residuals - Observed", xlab = "Residuals")
```

```
### test homogeneity of variances and extract levene test table to word ####

capture.output(leveneTest(Values~as.factor(Samples),data = Values_observed_anova), file = "Levene_observed.csv")

summary(ANOVA_observed)

### extract ANOVA table to word ####

capture.output(summary(ANOVA_observed), file = "ANOVA_observed.csv")

###### Chao1 ######

Values_chao1_anova<-Boxplot_Alpha_Div[11:20,-2]

ANOVA_chao1<-aov(Values~as.factor(Samples), data = Values_chao1_anova)

### plot residuals #####

res_chao1<-ANOVA_chao1$residuals

hist(res_chao1,main = "Histogram of residuals - Chao1", xlab = "Residuals")

### test homogeneity of variances and extract levene test table to word ####

capture.output(leveneTest(Values~as.factor(Samples),data = Values_chao1_anova), file = "Levene_chao1.csv")

summary(ANOVA_chao1)

### extract ANOVA table to word ####

capture.output(summary(ANOVA_chao1), file = "ANOVA_chao1.csv")

###### Shannon ######
```

```
Values_shannon_anova<-Boxplot_Alpha_Div[31:40,-2]

ANOVA_shannon<-aov(Values~as.factor(Samples), data = Values_shannon_anova)


### plot residuals #####

res_shannon<-ANOVA_shannon$residuals

hist(res_shannon,main = "Histogram of residuals - Shannon", xlab = "Residuals")


### test homogeneity of variances and extract levene test table to word ####

capture.output(leveneTest(Values~as.factor(Samples),data = Values_shannon_anova),
file = "Levene_shannon.csv")


summary(ANOVA_shannon)


### extract ANOVA table to word ####

capture.output(summary(ANOVA_shannon), file = "ANOVA_Shannon.csv")


###### ACE ######


Values_ACE_anova<-Boxplot_Alpha_Div[21:30,-2]

ANOVA_ACE<-aov(Values~as.factor(Samples), data = Values_ACE_anova)


### plot residuals #####

res_ACE<-ANOVA_ACE$residuals

hist(res_ACE,main = "Histogram of residuals - ACE", xlab = "Residuals")


### test homogeneity of variances and extract levene test table to word ####


capture.output(leveneTest(Values~as.factor(Samples),data = Values_ACE_anova), file =
"Levene_ACE.csv")
```

```
summary(ANOVA_ACE)

### extract ANOVA table to word ####
capture.output(summary(ANOVA_ACE), file = "ANOVA_ACE.csv")

############ Tukey Honest Post-HOc-Test ##########

###Observed####

TukeyHSD(ANOVA_observed)
capture.output(TukeyHSD(ANOVA_observed), file = "Tukey_observed.csv")

####Chao1#####

TukeyHSD(ANOVA_chao1)
capture.output(TukeyHSD(ANOVA_chao1), file = "Tukey_chao1.csv")

####ACE#####

TukeyHSD(ANOVA_ACE)
capture.output(TukeyHSD(ANOVA_ACE), file = "Tukey_ACE.csv")

####Shannon Wiener#####

TukeyHSD(ANOVA_shannon)
capture.output(TukeyHSD(ANOVA_shannon), file = "Tukey_shannon.csv")
```

```
##############Taxonomic profiles – Stacked bar charts##########
##############Tax_glom - Count taxa in each taxonomic rank##################

colnames(tax_table(Physeq))
tax_domain<-tax_glom(Physeq, taxrank = "Domain")

tax_phyla<-tax_glom(Physeq, taxrank = "Phyla")
tax_class<-tax_glom(Physeq, taxrank = "Class")
tax_order<-tax_glom(Physeq, taxrank = "Order")
tax_family<-tax_glom(Physeq, taxrank = "Family")
tax_genus<-tax_glom(Physeq, taxrank = "Genus")

tax_domain
otu_table(tax_domain)
tax_phyla
otu_table(tax_phyla)
tax_class
otu_table(tax_class)
tax_order
otu_table(tax_order)
tax_family
otu_table(tax_family)
tax_genus
otu_table(tax_genus)
######################## Stacked Bar Charts
####################################
####### The Plotting - Stacked bar charts - Phyla
##################################
```

```
library(ggplot2)
library(dplyr)

physeq_Phyla <- Physeq.rare %>%
  tax_glom(taxrank = "Phyla") %>%                    # agglomerate at taxa level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel. abundance
  psmelt() %>%                                       # Melt to long format
  filter(Abundance > 0.02) %>%                       # Filter out low abundance taxa
  arrange(Phyla)

# Set colors for plotting
Phyla_colors <- c(
  "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578","#CD9BCD")

##Reordering the x-axis

physeq_Phyla$Sample<-as.character(physeq_Phyla$Sample)
physeq_Phyla$Sample<-factor(physeq_Phyla$Sample, levels = c("SW.01",
"SW.02","SW.03","SW.04", "In.S.01", "In.S.02", "In.S.03","PW.01", "PW.02",
"PW.03"))

##Plotting ######

ggplot(physeq_Phyla, aes(x = Sample, y = Abundance, fill = Phyla)) +
  geom_bar(stat = "identity", position = "fill") +
  scale_fill_manual(values = Phyla_colors)+ggtitle("Relavtive Phylum-Composition") +
theme(plot.title = element_text(hjust = 0.5))

####### The Plotting - Stacked bar charts - Class
###################################
```

```
physeq_Class <- Physeq.rare %>%
  tax_glom(taxrank = "Class") %>%                    # agglomerate at taxa level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel. abundance
  psmelt() %>%                                       # Melt to long format
  filter(Abundance > 0.02) %>%                       # Filter out low abundance taxa
  arrange(Class)

# Set colors for plotting
class_colors <- c(
  "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578","#CD9BCD",
  "#AD6F3B")

##Reordering the x-axis

physeq_Class$Sample<-as.character(physeq_Class$Sample)
physeq_Class$Sample<-factor(physeq_Class$Sample, levels = c("SW.01",
"SW.02","SW.03","SW.04", "In.S.01", "In.S.02", "In.S.03","PW.01", "PW.02",
"PW.03"))

##Plotting ######

ggplot(physeq_Class, aes(x = Sample, y = Abundance, fill = Class)) +
  geom_bar(stat = "identity") +
  scale_fill_manual(values = class_colors)+ggtitle("Relative Class-
Composition")+theme(plot.title = element_text(hjust = 0.5), legend.text =
element_text(size = 8), legend.key.size = unit(0.5, "cm"))

####### The Plotting - Stacked bar charts - Order
###################################
```

```
physeq_Order <- Physeq.rare %>%
  tax_glom(taxrank = "Order") %>%                    # agglomerate at taxa level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel. abundance
  psmelt() %>%                                       # Melt to long format
  filter(Abundance > 0.02) %>%                       # Filter out low abundance taxa
  arrange(Order)

# Set colors for plotting
order_colors <- c(
  "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578","#CD9BCD",
  "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",
  "#8569D5", "#5E738F")

##Reordering the x-axis

physeq_Order$Sample<-as.character(physeq_Order$Sample)
physeq_Order$Sample<-factor(physeq_Order$Sample, levels = c("SW.01",
"SW.02","SW.03","SW.04", "In.S.01", "In.S.02", "In.S.03","PW.01", "PW.02",
"PW.03"))

###Plotting

ggplot(physeq_Order, aes(x = Sample, y = Abundance, fill = Order)) +
  geom_bar(stat = "identity") +
  scale_fill_manual(values = order_colors)+ggtitle("Relative Order-
Composition")+theme(plot.title = element_text(hjust = 0.5),legend.text =
element_text(size = 8), legend.key.size = unit(0.5, "cm"))
```

```
####### The Plotting - Stacked bar charts - Family
###################################

physeq_Family <- Physeq.rare %>%
  tax_glom(taxrank = "Family") %>%                    # agglomerate at taxa level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel. abundance
  psmelt() %>%                                        # Melt to long format
 filter(Abundance > 0.02) %>%                         # Filter out low abundance taxa
  arrange(Family)

# Set colors for plotting
family_colors <- c(
  "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578","#CD9BCD",
  "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",
  "#8569D5", "#5E738F","#D1A33D","forestgreen","firebrick", "dodgerblue")

physeq_Family$Sample<-as.character(physeq_Family$Sample)
physeq_Family$Sample<-factor(physeq_Family$Sample, levels = c("SW.01",
"SW.02","SW.03","SW.04", "In.S.01", "In.S.02", "In.S.03","PW.01", "PW.02",
"PW.03"))

###Plotting

ggplot(physeq_Family, aes(x = Sample, y = Abundance, fill = Family)) +
  geom_bar(stat = "identity") +
  scale_fill_manual(values = family_colors)+ggtitle("Relative Family-
Composition")+theme(plot.title = element_text(hjust = 0.5),legend.text =
element_text(size = 8), legend.key.size = unit(0.5, "cm"))
```

```
  ####### The Plotting - Stacked bar charts - Genus
##################################


  physeq_Genus <- Physeq.rare %>%

    tax_glom(taxrank = "Genus") %>%                    # agglomerate at taxa level

    transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel.
abundance

    psmelt() %>%                                  # Melt to long format

    filter(Abundance > 0.02) %>%                       # Filter out low abundance taxa

    arrange(Genus)


  # Set colors for plotting

  genus_colors <- c(

    "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578","#CD9BCD",

    "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",

    "#8569D5",
"#5E738F","#D1A33D","darkmagenta","darkred","darkseagreen4","gray1","yellow","gre
en","red")


  ### put the x-axis in the correct order

  physeq_Genus$Sample<-as.character(physeq_Genus$Sample)

  physeq_Genus$Sample<-factor(physeq_Genus$Sample, levels = c("SW.01",
"SW.02","SW.03","SW.04", "In.S.01", "In.S.02", "In.S.03","PW.01", "PW.02",
"PW.03"))


  ###Plotting


  ggplot(physeq_Genus, aes(x = Sample, y = Abundance, fill = Genus)) +

    geom_bar(stat = "identity") +
```

```
    scale_fill_manual(values = genus_colors)+ggtitle("Relative Genus-
Composition")+theme(plot.title = element_text(hjust = 0.5),legend.text =
element_text(size = 8), legend.key.size = unit(0.5, "cm"))


  ggplot(physeq_Genus, aes(x = Sample, y = Abundance, fill = Genus)) +

    geom_bar(stat = "identity") +

    scale_fill_manual(values = genus_colors)+ggtitle("Relative Genus-Composition") +
guides(fill=guide_legend(ncol=1))+theme(plot.title = element_text(hjust =
0.5),legend.text = element_text(size = 8), legend.key.size = unit(0.5, "cm"))


#### Creating the heatmaps #######

### Subset Order Oceanospirillales & Rhodobacterales for heatmap and plot#######


Heat_Oceanospirillales_Joined<-tax_glom(Heat_Oceanospirillales, taxrank = "Genus")

plot_heatmap(Heat_Oceanospirillales,low="white", high="#FF3300",method = "Null",
taxa.label = "Genus", sample.order = sample_names(Physeq.rare), na.value = "white",
title = "Heatmap - Oceanospirillales") + theme(plot.title = element_text(hjust = 0.5))

Heatmap_Oceanospirillales<-plot_heatmap(Heat_Oceanospirillales_Joined,low="white",
high="#FF3300",method = "Null", taxa.label = "Genus", sample.order =
sample_names(Physeq.rare), na.value = "white", title = "Heatmap - Oceanospirillales") +
theme(plot.title = element_text(hjust = 0.5))

Heatmap_Oceanospirillales + theme(axis.text.y = element_text(size = 7)) +
scale_y_discrete(name="Genus", labels = c("uncl. OM182
clade","Litoricola","Balneatrix","uncl. SAR86 clade","Neptuniibacter","uncl.
Oceanospirillales","uncl. SS1-B-06-26","uncl. CrystalBog021C3","uncl.
ZD0405","Endozoicomonas","Pseudohongiells","Kangialla"))


Heat_Rhodobacterales<-subset_taxa(Physeq.rare, Order=="Rhodobacterales")

plot_heatmap(Heat_Rhodobacterales,low="white", high="#FF3300",method = "Null",
taxa.label = "Genus", sample.order = sample_names(Physeq.rare), na.value = "white",
title = "Heatmap - Oceanospirillales") + theme(plot.title = element_text(hjust = 0.5))


Heat_Rhodobacterales_Joined<-tax_glom(Heat_Rhodobacterales, taxrank = "Genus")
```

```
Heatmap_Rhodobacterales<-plot_heatmap(Heat_Rhodobacterales_Joined,low="white", high="#FF3300",method = "Null", taxa.label = "Genus", sample.order = sample_names(Physeq.rare), na.value = "white", title = "Heatmap - Rhodobacterales") + theme(plot.title = element_text(hjust = 0.5))


Heatmap_Rhodobacterales + scale_y_discrete(name="Genus", labels = c("uncl. Rhodobacteraceae",
"Ascidiaceihabitans","Amylibacter","Breoghania","Labrenzia","Planktomarina","Shimia","Ruegeria","Sedimentitalea","Sulfitobacter","Roseovarius","Pseudohaeobacter","Halocynthiibacter"))


############################ Ordination ####################

#####Hellinger Transformation##########


Rare_OTU <- read_csv("~/University/Master Thesis/E labiata 16S Amplicon Sequencing Data/R - Programming/Rare.OTU.Table")

Rare_OTU<-as.data.frame(Rare_OTU)

x<-Rare_OTU[,1]

Rare_OTU<-Rare_OTU[,-1]

rownames(Rare_OTU)<-x

remove(x)

Hellinger<-decostand(Rare_OTU,"hellinger", MARGIN = 2)

write.csv(Hellinger, "Hellinger_Trans")


### Now create a new Phyloseq object including the hellinger transformed data

### and then plot the distance matrix below


OTU1 = otu_table(Hellinger, taxa_are_rows = TRUE)

TAX = tax_table(Phyla_Taxa_Matrix)


##### Create the Sample Matrix for Phyloseq##############
```

```
SampleData_Phyloseqxlsx <- read_excel("~/University/Master Thesis/E labiata 16S Amplicon Sequencing Data/R - Programming/SampleData_Phyloseqxlsx.xlsx")

SampleData<-as.data.frame(SampleData_Phyloseqxlsx)

SampleDataRow<-SampleData[,-1]

row.names(SampleData)<-SampleDataRow

Sampl=sample_data(SampleData)


#### Create the phyloseq object#############3


Physeq_Hellinger<-phyloseq(OTU1,TAX, Sampl)


### #####Plot the SampleID, Uniplot ###################


or<-ordinate(Physeq_Hellinger, "euclidean", method = "PCoA")

Ordination_SampleID<-plot_ordination(Physeq_Hellinger, or, label = "SampleID", title = "Principle Coordinates Analysis")

Ordination_SampleID + theme(plot.title = element_text(hjust = 0.5))


#### Distinguish the samples, because points are too close together to read them


Ordination_SampleID<-plot_ordination(Physeq_Hellinger, or, title = "Principle Coordinates Analysis", shape = "SampleID")


shapes <-c(1,2,3,4,5,6,7,8,9,10) ### we set 10 different shapes to distinguish the samples and retrieve the correct SampleID


Ordination_SampleID + theme(plot.title = element_text(hjust = 0.5)) + scale_shape_manual(values = shapes)
```

```
##### Include the 20 most abundant taxa from the whole sample



################## Non Rarefied##################



######## Set colors new ###############

ordination_colors<- c(
  "black", "orange","#DA5724", "#508578","#CD9BCD",
  "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",
  "#8569D5", "#5E738F","#D1A33D","forestgreen","firebrick",
"dodgerblue","yellow","gray1","darkblue","green","red")



Table_Otu<-otu_table(Physeq)

Hellinger<-decostand(Table_Otu, "hellinger", MARGIN = 2)

OTU1 = otu_table(Hellinger, taxa_are_rows = TRUE)

TAX = tax_table(Phyla_Taxa_Matrix)

Physeq_Hellinger<-phyloseq(OTU1,TAX, Sampl)

taxa_pruned_20<-prune_taxa(names(sort(taxa_sums(Physeq_Hellinger),TRUE)[1:20]),
Physeq_Hellinger)

Ordination_20<-plot_ordination(taxa_pruned_20, or, type = "biplot",color = "Genus",
label = "SampleID", title = "PCoA: Euclidean Distance")

Ordination_20 + theme(plot.title = element_text(hjust = 0.5)) +
scale_color_manual(values = ordination_colors) + theme_classic()



##############Final Plot of Ordination ##########################
```

```
Ordination_20<-plot_ordination(taxa_pruned_20, or, type = "biplot",color = "Genus",
title = "PCoA: Euclidean Distance")


Ordination_20 + theme_classic()+ theme(plot.title = element_text(hjust = 0.5), axis.text =
element_text(size=12, colour = "black"), legend.text = element_text(size =
3),legend.position = "none") + scale_color_manual(values = ordination_colors)

########################Permutation Test##############################


## create distance matrix - euclidean


Dist_Samples<-distance(Physeq_Hellinger, method = "euclidean", type = "samples")


### Create a matrix of the Distance matrix to export in excel

Dist_Samples_Matrix<-as.matrix(distance(Physeq_Hellinger, method = "euclidean", type
= "samples"))

write.csv(Dist_Samples_Matrix, file = "Distance_Matrix_Euclidean")


## turn sample_data into a dataframe


df = data.frame(sample_data(Physeq_Hellinger))

Distance_Matrix<-as.data.frame(Dist_Samples)

Permutation_Result<-adonis(Dist_Samples ~ Environment, data = df)


### extract for excel


Permutation_Test<-Permutation_Result$aov.tab

write.csv(Permutation_Test, "Permutation_Test")
```

```
#### Clustering


Cluster_Dis<-hclust(Dist_Samples, method = "average")
plot(Cluster_Dis)



### clustering with pvclust#########
install.packages("pvclust")

library(pvclust)
library(phyloseq)

pvclust_matrix<-as.data.frame(otu_table(Physeq_Hellinger))
pvclust_matrix<-as.matrix(pvclust_matrix)
pvc_result<-pvclust(pvclust_matrix, method.hclust = "average",method.dist =
"euclidean")
plot(pvc_result, title(""), main = "Cluster dendrogram with AU/BP values (%)")
```