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Exploration of *Ulva*-holobiont diversity in Portugal: Any lesson to learn for cultivation and use?

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2020

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Mestrado em Biologia Marinha

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Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Signature

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Acknowledgements

I would like to thank my first referee Dr. Aschwin Hillebrand Engelen for giving me the opportunity to conduct my Master thesis within the framework of the CCMAR and for introducing me to the world of seaweed ecology and microbiology. I am very grateful for his supportive and patient supervision during the last year, it has been an inspiring journey. A big thanks to Dr. Tânia Aires for helping me with the molecular biological tasks, for her guidance in the laboratory and for agreeing to be my second referee. As well, I would like to thank Sophie Steinhagen for sharing her reference sequences and phylogenetic advice with me. A deep gratitude to my friend Robert Priester who helped me with the sampling process and making of the herbarium. I would also like to thank Justine Nathan and Leona Ritter von Stein who helped me with important tasks along the way. Furthermore, a big thanks to Nicolas Blanc for translating my Portugese resumo. Lastly, I would like to thank the rest of my friends and my family for helping me to stay positive in times of doubt.

Resumo

Nesta era do Antropoceno, entre desafios relacionados com a sobrepopulação e alterações climáticas, a necessidade de substituir os métodos tradicionais de exploração de recursos do nosso planeta por alternativas sustentáveis é cada vez mais urgente. Uma alternativa promissora para contrapor os efeitos adversos de práticas convencionais de agricultura é a aquacultura de macroalgas. Além do seu papel crucial como produtoras primárias, criadoras de habitat e biorremediadoras, as macroalgas podem ajudar com a sequestração de carbono. Estas representam também uma fonte sustentável e valiosa do ponto de vista comercial enquanto alimento para humanos ou para animais, e podem ser aplicadas a outras áreas como higiene pessoal, medicação, e produção de biocombustíveis. Até ao momento têm sido sobretudo países asiáticos a utilizar uma grande variedade de macroalgas, enquanto que na Europa a potencial diversidade que pode ser utilizada para cultivo ainda não foi devidamente explorada.

O género de algas verdes *Ulva* é particularmente interessante para a indústria alimentar devido ao seu elevado valor nutricional. Adicionalmente, tem sido alvo de uma crescente atenção científica devido à sua recente aplicação enquanto modelo para o estudo de interações entre macroalgas e bactérias. Comunidades bacterianas associadas a macroalgas (microbiomas) têm sido reconhecidas por influenciar processos metabólicos essenciais entre o sistema alga-bactéria (holobionte). Certos tipos de bactérias providenciam ao hospedeiro vitaminas e fito-hormonas para crescimento e morfogénese, enquanto outros aparentam facilitar a adaptação a stressores ambientais. Contudo, a questão de como é determinada a composição do microbioma continua a ser um debate controverso e existem diferentes teorias para explicar este fenómeno. A *teoria da lotaria* baseia a composição do microbioma em processos estocásticos, a *especificidade ao hospedeiro* propõe que a espécie hospedeira determina que bactérias são “recrutadas”, enquanto a *especificidade funcional ao hospedeiro* indica que as bactérias se juntam de acordo com as suas funções e não à sua taxonomia. Finalmente, fatores ambientais também mostraram ser importantes na composição do microbioma. Embora todos os fatores indicados mostrarem ter importância, pouco se sabe sobre o fator principal que determina a composição de microbioma no género *Ulva*.

Assim, neste estudo, diferentes espécies de *Ulva* foram recolhidas ao longo da costa sul e sudoeste de Portugal em diferentes ambientes e identificadas através do gene de alongamento do cloroplasto (tufA), e os seus microbiomas associados foram analisados através da determinação do perfil do gene de ARNr 16S. Numa etapa mais avançada, os dados filogenéticos foram combinados com os dados metagenómicos para avaliar o nível de especificidade ao hospedeiro e/ou região. Foi dada atenção especial a abundâncias de grupos particulares de bactérias: *taxa* produtores de vitamina B_{12} (benéficos) e “ambivalentes” (potencialmente prejudiciais). Em conclusão, estes aspectos foram analisados de forma a perceber que lições podem ser tiradas para o cultivo futuro e uso de diferentes holobiontes *Ulva.*

Seis espécies diferentes do género *Ulva* foram identificadas com sucesso com o uso do gene marcador *tuf*A: *U. rigida*, *U. compressa*, *U. californica/flexuosa*, *U. australis, Ulva* sp.1, *Ulva* sp.2. A espécie não-indígena *U. australis* foi registada pela primeira vez em Portugal, assim como duas novas entidades. A suposição que *U. californica* e *U. flexuosa* formam um complexo (i.e. *U. californica/flexuosa*) foi verificada, e a distinção genética da *U. rigida* relativamente às suas parentes *U. laetevirens* e *U. lacutca* foi desenvolvida. Os microbiomas examinados neste estudo diferenciaram-se na sua composição e diversidade entre espécies *Ulva* e foram dominados maioritariamente pelas ordens Flavobactérias, Rhodobactérias, Caulobactérias, e Pirellulales. A última destas, já identificada como detentora de um conjunto de genes relacionados com resposta a stresses ambientais (*i.e. Rhodopirellula*), foi especialmente característica da *U. compressa*. Foi identificada especificidade ao hospedeiro clara e notável para *U. rigida* e *U. compressa*, respectivamente, com sinais de especificidade secundária à região a um nível intra-hospedeiro, predominantemente para *U. compressa*. A possibilidade de determinar a composição do microbioma com base unicamente em processos estocásticos (lotaria) ou apenas em fatores ambientais pôde ser descartada. A única excepção foi o microbioma de *U. californica/flexuosa* de uma bacia artificial onde as condições ambientais distintivas aparentam ter tido um elevado impacto na composição e levaram a uma elevada abundância de bactérias benéficas (Flavobacteriales).

Seguindo o raciocínio acima utilizado, as espécies *U. compressa*, *U. rigida* e *U. californica/flexuosa* foram propostas para ser de interesse especial para uso comercial. Com base numa elevada quantidade de *Rhodopirellula* e uma boa representação de *Dinoroseobacter*, *U. compressa* é assim recomendada para ser cultivada para consumo humano, conforme sugerido por estudos prévios. Devido à sua especificidade ao hospedeiro, a *U. rigida* poderá ser uma candidata apropriada para usos industriais para fornecer uma quantidade estável de compostos provenientes de bactérias específicas. A *U. californica/flexuosa* poderá ser uma candidata adequada para a produção de vitamina B_{12} devido à possibilidade da sua capacidade de elevar a quantidade de Flavobactérias sob certas condições ambientais. No seu todo, estas descobertas têm importantes implicações pois a especificidade ao hospedeiro permite o uso de microbiomas como uma ferramenta de delimitação de espécies, traceamento de espécies não-indígenas (e. g. *U. australis*) e usos industriais para obter compostos específicos derivados de bactérias em *Ulva* cultivada.

Palavras-chave: *Ulva*, *tuf*A, 16S rRNA, holobionte, microbioma, cultivo de macroalgas

Abstract

In the context of the ever-growing interest in seaweed aquaculture as sustainable alternative to traditional farming practices, here, the potential of the green seaweed genus *Ulva* for cultivation and use was assessed. *Ulva* is particularly interesting for science and industry owing to its high nutritional value, variety of applications, and use as model organism to study seaweed-bacterial interactions. Seaweed associated bacterial communities (microbiomes) have been recognized to influence essential metabolic processes within the alga-bacteria system (holobiont). How the microbiome composition is determined remains a controversial debate and different theories exist to explain the process (*i.e.* lottery, host-specificity, "functional host-specificity", and environmental factors). Hence, this study aimed to assess the importance of the host and/or region for the determination of the microbiome by means of combining phylogenetic data based on DNA barcoding of the chloroplast elongation factor (*tufA*) gene of various species of *Ulva* from Portugal with metagenomic data of their respective microbiomes, analysed *via* 16S rRNA gene profiling. Special attention was paid to particular bacterial groups, vitamin B_{12} producers (beneficial) and "the ambivalent" (potentially harmful). The different aspects were viewed under the collective question of what lessons could be learned for future cultivation and use of these holobionts. Six different *Ulva* species (*U. rigida*, *U. compressa*, *U. californica/flexuosa*, *U. australis, Ulva* sp.1, *Ulva* sp.2) were identified, among them the non-indigenous species (NIS) *U. australis*, recorded for the first time in Portugal, and two potentially new entities. Host-specificity emerged as primary factor in at least two species with signs of secondary region-specificity on a within-host level, whereas a determination purely based on lottery or environmental factors could be ruled out. Additionally, microbiomes were unobtrusive in the abundance of the ambivalent bacteria and diverse in common vitamin B_{12} producers, suggesting *Ulva* once more suitable for consumption. Altogether, these findings have important implications for using the microbiome as additional species-delimitation tool, tracing of NIS and industrial strategies to increase specific bacterial-derived target compounds in farmed *Ulva.*

Keywords: *Ulva*, *tuf*A, 16S rRNA, holobiont, microbiome, seaweed cultivation

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Abbreviations

NIS	Non-indigenous species
IMTA	Integrated Multitrophic Aquaculture
OMA	Ocean Macroalgal Afforestation
PUFAs	Poly-unsaturated fatty acids
CFB	*Cytophaga-Falvobacterium-Bacteriodes*
IAA	Indole-3-acetic acid
QS	Quorum sensing
OTU	Operational Taxonomic Unit
BI	Bayesian inference
MCMC	Markov Chain Monte Carlo

Content

1 General introduction

1.1 Potential of seaweed farming

In the era of Anthropocene, caught between overpopulation and climate change related challenges, the need to replace our traditional methods of exploiting our planets resources with sustainable alternatives is becoming more pressing. Human runaway population growth is projected to reach 9.4 billion by 2070, and with it the demand for crops and high-quality protein will continue to rise (Alexandratos and Bruinsma, 2012). This will result in a general intensification of agriculture and doubling of meat-production by 2050 devastating the environment as our waste production, pollution and land use will aggravate alongside. The exploitation of the marine environment heads a similar way as wild stocks are vastly collapsing (Cochrane *et al.*, 2009). Unfortunately, producing protein from fin-fish aquaculture also causes environmental problems as farming in coastal waters leads to the eutrophication of estuaries, disease transmission and introduction of non-indigenous species (NIS) (Gowen, 1994). Additionally, many farmed species are not robust enough to cope with the adverse effects of climate change such as increasing seawater temperatures (Cochrane *et al.*, 2009).

A promising alternative to counter these adverse effects is the farming of seaweed. In 2012, N'Yeurt and colleagues concluded that large scale Ocean Macroalgal Afforestation (OMA) could potentially solve a variety of the most severe environmental issues of today. If 9 % of the ocean's surface would be covered by seaweed farms, enhanced productivity through Integraded Multitrophic Aquacalture (IMTA) could potentially yield 200 kilograms of sustainably farmed fish per year, per person, for 10 billion people (Alvarado-Morales *et al.*, 2012). At the same time, 19 gigatons of CO_2 per year could be drawn from the atmosphere and enough biomethane could be produced to cover the total requirement of fossil fuels. The generated digestate from the biogas production could then be used as mineral fertilisers (Alvarado-Morales *et al.*, 2012). Moreover, macroalgal forests would decrease ocean acidification through enhanced primary production; not least increase marine biodiversity through the formation of habitats and provision of nursery grounds and shelter (Bulleri *et al.*, 2002).

In recent years, further studies have proven seaweeds have remarkable benefits when incorporated into human food and animal feed. As a supplement in fish meal, they enhance disease resistance in farmed fish (Xie *et al.*, 2018), whereas in sheep, a supplementation of conventional feed with the red seaweeds *Asparagopsis*, for instance can reduce

CH_4 emissions by up to 80% (Li *et al.*, 2018). For human nutrition, seaweeds are optimal in many ways as they contain high levels of important minerals, proteins, dietary fibres and PUFAs (Poly-unsaturated fatty acids) as well as vitamins. When consumed regularly, seaweeds can exhibit anti-obesity, anti-inflammatory, and carcinopreventitive effects (Wijesinghe and Jeon, 2012).

Having a long tradition in Asian culture as biomedicine and "sea vegetable", countries like Japan, China and the Republic of South Korea comprise the largest consumers world-wide and in other parts of the world the requirement for seaweed is vastly increasing (McHugh, 2003). The global production, culture and wild-harvest, doubled from almost 15 million tonnes in 2005 to over 30 million tonnes in 2015 (Ferdouse *et al.*, 2018). In Europe, however, seaweeds are rarely produced and if so mainly for the extraction of hydrocolloids as they still lack popularity as a food ingredient (Fleurence *et al.*, 2012).

Marine macroalgae are of great diversity; about 12,000 have been described to date (Guiry, 2012). Worldwide, solely 220 species are of commercial value, of which ten are cultivated in notable amounts, such as the brown seaweeds (*Laminaria* ssp., *Undaria* ssp. and *Sargassum* ssp.); red seaweeds (*Porphyra* spp., *Eucheuma* spp., *Kappaphycus* spp. and *Gracilaria* spp.); and green seaweeds (*Ulva/Enteromorpha* spp., *Monostroma* spp. and *Caulerpa* spp.) (Ferdouse *et al.*, 2018). Most of the seaweeds in Europe, mainly species of *Laminaria* and *Ascophyllum,* are harvested from wild populations, whereas for cultivation, solely *Ulva* spp. and *Gracilaria* spp. are used (Buschmann *et al.*, 2017). However, the rising demand for macroalgae in Europe and other non-Asian countries means current wild-harvest and aquaculture practices only based on the aforementioned species will not be able to maintain their current production trajectories to meet this demand (McHugh, 2003). Fortunately, given the biodiverse nature of marine macroalgae, their potential for increased cultivation and consumption is soon to be realised.

1.2 The green seaweed genus *Ulva*

1.2.1 Characteristics and traits

Among green algae (Chlorophyta), the genus *Ulva* (Linnaeus, 1753) (class *Ulvophyceae*, order *Ulvales*, family *Ulvacea*), partly referred to as "sea lettuce" (Figure 1), is one of the most abundant seaweeds of coastal benthic communities around the world. Presently, there are 403 species registered in Algaebase of which 131 have been flagged as taxonomically accepted. Additionally, 201 infraspecific names have been recognized (Algaebase, www.algaebase.org, accessed on 30/04/20). *Ulva* particularly holds promising features for valuable uses. Their high levels of essential amino acids, PUFAs and vitamin B_{12} make them a suitable alternative nutrition source for vegetarians and vegans (MacArtain et al., 2007). For instance, *U. pertusa* contains valine, leucine, lysine and histidine in a notably high amount (Fleurence, 1999). Another species, *U. mutabilis*, was suggested as a feedstock due to their high production of PUFAs (Alsufyani et al., 2014). Seaweeds are one of the few vegetable sources of vitamin B_{12}. For instance, 8 g of dried *U. lactuca* can provide this vitamin in excess of the recommended dietary allowances for Ireland of 1.4 µg/day (MacArtain *et al.*, 2007).

Generally, *Ulva* can be found at the top of the sublittoral zone, either free-floating in sheltered bays with calm waters and sandy bottoms or attached to rocks or different substrate. Their holdfast consists of several basal cells forming an adhesive disc, while downgrowing filaments provide anchoring. Some species are blade-forming (Figure 1), others tubular with a varying size between 2 - 100 cm (McHugh, 2003; Guiry, 2012; Bunker *et al.*, 2017). Owing to the opportunistic lifestyle of many members, *Ulva* spp. can tolerate a wide range of salinity and light regimes (Steinhagen, Karez and Weinberger, 2018). Moreover, it frequently occurs in eutrophicated waters due to its particular affinities for growth in high nitrogen concentrations, where it accumulates in green tides (Smetacek and Zingone, 2013; Bolton *et al.*, 2016). During its life history *Ulva* alternates between a diploid sporophytic and haploid gametophytic phase. Both mobile flagellate stages attach themselves to substrate, secrete cell walls and form isomorphic phenotypes (Ohno, 1997). Seasonal fluctuations, driving the changes of environmental factors like temperature and salinity may affect the growth pattern as well as the metabolite content (Ohno, 1997; Fleurence, 1999). After the reproductive phase and subsequent release of the gametes, the growth of *Ulva* spp. is terminated and part of the thallus slowly starts to degrade itself, notably reducing productivity (Oza and Sreenivasa Rao, 1977).

In summary, their metabolite content, relatively simple life history traits, stress resilience and ability to thrive unattached in sheltered waters with particular affinities for growth in high nitrogen concentrations makes them perfect aquaculture candidates (Bolton *et al.*, 2009, 2016; Steinhagen, Karez and Weinberger, 2018).

Figure 1. Blade-like morphotype ("sea lettuce") of *Ulva rigida*.

1.2.2 Taxonomic ambiguity

Despite its ubiquitous distribution and industrial potential, the genus remains largely understudied, partly because its taxonomic identification solely based on traditional morphological characteristics is problematic (Wichard and Oertel, 2010; Steinhagen, Karez and Weinberger, 2018). In the past, different morphotypes have been mistaken for distinct genera due to phenotypic plasticity (*i.e. Ulva/Enteromorph*a, now *Ulva*); or distinct species *(i.e.* the cryptic clade *mutabilis/pseudocurvata/compressa*, now synchronized as *U. compressa*) (Wichard and Oertel, 2010; Steinhagen, Karez and Weinberger, 2018; Steinhagen *et al.*, 2019). Other species are still debated whether to be conspecifics or not and are grouped together as species-complex (i.e., *flexuosa/californica)* (Steinhagen, Karez and Weinberger, 2018).

Over the last twenty years, however, the streamlining of modern molecular identification techniques gave rise to an increasing number of systematic studies based on DNA sequence data. Consequently, many misidentifications were revealed and led to taxonomical rearrangements within the genus (Blomster, Maggs and Stanhope, 1998; Hayden *et al.*, 2003; Heesch *et al.*, 2009; Saunders and Kucera, 2010; Kirkendale, Saunders and

Winberg, 2013; Chávez-Sánchez *et al.*, 2019; Steinhagen *et al.*, 2019). The first genetic barcodings were implemented using the internal transcribed spacer region of the ribosomal cistron (ITS) (Blomster, Maggs and Stanhope, 1998), while more recent studies targeted the rubisco large subunit gene (*rbc*L) (Hayden and Robert Waaland, 2002; Heesch *et al.*, 2009). However, the amplification success was shown to be hampered for both molecular markers. Double bands for ITS occurred widely among green algae lineages, indicating divergent copies (Saunders and Kucera, 2010), whereas low levels of genetic diversity within the *rbc*L gene hindered the species delimitation (Heesch *et al.*, 2009). Although the amount of DNA sequence data is growing, a curated reference database for Chlorophyta, particularly *Ulva*, is still non-existent. Currently, the genetic species identification relies on GenBank as reference database, where in fact high numbers of sequences are incorrectly identified. This applies for the species *U. rigida*, *U. laetevirens* and *U. lactuca*, which frequently are misidentified due to discrepancies in the database (Sfriso, 2010).

Recently, another molecular marker, the plastid encoded elongation factor gene (*tuf*A), has been evaluated as a reliable species delimitation tool within the order of *Ulvales* spp. (Saunders and Kucera, 2010; Chávez-Sánchez *et al.*, 2019). Although the database for *tuf*A is relatively small, compared to the aforementioned marker genes, uploaded sequences were mostly peer reviewed and carefully annotated (Steinhagen, Karez and Weinberger, 2018). Thus, in this study, the *tufA* marker gene was used to generate the most accurate genetic identification possible within the genus *Ulva*.

1.2.3 Diversity in Portugal

According to the Portuguese Seaweed Website MACIO (www.macoi.ci.uc.pt; accessed 16th of October 2019) and the list of marine algae recorded by Araújo *et al.*, 2009, a variety of *Ulva*-species can be found in Portuguese waters, such as: *U. bifrons*, *U. clathrate*, *U. compressa*, *U. curvata*, *U. flexuosa*, *U. intestinalis*, *U. lacutca*, *U. linza*, *U. prolifera*, *U. pseudocurvata*, *U. pseudolinza*, *U. rhacodes*, *U. rigida*, *U. scandinavica*, *U. simplex.* Moreover, records suggest that the NIS, *U. australis*, native to the Pacific, successfully established on the Iberian peninsula (Couceiro, Cremades and Barreiro, 2011). However, despite the case of *U. australis*, which was barcoded *via* amplification of the ribosomal ITS region, the above-mentioned species identifications were solely based on morphological characteristics and, hence, are rather unreliable.

In earth ponds of the Ria Formosa lagoon, in the south of Portugal, multiple species were previously barcoded using ITS: *U. flexuosa*, *U. clathrata*, *U. intestinalis*, *U. sapora* and *U. torta*, as well as *U. prolifera* (Favot, 2016). Based on *rbcL,* another study found the species *U. rigida*, *U. rotundata*, *U. ohnoi* and *U. compressa* in the Ria Formosa (Alsufyani *et al*. 2014). Although, barcoding yields more reliable taxonomic data when compared to macroscopic analysis, results obtained using ITS or *rbcL* should be interpreted with caution as for above-mentioned reasons (see chapter 1.2.2). Recently, more systematic studies were carried out on the basis of *tufA* (Saunders and Kucera, 2010; Chávez-Sánchez *et al.*, 2019). Nevertheless, barcoding data in Portugal remains sparse and the taxonomical ambiguity underlines the importance of further molecular identification of this genus.

1.2.4 Cultivation and use

The cultivation of *Ulva* for human consumption began in Japan around 1985, as demands for the edible green seaweed exceeded the capacity of wild stocks. Eastern Asian countries are still the largest producers of 'aonori', the commercial product made from *U. intestinalis*, *U. prolifera* and *U. compressa* (Ohno, 1997). Since 1990, these species have also been authorised for human food in France and are becoming more and more popular (Fleurence *et al.*, 2012). In other countries outside of Asia, *Ulva* starts to be recognized as a promising aquaculture candidate (Ray, 2006; Bolton *et al.*, 2009; Fleurence *et al.*, 2012; Gao *et al.*, 2017; Fernandes *et al.*, 2019). Pilot commercial systems have been installed in Brazil and the Netherlands to investigate the feasibility of *U. flexuosa* and *U. lactuca* (Ginneken and Vries, 2011; Castelar, Reis and dos Santos Calheiros, 2014). In South Africa, *Ulva* is grown as commercial on-land crop in combination with IMTA. Despite local ambiguities about *Ulva*-taxonomy, farmed species are most likely *U. rigida* and *U. lacutca* (Bolton *et al.*, 2009). In Israel, pond cultivation under a similar IMTA concept has been developed to produce a variety of cosmetic and health related products (Neori *et al.*, 2004; www.seakura.net, accessed 30/04/20). Furthermore, first attempts to cultivate *Ulva* offshore in the Mediterranean have been made (Chemodanov *et al.*, 2019). In Ílhavo, northern Portugal, the company ALGA+ ® produces *U. rigida* for cosmetics and food, as does the company La Huerta Marina in Huelva, southern Spain, (www.algaplus.pt; www.lahuertamarina.com, accessed 30/04/20). *Ulva*-production is often hindered by the degradation of the thallus following a reproductive event (compare chapter 1.2.1; Oza and Sreenivasa Rao, 1977). However, more effort has recently been directed towards the development of new sterile strains (Gao *et al.*, 2017; Waaland, 1979). Results have demonstrated that a sterile strain of *U. rigida* increases the productivity by preventing gamete production. Additionally, carbon capture and wastewater bioremediation capacity are higher due to rapid and sustained growth combined with elevated nutrient uptake. Furthermore, enhanced concentrations of PUFAs in sterile strains offer benefits for food applications (Gao *et al.*, 2017).

Besides cultivation, another sustainable option of obtaining *Ulva*-biomass arose in very recent years. Green tides, caused by eutrophication of estuarine areas, have become more frequent. These events have detrimental effects on coastal ecosystems and tourism. If not removed in time, they can produce toxic hydrogen sulphide. However, when predicted, the occurrence of green tides provides a unique opportunity to collect the biomass for industrial purposes (Smetacek and Zingone, 2013; Reisky *et al.*, 2019). In the past, the efficient use of green tides for extraction of bioactive compounds was hindered by missing knowledge about enzymatic pathways and tools for pre-digestion and extraction. In recent years, studies found that microbes equipped with specific lyases can successfully digest and convert the seaweed-polysaccharides into bioethanol (Reisky *et al.*, 2019). On the coast of Brittany, green tides are now harvested for the development of a range of bioproducts (Bolton *et al.*, 2009; https://www.olmix.com/news/ulvans-project, accessed 30/04/20).

Despite the growing potential of seaweeds, the rapid expansion of the industry could easily drop into similar pitfalls previously experienced in agriculture, fish, and shellfish farming. If not developed in a careful sustainable way, mistakes in seaweed farm management such as the illegal use of algicides and pesticides or the outbreak and subsequent spread of NIS could have detrimental effects on the surrounding marine ecosystems (Cottier-Cook *et al.*, 2017). Solely using sterile strains, placing the crops too close together or monoculture in general, would promote genetic impoverishment and leave the seaweeds more susceptible to disease transfer, consequently promoting the spread of pathogens (Cottier-Cook *et al.*, 2017). Therefore, it is of increasing importance for successful seaweed farming to consider the microbial component, especially bacterial.

1.3 Seaweed microbiomes

In the oceans, microbes are easily dispersed due to rapid diffusion of the cells in seawater. Therefore, they accumulate in microscale patches forming biofilms (Dittami *et al.*, 2016). These large, diverse microbial communities consisting of bacteria, archaea, and fungi are referred to as microbiomes. Like with most other eukaryotic organisms, microbial communities are also associated to algae (Egan *et al.*, 2012). Here, the focus lies on the bacterial component of microbiomes. Algae excrete exudates, extracellular products, outward from their thalli into a layer of surrounding seawater, the "phycosphere" (Bell and Mitchell, 1972), in which bacteria subsist on these exudates (Dittami *et al.*, 2016). In turn, these bacteria can enhance the metabolic abilities of the algae by the production of specific compounds on their side (Dittami, Eveillard and Tonon, 2014). Endophytic bacteria inhabit the thallus while epiphytic bacteria occupy the surface of its host. The bacterial community forming the microbiome can interact with its host in various ways. A wide range from beneficial (mutualistic) over neutral (commensal) to harmful (parasitic) relationships exist (Hollants *et al.*, 2013). Knowledge about important algae-bacterial interactions mainly exists in the context of phytoplankton (Amin, Parker and Armbrust, 2012). In diatoms, for instance, *Sulfitobacter* sp. promotes diatom cell division *via* secretion of the hormone indole-3-acetic acid (IAA) (Amin, Parker and Armbrust, 2012).

Similar bacterial-dependent interactions can be observed in seaweeds. Associated bacteria have been shown to be essential for growth by providing the host with important vitamins and promote the healthy development of seaweed morphology (Croft *et al.*, 2005; Spoerner *et al.*, 2012; Wichard *et al.*, 2015; Ghaderiardakani, Coates and Wichard, 2017). Moreover, they enable seaweeds to tolerate wider ranges of salinity (Dittami *et al.*, 2016) and contribute to host defence against herbivory and biofouling (Egan *et al.*, 2012). Additionally, it is suggested, that they facilitate the adaptation to environmental stressors (Aires, Serrão and Engelen, 2016).

It becomes increasingly evident that bacterial communities not only shape the seaweed-biology but also partly evolved together. Major groups of algae laterally acquired important metabolic processes from bacteria i.e. the cellulose synthesis in red algae (Singh and Reddy, 2014; Dittami *et al.*, 2016). Therefore, it is now widely recognized that bacteria and seaweed should be studied as one unit, referred to as "holobiont" (Rosenberg *et al.*, 2009).

1.4 *Ulva*-Holobiont

Ulva provides a unique opportunity to study microbiomes as the availability of sterile gametes facilitated the decoding of some of the bacterial-dependent processes (Spoerner *et al.*, 2012). Many have already been described in this genus and associated bacterial communities of some representative species are relatively well studied (Burke *et al.*, 2011; Egan *et al.*, 2012; Wichard *et al.*, 2015). Like in most other seaweeds, common high-level taxonomic groups of epiphytic bacteria associated to *Ulva* are *Gammaproteobacteria, Flavobacteriaceae, Rhodobacteriaceae, Sphingomonadaceae, Planctomycetacea,* as well as *Saprospiraceae* and *Actinobacteria* (Longford *et al.*, 2007; Staufenberger *et al.*, 2008; Burke *et al.*, 2011). The investigation of microbiomes of *U. australis* individuals by Tujula *et al.*, 2010 revealed that the bacterial community was dominated by *Alphaproteobacteria* and *Bacteroidetes*, while a large number of *Alphaproteobacteria* belonged to the *Roseobacter* clade (Tujula *et al.*, 2010).

In most Ulvales, bacteria from the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group and *Roseobacter* clade are responsible for the development of normal macroalgal morphology (Matsuo *et al.*, 2005; Spoerner *et al.*, 2012). *Maribacter* from the CFB group, for instance, produces thallusin, a compound necessary to induce germination (Matsuo *et al.*, 2005), whereas another enables the conversion of tryptophan into the phytohormone IAA, essential for growth (Fries and Āberg, 1978). Furthermore, bacteria also appear to be able to partly control the colonization process. Zoospores of *Ulva* appear to exploit a bacterial sensory system, named quorum sensing (QS) (Joint *et al.*, 2002). In QS a signal molecule, *N*-acylhomoserine lactones (AHLs) is produced by the bacteria which serves to attract bacteria until a threshold concentration that switches on target genes. Zoospores of *Ulva* appear to sense AHL and attach to specific bacterial cells in biofilms on surfaces (Joint *et al.*, 2002). In the case that preferable conditions for settlement of the spores are not met, germination can be inhibited by bacteria such as *Pseudoalteromonas tunicate*, *Shewanella oneidensis* and *Alteromonas* spp.. These taxa were shown to produce antialgal peptides to inhibit zoospore germination in *U. australis* and *U. lactuca* (Bhattarai *et al.*, 2007; Goecke *et al.*, 2010).

1.4.1 Specificity paradox

The question of what determines the bacterial composition of seaweed-microbiomes is an ongoing discussion. Generally, there are two controversial phenomena both supported by scientific evidence. Some studies revealed that each species of seaweed harbours its own unique community of associated bacteria, referred to as "host-specificity" (Egan *et al.*, 2012). In support of host-specificity, various studies showed that microbiomes belonging to one and the same seaweed species from different geographic sites were more similar to each other than to other coexisting species of the local community or the surrounding seawater (Lachnit *et al.*, 2009; de Oliveira *et al.*, 2012; Eigemann *et al.*, 2013; Aires, Serrão and Engelen, 2016). This suggests that the associated bacterial community is assembled due to the specific surface character and exudates of its host.

In contrast, other studies found that the microbiomes of three *Ulva* species were different among individuals of the same species and between species across space (ranging from a few meters to hundreds of meters distance) and time (Burke *et al.*, 2011; Roth-Schulze *et al.*, 2018). The authors proposed the competitive lottery theory (Sale, 1977) as an explanation, implying the composition of the microbiome is determined by chance. Nonetheless, both studies found a high similarity of functional genes within the microbiome of the same species, suggesting functional redundancy (*i.e.* many different bacterial species share the same functions). In summary, the composition of the associated bacterial community is suggested to be determined randomly through lottery and assembled based on function rather than taxonomy, suggesting "functional host-specificity".

However, the above studies were solely conducted with three different *Ulva* species, *U. australis*, *U. rigida* and *U. ohnoi*. Hence, the information might not be enough to suggest that there is generally no host-specificity within species of the genus *Ulva.* Even though not highlighted in the study of Roth-Schulze *et al.* (2018), the cladogram based on similarities of bacterial community composition shows evidence of host-specificity. In both cases of *U. rigida* and *U. australis*, samples grouped by host-species, even though collected from two different sites in Spain. However, samples were collected from two adjacent rockpools of each of the two countries (Spain and Australia). Given the fact that mixing of the water between the rockpools was most likely possible plus the small number of sampling sites, it is advisable to include a higher number of separated sampling sites to verify the results of the above-mentioned studies.

At the same time, biogeography seems to play a role in the determination of microbiomes as local environmental factors shape the bacterial composition to some degree (Campbell *et al.* 2015). Climate change-related stress, for instance, can disrupt the microbiome and result in a shift of the bacterial community (Aires *et al.*, 2018). In the above-mentioned study of Roth-Schulze *et al.* (2018), microbiomes of *U. australis* from Spain and Australia grouped by sampling site rather than host-species, suggesting the local environmental factors primarily influenced the microbiome composition . However, species of the study were identified *via* comparison of *rbCL* sequences with GenBank homologies. Considering the lack of accuracy of GenBank sequences, it is possible that *U. australis* individuals from the two different sites are actually different species.

Host-specificity in *Ulva* could be advantageous for scientific and commercial purposes. As for scientific advantages, host-specificity would suggest that the microbiome composition could be used as an additional tool for species delimitation. Furthermore, bacterial communities could be used to trace the origin of NIS of *Ulva*, as demonstrated in the case of *Caulerpa racemosa* (Aires *et al.*, 2013). In a commercial context, the discovery of host- specific bacterial interactions would mean the implementation of a new type of optimal growth performance indicator - the microbiome of *Ulva*-species. The host-mediated regulation of the microbiome, thus, constant exposure to beneficial bacterial taxa would mean a stable amount of bacteria-derived target compounds could be extracted with an increase in biomass accompanied by a decrease in farming effort, ultimately lowering costs.

On the other hand, a more lottery and/or functional host-specificity based assembly would facilitate microbiome engineering. The same bacterium or different bacteria with the same function could be used to manipulate microbiomes into favourable compositions across different species of *Ulva*. However, for this approach, controlled conditions are necessary to keep the bacterium of interest a stable member of the microbiome, which is especially challenging for epiphytic communities (Mueller and Sachs, 2015). At the same time, the manipulation of the microbiome would bare the risk of affecting the abundance and functions of other important bacterial members (Mueller and Sachs, 2015).

1.4.2 Particular bacterial groups

1.4.2.1 Vitamin B_{12} producers

Vitamin B_{12} (cobalamin), essential for the biosynthesis of methionine can only be synthesized by prokaryotes. Most eukaryotic organisms lack the enzyme to produce this vitamin *de novo*. Consequently, many seaweed species are dependent on an exogenous source for vitamin B_{12}. In 2005, Croft and colleagues found that this source is in fact cobalamin producing bacteria (i.e. *Halomonas* spp.). More than about one-third of the Chlorophyta surveyed by Croft and colleagues turned out to be vitamin B_{12} dependent. Very recently, another study underlined the mutualistic interaction in the synthesis of important B-vitamins as their cross-exchange was revealed between the picoeukaryotic alga *Ostreococcus tauri* and the bacterium *Dinoroseobacter shibae* (Cooper *et al.*, 2019). The same bacterium was also found in microbiomes of *U. rigida* (Ismail *et al.*, 2018).

Algae that require an external supply of vitamin B_{12} encode for the B_{12}-dependent methionine synthetase enzyme (METH) which is dependent on B_{12} as a co-factor, hence cannot produce it *de novo*. Whereas algae that are independent on an exogenous source for cobalamin, encode for the alternative methionine synthetase (METE) that does not need B_{12} as co-factor, hence can produce it *de novo* (Wagner-Döbler *et al.*, 2010; Cooper *et al.*, 2019). In fact, *U. compressa* is encoding for METH, rather than METE, implying that the alga is dependent on bacteria as a source of vitamin B_{12} synthesis for growth. (www.bioinformatics.psb.ugent.be/orcae/annotation/Ulvmu/current/UM066_0024.1; accessed 27th November 2019).

Considering the above it is likely that most species of *Ulva* host a variety and abundance of cobalamin producing bacteria. From a food industry point of view, these species would be a promising source of vitamin B_{12} as well as opportunity to elevate and harness the bacteria (Cavari and Grossowicz, 1977). Moreover, a high vitamin B_{12} content was shown to increase productivity in algae (Tandon, Jin and Huang, 2017), which would be of great advantage for cultivation. Asides *Halomonas* sp. and *Dinoroseobacter shibae*, several other taxa were found to produce vitamin B_{12}: *Aerobacter, Agrobacterium, Alcaligenes, Azotobacter, Bacillus, Clostridium, Corynebacterium, Flavobacterium, Micromonospora, Mycobacterium, Norcardia, Propionibacterium, Protaminobacter, Proteus, Pseudomonas, Rhizobium, Salmonella, Serratia, Streptomyces, Streptococcus* and *Xanthomonas* (Perlman 1959 in Martens et al., 2002). Industrially, mainly *Propionibacterium shermanii* and *Pseudomonas denitrificans* strains are employed for vitamin B_{12} production (Martens et al., 2002).

1.4.2.2 The ambivalent

As with terrestrial plants, seaweeds are susceptible to diseases caused by a wide range of microorganisms. Due to climate change-related stress, disease outbreaks in both the natural environment and in aquaculture have become more frequent (Cochrane *et al.*, 2009; Gachon *et al.*, 2010; Campbell *et al.*, 2011). Stress can disturb the seaweed-holobiont and potentially result in a shift from a healthy microbiome, with mainly beneficial or neutral bacteria, to a more vulnerable microbiome with more harmful bacteria (Vairappan *et al.*, 2001; Egan *et al.*, 2014; Aires *et al.*, 2018). Additionally, mismanagement in farming practices such as monoculture or cultivation in high densities has increased disease transmission in recent years (Cottier-Cook *et al.*, 2017). A severe outbreak that occurred in the Philippines between 2011 and 2013 as a large proportion of farmed *Kappaphycus* and *Eucheuma* was infected by the Ice-ice disease, causing an estimated loss of over US$ 310 million. The three bacterial pathogens that caused the bleaching of the macroalgal thallus were newly identified as members of the *Alteromonas*, *Aquimarina* and *Agarivorans* genera (Kumar *et al.*, 2016). Other common bacterial pathogens known to cause rot symptoms and galls in seaweeds include the taxa *Cytophaga, Flavobacterium, Pseudomonas, Pseudoalteromonas, Roseobacter, Saprospira* and *Vibrio* (Gachon *et al.*, 2010).

Associated pathogenic bacteria may be commensal or even beneficial under normal conditions but can get virulent when the host is exposed to stress such as temperature increase (Vairappan *et al.*, 2001; Egan *et al.*, 2012). This was observed in the case of the bleaching disease in *Delisea pulchra* (Case *et al.*, 2011). The red alga produces a range of furanones, secondary metabolites, that act as a defence against colonisation of unwanted epiphytic organisms and herbivores. During summer months, when the seawater temperature rises *D. pulchra* loses its protective furanones. That is when otherwise commensal associated bacteria of the *Roseobacter* clade, permanently present in the microbiome, become harmful and cause bleaching of the thallus. Therefore, temperature might play a crucial role in seaweed-holobiont health as an intact chemical defence can normally suppress the expression of virulence genes (Egan *et al.*, 2012).

It is difficult to tell when these opportunistic and potentially pathogenic bacteria become parasitic without investigating virulence genes. Nonetheless, pathogenicity is often linked with cell-wall degrading processes (Egan *et al.*, 2012, 2014). These bacteria may play a crucial role in disease progression by producing depolymerizing enzymes, which further weaken already damaged tissue (Egan *et al.*, 2014). Along with the causative agent of the perforation disease that affected experimental cultures of *Ulva* in Israel in 1989, an unidentified cell-wall degrading bacterium was also found (Colorni, 1989; Del Campo, García-Reina and Correa, 1998). Regarding the correlation between these two types of bacteria, this study will, in addition to potentially pathogenic taxa, also include cell-wall degrading taxa. Genera that have been linked with seaweed degradation and decay of algal blooms are for instance *Vibrio, Alteromonas, Cytophaga, Flavobacterium* and *Pseudoalteromonas* (Teeling *et al.*, 2012; Kopel *et al.*, 2016; Reisky *et al.*, 2019).

On the other hand, cell wall degrading bacterial taxa also represent a promising tool for the seaweed industry. The use of algae still is hindered by a limited knowledge of the microbial pathways to convert algal polysaccharides into fermentable oligo- and monosaccharides. A recent study by Reisky *et al.*, 2019 brought the industry a step closer to unlocking seaweeds for applications such as the fermentation of algal biomass into bioethanol or other valuable compounds. They described the metabolic pathway of an *Ulva*-associated bacterium from the Flavobacteriaceae family, *Formosa agariphila* to degrade ulvan, the main cell wall polysaccharide. Including other seaweed derived polysaccharides, such as alginates, agars and carrageenans, ulvans are widely used in the food industry. Unfortunately, it is resistant to fermentation or only weakly degraded by human intestinal bacteria (Michel and Macfarlane, 1996).

Over the last few years, the link between the dietary uptake in humans and livestock and the compositions of their gut microbiome has gained more attention (Turnbaugh *et al.*, 2007; Pluvinage *et al.*, 2018; Kazir *et al.*, 2019). New insights suggest that the digestive fate of these seaweed polysaccharides can be influenced (Pluvinage *et al.*, 2018). Generally, the gut microbiome is shaped by the type of food that is eaten, hence, the digestive system responses according to the type of sugars that must be processed. This opens the opportunity to manipulate the composition of the microbiome and select for specific bacteria that can degrade indigestible dietary carbohydrates from seaweeds. The authors propose that, metabolic pathways such as the agarose metabolism in a diet could be used to keep a probiotic bacterium of interest, involved in that metabolism, as a member of the microbiome. Moreover, the bacterium might eventually be laterally acquired as a permanent human intestinal symbiont as already proven in the gut flora of Japanese citizens and seagrass consuming iguanas (Sonnenburg, 2010; Hong *et al.*, 2011). This way valuable dietary compounds of the seaweed could become more bioavailable for humans and livestock (Pluvinage *et al.*, 2018).

For reasons of simplification and considering that both groups addressed in this study (potentially pathogenic bacteria and cell-wall degrading bacteria) can be advantageous or disadvantageous depending on the circumstance, here, they will be referred to by the collective term "the ambivalent".

1.5 Thesis aims and hypotheses

In the context of overpopulation and global change, providing alternative food sources for people and livestock and to sustainably manage our planet's resources is more important than ever. Through seaweed cultivation, not only adverse effects of conventional food production could be reduced but it could also contribute to solving many other problems associated with sustainability. However, in Europe, seaweeds are rarely produced, and industries rely on harvesting natural stocks to a large extent (Buschmann et al., 2017;

Ferdouse et al., 2018). In this light, it is crucial to continue research effort in exploring the diversity of culturable seaweeds and how to successfully and sustainably farm them.

Previous studies have collected phylogenetic data about the scientifically and commercially interesting *Ulva*-species occurring in the Ria Formosa lagoon in southern Portugal. Additional phylogenetic data, generated *via* barcoding based on the *tufA* marker gene, will extend the existing taxonomic knowledge about *Ulva*-species from the Ria Formosa in the south towards the open coast in the southwest. Thus, this work will contribute to further biodiversity insights about the green seaweed genus *Ulva* in Portuguese waters.

Considering the importance of the bacterial component for the seaweed, in this study, the combined holobiont-system is considered for addressing scientific questions. Up to date knowledge remains insufficient to evaluate what factor is primarily shaping the microbiome, the host, or the environment. Hence, it remains unclear if there is host-specificity or a combination of lottery and functional host-specificity, raising the question: *To what extent are the microbiomes of Ulva linked to the host-phylogeny and/or to the geographic region?* The reinvestigation of these question holds important implications for cultivation and use, as new insights could contribute to the optimisation of harnessing or controlling surface-associated microbial communities as well as lead to the development of additional tools for species delimitation and tracing of NIS.

Certain bacterial groups within the microbiome are of particular interest from the scientific as well as the industrial perspective. Vitamin B_{12} producing bacteria are essential for development and growth of the seaweed. Hence, they are important players to influence the biomass of the seaweed in both the natural environment as well as aquaculture settings. Since some species of *Ulva* exhibit faster growth than others, it is likely that the abundance and diversity of vitamin B_{12} producing bacteria is primarily influenced by the host. Another group of bacteria, potentially harmful members of the microbiome (here addressed as "the ambivalent"), should receive more attention, given that diseases are currently increasing in both aquaculture and the natural environment (Gachon et al., 2010; Egan et al., 2013). Since environmental factors can shift the microbiome towards more pathogenic and cell-wall degrading bacteria, it is suspected that the geographical region is primarily influencing the abundance and diversity of the ambivalent.

Altogether, three aspects of the commercially interesting green seaweed *Ulva* will be explored in this study: i) the biodiversity and phylogeny of the genus from different regions along the south and southwestern coast of Portugal, ii) to what degree the microbiome is linked to the host-species and the region, and iii) how the relative abundance of vitamin B_{12} producers and the ambivalent differs according to the host-species or region. In conclusion, these aspects will be viewed under the collective view of the lessons that can be learned for future cultivation and use of different *Ulva*-holobionts. Against this background, the following objectives were pursued, and hypotheses tested:

(1) DNA barcoding *via tufA* contributes to capturing the biodiversity of the genus *Ulva* in southern and southwestern Portugal

(2) Combined phylogeny offers new insights to what extent the microbiome is linked to the host-species and geographic region:

Hypothesis 1: The associated bacterial communities of *Ulva* are primarily host specific.

Hypothesis 2: There is a degree of region-specificity in the bacterial communities associated to *Ulva.*

(3) The abundance of certain bacterial groups in microbiomes of different *Ulva*-specimen and how they are influenced, affects the potential for cultivation and use:

Hypothesis 1: The abundance of vitamin B12 producing bacteria is rather influenced by the host-species, whereas the abundance of the ambivalent is rather influenced by the geographic region.

2 Material and methods

2.1 Fieldwork

2.1.1 Sampling sites

All samples were collected during low tide at the beginning of March 2019 along the southern and southwestern Portuguese coast. Specimens were sampled from eight sampling sites (Figure 3) across four regions within different habitats (Figure 2): a) coastal lagoon - Ria Formosa (R), b) intertidal pools – Albufeira (A and V), c) open west coast – south of Sines (W), and d) Sado estuary – Arrábida (Ar) .

Figure 2. Different habitats of the four sampling regions: a) soft bottom in Ria Formosa (Ilha de Faro, 36°59'53.4"N 7°58'40.5"W); b) intertidal pools in Albufeira (Praia dos Aveiros, 37°5'0,68"N8°13'53,45"W); c) rocky shore of open west coast (Praia da Ilha do Pessegueiro, 37°49'42.0"N 8°47'30.6"W); and d) sandy beach with boulders in Arrábida (Praia dos Galapos, 38°29'04.5"N 8°57'20.4"W).

The Ria Formosa lagoon (Figure 2a) is a 55 km long coastal lagoon in the Algarve, southern Portugal, approximately 16,3000 ha in size (Monteiro, 1989). It has been recognised as an important natural wetland with great economic value for tourism, aquaculture and fisheries. In 1987, it was declared a natural park and is now a Ramsar and Natura

2000 site (Ribeiro *et al.*, 2008). Generally, the lagoon is shallow with soft substrate, consisting of salt marsh and tideway channels; the latter of which has a maximum depth of 6 m at low tide. It has six inlets leading to the sea, both natural and artificial. The system has semi-diurnal tides, with 50-75% of water volume exchanged during each tide (ranging between 1.5-3.7 m) (Águas, 1986). Water temperatures vary from 12 – 28°C (Sprung, 1994). Samples in the region of Ria Formosa were collected in the area of Ramalhete (R1-3), field station of the Universidade do Algarve (37°00'19.4"N 7°58'02.8"W). Sample R4 was collected in the area of Quinta do Lago (37°00'19.4"N 7°58'02.8"W), whereas samples R5-12 and R22-26 were collected on Ilha de Faro (37°00'19.4"N 7°58'02.8"W). Precise descriptions of all sampling sites can be viewed in the meta data recorded in Appendix 1.

The Arrábida Marine Park (Figure 2d) is a stretch of protected coastline (53 km^2) on the west coast of Portugal, adjoining the Arrábida Nature Park, designated in 1998 (Horta e Costa *et al.*, 2013). It is situated between the Sado estuary with the city of Setúbal in the east and Cape Espichel in the west. The marine park plays an important role for commercial and recreational activities as it faces south and is therefore protected against north-west winds and waves (Horta e Costa *et al.*, 2013). As an oceanographic transition zone between cold Atlantic water and warmer Mediterranean waters, it frequently exhibits upwelling in summer months (Wooster *et al.*, 1976). The steep intertidal zone is built of subtidal rocky reefs with boulders originated from the erosion of the coast and mixed sandy (Figure 2d), creating a complex habitat for a diversity of fish, algae and invertebrate species. North and south of the park, extensive sandy shores prevail, adding to contrasting coastal features making this area a unique biogeographic region (Henriques et al. 1999, Gonçalves et al. 2003). Annual seawater temperatures range between 13.6 – 22.3°C (measured in Sesimbra, Setúbal; www.seatemperature.org; accessed on 21st November 2019). Samples Ar37-39 and Ar41-44 were collected at Praia da Figueirinha (38°29'06.2"N 8°56'32.5"W) and Praia dos Galapos (38°29'04.5"N 8°57'20.4"W), respectively (Figure 3a).

The two regions Albufeira coast, west of Faro, and the open west coast, south of Sines (Figure 3a), represent intermediate habitats between both the above-mentioned natural parks, Ria Formosa and Arrábida. While a wide area of the coast of Albufeira is dominated by shallow intertidal pools, building a coherent subtidal rocky reef of bed-and beachrock (Figure 2b), the west coast is more similar to Arrábida, with nestled beaches equipped with big boulders and intermitted sandy substrate (Figure 2d). Annual water temperatures of both regions range from 14.3 – 22.5°C (measured in Albufeira and Sines; www.seatemperature.org; accessed on 21st November 2019). Samples A13-20 and V21, were collected at Praia dos Aveiros (37°5'0,68"N8°13'53,45"W) and at the pier of Vilamoura, Praia da Rocha Baixinha (37,071999-8,12522'4), respectively (Figure 3a). Samples in the region of the open west coast, W28-30 and W34-36, were collected at Praia da Ilha do Pessegueiro, south of Sines (37°49'42.0"N 8°47'30.6"W) (Figure 3c).

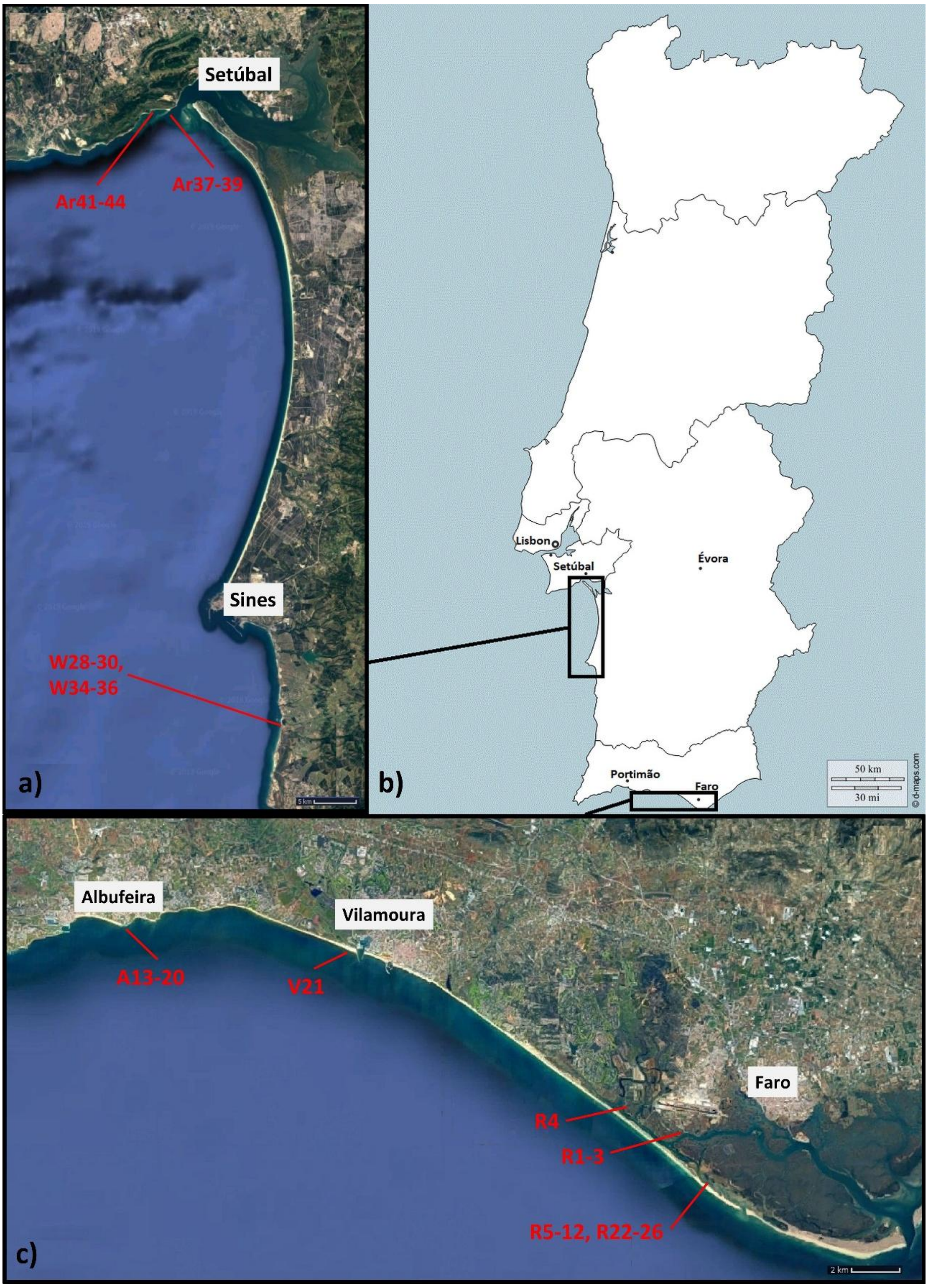

Figure 3. Map of Portugal (b) with two enlarged sections, showing the sampling sites along the southwestern (a) and southern Portuguese coast (c); a) shows three sampling sites in Arrábida and at the west coast, of samples: **Ar37-39** (Praia da Figueirinha, 38°29'06.2"N 8°56'32.5"W), **Ar41-44** (Praia de Galapos, 38°29'04.5"N 8°57'20.4"W), **W28-30** and **W34-36** (Praia da Ilha do Pessegueiro, 37°49'42.0"N 8°47'30.6"W); c) shows five sampling sites in the regions of Albufeira and Ria Formosa of samples: **A13-**

20 (Praia dos Aveiros, 37°5'0,68"N8°13'53,45"W), **V21** (Praia da Rocha Baixinha, 37,071999-8,12522'4), **R4** (Quinta do Lago, 37°00'19.4"N 7°58'02.8"W), **R1-3** (Ramalhete, 37°00'19.4"N 7°58'02.8"W), **R5-12** (Ilha de Faro, 36°59'53.4"N 7°58'40.5"W), **R22-26** (Ilha de Faro, 37°00'19.4"N 7°58'02.8"W); www.maps.google.de, accessed on 19th November, 2019).

2.1.2 Sampling procedure

As many morphologically different *Ulva*-specimens as possible, including probable replicates for each (macroscopic identification in the field was not possible due to cryptic morphology of *Ulva*), were collected for i) the preparation of an herbarium, ii) DNA-barcoding for species identification, and iii) microbiome analysis. Samples were collected at seawater temperatures ranging from 16.3 – 21°C and salinities from 31.1 – 40.1 PSU. Free floating or attached individuals were found either entirely surfaced or submerged until a depth of 70 cm. Individuals without signs of bleaching or evident damage of the thallus were collected.

The sampling protocol was consistent for all sampling sites. The seaweed thalli were carefully detached from their holdfasts or collected including their attachment if of transportable size. Samples were rinsed at the respective place of growth to avoid possible contamination by foreign seawater while removing epiphytes and invertebrates. Nitrile gloves were worn throughout the procedure. Subsequently, pieces of approximately 1 cm^2 where detached from the side or apical areas of the blades. In order to minimize the disruption of the microbiome and contamination with foreign bacteria, the separated sections of leaves were clipped with the lid of a previously autoclaved Eppendorf tube, excluding any parts of the thallus that was previously touched with gloves. The samples were immediately put on ice to minimize changes in the microbiome and directly transported to the laboratories of the University of Algarve. After clipping the microbiome samples, the same individuals of *Ulva* were stored in 3 L zip lock bags and kept in the laboratory for the creation of the herbarium.

Metadata (*i.e.* habitat characteristics and environmental data), was recorded for each of the eight sampling sites within the four regions (see Appendix 1). In the laboratory, samples were stored at -80°C to inhibit any bacterial activity. As further preparation of the samples for DNA-extraction, samples were placed into a lyophilizer (ModulyoD Freeze Dryer, Thermo Fisher Scientific^{TM}) at -44°C for 24 – 48 h for freeze-drying.

2.2 Herbarium

A single representative leaf of each *Ulva*-specimen, including the basal plate if possible, was collected for the herbarium (see Appendix 2). During the transport to the laboratory facilities all samples were kept in 1 litre bottles filled with seawater to preserve their natural form. Prior to further processing, the herbarium samples were stored in a Phytoclima ®. For the preservation on paper, leaves were placed in a water container with a submerged 100g/m^2 blank paper. While slowly withdrawing the paper from one side of the container, the desired position of the leaf was ensured using forceps. The wet papers were placed on cardboard with absorbent paper. A layer of wax paper was placed on the top surface to prevent the leaf sticking to the drying layers before covering it with additional paper. The samples where then pressed over the course of three days while replacing damp tissues with dry ones. Once completely dried, the herbarium pages were digitized using a scanner and were labelled with a scale and the corresponding sampling site as well as the sampling date.

2.3 Molecular biology

2.3.1 DNA extraction

Freeze-dried tissue samples were transferred under sterile conditions into round bottom Eppendorf tubes with tungsten beads and underwent beadbeating on a tissue grinder (TissueLyser - II QIAGEN™) at a maximum frequency of 30s^{-1} for 3 minutes. DNA was extracted from all 44 samples (Figure 4a) using the Quick–gDNA Miniprep extraction kit (Zymo Research^{TM}, Irvine, CA, USA) according to the manufacturers protocol for "Solid Tissue Samples" (page 4 of the instruction manual). As the first step of the protocol, 500 µl of Genomic Lysis Buffer, containing β-Mercaptoethanol at concentrations of 0.5%, was added to the grinded tissue and vortexed to obtain a homogenized liquid mixture of the samples. Subsequent steps were carried out as instructed in the manufacturer's manual. DNA was stored at -20°C in newly prepared Eppendorf tubes until further processing. Gloves were worn during the whole procedure to avoid contamination.

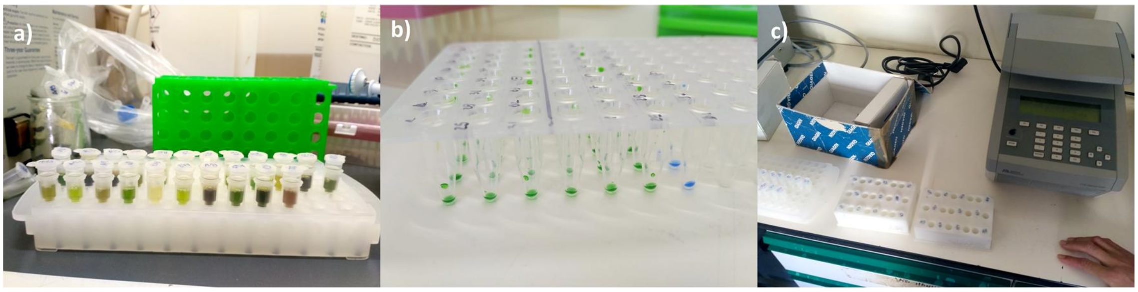

Figure 4. Molecular biological equipment: a) Eppendorf tubes with samples during DNA extraction; b) PCR plate and c) Thermocycler (Applied Biosystems$^{\text{TM}}$)

2.3.2 DNA barcoding

The barcoding was carried out using the chloroplast-encoded elongation factor and marker gene *tufA*. For PCR amplification of *tuf*A the primers *tuf*AF (5'-TGAAACAGAAMAWCGTCATTATGC-3') and *tuf*AR (5'-CCTTCNCGAATMGCRAAWCGC-3') (Famà *et al.*, 2002) were used. The reaction volume of 20 µl PCR-Mastermix was prepared for a total of 70 samples, consisting of 66 *Ulva* sp. samples (of which 44 were used in this study), two positive controls of *Caulerpa* sp. and a negative control. The PCR reaction was prepared for a total volume of 20 µl consisting of 1x of the 2 PCR Buffer mix (already containing $MgCl_2$), 0.25 mM of each dNTP, 0.2 µM of *tufA*F and *tufA*R, 0.2 µl of *Taq* DNA Polymerase (Advantage R2; Clontech™, Mountain View, CA, USA) and 1 µl of the DNA template (without dilution). The remaining volume was filled out with water (Sigma-Aldrich®) and loaded into the PCR plate (Figure 4b). All PCR-equipment was previously placed under UV light to avoid DNA contamination. The reaction took place under the following PCR profile: initial denaturation at 95°C for 5 minutes, 37 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute. The final extension was conducted at 72°C for 5 minutes. Amplification success of the target region was checked in a 1% gel-electrophoresis (using Tris-EDTA-Acetic acid and regular grade agarose). The lanes were loaded with 5 µl of PCR product, 2 µl loading dye and 1 µl gel red (dilution 1/500). Successfully amplified PCR products were then outsourced for Sanger sequencing and further visualized and edited using Geneious (http://www.geneious.com, Kearse *et al.*, 2012).

2.3.3 16S rRNA amplification of the microbiome

The total bacterial *16S rRNA* gene was amplified by PCR using the universal primers 27F and 1492R with the following changes to the original protocol (Lane, 1991): an initial denaturation at 95ºC for 2 min, 35 cycles of denaturation at 95ºC for 20 s, annealing at 55ºC for 20 s, and extension at 72ºC for 90 s, with a final extension was at 72ºC for 3 min. The 25 ml reaction mixture contained 250 mM dNTPs, 0.6 mM of each primer, 1x 2 PCR buffer mix, 2 ml of template DNA (with a final concentration of about 10 ng/ml) and 0.3 ml of *Taq* DNA polymerase (Advantage R2; Clontech™, Mountain View, CA, USA). As before, all PCR-equipment was previously placed under UV light eliminate any chance of DNA contamination. The PCR was carried out in a thermocycler (Applied Biosystems^{TM}; Figure 4c) set to the following temperature profile: an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 20 s, primer annealing at 55°C for 20 s, and extension at 72°C for 90 s. Final elongation was at 72°C for 3 min. In order to ensure the successful amplification of the 1400 bp DNA-fragment, all PCR products were checked by gel electrophoresis as described previously.

Amplified DNA was sent to Molecular Research (MR DNA; Shallowater, Texas, USA) where a nested PCR was performed prior to sequencing. The modified 8 bp key-tagged primer 799F along with the reverse primer 1193R, covering the regions V5-V7 from 16S-rRNA, amplifying a fragment of ~400 bp, were used to avoid chloroplast cross amplification (Bodenhausen, Horton & Bergelson, 2013). PCR conditions were as followed: 95ºC for 3 min, 10 cycles of 95ºC for 20 s, 50ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC for 3 min. Samples were pooled together in equal proportions based on their molecular weight (calculated based on the size of the amplicon) and DNA concentrations (using Qubit; Invitrogen, Carlsbad, CA, USA) and purified using calibrated Agencourt AMPure XP beads. DNA libraries were prepared by following the Illumina TruSeq DNA library preparation protocol. The paired-end (2x250 bp) sequencing was performed at Molecular Research (MR DNA; Shallowater, Texas, USA) on a MiSeq following the manufacturers guidelines.

2.4 Sequence analysis and bioinformatics

2.4.1 DNA barcoding and phylogenetic analysis

Ulva samples were processed for species delimitation and identification based on the *tufA* marker gene. Sequencing data was manually edited and assembled in the bioinformatics software and molecular analysis program Geneious Prime (v. 2019.2.1). Subsequently, the nucleotide sequences obtained were evaluated using the Basic Local Alignment Search Tool (BLAST). Where comparison of the nucleotide sequences with the database showed unclear results, peer reviewed sequences of a previous study on *Ulva*-taxonomy from the Baltic coast (Steinhagen, Karez and Weinberger, 2018) were used for the comparison. The Global Alignment with free end gaps and Cost Matrix of 65% similarity (5.0/4.0) was chosen as alignment type. A phylogenetic tree, including with the above-mentioned sequences, was created and used for species delimitation. Pairwise distances of the *tuf*A sequences between species were generated by Bayesian inference (BI). The analysis was conducted with MrBayes (v. 3.2.6) (Huelsenbeck and Ronquist, 2001) using the Markov chain Monte Carlo (MCMC) method with four heated chains of 1,100,000 length and temperature of 0.2; the subsampling frequency was 200, for a burn-in length of 100,000. The BI phylogenetic tree was rooted by the outgroup *Ulvales* sp. (Sample R4) and implemented with Geneious Prime (v. 2019.2.1). The posterior probabilities of the branches (between 0 and 1) were considered a measure of support, and values below 0.95 were considered with low support. Delimited species names where then used as a factor for further statistical analysis in PRIMER-E (v. 6.1.11).

2.4.2 Microbiome analysis

A total of 3,204,094 partial 16S rRNA gene sequences were obtained from the 44 samples. The pipeline Quantitative Insights into Microbial Ecology (QIIME version 1.8.0; Caporaso *et al.*, 2010) was used as quality control and to cluster the sequences into Operational Taxonomic Units (OTUs). Selected high-quality sequences were clustered into OTUs within reads using the open-reference OTU picking method. Representative sequences for each OTU were selected using the "most-abundant" method and OTU sequence alignment was carried out using PyNAST (Caporaso *et al.*, 2010). Taxonomic assignments were done using the UCLUST method (Edgar, 2010) with a 97% confidence threshold. To assign each OTU to the closest matching described taxon, searches were performed against the SILVA taxonomy database (version 128) for16S rRNA (Quast *et al.*, 2013),and sequences were putatively assigned to a described taxon with a minimum threshold of 0.001 (default value). Eukaryotes (i.e., chloroplasts and mitochondria) matching sequences and unassigned sequences were excluded from the OTU table in downstream analyses as well as rare OTUs (singletons and doubletons). The OTU table was rarefied to the minimum number of sequences (37,480) to be used in all the statistical analysis.

2.5 Visualisation and statistical analysis

For multivariate analysis of the bacterial communities, OTU tables for each sample with different regions and species as factors were imported into PRIMER-E (v. 6.1.11). The program was used to calculate a Bray-Curtis similarity matrix after square root transformation, which was used to build a dendrogram for hierarchical clustering (group-average linking) of OTU counts. The phylogenetic data of the microbiome and species of *Ulva* were joint to construct a mirrored phylogenetic tree of both data sets using Microsoft PowerPoint. The bacterial composition was visualized by a canonical analysis of principal coordinates (CAP) with the PERMANOVA+ (v. 1.0.1) add on for PRIMER-E (v. 6.1.11) (Anderson, Gorley and Clarke, 2008). In order to visualise and examine the influence of certain variables, OTUs most contributing to dissimilarities between groupings, as well as environmental data (temperature and salinity) were used as correlation factors. In order to identify the ten most abundant bacterial orders among the different *Ulva* species and regions, a stack chart was constructed using Microsoft Excel. The same program was used to visualize alpha diversity of associated bacterial communities (Shannon Wiener Index and Species Richness) and to construct bar charts the relative abundances of particular bacterial groups (vitamin B_{12} producers and the ambivalent).

For statistical analysis permutational multivariate analyses of variances (PERMANOVA) were carried out in PERMANOVA+ for PRIMER-E (v. 6.1.11). A two-factorial PERMANOVA main test was performed with region and species as fixed factors under 999 random permutations of residuals. *P*-values were considered significant with an alpha level of 5% (P_{Perm}), and for low numbers of permutations (< 800) the Monte Carlo test was applied (P_{MC}). Posteriori pairwise PERMANOVA tests with the combined factor of region and species were carried out for both levels, species and region, to identify differences between factor levels. For further in-depth analysis, a similarity percentage breakdown (SIMPER) was conducted to identify the OTUs contributing most to dissimilarities between the factors.

3 Results

3.1 Molecular data and phylogenetic analysis of *Ulva*

Altogether, 43 of the 44 genetically processed samples were successfully sequenced for species delimitation and identification based on the *tuf*A marker gene. Five samples where not considered for further analysis due to their identification as the red seaweed *Porphyra umbilicalis*. The high-quality consensus sequences of the remaining 39 samples, assigned to the genus *Ulva* and order *Ulvales* were used for further phylogenetic analysis.

In total, six distinct species of the genus *Ulva* could be delimited: *U. australis*, *U. compressa*, *U. californica/flexuosa*, *U. rigida*, *Ulva* sp.1 and *Ulva* sp.2 (Figure 5a). Based on peer reviewed and GenBank sequences, all but two species (*Ulva* sp.1 and *Ulva* sp.2) were successfully identified. The phylogenetic tree separated the investigated sequences into 7 entities. The first entity, containing sequences belonging to the outgroup, was identified as the order *Ulvales* sp. from the Ria Formosa lagoon. The outgroup clearly separated from all the remaining *Ulva* entities. *U. australis* was exclusively found in the region of Arrábida. All sequences of the third entity were allocated to *U. compressa* which was found in the Ria Formosa and the west coast. Each region exhibited a different morphotype; tubular and blade-like fronds, respectively. *U. californica* and *U. flexuosa*, from Arrábida and the Ria Formosa, respectively, were considered a species complex. *U. rigida* was found in all regions despite Arrábida and the only species found in the region of Albufeira. The two species, *Ulva* sp.2 from the Ria Formosa and *Ulva* sp.1 from the West coast could not be identified, neither by the peer reviewed nor by GenBank sequences. Despite the difference in their sequences, according to GenBank sequences both species were most similar to *Ulva lactuca* (Grade score ~ 98%). However, this was not reflected in the phylogenetic analysis in which *U. rigida* was the closest related species (support values < 70; Figure 5a). Detailed descriptions of respective species and their morphotypes can be viewed in the herbarium (Appendix 2).

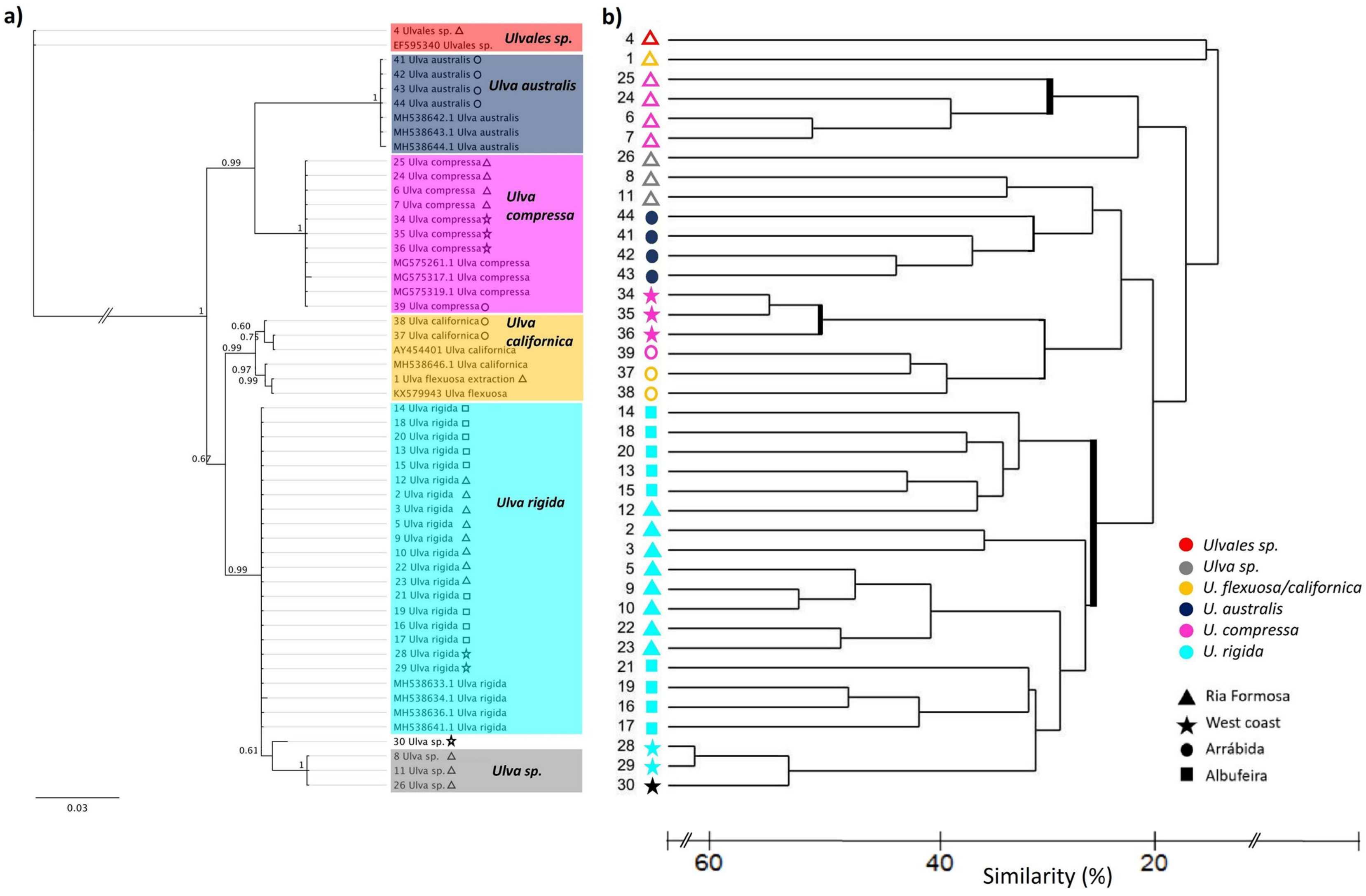
a)
4 Ulvales sp. △
EF595340 Ulvales sp.
Ulvales sp.
41 Ulva australis ○
42 Ulva australis ○
43 Ulva australis ○
44 Ulva australis ○
MH538642.1 Ulva australis
MH538643.1 Ulva australis
MH538644.1 Ulva australis
Ulva australis
25 Ulva compressa △
24 Ulva compressa △
6 Ulva compressa △
7 Ulva compressa △
34 Ulva compressa ☆
35 Ulva compressa ☆
36 Ulva compressa ☆
MG575261.1 Ulva compressa
MG575317.1 Ulva compressa
MG575319.1 Ulva compressa
39 Ulva compressa ○
Ulva compressa
38 Ulva californica ○
37 Ulva californica ○
AY454401 Ulva californica
MH538646.1 Ulva californica
1 Ulva flexuosa extraction △
KX579943 Ulva flexuosa
Ulva californica
14 Ulva rigida □
18 Ulva rigida □
20 Ulva rigida □
13 Ulva rigida □
15 Ulva rigida □
12 Ulva rigida △
2 Ulva rigida △
3 Ulva rigida △
5 Ulva rigida △
9 Ulva rigida △
10 Ulva rigida △
22 Ulva rigida △
23 Ulva rigida △
21 Ulva rigida □
19 Ulva rigida □
16 Ulva rigida □
17 Ulva rigida □
28 Ulva rigida ☆
29 Ulva rigida ☆
MH538633.1 Ulva rigida
MH538634.1 Ulva rigida
MH538636.1 Ulva rigida
MH538641.1 Ulva rigida
Ulva rigida
30 Ulva sp. ☆
8 Ulva sp. △
11 Ulva sp. △
26 Ulva sp. △
Ulva sp.
1
0.99
1
1
0.60
0.75
0.99
0.97
0.99
0.67
0.99
0.61
1
0.03
b)
4
1
25
24
6
7
26
8
11
44
41
42
43
34
35
36
39
37
38
14
18
20
13
15
12
2
3
5
9
10
22
23
21
19
16
17
28
29
30
60
40
20
Similarity (%)
● *Ulvales sp.*
● *Ulva sp.*
● *U. flexuosa/californica*
● *U. australis*
● *U. compressa*
● *U. rigida*
▲ Ria Formosa
★ West coast
● Arrábida
■ Albufeira

Figure 5. Mirrored phylogenies of *Ulva*, shown as a) BI phylogenetic tree using MCMC (MrBayes) based on *tuf*A sequences, delimiting the different species; and b) cladogram of their respective associated microbiomes based on a Bray-Curtis similarities of OTU abundances. GeneBank sequence indications start with letters "A","E", "K", whereas peer reviewed sequences start with "MG" and "MH". Support values of branches correspond to posterior probabilities. Branch lengths are drawn proportional to the amount of sequence differences. Colours depicted in the legend (right side of the figure) indicate *Ulva* species, symbols indicate regions, whereas outlined vs filled symbols indicates tubular or blade-like morphotype, respectively. Black bars show significant differences between and within respective groups.

3.2 Microbiome analysis

The complete dataset of 16s rRNA sequences resulted in a total of 2,583,159 high-quality sequences (after quality control, *i.e.* removal of unassigned sequences, rare OTUs and eukaryotes matching sequences) belonging to a total of 34,002 unique OTUs. The number of sequences obtained per sample ranged from 104,534 to 37,480. All samples reached a similar level of OTU richness and the rarefaction curves were close to saturation at the maximum number of reads (Figure 6). In all analyses, the rarefied dataset with the minimum number of 37,480 sequences was used.

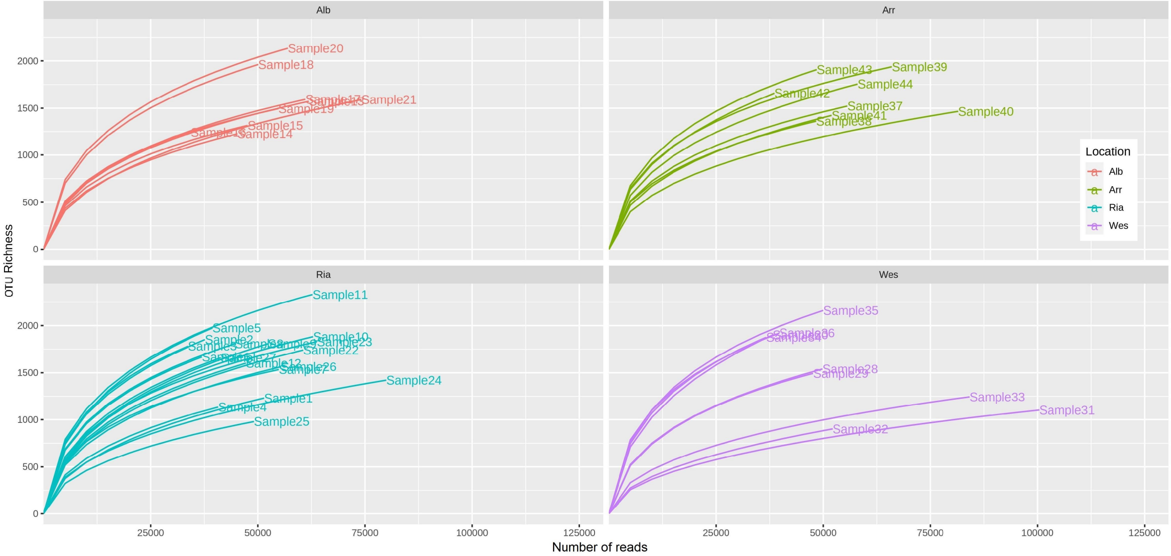

Figure 6. Rarefaction curve, showing the number of OTUs (OTU richness) detected *vs* the number of sequences per sample in each of four regions (Alb – Albufeira, Arr – Arrábida, Ria – Ria Formosa, Wes – West coast).

3.2.1 Bacterial diversity and composition

Considering that not all *Ulva* species were found in each region and numbers of replicate samples for each species varied greatly, all applied statistics using the single factors "Re" and "Sp" were masked by the effect of unequal distribution. Therefore, only the combined factor "RexSpe" was considered for further analyses. Alpha-diversity of *Ulva*-microbiomes was expressed by species richness and the Shannon Wiener index (Figure 7). Species richness of *U. compressa* microbiomes was higher at the West coast compared to those at the Ria Formosa and Arrábida as well as compared to *U. rigida* from the West coast (Table 1, Fig. 7). Within the interaction of Region and Species (combined factor "RexSpe") for the Shannon Wiener index, only low to no significant differences were found. However, within the combined factor for species richness, significant differences were recorded (Table 1).

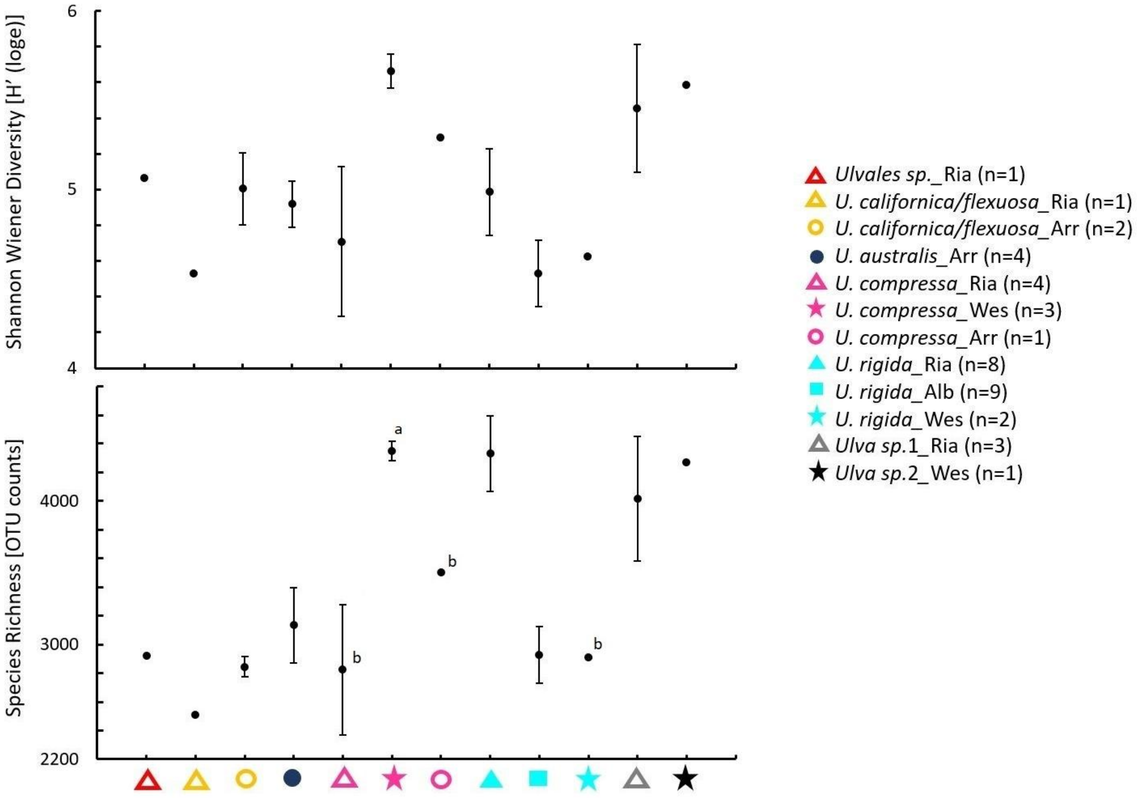

Figure 7. Alpha diversity of *Ulva*-associated microbiomes, expressed as Shannon Wiener index (top) and Species Richness (bottom). As in previously figures, colours indicate species of *Ulva*, symbols indicate regions and outlined *vs* filled indicate morphotypes. Significant differences are indicated as letters, no labels correspond to no significance, whereas error bars show standard errors.

Table 1. PERMANOVA table of main tests for alpha diversity (Shannon Wiener index and species richness), as well as pair-wise tests for the combined factor "RexSp" in species richness. Significant *P*-values used are highlighted in bold.

PERMANOVA main tests	
Shannon Wiener index	$\boldsymbol{P_{\mathrm{perm}}}$
Re	0.705
Sp	0.668
RexSp	**0.047**
Species richness	
Re	0.614
Sp	0.301
RexSp	**0.011**

PERMANOVA pair-wise tests for species richness	
Term 'RexSp' for pairs of levels of factor 'Region'	$\boldsymbol{P_{MC}}$
Within level 'Wes' of factor 'Region'	
Ulva rigida, Ulva compressa	**0.000**
Ulva rigida, Ulva sp.2	**0.003**
Within level 'Ulva compressa' of factor 'Species'	
Ria, Wes	**0.040**
Wes, Arr	**0.025**

The 16S rRNA gene libraries of *Ulva* predominantly consisted of sequences from the bacterial phyla Bacteroidetes (mainly from the Bacteroidia class), Proteobacteria (mainly Alpha- and Gammaproteobacteria), Planctomycetes (mainly Planctomycetia), and Actinobacteria (mainly Acidimicrobiia). Across all examined *Ulva* species, the ten most abundant orders were Flavobacteriales (31.5 %), Rhodobacterales (18.6 %), Pirellulales (18.0 %), Caulobacterales (11.4 %), Chitinophagales (5.6 %), Thiohalorhabdales (6.7 %), Cellvibrionales (3.5 %), Alteromonadales (2.2 %), as well as Sphingomonadales (1.6 %) and Microtrichales (0.9 %) (). The orders Flavobacteriales, Rhodobacterales, Pirellulales and Caulobacterales were most abundant, contributing up to 90 % of the ten most abundant orders.

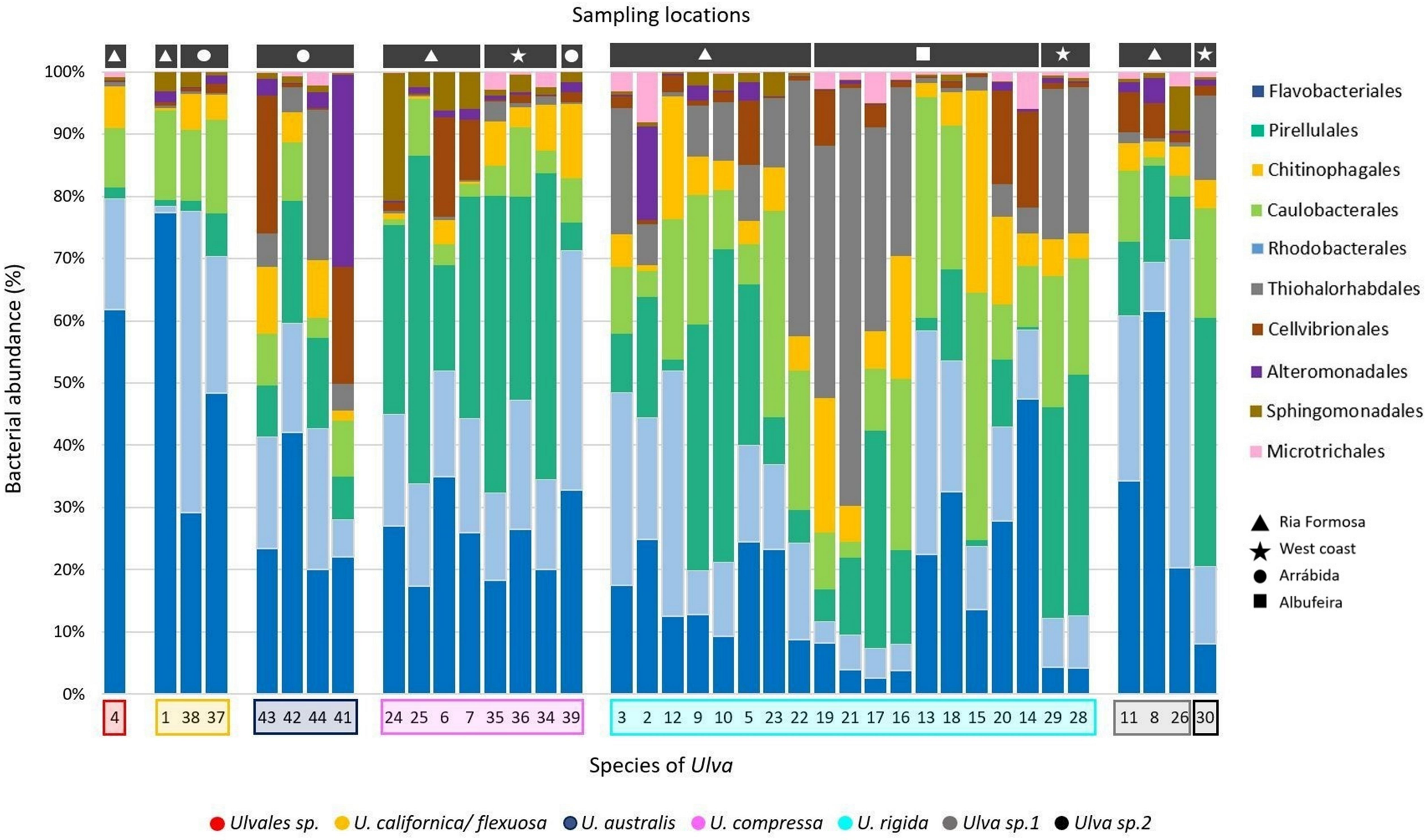

Figure 8. Relative abundance of the ten most abundant bacterial orders associated to seven *Ulva* species (x-axis colours) collected from four regions (top symbols) along the southwest coast of Portugal.

Microbiomes of *Ulva* appeared to be distinctive in their composition as at least two bacterial orders were characteristic for each species. Associated bacterial communities of the species complex *U. californica/flexuosa* seemed to be especially dominated by *Flavobacteriales* (51.5 %) when compared to the other *Ulva*-species. Furthermore, their microbiomes were high in *Rhodobacterales* (24.0%) and *Caulobacterales* (13.6%). The otherwise abundant *Pirellulales* seemed to be less well represented in this species (3.1 %) (Figure 8). *U. australis* was dominated by *Flavobacteriales* (26.8%) and *Rhodobacterales* (16.0%). The bacterial families *Cellvibrionales* (10.5%) and *Alteromonadales* (9.2%) appered to be most abundant in this species of *Ulva*. *Pirellulales* (33.2%) were very abundant in *U. compressa* samples. Moreover, this *Ulva* species seemed to have the highest abundance of *Sphingomonadales* (4.7%) of all *Ulva*-species.

In microbiomes of *U. rigida*, the four most abundant orders mentioned above were present in relatively equal proportions. Bacterial orders of *Thiohalorhabdales* (17.6%) and *Chitinophagales* (9.2%) as well as *Microtrichales* (1.7%) seemed to be the highest in this species. Unidentified *Ulva* sp.1 was dominated by *Flavobacteriales* (30.6%) and *Rhodobacterales* (25.1%), which seemed to be the most abundant in this *Ulva*-species compared to the other ones. Unidentified *Ulva* sp.2 contained the highest numbers of *Pirellulales* (39.8%) of all species of *Ulva* (Figure 8).

Regional differences were recorded between samples of *U. compressa* from the Ria Formosa and those from the other two regions (West coast and Arrábida) as the former seemed to be more abundant in Sphingomonadales (Figure 8). *Microtrichales* in samples of the West coast, were negligible in samples from the Ria Formosa and Arrábida. In the case of *U. rigida*, *Thiohalorhabdales* appeared to be especially well represented in a subgroup of samples from Albufeira, as well as in both samples from the West coast (Figure 8).

3.2.2 Host- and/or region-specificity

The degree of host-specificity and/or region-specificity of *Ulva*-associated microbiomes was assessed by comparing their similarity-based clustering according to species and region (Figure 5b and Figure 9). PERMANOVA results showed a statistically significant interaction between region and species ("RexSp", Table 2).

Bacterial communities of *U. compressa* from the Ria Formosa differed from those from the West coast (Table 2, Figure 5b). This difference was mainly due to almost 20 times more *Blastopirellula* from the family of Pirellulaceae (contribution 0.4%, SIMPER analysis, Table 3) in microbiomes of *U. compressa* from the West coast compared to conspecifics from the Ria Formosa. Bacterial communities of *U. compressa* from the West coast clustered together with the microbiome of the same species, originating from Arrábida, as well as microbiomes of *U. californica/flexuosa* from the same region (Figure 5b). However, in the canonical analysis of principal components (CAP) microbiomes of *U. compressa* clearly clustered according to host-species (Figure 9). OTUs exhibiting a strong positive vectorial correlation with this species of *Ulva* were members of *Rhodopirellula* from the family of Pirellulaceae (vector 1 and 2, New.CleanUp.ReferenceOTU8863 and New.CleanUp.ReferenceOTU31502, Figure 9).

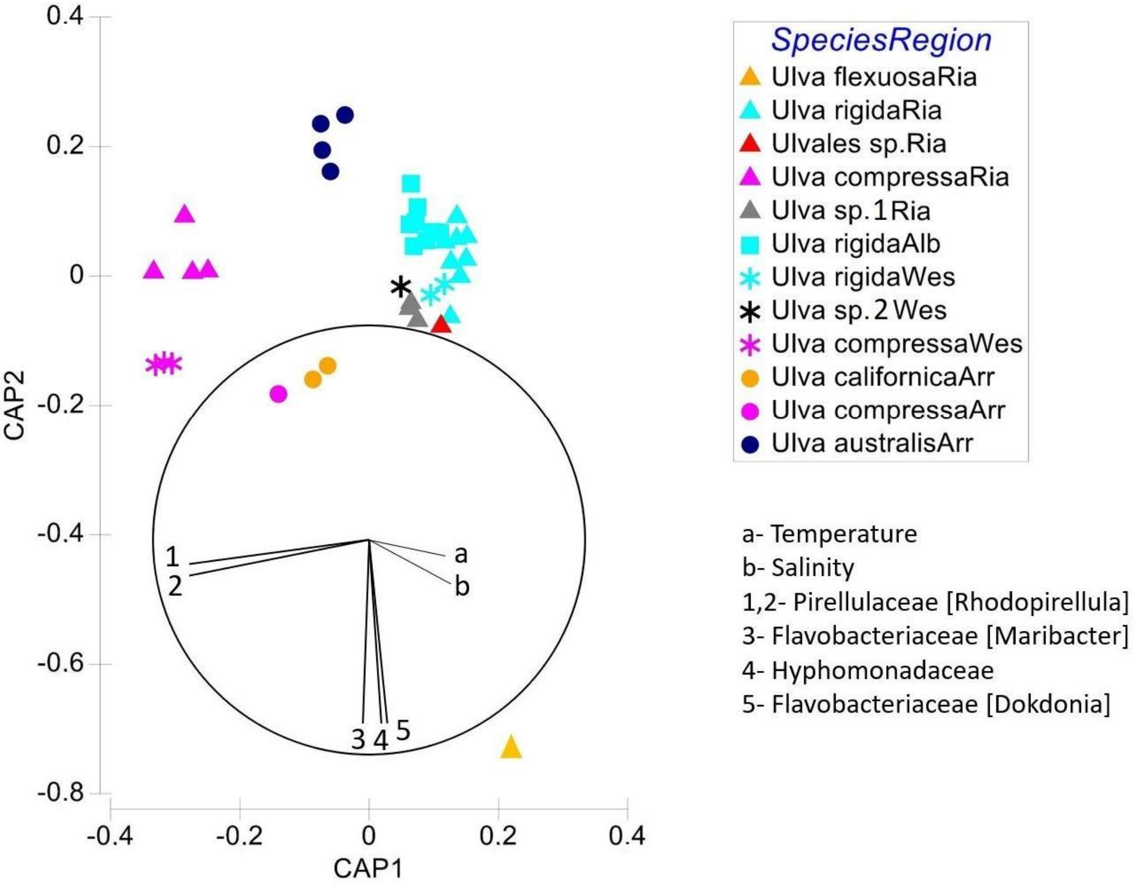

Figure 9. Canonical Analysis of Principle coordinates (CAP) (based on Bray–Curtis distances of OTU abundances) of associated bacterial communities of different *Ulva* species (colours) from different regions (symbols). Vectors show Pearson correlations (> 0.84) with OTUs (1-5) and environmental factors (a, b), with the circle representing the maximum correlation of 1.

All microbiomes associated to *U. rigida* from the three different regions (Ria Formosa, Albufeira and the west coast) grouped by host-species at approximately 25%

similarity (Figure 5b). This pattern was also supported by the CAP, as all microbiomes of *U. rigida* clustered together with a strong negative correlation of members of *Rhodopirellula* from the Pirellulaceae (vector 1 and 2, OTU IDs see above, Figure 9). However, at around 30% similarity, bacterial communities of *U. rigida* also showed a pattern grouped by region (Figure 5b). There was a significant difference between bacterial communities from the Ria Formosa and Albufeira, the Ria Formosa and the west coast, as well as between communities from the west coast and Albufeira (Table 2). The main difference between *U. rigida* from the Ria Formosa and Albufeira was due to slightly more *Granulosicoccus* (Thiohalorhabdaceae) in microbiomes of *U. rigida* from the Ria Formosa than from Albufeira (Table 3. SIMPER analysis of percentages for combined factor "RexSp"). The difference between *U. rigida* from the Ria Formosa and the West coast was mainly due to almost six times more *Loktanella* (Rhodobacteraceae) in microbiomes from the Ria Formosa (Table 3). Bacterial communities of *U. rigida* from the West coast were around one half more abundant in *Granulosicoccus* than in microbiomes of their conspecifics from Albufeira (Table 3).

Although, pair-wise differences in microbiomes among *Ulva* species could not be detected in all cases as explained previously, the distribution and replication of *U. rigida* and *U. compressa* samples was enough to test for differences within the combined factor "RexSp" of both species. Within the Ria Formosa, bacterial communities of *U. rigida* differed from the communities of *U. compressa* (Table 2). The difference was mainly due to almost five times more *Blastopirellula* in *U. compressa* compared to *U. rigida* (Table 3). The difference within the West coast between bacterial communities of *U. rigida* and *U. compressa* (Table 3) was, again, due to *Blastopirellula*, which was almost four times more abundant in *U. rigida* compared to *U. compressa* (Table 3).

For the remaining species *U. californica/flexuosa*, *U. australis*, *Ulva* sp.1 and *Ulva* sp.2, no statistical results could be obtained. However, notable trends within the clustering of their associated bacterial communities was registered. Microbiomes of *U. australis*, found in Arrábida, formed an individual cluster in both analyses (Figure 5b and Figure 9). Vectors depicted in the CAP indicate a strong negative correlation of this species of *Ulva* with *Maribacter* (New.CleanUp.ReferenceOTU884475, vector 3) and *Dokdonia* (CP002528.2383976.2385490, vector 5) from the Flavobacteriaceae, as well as Hyphomonadaceae (New.CleanUp.ReferenceOTU165513, vector 4) (Figure 9). Microbiomes of *U. californica/flexuosa* and *U. compressa* from the same region, clearly separated from the above with a weaker negative correlation with the same OTUs (vector 3, 4 and 5;

Figure 9). Sample 1 (*U. californica/flexuosa*) originated from an enclosed artificial pond of the Ramalhete fieldstation in the Ria Formosa. Considering that it was the only one to exhibit a strong positive correlation with the above-mentioned OTUs, it is assumed as this sample alone is pivotal for the correlation. Furthermore, it also clustered separately in the cladogram (Table 2b), and, therefore, can be considered an outlier with a distinct microbiome.

Table 2. PERMANOVA table of main test and pair-wise differences between microbiome-similarities (*i.e.* OTU abundances) of *Ulva* species. *P*-values used are highlighted in bold.

PERMANOVA main test for microbiome similarity	P_{perm}	**Unique perms**	P_{MC}
Re	**0.001**	999	0.001
Sp	**0.001**	997	0.001
RexSp	**0.002**	998	0.005
PERMANOVA pair-wise tests			
Term 'RexSp' for pairs of levels of factor 'Species'			
Within level 'Ria' of factor 'Region'			
Ulva rigida, Ulva compressa	0.001	422	**0.005**
Within level 'Wes' of factor 'Region'			
Ulva rigida, Ulva compressa	0.097	10	**0.036**
Term 'RexSp' for pairs of levels of factor 'Region'			
Within level '*Ulva rigida*' of factor 'Species'			
Ria, Alb	**0.001**	979	0.017
Ria, Wes	0.025	45	**0.041**
Alb, Wes	0.017	55	**0.042**
Within level '*Ulva compressa*' of factor 'Species'			
Ria, Wes	0.032	35	**0.031**

Table 3. SIMPER analysis of percentages for combined factor "RexSp" to determine OTUs contributing most to dissimilarities between microbiomes of the different species of *Ulva* and from different regions.

SIMPER of RexSp (One-Way Analysis)

Family (Genus); OTU ID	**Av. abundance**	**Av. abundance**	**Contribution**
Pirellulaceae (*Blastopirellula*); FN822210.1.1536	*U. rigida* - Ria 13.2	*U. compressa* - Ria 63.02	0.6
Rhodobacteraceae (*Loktanella*); JX984093.1.1225	*U. rigida* - Ria 50.3	*U. rigida* - Wes 8.6	0.5
Pirellulaceae (*Blastopirellula*); KX213854.1.1372	*U. compressa* - Ria 2.0	*U. compressa* - Wes 39.1	0.4
Thiohalorhabdaceae (*Granulosicoccus*); JF344155.1.1507	*U. rigida* - Ria 49.1	*U. rigida* - Alb 47.3	0.5
Thiohalorhabdaceae (*Granulosicoccus*); JF344155.1.1507	*U. rigida* - Alb 47.3	*U. rigida* - Wes 67.1	0.5
Pirellulaceae (*Blastopirellula*); DQ269069.1.1398	*U. rigida* - Wes 73.1	*U. compressa* - Wes 18.5	0.6

Microbiomes of *Ulvales* sp. (sample 4) and *Ulva* sp.1 (sample 26) both from the Ria Formosa region, clustered separately in the cladogram (Figure 5b). In the CAP, however, the microbiome of *Ulvales* sp. and all the bacterial communities of *Ulva* sp.1 grouped together with the ones of *U. rigida*, with a strong negative correlation of the above-mentioned *Rhodopirellula* (vector 1 and 2, Figure 9). In coherence with the mirrored tree, the microbiome belonging to *Ulva* sp.2 (sample 30) also clustered together with bacterial communities of *U. rigida* in the CAP (Figure 5b and Figure 9).

Environmental factors (water temperature and salinity) differed among regions (PERMANOVA main test, $P_{\text{perm}} = 0.001$,) with water temperature mainly contributing to the difference (SIMPER analysis, contribution 58-100%,). The regions of Ria Formosa, Albufeira and the west coast exhibited similar conditions, and differed in environmental conditions prevailing at Arrábida (PERMANOVA pair-wise test; $P_{\text{perm}} = 0.001$).

3.3 Abundance of particular bacterial groups

3.3.1 Vitamin B_{12} producers

The relative abundance of vitamin B_{12} producing bacteria (relative abundances of OTU counts per species) in microbiomes did not differ among *Ulva*-species (Table 4). Nevertheless, some trends could be observed (Figure 10a). The most abundant species in terms of vitamin B_{12} producers appeared to be *U. californica/flexuosa* followed by *U. compressa* (both mainly due to *Flavobacterium*). The highest abundance of *Dinoroseobacter* appeared to be in microbiomes of *U. compressa*. *Pseudomonas* seemed to be more abundant in species of *Ulva* than in *Ulvales* and appeared to be most abundant in microbiomes of *U. australis*. The most diverse *Ulva* species seemed to be *U. rigida* and *Ulva sp.1*, with *Bacillus* and *Halomonas* only present in these two species, respectively (Figure 10a). The relative abundance of vitamin B_{12} producers in microbiomes did not differ among geographical regions (Table 4). Nevertheless, some trends could be observed (Figure 10b). The most abundant region in terms of vitamin B_{12} producers appeared to be Arrábida, mainly due to *Streptococcus*. The most diverse region seemed to be the Ria Formosa with all members present despite *Streptococcus* and four genera exclusively present in microbiomes from that region (*Bacillus* spp., *Clostridium*, *Halomonas*, *Serratia*). In samples of the other regions, the bacterial group appeared to be only represented by about half of the members (Figure 10b).

Table 4. PERMANOVA table of main test to determine differences in relative abundance of particular bacterial groups (i.e. Vitamin B_{12} producers and the ambivalent) between species of *Ulva*.

PERMANOVA main test	P_{perm}	**Unique perms**	P_{MC}
Vitamin B_{12} producers			
Re	0.786	998	0.839
Sp	0.455	998	0.455
RexSp	0.249	998	0.212
The ambivalent			
Re	0.741	998	0.762
Sp	0.538	999	0.567
RexSp	0.396	997	0.382

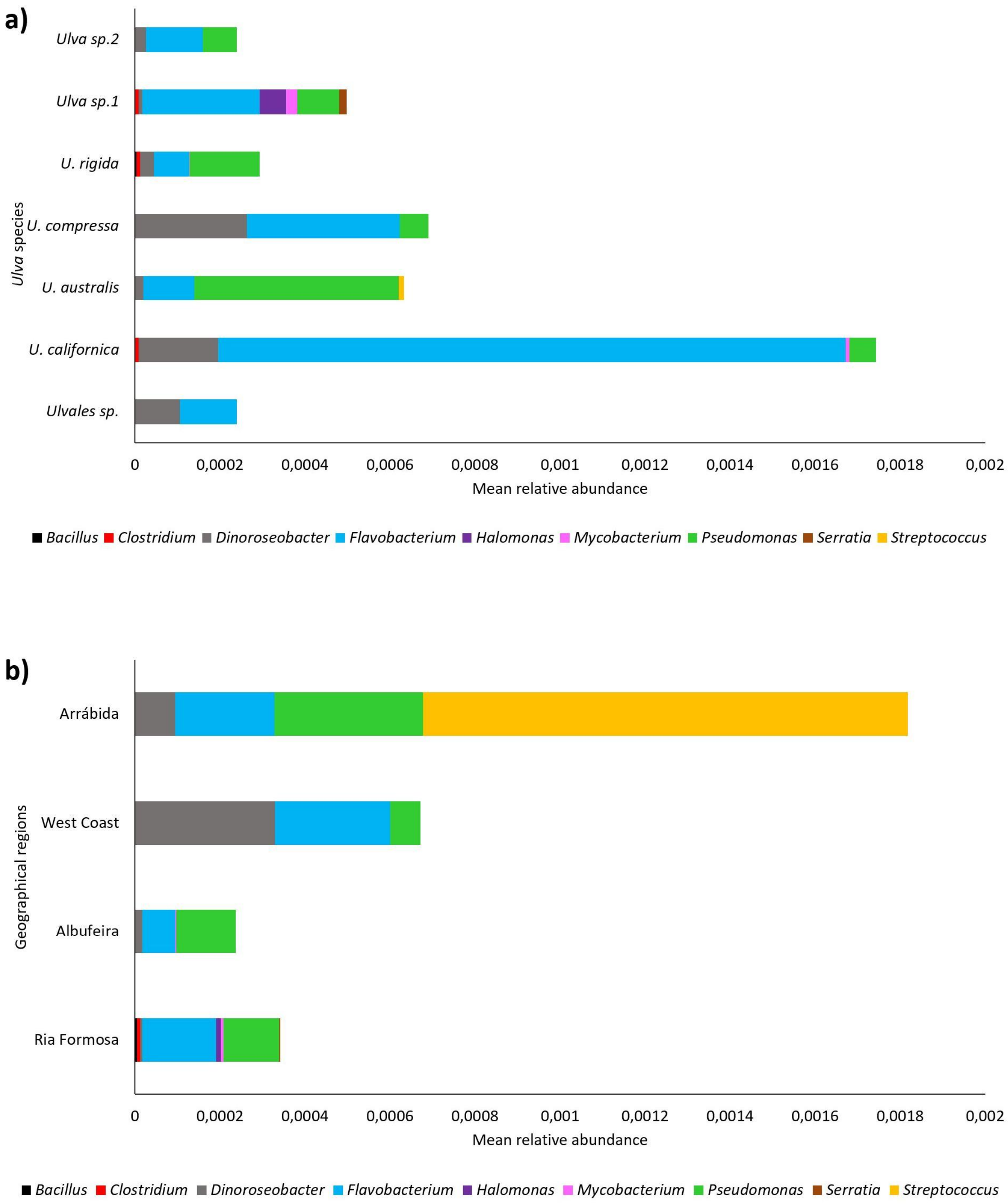

Figure 10. Relative abundance of vitamin B_{12} producing bacteria in microbiomes of a) different *Ulva*-species, and b) different regions. Values are expressed in percentage of the total number of OTU counts per species of *Ulva* and per region, respectively.

3.3.2 The ambivalent

The relative abundance of the ambivalent bacteria (compare chapter 1.1.4.2) in microbiomes did not differ among *Ulva*-species (Table 4). Nevertheless, some trends could be observed (Figure 11a). The most abundant species in terms of the ambivalent appeared to be *Ulva* sp. 1 followed by *U. rigida*, mainly due to *Pseudoalteromonas* and *Vibrio*, respectively. *Aquimarina* seemed to be the most abundant in bacterial communities of *U. californica/flexuosa* and relatively well represented though out all other species. *Agarivorans* seemed to be most abundant in microbiomes of *Ulva* sp. 2 and *Roseobacter* in microbiomes of *U. compressa* (Figure 11a).

The relative abundance of the ambivalent in microbiomes did not differ among geographical regions (Table 4). Nevertheless, some trends could be observed (Figure 11b). The most abundant region in terms of the ambivalent appeared to be the Ria Formosa, mainly due to the genera *Pseudoalteromonas* and *Alteromonas*. *Agarivorans* was only present in the regions of Ria Formosa and West coast. *Aquimarina*, *Flavobacterium* and *Pseudomonas* seemed to be most abundant in Arrábida. *Roseobacter* seemed to be the most abundant in the West coast, whereas *Vibrio* in the Ria Formosa (Figure 11b).

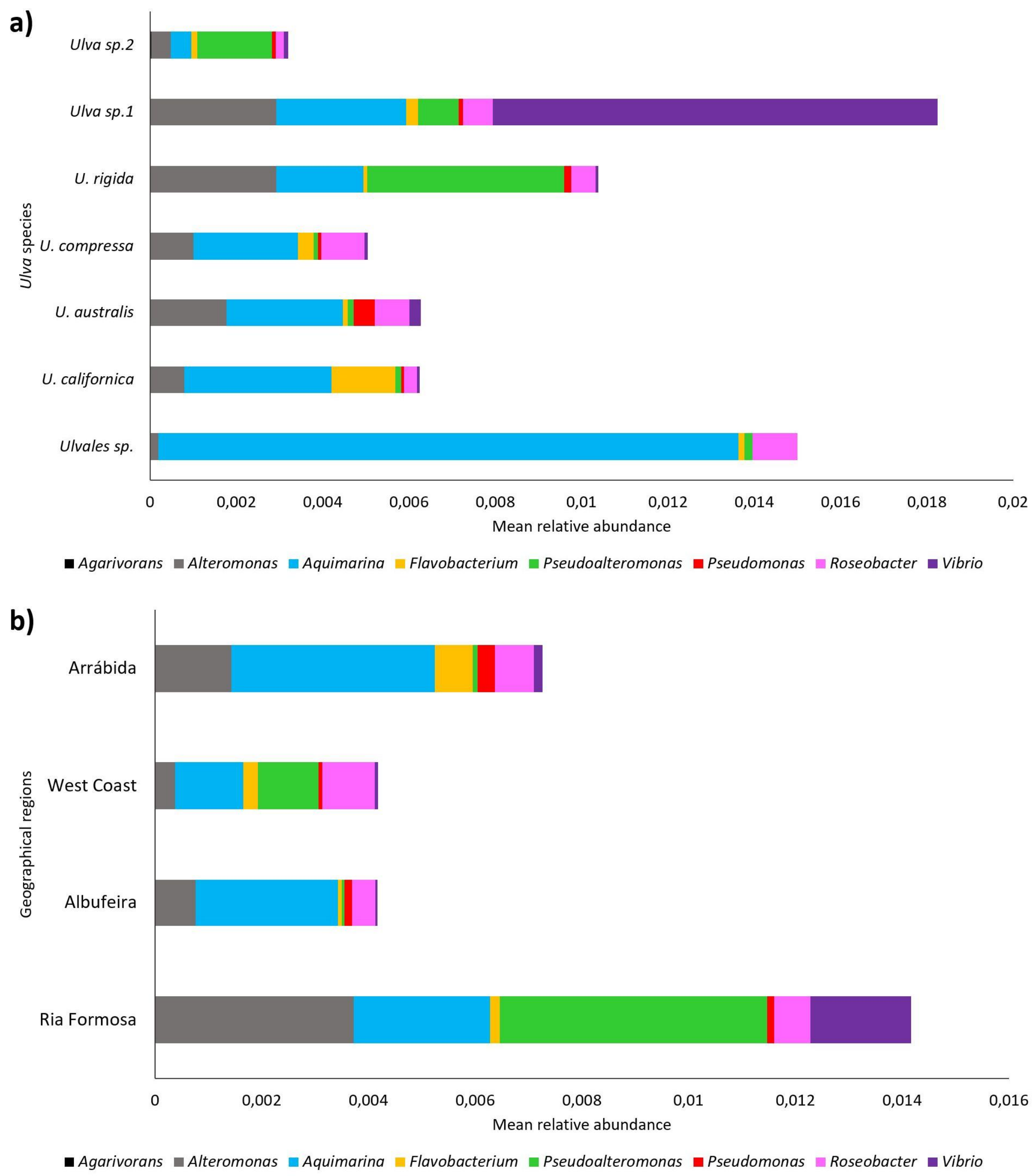

Figure 11. Relative abundance of the ambivalent bacteria (genus level) in microbiomes of a) different *Ulva*-species, and b) different regions. Values are expressed in percentage of the total number of OTU counts per species of Ulva and per region, respectively.

4 Discussion

4.1 *Ulva* diversity of southern and southwestern Portugal

This study is, to our knowledge, the first using DNA-barcoding based on the *tuf*A marker gene to identify species of *Ulva* in Portugal. The phylogenetic analysis of specimen collected along the southern and southwestern Portuguese coast across four different regions resulted in a total of six distinct species of *Ulva*: *U. californica/flexuosa*, *U. australis*, *U. compressa*, *U. rigida*, and two unidentified species, *Ulva* sp. 1 and *Ulva* sp. 2. All detected species were previously identified in Portugal by Araújo *et al.* (2009) (see chapter 1.2.3), except for *U. australis*. However, these past identifications were solely based on morphological character traits. Hence, this study consolidates the to date existent information about the diversity of *Ulva* in south and southwestern Portugal. This study is the first to ever record the occurrence of *U. australis* in Portugal. So far, the NIS was only found at the north and south coast of Spain, probably leading back to separate introduction events in the context of commercial shellfish trade (Couceiro, Cremades and Barreiro, 2011). The source and vector of the introduction of *U. australis* into Portuguese waters and whether it resulted from a single introduction event, followed by regional spread, or from multiple introductions, remains to be investigated.

Furthermore, this study led to new insights about the two ambiguous cases of *U. rigida* and *U. californica/flexuosa.* With the help of peer reviewed sequences, the former was clearly classified as *U. rigida*. Although, previously, this species was often misidentified as *U. laetevirens* or *U. lactuca* in Portugal and the Mediterranean region due to discrepancies in GenBank (Sfriso, 2010). The species *U. californica* and *U. flexuosa*, still debated whether to be conspecifics or not, here, grouped together as a species-complex (i.e., *U. flexuosa/californica).* The same pattern was also observed by Steinhagen, Karez and Weinberger (2018), supporting the theory that these two species of *Ulva* are conspecifics.

The two species *Ulva* sp.1 and *Ulva* sp.2 could not be resolved systematically, neither by the peer reviewed nor the GenBank sequences. The BLAST search suggested sequences assigned to *U. lactuca* as closest matches, although the peer reviewed sequences of *U. lactuca* did not match with either of them. The closest related species of both *Ulva* sp.1 and *Ulva* sp.2 appeared to be *U. rigida*. Therefore, it is likely that the GenBank sequences are in fact wrongly assigned. Considering that *U. lacutca* is often confused with *U. laetevirens* and *U. rigida*, it likely that sequences of one of the unidentified *Ulva*-species can be assigned to *U. laetevirens.* Further work needs to investigate whether this allocation can be confirmed or both unidentified species are new entities.

4.2 Bacterial diversity and composition

In support of previous studies of surface-associated bacterial communities of seaweeds (Longford *et al.*, 2007; Staufenberger *et al.*, 2008; Tujula *et al.*, 2010; Burke *et al.*, 2011), microbiomes of *Ulva* were characterised by members of the four phyla Bacteroidetes (Bacteroidia), Proteobacteria (Alpha- and Gammaproteobacteria), Planctomycetes (Planctomycetia) and Actinobacteria (Acidimicrobiia). On an order-level, examined *Ulva*-microbiomes in this study were mainly dominated by Flavobacteriales, Rhodobacterales, Caulobacterales, as well as Pirellulales.

In broad agreement with associated bacterial communities previously identified by Burke *et al.*, 2011, here, microbiomes of *U. australis* were dominated by Flavobacteriales and Rhodobacterales. Their importance in microbiomes across many *Ulva*-species is widely known. As discussed in chapter 1.4, members of Flavobacteriales and Rhodobacterales are involved in various *Ulva*-related metabolic processes (morphogenesis, degradation of polymers and production of vitamins) (Matsuo *et al.*, 2005; Wichard *et al.*, 2015; Kumar *et al.*, 2016; Aires *et al.*, 2018; Reisky *et al.*, 2019). Some are pathogens and related to stress conditions in diseased marine organisms, while others were shown to have antifouling capacities (Egan *et al.*, 2001, 2012).

Although, in broad agreement with findings by Burke *et al.*, 2011, *U. australis*-microbiomes in this study displayed some characteristic differences. Cellvibrionales and Alteromonadales were notably more abundant. The latter are for instance involved in the inhibition of germination (Bhattarai *et al.*, 2007; Goecke *et al.*, 2010; see chapter 1.4). Some members of Alteromonadales are known to be causative agents of various diseases such as the bleaching disease (Kumar *et al.*, 2016; see chapter 1.4.2.2), while others have been shown to degrade complex polymers by the production of ulvan lyases (Teeling *et al.*, 2012; Kopel *et al.*, 2016). Thus, the high abundance of Alteromonadales in presently examined *U. australis* could indicate that individuals were diseased, or germination repressed as environmental conditions were not favourable. Considering only healthy tissue was used in this study, the latter case is more likely. In contrast to existing data of the study mentioned above, Sphingomonadaceae (Sphingomonadales), Saprospiraceae (Saprospirales) and Planctomycetaceae (Planctomycetales) did not comprise dominant taxa in microbiomes of *U. australis*. Saprospiraceae were demonstrated to be important in the breakdown of complex organic compounds in wastewater (McIlroy and Nielsen, 2014). Their weak representation in here examined microbiomes might be an indication of lower organic matter content in the sampling region of Arrábida. Owing to functional redundancy (see chapter 1.4.1), Saprospiraceae along with the other two taxa, were most probably replaced by other bacteria taking on similar roles, explaining their lower abundances when compared to previous studies.

In accordance with previous results by the researchers Bolinches, Lemos and Barja, 1988, Caulobacterales (along with Flavobacteriales) were identified as predominant taxa in *U. rigida* (Bolinches, Lemos and Barja, 1988). Caulobacterales are typical symbionts of aquatic organisms and wide spread on marine surfaces in general (Pujalte *et al.*, 2014; Singh and Reddy, 2014; Cooper *et al.*, 2019). In this study, microbiomes of *U. rigida* were furthermore characterised by Chitinophagales and Thiohalorhabdales. Although, on class-level, these findings are in accordance with results of Ismail *et al.*, 2018, which revealed Gammaproteobacteria as highly abundant in *U. rigida*. Additionally, Thiohalorhabdales were found in associated bacterial communities of the red seaweed *Asparagopsis taxiformis* (Greff *et al.*, 2017).

The order Pirellulales – particularly abundant in samples of *U. compressa* – has not yet been reported as one of the most dominant orders in microbiomes of *Ulva*. However, during summer months, a high abundance was previously observed in the brown seaweed *Sargassum muticum* from the Portuguese coast (Serebryakova *et al.*, 2018). Members of Pirellulales were shown to hold a set of genes related to environmental stress response (*i.e.* regulation of temperature and salt stress)(Wecker *et al.*, 2009). Moreover, *U. compressa* was characteristic for Sphingomonadales, particularly samples from the Ria Formosa region. Although, *U. compressa* recently evolved into a model organism to study microbiomes and morphogenesis of green seaweeds, to our knowledge, this study represents the most detailed description of bacterial communities associated to this species of *Ulva*. The same applies for microbiomes of the species-complex *U. californica/flexuosa*. Associated bacterial communities of *U. californica/flexuosa* were, particularly abundant in Flavobacteriales. Hence, this research not only confirms existing results about the composition of already known *Ulva*-microbiomes but provides new insights into associated bacterial members of newley examined *Ulva*-species.

4.3 Assessment of host- and/or region-specificity

The assessment of the degree of host- and/or region-specificity of microbiomes of different species of *Ulva* across different regions along the southern and southwestern coast of Portugal revealed a primarily host-specific pattern in at least two of the examined species. In support of previous studies (Lachnit *et al.*, 2009; de Oliveira *et al.*, 2012; Eigemann *et al.*, 2013; Aires, Serrão and Engelen, 2016), the present findings suggest the seaweed-host as the primary factor to determine the microbiome composition. Although, the environmental conditions (of which geographic region is a proxy) also appeared to influence the bacterial assembly resulting in different degrees of region-specificity on a within-host level.

In contrast to the study by Roth-Schulze *et al.*, 2018, that found no to little support of host-specificity, here examined microbiomes of *U. rigida* had a distinctive bacterial composition unique to the species, regardless of the geographical region. Although, statistically, the uniqueness of *U. rigida* microbiomes was only proven in comparison to bacterial communities of co-occurring *U. compressa*. Nevertheless, the large sample size and clear separation from samples of the remaining species in both similarity analyses support the host-specific pattern. Moreover, in the study by Roth-Schulze *et al.*, 2018 solely samples from two adjacent locations were analysed, whereas, here, examined microbiomes originated from three different regions with a geographic distance ranging from ~30 to over 200 km, minimizing the chance of bacterial interaction among the samples and increasing the statistical resolution. At the same time, microbiomes of *U. rigida* were also influenced by the environment as they exhibited a degree of region-specificity on a within-host level. These regional differences were mainly due to a higher abundance of *Granulosicoccus* (Thiohalorhabdales) and *Loktanella* (Rhodobacterales) in samples from the Rio Formosa compared to the other regions. Still, the environment appeared to play a secondary role in the assembly of associated bacterial communities in this species of *Ulva*.

Similar conclusions can be drawn in the case of *U. compressa*. Microbiomes were distinct from the ones of co-occurring *U. rigida* and clearly separated from the remaining samples, suggesting a primarily host-specific pattern. The differentiation from microbiomes of *U. rigida* was mainly due to the close affiliation of *U. compressa* with *Rhodopirellula* (Pirellulales). As previously mentioned in chapter 4.2, Pirellulales are related to environmental stress-response. The genus *Rhodopirellula* was shown to up-regulate the expression of heat or cold shock protein in relation to sudden temperature shifts

(Wecker *et al.*, 2009). Hence, the high abundance of *Rhodopirellula* in microbiomes of *U. compressa* might be an adaptation to quick environmental changes in the intertidal zone. In one of the analyses, the bacterial communities of *U. compressa* appeared to be more region-specific when compared to *U. rigida*. Regarding the otherwise host-specific pattern, here, two region-unrelated theories to explain the greater variability in microbiomes of *U. compressa* are proposed. During the time of sampling, specimen from the Ria Formosa region were submerged, whereas the ones from the west coast were emerged. Considering that the latter were temporarily exposed to extreme environmental stress (*i.e.* strong irradiance and draught), a higher abundance of stress tolerant Pirellulales in emerged samples could be responsible for the differences between regions. In fact, a higher abundance of *Blastopirellula* in samples of emerged *U. compressa* from the west coast compared to samples from the Ria Formosa contributed the most to the difference between microbiomes from the two regions. *Blastopirellula* was shown to tolerate salt stress, easily caused in periods of air exposure (Schwibbert *et al.*, 2011). Thus, a tide-related temporal change of the microbiome, rather than region-specificity, is likely the cause of the greater variability in microbiomes of *U. compressa*. This insight strongly supports the hypothesis of Aires, Serrão and Engelen, 2016, that the microbiome facilitates adaptation to environmental stressors.

Another theory, explaining the variability would be morphotype-induced assembly of the associated bacterial composition. As previously assumed for this species of *Ulva* (Steinhagen et al., 2019), and proven in several other representatives of the genus (Hayden et al., 2003), *U. compressa* exhibited different morphotypes (*i.e.* tubular or sheet-like fronds) in the two major regions of occurrence. The seaweed-morphology might act as selective force on the composition of the bacterial community, as already hypothesized by Egan *et al.*, 2000. Thus, the combination of the two factors, morphotype and environment, might result in more variable microbiomes in comparison to *U. rigida,* that was only found in blade-like fronds. Regardless of a more evident variability, it is likely that microbiomes of *U. compressa* are primarily determined by the host.

As the main limitation of this work, the assessment of the remaining *Ulva*-species was compromised by a small sample size and/or the effect of unequal distribution. Microbiomes of *U. australis* appeared to be distinct in both analyses. However, all samples originated from one region, exclusively, preventing to draw further conclusions. The same was true for both unidentified species (*Ulva* sp.1 and *Ulva* sp.2). Moreover, results concerning microbiomes of *Ulva* sp.1 were ambiguous as their associated bacterial communities grouped with different species depending on the type of analysis.

The sample size for *U. californica/flexuosa* also were too small for the assessment of host- and/or region-specificity, although samples were found in two regions. The microbiome sampled from the artificial pond was considered an outlier as the exceptional environmental conditions of the enclosed artificial pond (*i.e.* elevated salinity and possibly oligotroph conditions due to less tidal mixing) lead to an essentially unique bacterial community. Nevertheless, this result highlights how drastically the associated bacterial community can be influenced by environmental conditions. *U. californica/flexuosa*-microbiomes particularly showed close affiliation with *Dokdonia* and *Maribacter* (Flavobacteriales) and Hyphomonadaceae (Rhodobacterales). *Dokdonia*, a photoheterotrophic bacterium, uses the light absorbent protein proteorhodopsin which grants the bacterium to be less dependent on nutrients (González *et al.*, 2011). Hyphomonadaceae reduce nitrate and are common bacteria in oligotrophic niches (Abraham and Rohde, 2014). The genus *Maribacter* is a halotolerant taxon and has been reported to occur in evaporitic habitats (Nedashkovskaya *et al.*, 2004; Dorador *et al.*, 2009). Considering that the sample from the artificial pond alone was most likely responsible for the strong positive correlation with these taxa, their recruitment might be an environmental adaptation to the elevated salinity and possbile oligotroph conditions of the artificial pond.

All things considered, the assessment of bacterial specificity found support of host-specificity in surface-associated bacterial communities of at least two species of *Ulva* (*U. rigida* and *U. compressa*). The host-specificity of *U. rigida* seemed tighter when compared to the remaining species, suggesting a slightly more stable microbiome. *U. rigida* was recently shown to have specific characteristics making them more robust and enable them to form green tides (Fort *et al.*, 2020). The microbiome of a more environmentally robust *Ulva*-species most likely will not get disrupted so easily when compared to a more sensitive one. As microbiome stability is considered critical for good health in other holobionts (Kerényi *et al.*, 2013; Coyte, Schluter and Foster, 2015), it can be speculated that a host-specific *Ulva*-microbiome might enhance seaweed-fitness, hence, select for more environmentally robust *Ulva* species. However, the above argumentation is purely based on speculation and the validity of this assumption remains to be investigated.

4.4 Particular bacterial groups

4.4.1 Vitamin B_{12} producers

The analysis of vitamin B_{12} producers in *Ulva*-associated bacterial communities revealed that most bacterial taxa comprising this group were present across the different species. In line with previous findings by Ismail *et al.*, 2018 the genera *Bacillus*, *Halomonas*, *Pseudomonas* and *Streptococcus* were present in microbiomes of *Ulva*. Although, here, only *Pseudomonas*, along with *Dinoroseobacter* and *Flavobacterium*, was common across Ulva species. Considering the employment of *Pseudomonas* for the industrial production of vitamin B_{12} (Martens et al., 2002; see chapter 1.4.2.1), it represents a promising source of B_{12} in *Ulva*-microbiomes. *Dinoroseobacter*, demonstrated to produce vitamin B_{12} in the green algae *Ostreococcus tauri* (Cooper *et al.*, 2019), was recently suggested to play a similar role in *U. rigida* (Ismail *et al.*, 2018). Results of this study confirm its presence in microbiomes of *U. rigida* and *U. compressa*. Along with the fact that *U. compressa* encodes for METH, the B_{12}-dependent methionine synthetase (see chapter 1.4.2.1), it can be suggested that both species of *Ulva* partly depend on *Dinoroseobacter* to produce B_{12}.

Although some trends could be recognized, assumptions about the differences in relative abundance or diversity of members of this group could not be supported, statistically. The same limitations apply for differences in bacterial abundances among regions. Moreover, the approach selected here was, again, compromised by the effect of unequal distribution. For instance, the seemingly higher diversity of B_{12} producing taxa in microbiomes from the Ria Formosa could be due to *Ulva* sp.1 and *U. rigida* (in which almost all B_{12} producers were present) that mainly originated from that region. Therefore, the hypothesis concerning this chapter – whether this particular bacterial group is rather influenced by the host then by the region – could not be answered.

However, extreme environmental conditions such as in the artificial pond, might influence the abundance of B_{12} producers. In microbiomes of *U. californica/flexuosa*, which were strongly influenced by the sample originating from the artificial pond, *Flavobacterium* appeared to be notably well represented. Thus, the elevated abundance of Flavobacteriales might imply a potential enhanced production of Vitamin B_{12}. In that case the vitamin B_{12} producers would rather be influenced by the environmental conditions. Although, generally, the correlation between a high relative abundance of vitamin B_{12}

producing bacteria and consequently elevated production of B_{12} remains to be investigated further.

4.4.2 The ambivalent

The ambivalent group of bacteria seemed to be stable across species and regions as all members (see chapter 3.3.2) but *Agarivorans* were present in microbiomes of each *Ulva* species. Several genera were previously identified in associated bacterial communities of *U. rigida* (*Alteromonas*, *Pseudoalteromonas, Pseudomonas* and *Vibrio* in Ismail *et al.*, 2018). In line with assumptions made in chapter 1.4.2.2, *Ulva*-microbiomes harbour common seaweed pathogens such as all three genera (*Agarivorans*, *Aquimarina* and *Alteromonas*) previously identified as causative agents of the ice-ice disease (Kumar *et al.*, 2016). In this light it is possible that not only red seaweeds such as *Kappaphycus* and *Eucheuma* can be affected but *Ulva* might also be at risk of a similar disease, under certain conditions. *Roseobacter* represents a similar case as the inducer of the bleaching disease in the red seaweed *D. pulchra* (Egan *et al.*, 2012), was identified in all examined *Ulva* species. Hence, it is possible that the normally commensal or even beneficial *Roseobacter* (phytohormones for morphogenesis, see chapter 1.4) could also represent a threat to *Ulva*, when under environmental stress. Moreover, *Flavobacterium, Pseudomonas, Pseudoalteromonas* and *Vibrio* common bacterial pathogens known to cause rot symptoms and galls in seaweeds (Gachon *et al.*, 2010) were also present in *Ulva*-microbiomes.

Aquimarina, *Alteromonas* and *Pseudoalteromonas* appeared to be the most common members within the ambivalent. However, no statistical support could be provided concerning the differences in relative abundance of the ambivalent taxa among species of *Ulva*; neither among regions. Considering these limitations, the hypothesis that the relative abundance of the ambivalent is rather influenced by the region than by the host-species, could not be confirmed. Although, it is worth pointing out that contrary to the expectations, microbiomes of *U. californica/flexuosa* that were under the influence of more extreme environmental conditions of the artificial pond, were not particularly abundant in any of the ambivalent members. Whereas, *Ulva* sp.1 seemed to be more abundant in the ambivalent, mainly due to *Vibrio*. These trends could lead to the speculation that the relative abundance of the ambivalent is rather influenced by the host-species, at least under "normal" environmental conditions.

Overall, the presence of the ambivalent across microbiomes of all examined *Ulva*-species implies the potential risk of a disruption of a healthy bacterial community.

However, it appears that extreme environmental conditions are needed for a shift to take place as no effect of the geographical region could be registered. Regarding the ambivalence of the bacterial genera, more species need to be isolated and the functions investigated further (*i.e. Flavobacterium* and *Pseudomonas*, considered in vitamin B_{12} producers and the ambivalent). Moreover, it is important to direct more research effort towards the determination the environmental conditions of the tipping point, when certain bacteria tend to turn from beneficial or commensal to degrading or pathogenic.

5 Conclusion and future remarks

In this work, the combined approach of DNA barcoding *via* the chloroplast-encoded elongation factor Tu (*tuf*A) and 16S ribosomal RNA gene amplification was used to analyse the holobiont-diversity of the green seaweed genus *Ulva* from four different regions along the southern and southwestern coast of Portugal. In view of potential lessons to learn for science and industry, the degree of host- and/or region-specificity in microbiomes of different species of *Ulva* was assessed and a closer look taken at the abundance of particular bacterial groups of interest.

So far, this study represents the first classification record of species of *Ulva via tuf*A in the region of Portugal. The marker gene was successfully used to delimit six different species within the genus *Ulva* (*U. rigida*, *U. compressa*, *U. californica/flexuosa*, *U. australis, Ulva* sp.1, *Ulva* sp.2) of which two seem to be new entities. Further steps should be taken to classify the two unidentified species. The non-indigenous species (NIS) *U. australis* was registered for the first time in Portugal. Thus, it is of scientific and conservational interest to further investigate the source of introduction and degree of spread of this NIS along the Portuguese coast. With the help of this study, previous assumptions that *U. californica* and *U. flexuosa* can be combined a species complex (i.e. *U. californica/flexuosa*) could be verified. This insight should be a call to further investigate their degree of relatedness in order to standardize nomenclature for these species and eliminate GenBank discrepancies. In the context of another taxonomic confusion, this study contributes to clarify the genetic distinction of the often-misidentified *U. rigida* from its relatives *U. laetevirens* and *U. lacutca*. Additionally, present results could confirm the longstanding assumption that *U. compressa* is polymorph and exhibits two different morphotypes (*i.e.* tubular and blade-like fronds).

Clear to notable host-specificity emerged in *U. rigida* and *U. compressa*, respectively, with signs of secondary region-specificity, predominantly in *U. compressa*. Although, here, two alternative theories were proposed to explain this pattern (tide-related changes and morphotype-induced assembly of the microbiome). Microbiome composition purely based on stochastic processes (*i.e.* lottery) could be ruled out. Taken the above insights into account, it can be concluded that the *Ulva*-host most likely plays a primary role over environmental conditions (of which geographic region is a proxy) in the determination of the microbiome composition. In one case (*U. californica/flexuosa*), however, the environment seemed to have a primary impact on the microbiome composition. Considering the overall different environmental factors of the artificial artificial pond that effected the bacterial communities of this species, this case most likely is an exception. Regarding the primarily host-specific pattern in *U. rigida*, this species was suggested to be relatively microbiome-stable. Considering the specific genetic make-up rendering it to be a common green-tide species, here, it was speculated that host-specificity increases seaweed-fitness and might select for environmental robustness. In order to evaluate the validity of this speculation, however, more information should be gathered regarding the link between microbiome-stability and environmental robustness in seaweeds.

Resulting from the above findings, important conclusions can be drawn for the cultivation and use of the respective *Ulva*-species and the genus *Ulva* in general. Considering that all holobionts were in a healthy condition as the seaweed-thalli were intact, the relative abundance of potentially harmful bacteria (the ambivalent) was low and a variety of beneficial bacteria (vitamin B_{12} producers) was found in associated microbial communities, it can be confirmed once more that *Ulva* is a suitable genus for consumption. Depending on the degree of host-specificity different strategies could be used to increase specific bacterial-derived target compounds. In species such as *U. rigida* certain bacteria most probably remain relatively stable in the microbiome even under environmental changes. This would mean a secured amount of target compounds and little risk of diseases. The microbiomes of *Ulva* sp.1, on the other hand, could probably be manipulated easier to increase a target compound, due to the little to no host-specificity. Here, the species *U. compressa*, *U. rigida* and *U. californica/flexuosa* are proposed to be of specific interest for commercial use. Based on a high amount of environmentally stable Pirellulales such as *Rhodopirellula* and a good representation of the vitamin B_{12} producer *Dinoroseobacter* in microbiomes of *U. compressa,* this species is recommended to be cultivated for human consumption as previously suggested by Aguilera-Morales *et al.* (2005). Owing to their host-specificity and general robustness, *U. rigida* might be suitable for industrial strategies to yield a stable amount of specific bacterial-derived target compounds and to remediate sewage treatments from effluents as this species would probably withstand the conditions encountered in the processing of the wastewater as previously suggested by Gao *et al.* (2017). *U. californica/flexuosa*, suspected to be able to elevate the abundance of beneficial bacteria (Flavobacteriales such as *Dokdonia*, *Maribacter* and *Flavobacterium*) under certain environmental conditions, might be a suitable candidate for the production of vitamin B_{12}. Considering the good representation of a variety of ambivalent bacteria in *Ulva* sp.1 this species might have the potential for isolate and harnessing degraders together with their enzymatic toolkit.

In conclusion, this thesis has met its objectives and gave rise to many questions in need of further investigation. Generally, the results to shed more light on the determination of the microbiome composition in species of *Ulva* are promising and should be validated by a larger sample size. The following directions are suggested for future studies: the tracing of the introduction event of *U. australis*, host-specificity to promote environmental robustness, and the influence of artificial environmental factors on growth and vitamin B_{12} production. Research into resolving the two unidentified *Ulva*-speices and verification of the assumed functions of members of the particular bacterial groups is already underway.

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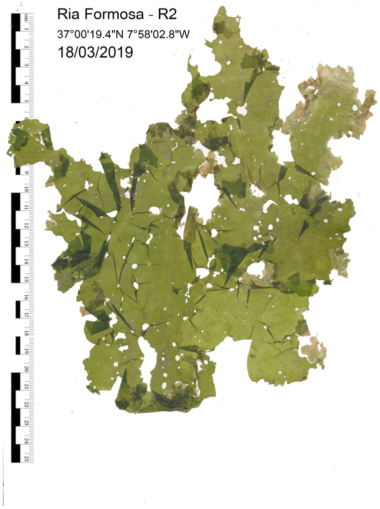
Appendices

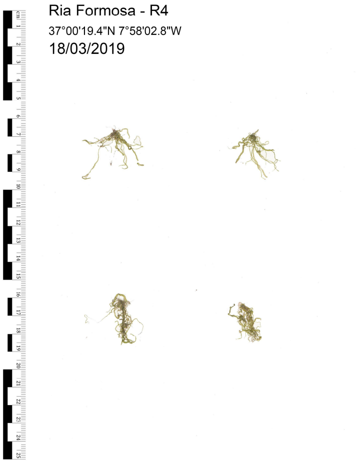
Appendix 1. Metadata, containing the detailed description of *Ulva* samples and respective environmental parameters.

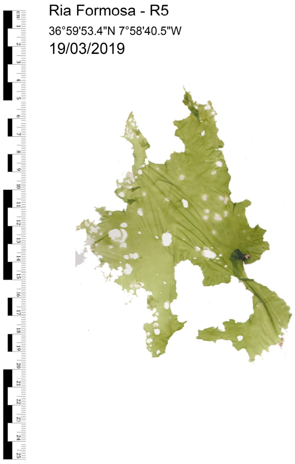
#	Sample	Species	Region	Location	Coordinates	Morphotype
1	R1	*Ulva flexuosa*	Ria	Ramalhete	37°00'19.4"N 7°58'02.8"W	Tubular
2	R2	*Ulva rigida*	Ria	Ramalhete	37°00'19.4"N 7°58'02.8"W	Blade
3	R3	*Ulva rigida*	Ria	Ramalhete	37°00'19.4"N 7°58'02.8"W	Blade
4	R4	*Ulvales* sp.	Ria	Quinta do Lago	37°00'19.4"N 7°58'02.8"W	Tubular
5	R5	*Ulva rigida*	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Blade
6	R6	*Ulva compressa*	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Tubular
7	R7	*Ulva compressa*	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Tubular
8	R8	*Ulva* sp.1	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Tubular
9	R9	*Ulva rigida*	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Blade
10	R10	*Ulva rigida*	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Blade
11	R11	Ulva sp.1	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Tubular
12	R12	*Ulva rigida*	Ria	Ilha de Faro	36°59'53.4"N 7°58'40.5"W	Blade
13	A13	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
14	A14	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
15	A15	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
16	A16	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
17	A17	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
18	A18	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
19	A19	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
20	A20	*Ulva rigida*	Alb	Praia dos Aveiros	37°5'0,68"N8°13'53,45"W	Blade
21	V21	*Ulva rigida*	Alb	Praia da Rocha Baixinha	37,071999-8,12522'4	Blade
22	R22	*Ulva rigida*	Ria	Ilha de Faro	37°00'19.4"N 7°58'02.8"W	Blade
23	R23	*Ulva rigida*	Ria	Ilha de Faro	37°00'19.4"N 7°58'02.8"W	Blade
24	R24	*Ulva compressa*	Ria	Ilha de Faro	37°00'19.4"N 7°58'02.8"W	Tubular
25	R25	*Ulva compressa*	Ria	Ilha de Faro	37°00'19.4"N 7°58'02.8"W	Tubular
26	R26	Ulva sp.1	Ria	Ilha de Faro	37°00'19.4"N 7°58'02.8"W	Tubular
28	W28	*Ulva rigida*	Wes	Praia da Ilha do Pessegueiro	37°49'42.0"N 8°47'30.6"W	Blade
29	W29	*Ulva rigida*	Wes	Praia da Ilha do Pessegueiro	37°49'42.0"N 8°47'30.6"W	Blade
30	W30	Ulva sp.2	Wes	Praia da Ilha do Pessegueiro	37°49'42.0"N 8°47'30.6"W	Blade
34	W34	*Ulva compressa*	Wes	Praia da Ilha do Pessegueiro	37°49'42.0"N 8°47'30.6"W	Blade
35	W35	*Ulva compressa*	Wes	Praia da Ilha do Pessegueiro	37°49'42.0"N 8°47'30.6"W	Blade
36	W36	*Ulva compressa*	Wes	Praia da Ilha do Pessegueiro	37°49'42.0"N 8°47'30.6"W	Blade
37	Ar37	*Ulva californica*	Arr	Praia da Figueirinha	38°29'06.2"N 8°56'32.5"W	Tubular
38	Ar38	*Ulva californica*	Arr	Praia da Figueirinha	38°29'06.2"N 8°56'32.5"W	Tubular
39	Ar39	*Ulva compressa*	Arr	Praia da Figueirinha	38°29'06.2"N 8°56'32.5"W	Tubular
41	Ar41	*Ulva australis*	Arr	Praia dos Galapos	38°29'04.5"N 8°57'20.4"W	Blade
42	Ar42	*Ulva australis*	Arr	Praia dos Galapos	38°29'04.5"N 8°57'20.4"W	Blade
43	Ar43	*Ulva australis*	Arr	Praia dos Galapos	38°29'04.5"N 8°57'20.4"W	Blade
44	Ar44	*Ulva australis*	Arr	Praia dos Galapos	38°29'04.5"N 8°57'20.4"W	Blade

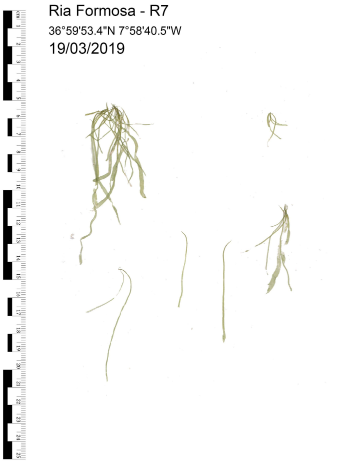
#	Morphology	Temperature (°C)	Salinity PSU	Substrat type	Water body	Depth (cm)
1	Elongated sheets	19.40	36.50	Silt	Salina	50
2	Big round sheets	18.60	32.70	Sand	Big channel	50
3	Big round sheets	21.00	40.10	Silt	Tidal pond	10
4	Small filamentous tubes curled	18.50	33.70	Rock	Big channel	70
5	Big round sheets	15.70	35.00	Sand	Puddle	10
6	Big long tubes	15.70	35.00	Sand	Puddle	10
7	Medium long tubes	16.40	34.60	Sand	Puddle	10
8	Long filamentous branched	16.30	35.40	Sand	Puddle	<10
9	Big round sheets	17.60	35.10	Sand	Puddle	<10
10	Big round sheets	17.00	34.80	Sand	Small channel	40
11	Long filamentous branched	17.60	35.50	Plastic (Ponton)	Main channel	20
12	Rose	17.60	35.50	Plastic (Ponton)	Main channel	20
13	Small round sheets	17.40	34.50	Rock	Intertidal rock pool	1
14	Rose	17.50	34.50	Rock	Intertidal rock pool	1
15	Rose	16.00	34.50	Rock	Intertidal rock pool	15
16	Rose	18.00	34.50	Rock	Intertidal rock pool	<1
17	Rose	18.60	34.50	Rock	Intertidal rock pool	15
18	Rose	18.50	34.50	Rock	Intertidal rock pool	<1
19	Rose	18.50	34.50	Rock	Intertidal rock pool	5
20	Rose	18.50	34.50	Rock	Intertidal rock pool	5
21	Small round sheet	16.50	34.60	Concrete (Pier)	Open intertidal	1
22	Big round sheets	20.00	34.60	Silt	Puddle	10
23	Big round sheets	20.00	34.60	Silt	Puddle	10
24	Medium long tubes	20.00	34.60	Silt	Puddle	10
25	Medium long tubes	20.00	34.60	Silt	Puddle	10
26	Long filamentous branched	20.00	34.60	Silt	Puddle	10
28	Small elongated sheets and tubes	18.40	35.30	Rock	Intertidal rock pool	4
29	Small elongated sheets and tubes	18.40	35.30	Rock	Intertidal rock pool	4
30	Small elongated sheets and tubes	18.40	35.30	Rock	Intertidal rock pool	4
34	Small elongated sheets and tubes	16,6 (h2o), 18 (o2)	34.20	Rock	Intertidal rock pool	0
35	Small elongated sheets and tubes	16,6 (h2o), 18 (o2)	34.20	Rock	Intertidal rock pool	0
36	Small elongated sheets and tubes	16,6 (h2o), 18 (o2)	34.20	Rock	Intertidal rock pool	0
37	Small filamentous tubes	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0
38	Small filamentous tubes	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0
39	Small filamentous tubes	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0
41	Rose	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0
42	Rose	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0
43	Rose	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0
44	Small sheet	16,8 (h2o), 21,7 (o2)	31.10	Rock	Open intertidal	0

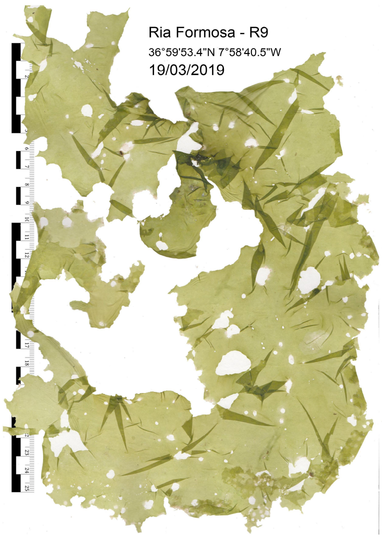
Appendix 2. Herbarium of *Ulva* species (electronical scans of respective specimens).

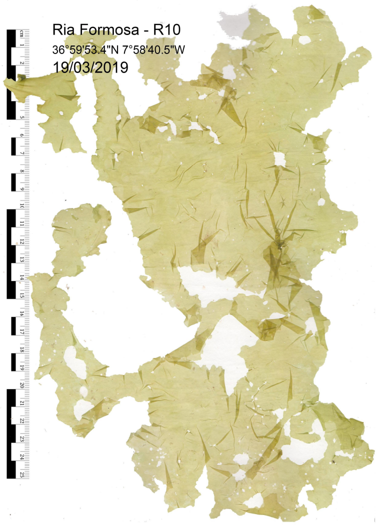




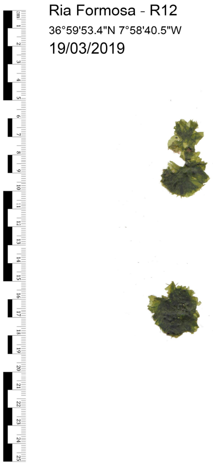








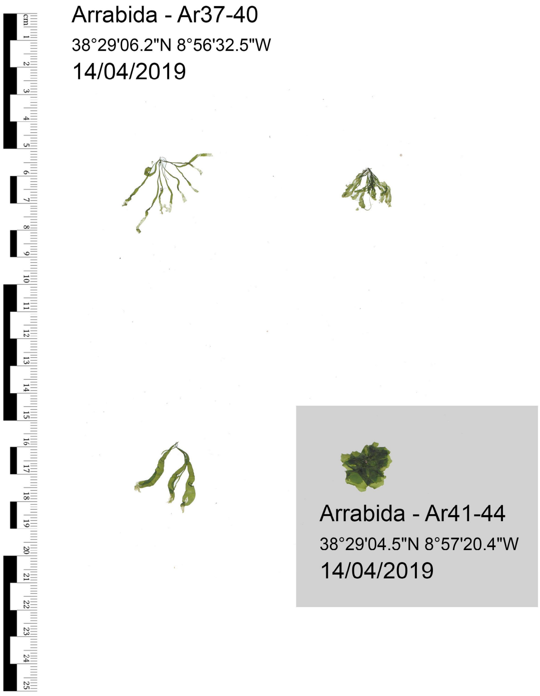
Ria Formosa - R11
36°59'53.4"N 7°58'40.5"W
19/03/2019



Ria Formosa - R12
36°59'53.4"N 7°58'40.5"W
19/03/2019

Praia Aveiros - V21
37°07'19.9"N 8°12'52.2"W
19/03/2019

Praia da Ilha do Pessegueiro - W34-36
37°49'42.0"N 8°47'30.6"W
14/04/2019



Arrabida - Ar37-40
38°29'06.2"N 8°56'32.5"W
14/04/2019

Arrabida - Ar41-44
38°29'04.5"N 8°57'20.4"W
14/04/2019