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# The influence of bacteria on the adaptation to changing environments in *Ectocarpus*: a systems biology approach

Hetty Kleinjan

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Hetty Kleinjan. The influence of bacteria on the adaptation to changing environments in *Ectocarpus*: a systems biology approach. Molecular biology. Sorbonne Université, 2018. English. NNT : 2018SORUS267 . tel-02868508

**HAL Id: tel-02868508**

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# Sorbonne Université

ED 227 - Sciences de la Nature et de l'Homme : écologie & évolution

*Laboratoire de Biologie Intégrative des Modèles Marins*

*Equipe Biologie des algues et interactions avec l'environnement*

## **The influence of bacteria on the adaptation to changing environments in *Ectocarpus*: a systems biology approach**

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Thèse de doctorat en Biologie Marine

Dirigée par Simon Dittami et Catherine Boyen

Présentée et soutenue publiquement le 24 septembre 2018

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*To my sister, the biggest hero of all time*

# Acknowledgments

All good things must come to end and today, although I hardly believe it and I don't dare to say it out loud yet, is the end of my thesis, or at least the writing of it. I hereby wish to express my gratitude to everyone who has contributed to this work, or to the process of getting here in any other way.

First and foremost, I wish to thank my supervisors, Simon and Catherine, for giving me the opportunity to start the PhD, and also for their continued support until this very moment. Special thanks to Simon, for putting faith in me as a person and as a scientist. I could not have wished for anyone more kind, more motivating, or more efficient in proofreading ;-)

I would like to thank the members of my thesis committee, Thomas Wichard, Christian Jeanthon, and Christophe Destombe, for their valuable input and scientific guidance throughout the three years. Also a pre-thank you (if you can say that) for the members of my jury, Fabrice Not, Suhelen Egan, Ute Hentschel Humeida, David Green, and Aschwin Engelen. I hope you enjoy reading the manuscript!

A great thanks to all collaborators in this project. Thomas Wichard, who let me work in his laboratory to do metabolomics. Christian Jeanthon, who helped during the cultivation experiments in the first year of my thesis, and introduced me to the challenges of marine microbiology. Thanks to Eileen Bresnan and Kevin MacKenzie for their hospitality and warm welcome when I arrived in Aberdeen earlier this year. I will never forget how excited we all were about the fusilli-shaped bacteria! Thanks to Anne Siegel, Clémence Frioux and Enora Fremy for their computational expertise and know-how on metabolic networks especially towards the end of the thesis (sorry for letting you wait for the data!).

Thanks to everyone at the ABiMs platform for letting me use the server and for solving all technical issues related to that. Special thanks to Erwan Corre for his input regarding the transcriptomic/metagenomic work and for making this project work from an informatic point of view.

I want to thank Laurence Darteville for her advice on how to best culture *Ectocarpus* with and without bacteria and for her help in the laboratory. It is very much appreciated!

A special thanks to Bertille Burgunter-Delamare for her enthusiasm and passion for her master project, which was truly amazing. The work you accomplished is very impressive and I'm very convinced you'll do great in your PhD!

I want to thank everyone in the ALFF consortium, and all supervisors, for putting together such a nice group of PhD students :-). I am very grateful to have met so many new people, from so many different places. The first post-PhD student meeting is already arranged, and I am convinced we will keep meeting each other, be it in an academic setting or just for fun (hopefully the latter of course). In particular, I want to thank Gianmaria Califano for always seeing the bright side of things (I truly need that!), and also for helping me with the analysis of the metabolite data.

Finally, I want to thank my friends who helped me take my mind off the thesis from time to time. Miriam, who has been a great friend since the very first moment we met in Bonn and was always there when I needed someone to talk to. Svenja, Martina, Margot, and Zujaila for making me laugh all the time :-). Nick and Jaro for playing soccer and games and for always being in for fun. Nick also for making me appreciate napping time!! Yao for sharing food and thoughts with me in the office. Simon B. for always being friendly in the morning and teaching me some very handy Unix tricks. Josselin, for just being there :D. Djoyce, who helped me realize that the even the tiniest steps will make you move forward. Martine, who made me apply to this project three years ago. Wiebren, for following me on this journey and for sticking around until the end.

Merci! Bedankt! Thank you!

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## Glossary

<b>Abiotic</b>	The non-living chemical and physical parts of the environment
<b>Auxotrophy</b>	The inability of an organism to synthesize a particular nutritional that is required for growth
<b>Axenic</b>	Deprived of bacteria, e.g. via antibiotic-treatment
<b>Biofilm</b>	An assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix.; result of <i>biofouling</i> .
<b>Biofouling</b>	See <i>epibiosis</i>
<b>Biotic</b>	Living components within an ecosystem.
<b>Coevolution</b>	Reciprocal evolution of interacting species
<b>Commensalism</b>	Type of symbiosis where one partner benefits while the other is unaffected
<b>Cross-feeding</b>	One species lives off the products of another species and vice versa
<b>Dysbiosis</b>	A shift in the microbiome from a stable state to a disturbed state e.g. by abiotic stress
<b>Epibiosis</b>	the settlement of (micro)organisms, <i>epibionts</i> , on other organisms that serves as a living substrate, the <i>basibiont</i> . In aquatic environments, <i>epibiosis</i> is omnipresent as a result of competition for nutrients and space to settle
<b>Heterotroph</b>	An organism that requires intake of nutrients such as carbon from an external source because they cannot produce it themselves.
<b>Holobiont</b>	A unit of biological organization composed of different species that function as one entity; the host plus all associated microorganisms;
<b>Hologenome</b>	The complete genetic content of the host genome, its organelles' genomes, and its microbiome
<b>Metabarcoding</b>	High-throughput DNA sequencing using universal primers to amplify specific regions in the DNA that serve as a taxonomic marker
<b>Metabolomics</b>	The analytical approaches used to study chemical processes involving metabolites, <i>i.e.</i> the products of metabolism
<b>Metagenome</b>	The collection of genomes and genes from the members of a microbiota

<b>Metatranscriptome</b>	Community-wide gene expression (RNA-seq) analysis via high-throughput sequencing of the complete set of transcripts from an environmental sample.
<b>Microbiome</b>	A collection of microorganisms and their collective genomes, that co-exist under certain environmental conditions;
<b>Microbiota</b>	The microbes in or on a host, including bacteria, archaea, viruses, protists, and fungi
<b>Microorganisms</b>	Microorganisms, or microbes, are a non-phylogenetic group of microscopic organisms, including bacteria, archaea, fungi, viruses, protozoa, and algae
<b>Mutualism</b>	Type of symbiosis where both partners benefit from the interaction
<b>Parasitism</b>	Type of symbiosis where one partners benefits at the cost of the host
<b>Stramenopiles</b>	A lineage of eukaryotes that comprises, amongst others, brown algae and diatoms
<b>Symbiosis</b>	Two or more species living closely together in a long-term relationship, a term that can be used independent of the outcome on host functioning

# **General Introduction**

# General introduction

## Holobionts in multicellular eukaryotes - challenges & key questions

Life of complex multicellular eukaryotes has evolved to depend on microorganisms. One advantage of symbiont acquisition lies in the fact that bacterial or other symbionts can provide host organisms with new or enhanced metabolic capacities. A noticeable example is that of the acquisition of aerobic respiration in eukaryotes, which became possible because of the uptake of an oxygen-using alphaproteobacterium and the conversion into the mitochondria over time. This event and the acquisition of photosynthetic symbionts is undoubtedly linked to the evolutionary success of eukaryotes, the proliferation in an oxygen-rich environment, and the eventual rise of complex eukaryotic life forms. Similar symbiotic acquisition events have followed, leading to the wide diversity of eukaryotic life forms found today (Douglas, 2014; McFall-Ngai, 2015).

Yet, symbionts can exert a variety of functions, and are not always beneficial to the host. Their effects can be described based on the effect the interaction has on both partners: mutualism indicates an interaction where both host and symbiont benefit from each other presence, whereas if the symbiont utilizes the host without benefiting or harming it, it is considered as a commensal. In contrast, if the host is harmed by the symbiont the interactions are categorized as parasitic (Leung and Poulin, 2008; Parfrey, Moreau and Russell, 2018). The distinction is, however, sometimes difficult since the cost and benefits of symbionts are not always measurable and may fluctuate over time due to abiotic and biotic changes (Leung and Poulin, 2008). Furthermore, obligatory symbionts depend on the host for essential function, while facultative symbionts do not. Likewise, some bacteria may be temporally acquired, while others have established long-lasting relationships (Figure 0-1).

In this context, the term Holobiont concept is frequently used (Figure 0-1), which considers the host and all its associated microbes as one functional entity, rather than individual partners (Margulis, 1991; Rosenberg *et al.*, 2007). This concept infers that the dependencies that occur between the host organism and its associated microorganisms are an integral part of host biology and one should consider all partners equally to get a complete and correct understanding of the ecological and biological features of the host in its environment (Webster, 2017), and in its evolutionary context (Zilber-Rosenberg and Rosenberg, 2008). Holobionts are inherently

complex as they are influenced by both the host genome and all symbiont genomes, and the environment acts on all partners individually (Carrier and Reitzel, 2017; Theis et al., 2016;). Thus, elucidating to what extent and how exactly different microorganisms contribute to holobiont functioning can be a challenging task.

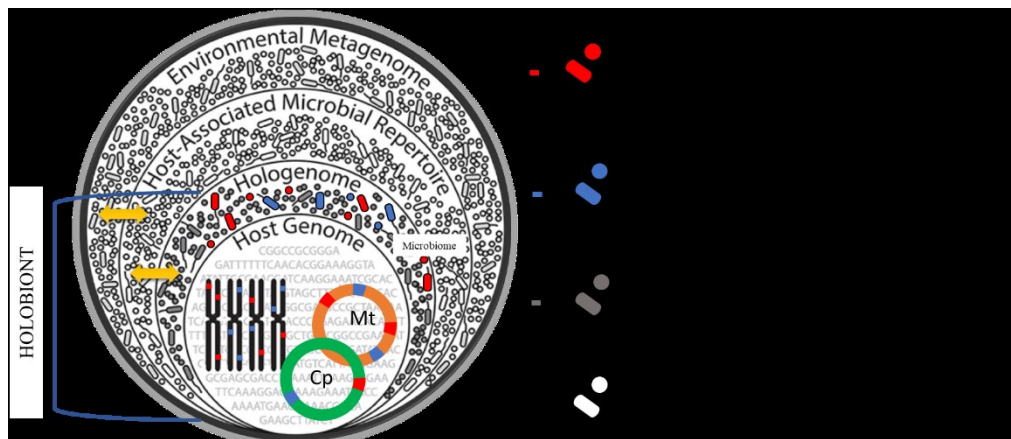


Figure 0-1 Schematic overview of the holobiont concept. Holobionts are comprised of the host and all symbionts, including those that have coevolved with the host and have established long-lasting relationship (blue), and those that did not coevolve but still affect the host (red). In grey are depicted the symbionts that do not affect the host (commensal) and in white the symbionts that are not part of the holobiont. The genomes (mitochondrial, Mt; chloroplast, Cp) of the host and all symbionts combined at any given time point, forms the hologenome. The hologenomic content varies among different environments increasing the complexity of the interactions. The collection of all possible hologenomes associated with the host during its life cycle is referred here as ‘the host-associated microbial repertoire’. Symbionts may be recruited from the environment to become part of the holobiont (yellow arrows). Figure is adapted from Carrier and Reitzel, 2017; Theis et al., 2016.

The significance of microorganisms on host biology and functioning raises questions about the underlying principles that shape these interactions (Parfrey, Moreau and Russell, 2018). For example: which taxa are the key drivers? How do host and symbionts communicate? Is there an exchange of chemical compounds? What is the outcome of the interaction for both partners? How is the symbiosis established? What functions do microorganisms provide to the host?

To answer these questions, one first needs to understand how microbiomes are structured. A description of the overall community composition and relative abundance of community members (who is there?) can help to define which part of the microbiome belongs to the ‘core’ microbiome; which taxa are environmentally/temporally acquired; and how both partners vary

under specific environmental conditions. The next step and one of the main challenges in holobiont research is, to move from purely descriptive towards functional studies (what do they do?).

## **Holobionts in terrestrial models**

Microorganisms affect every aspect of our lives – they are in us, on us and around us, and their activities are of vital importance to basically all processes on earth (Gilbert and Neufeld, 2014), including the functioning of complex multicellular eukaryotes, such as plant and animals. The human body, for example, harbors  $10^{13}$  intestinal bacteria (Sender, Fuchs and Milo, 2016) and many among them contribute to human metabolism in a beneficial way, *i.e.* via the excretion of digestive enzymes, production of essential vitamins, stimulation of host-intestinal immunity, and inhibition of colonization by pathogens (Turnbaugh *et al.*, 2007; Hooper and Macpherson, 2010; Bevins and Salzman, 2011). The human microbiome is, amongst others, shaped by diet (Sonnenburg and Bäckhed, 2016) and host physiology, and alterations in microbiome composition and diversity have been linked to the development of diseases (Pflughoeft and Versalovic, 2012).

Similarly, in plants, the rhizosphere (the layer of soil adjacent to the roots) can contain up to  $10^{11}$  microbial cells per gram of root tissue (Berendsen *et al.*, 2012). The diversity and activity of this community are impacted by plant root exudates and interactions with the surrounding soil, creating a complex and dynamic environment. In return for a steady carbon supply, bacterial symbionts can exert beneficial functions to the host (reviewed in Mendes *et al.*, 2013). Some of these “plant growth-promoting rhizobacteria” (PGPR) are known to fix nitrogen and provide it as ammonia to the plant host; others produce phytohormones, such as auxin, cytokines, and gibberellins, that stimulate root formation and thus plant growth (Doornbos, Van Loon and Bakker, 2012). Plant-associated microbes can also prevent colonization by pathogenic organisms, suppress disease (Mendes *et al.*, 2011), and enhance tolerance to drought and salinity stress (Yang, Kloepper and Ryu, 2009).

## **Holobionts in brown macroalgae - a unique group of multicellular eukaryotes**

Macroalgae, or seaweeds, are sessile, multicellular photosynthetic eukaryotic organisms, that live attached to rocks or other solid substrates in the intertidal zone in coastal waters, where they play an important ecological role, *i.e.* they contribute to primary production in the ocean (Duarte, Middelburg and Caraco, 2005), and function as ecosystem engineers by creating biodiversity hotspots for other marine organisms such as fish, invertebrates, and other seaweeds via provision of food and shelter (Bulleri *et al.*, 2002; Egan *et al.*, 2013; Thornber, Jones and Thomsen, 2016).

Macroalgae comprise three principal groups: red macroalgae (Rhodophyta), green macroalgae (Chlorophyta), and brown macroalgae (Phaeophyceae). They lack roots, stems, and leaves, which differentiates them from terrestrial plants. They were primarily distinguished by their color which is due to differences in the accessory pigments they use to capture light. However, the groups have evolved along different evolutionary paths and do not have a shared multicellular ancestor (Keeling, 2004; Palmer, Soltis and Chase, 2004; Baldauf, 2008; Cock, Peters and Coelho, 2011; Burki, 2014, 2017; Brodie *et al.*, 2017). Red and green macroalgae belong to the lineage of Archeplastida, a monophyletic clade comprising also the glaucophytes and terrestrial plants. Archeplastida originated as a result of primary endosymbiosis, where the uptake of a cyanobacterium (1.6 billion years ago) by a unicellular host led to the development of the plastid and thus photosynthetic activity. Brown macroalgae have originated from secondary endosymbiosis event, *i.e.* the uptake of a unicellular red alga, which developed into the plastid (Keeling, 2004). They belong to the group of stramenopiles and are thus closely related to the diatoms. The brown algae are a diverse group regarding morphology comprising giant kelps and filamentous algae such as *Ectocarpus*. Macroalgae are one of the five taxonomic groups that evolved complex multicellularity, which means that they have organized macroscopic body plans with multiple cell types that develop in specialized tissue. The fact that brown algae are only distantly related to other multicellular eukaryotes (Figure 0-2; Cock *et al.*, 2011) makes them an interesting group to study the evolutionary processes that led to the rise of multicellular eukaryotes. As a result, they display several unique features (Charrier *et al.*, 2007) including complex halogen (iodine) metabolism (La Barre *et al.*, 2010), cell-wall composition (Popper *et al.*, 2011), defense strategies (Ritter *et al.*, 2014), and high resistance to osmotic stressors (Thomas and Kirst, 1991). These unique metabolic features can provide us

with insights in the emergence of complex multicellularity, the physiological adaptations required for life in the intertidal, and how microorganisms have contributed to these two processes. In addition, there is a growing commercial interest in (brown) macroalgae as a source of nutrients, chemicals, and bioactive compounds (Wells *et al.*, 2017).

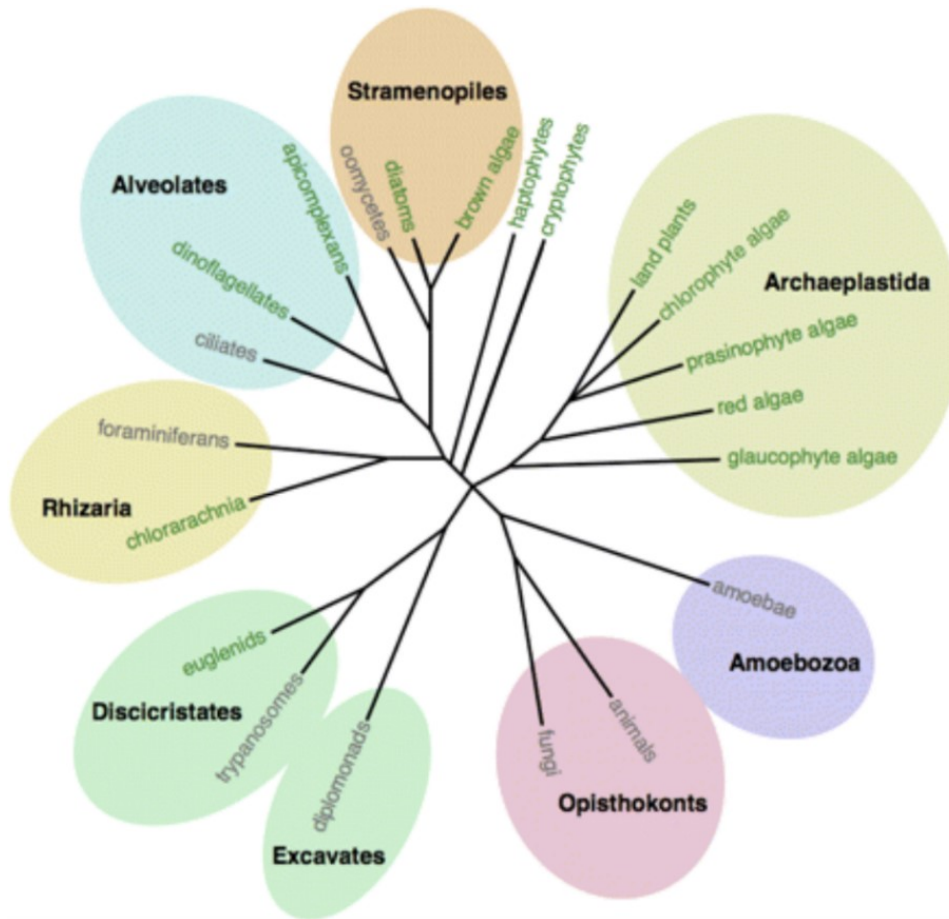


Figure 0-2 Simplified view on the eukaryotic tree of life. Crown taxa are indicated in different colors. The brown algae are in a separate clade compared to land plants, red algae and green algae (Archeplastida). Source: Cock *et al.*, 2011.

In the marine environment, multicellular organisms are also susceptible to microbial colonization and biofilm formation as they are constantly in contact with a variety of ambient free-living microbes (Harder, 2009; Wahl *et al.*, 2012). In fact, the number of microbes in the ocean is estimated to be  $1.2 \times 10^{29}$  (Whitman, Coleman and Wiebe, 1998), and one milliliter of seawater can contain up to  $10^6$  bacteria (Harder, 2009). The colonization pressure exerted by this pool of microorganism is large because they are all competing for nutritional resources and space to settle (Steinberg and De Nys, 2002; Wahl *et al.*, 2012). Macroalgae are particularly attractive for the settlement of marine microorganisms (prokaryotes, eukaryotes, diatoms, fungi, protozoa), because they actively excrete carbohydrates, such as alginates, carrageenan, and cellulose (Egan *et al.*, 2013; Michel *et al.*, 2010; Popper *et al.*, 2011) and other organic or



growth-promoting substances (Salaün *et al.*, 2012; Goecke, Thiel, *et al.*, 2013) that can be rapidly utilized by heterotrophic bacteria. These compounds serve as an energy source and promote settlement and growth of epibionts. Hence, a high number of intimately associated microorganisms (symbionts) can be found in association with seaweeds and (Figure 0-3) similar to the examples mentioned above (human gastrointestinal tract, plant rhizosphere), these can have profound effects on the biology of the algal host (Goecke *et al.*, 2010; Egan *et al.*, 2013; Hollants *et al.*, 2013; Singh and Reddy, 2016). Several relationships between algae and bacteria are obligatory, shown also by axenic culturing of algae, which often have aberrant morphological features or reduced growth. This points out that, for a complete and correct view on algal functioning, metabolism, and performance, bacterial interactions should be incorporated. Thus, in this context, the term ‘holobiont’ seems applicable: both algal host and associated microorganisms are treated as one functional entity (Egan *et al.*, 2013; Figure 0-3).

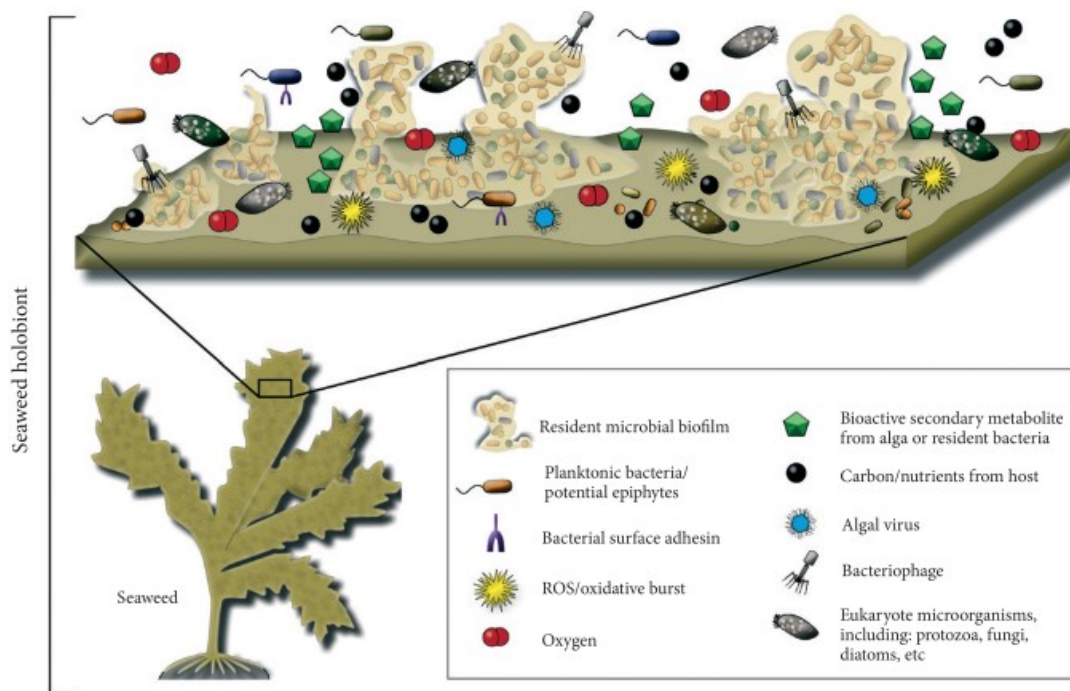


Figure 0-3 The seaweed surface is a complex environment shaped by host factors (e.g. via exudates, ROS) and microbial contributions (e.g. via secondary metabolites). Source: Egan *et al.* 2013.

## **Factors that shape the algal holobiont**

### **Algal defense mechanisms**

As explained in the introduction, macroalgal surfaces form an attractive surface for settlement of microorganisms, and those interactions can affect host physiology and host functioning. Uncontrolled colonization and biofilm formation may affect light penetration as well as nutrient and gas exchange, and thus indirectly affect photosynthetic activity (Wahl *et al.*, 2012; da Gama, Plouguerné and Pereira, 2014). Hence, the algal host needs some level of control, both to tolerate commensal bacteria that inhabit the surface and are of benefit to the host, but also to prevent/regulate colonization by opportunistic/pathogenic invaders. Algal antifouling mechanisms can be exerted via chemical defense mechanisms such as the production of antimicrobial compounds, the release of iodine, and oxidative bursts, or mechanical defense mechanisms, such as shedding of the biofilm-covered outer layer of the algal surface (da Gama, Plouguerné and Pereira, 2014).

### **Anti-microbial compounds**

Macroalgae need to control their microbiomes and one way to do this is to secrete chemical compounds/secondary metabolites that can inhibit or interfere with epibiont settlement (Steinberg and De Nys, 2002; Goecke *et al.*, 2010; Egan *et al.*, 2013; da Gama, Plouguerné and Pereira, 2014). Most of these compounds belong to the terpenes and halogenated compounds (da Gama, Plouguerné and Pereira, 2014).

Studies using whole brown algal tissue extracts, showed inhibition of bacterial growth and/or biofilm formation (Sieburth and Conover, 1965; Caccamese *et al.*, 1985; C Hellio *et al.*, 2001; Claire Hellio *et al.*, 2001; Viano *et al.*, 2009), and the inhibitory effect can be specific towards non-host derived bacteria (Saha *et al.*, 2011; Salaün *et al.*, 2012). In most studies, the exact compound remains to be elucidated, yet structural elucidations were sometimes accomplished. For example, in *Fucus vesiculosus*, fucoxanthin, DMSP, and proline were purified from cell surface extracts and shown to inhibit bacterial growth (Saha *et al.*, 2011, 2012; Lachnit *et al.*, 2013).

Most antifouling compounds are classified as either antimicrobial and/or biofilm inhibiting (Saha, Goecke and Bhadury, 2018). However, some (brown) macroalgal extracts were specifically specified as having an inhibitory effect on quorum sensing (Borchardt *et al.*, 2001; Dobretsov, Dahms and Qian, 2006; Kanagasabhapathy *et al.*, 2009; Goecke *et al.*, 2010; Cho,

2013; Batista *et al.*, 2014). Quorum sensing (QS) is a bacterial density-dependent gene regulatory mechanism, in which the production of signal molecules (called autoinducers) increases with cell density (Waters and Bassler, 2005). When the threshold is exceeded, a signaling cascade is triggered resulting in altered gene expression. QS is, amongst others, involved in the regulation of virulence, symbiosis, swarming and biofilm formation. Algae can selectively inhibit colonization, by producing compounds that inhibit or mimic quorum sensing signals, such as AHLs (Steinberg and De Nys, 2002; Goecke *et al.*, 2010). Examples of QS-inhibiting compounds are halogenated furanones ( derived from the red alga *Delisea pulchra*; Harder *et al.*, 2012; Hudson *et al.*, 2018; Manefield *et al.*, 2002), polybrominated heptanones (derived from the red alga *Bonnemaisonia asparagoides*, Nylund *et al.*, 2010), hypobromous acids (derived from the brown alga *Laminaria digitata*; Borchardt *et al.*, 2001), and dulcitol (derived from the brown alga *Spatoglossum* sp.; Dobretsov *et al.* 2010). More examples are given in Dahms and Dobretsov (2017).

Identifying compounds involved in chemical defense is challenging due to variation in surface metabolites over the seasons (Rickert *et al.*, 2016; Sieburth & Tootle 1981), between tissue parts (Küpper *et al.*, 1998), under changing environmental conditions (e.g. light/temperature Saha *et al.*, 2014), and according to geographical area (Sieburth and Conover, 1965). In addition, defense molecules can be produced by the algal host as well as by commensal microbes (Egan *et al.*, 2000; Goecke *et al.*, 2010; Batista *et al.*, 2014).

### **Oxidative burst and halogen metabolism**

Oxidative burst refers to the production of reactive oxygen species (ROS), such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sup>-</sup>) or hydrogen radicals (H), and functions as a non-specific defense mechanism commonly found in green, red and brown macroalgae (Küpper *et al.*, 2002; Goecke *et al.*, 2010), but also plants and animals. In algae, ROS are produced upon recognition of algal cell wall degradation products, e.g. oligopolysaccharides as a result of bacterial enzyme activity, or bacterial-derived peptides (Potin *et al.*, 2002; Küpper *et al.*, 2006).

Halides, such as iodine, chlorine or bromide, can function as ROS scavengers (Küpper *et al.*, 2008). Brown algae, especially species within the *Laminariales* (kelps), are known to accumulate iodine in the form of iodide (I<sup>-</sup>) in the extracellular matrix (apoplast). They can reach concentrations up to 30.000 times as high compared to the surrounding seawater (La Barre *et al.*, 2010). Iodides are released upon oxidative stress into the apoplast (Küpper *et al.*, 2008) and are able to rapidly oxidize ROS with the help of vanadium-dependent halogen

peroxidases (Küpper *et al.*, 2002; La Barre *et al.*, 2010). The resulting detoxification products (iodinated organic compounds, hypoiodous acid, diiodine), may play a role in the defense against biofouling (Potin *et al.*, 2002; Leblanc *et al.*, 2006; La Barre *et al.*, 2010). The high levels of iodine on the algal surface may create a selection force for iodine-metabolizing bacteria in the epibiont (Amachi, 2008; Barbeyron *et al.*, 2016).

### **Host-specificity of microbiomes**

To conclude, the algal holobiont is a continuously changing environment shaped by the host (cell wall composition, defense molecules), the microbiome (community composition, metabolites, enzymes) and environmental factors (salinity, temperature). Bacteria can respond differently to algal defense mechanism creating a way for the algae to put selective pressure and control the composition of the bacterial epibiont community, *i.e.* attract or repel bacteria dependent on their functions. This can result in specific interactions and co-dependencies between macroalgae and epibionts, where algae favor colonization by mutualists rather than commensals (Bengtsson *et al.*, 2011). Most algal microbiomes are highly specific (Staufenberger *et al.*, 2008; Lachnit *et al.*, 2009; Wang *et al.*, 2018), and different from the surrounding water column (Bengtsson, Sjøtun and Øvreås, 2010; Burke, Thomas, *et al.*, 2011; Mancuso *et al.*, 2016; Lemay *et al.*, 2018). Brown algae microbiomes are generally dominated by *Gammaproteobacteria*, *Alphaproteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, where the latter three phyla are usually less abundant (Hollants *et al.*, 2013; Florez *et al.*, 2017). At lower taxonomic levels (e.g. genus), the taxonomic differences are stronger (Dittami *et al.*, 2016; Florez *et al.*, 2017), resulting in high variation within one host species (Burke, Thomas, *et al.*, 2011). However, different bacterial taxa can have similar functions, and microbes seem to be rather selected by functionality than by taxonomy (Burke, Steinberg, *et al.*, 2011).

### **Bacterial contributions to macro-algal metabolism**

Bacteria can interact with algae beneficially in many different ways (Goecke *et al.*, 2010), and here I selected four examples of interactions that are of interest. They involve nutrients that are limited in the marine environment such as nitrogen, soluble iron, and vitamins, which may create a positive selection force for bacteria to increase the availability of those compounds. I also discuss possible interaction via morphogenetic compounds, as this is a well-described phenomenon in some macroalgae, e.g. *Ulva* and *Ectocarpus*.

## Nitrogen metabolism

Nitrogen (N) is an essential macronutrient required for the production of amino acids, purines, pyrimidines, amino-sugars, and amines. One way to bring nitrogen into the aquatic environment is via nitrogen fixation ( $N_2 \rightarrow NH_3$ ), which can be subsequently converted into ammonium ( $NH_4$ ) and nitrate ( $NO_3$ ). Macroalgae cannot fix  $N_2$  and they depend on external sources of nitrogen, for example in the form of nitrate, ammonium or organic nitrogen released by nitrogen-fixing bacteria (diazotrophs; Lobban and Harrison, 1994). Living closely associated with nitrogen-fixing bacteria is one solution to obtain sufficient levels of nitrogen. Nitrogenase activity, *i.e.* the capacity to convert  $N_2$  into  $NH_3$  was found in genomes of endophytic *Rhizobiales* isolated from *Caulerpa taxifolia* (Chisholm *et al.*, 1996). In *Sargassum*, cyanobacteria were shown to contribute to the algal nitrogen-supply (Phlips, Willis and Verchick, 1986). The supply of fixed nitrogen by *Rhizobacteria* is a well-described process in plants (Mendes, Garbeva and Raaijmakers, 2013). Additionally, bacterial symbionts can provide algae with nitrogen in the form of ammonium ( $NH_4$ ). For example, *Sulfitobacter* increased ammonium release (*i.e.* nitrogen reduction) upon co-cultivation with *Pseudonitzschia* and the diatom host was shown to have a preference for the bacterially derived ammonium over exogenous nitrate (Amin *et al.*, 2015). Such symbiotic interactions could potentially be beneficial to the alga because nitrate, the predominant form of nitrogen in coastal waters, is energetically more costly to assimilate than ammonium (Rees *et al.*, 2007). Bacterial communities on the surface of *Macrocystis* were shown to be enriched in nitrogen metabolism (e.g. nitrite and nitrate reductases), compared to bacteria in the surrounding water, suggesting a possible interaction between the kelp host and nitrogen reducing symbionts (Minich *et al.*, 2018).

## Siderophore uptake

Iron is an essential element for all organisms and involved in a range of biological processes, including nitrogen fixation/nitrate utilization, methanogenesis, respiration and oxygen transport, chlorophyll synthesis, gene regulation, and DNA synthesis (Keshtacher-Liebson, Hadar and Chen, 1995; Smith *et al.*, 2010). However, the predominant form of iron in the aquatic environment is Fe(III), which has low solubility and easily mineralizes in the form of iron oxide. This, in turn, limits the availability of soluble iron to marine organisms. Hence, iron is, next to nitrogen and phosphorus, one of the limiting factors for primary production in the ocean (Fung *et al.*, 2000; Vraspir and Butler, 2009; Giovannoni, 2017).

Siderophores, produced by bacteria, fungi, and cyanobacteria, are iron-chelating compounds, meaning that they bind to Fe(III) to create soluble iron-complexes and, as a result, increase the bioavailability of iron. Bacteria can uptake the siderophores themselves, however, siderophores can also be scavenged and processed by other organisms, as shown for dinoflagellates (*Marinobacter*; Amin et al., 2009) and green microalgae (*Halomonas*; Keshtacher-Liebson et al., 1995), suggestive of a mutualistic exchange of fixed carbon and complexed iron. Studies on iron acquisition in (brown) macroalgae are scarce. However, metagenomic analysis of microbial communities associated with *Macrocystis* showed an enrichment of genes related to iron acquisition compared to seawater communities (Minich *et al.*, 2018).

## Vitamins

Vitamin B12 is an organic compound and essential micronutrient for all organisms on earth because it serves as an enzymatic cofactor in methionine synthesis. Production of the vitamin B12 is restricted to prokaryotes. Recently, Croft and co-workers showed that vitamin B12 auxotrophy is common among uni- and multicellular algae. Among all the species investigated, 50% required an external supply of vitamin B12 (cobalamin) and among the 80 species of Stramenopiles that were investigated this was 59% (Croft, Warren and Smith, 2006). However, vitamin B12 levels in the natural environment cannot sustain algal growth (Croft *et al.*, 2005). It was thus hypothesized that bacterial symbionts could provide sufficient amounts of the vitamin to support algal growth (Helliwell *et al.*, 2011). Indeed, the vitamin B12-auxotrophic microalga *Porphyridium purpureum* (Rhodophyta) was able to grow on vitamin B12 produced and excreted by *Halomonas* sp. in co-cultures. In addition, bacterial growth was increased in co-cultures suggesting a mutualistic interaction (Croft *et al.*, 2005).

Early studies on the nutritional requirements of brown macroalgae (Boalch, 1961; Pedersén, 1969; Provasoli and Carlucci, 1974) showed that the addition of vitamin B12 to the algal medium could stimulate the growth of ten brown macroalgal species, including *Ectocarpus fasciculatus*, but there was no absolute requirement (Pedersén, 1969). More recently, it was shown that *Ectocarpus siliculosus* has both the vitamin B12-independent and dependent form of methionine synthase (Helliwell *et al.*, 2011). This suggests that the alga does not depend on the external supply of vitamin B12 for the working of the enzyme, but it may benefit from it if it is present.

It remains to be elucidated whether bacteria can sustain growth in brown macroalgae in a similar way as described for *P. purpureum* (Croft *et al.*, 2005). Hints that this may be possible can be

drawn from genomes obtained from algal-associated bacterial symbionts. Such a strategy was applied to a bacterial genome that was sequenced along with the *Ectocarpus siliculosus* genome (Cock *et al.*, 2010; Dittami *et al.*, 2014). This bacterium, *Candidatus* Phaeomarinobacter ectocarpi, can probably contribute to certain algal metabolic processes (nutrient assimilation, growth factors), however, it is not able to provide vitamin B12. Such a genomic approach creates new opportunities to investigate nutritional corporation between algae and bacterial symbionts, beyond the relatively well-studied example of vitamin B12. This approach is especially valuable for species that are difficult to obtain in axenic conditions, which is the case for brown macroalgae (Boalch, 2018; Fries, 1973).

### **Morphogenetic compounds**

In addition to the growth-promoting effect of bacteria on algae via the provision of nutrients, several bacteria influence morphology and development of macroalgal species. This was first observed in axenic algal cultures, which often show deformations compared to algae living with their natural microbiomes (Pedersén, 1968). In *Ectocarpus*, removal of symbiotic bacteria via antibiotic treatment has significant effects on algal growth and morphology (Tapia *et al.*, 2016). In axenic conditions, the algae have a ball-like appearance compared to the branched morphology when associated with full flora. Several bacteria, *i.e.* *Marinobacter* sp., *Halomonas* sp. and *Roseobacter* sp., isolated from the *Ectocarpus* surface were shown to have morphogenetic activity (Tapia *et al.*, 2016); they were able to restore the branched morphotype to a similar extent as the full bacterial inoculum (Figure 0-4).

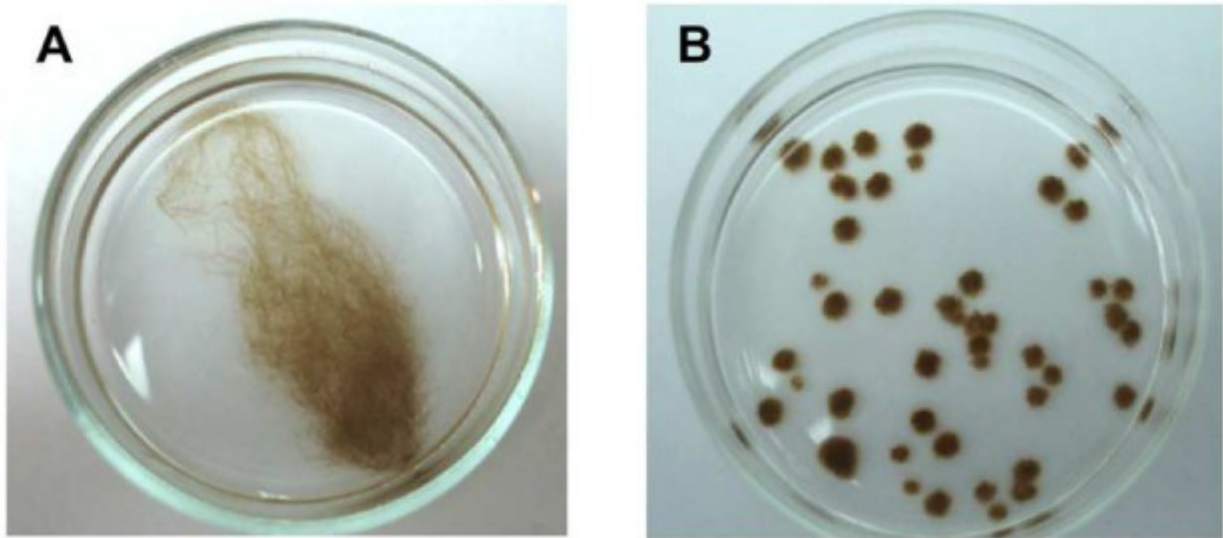


Figure 0-4 *Ectocarpus siliculosus* in seawater associated with its full microbiome (A) and after treatment with antibiotics (B); Source: Tapia et al., 2016

The effect of bacteria on morphology in *Ectocarpus* may be mediated by phytohormones. Phytohormones are signaling molecules in plants, that regulate plant growth, development, and reproduction. The same molecules may also be involved in regulation of stress response to abiotic changes. Examples of chemicals that can function as phytohormones in algae are auxin, cytokines, abscisic acid, gibberellins, jasmonic acid, and polyamines (Tarakhovskaya, Maslov and Shishova, 2007). Some of those were shown to affect the development of *Ectocarpus*. For example, the cytokine kinetin was required for normal growth in *Ectocarpus fasciculatus* (Pedersén, 1968, 1973) and, similarly, auxins affected cell differentiation in *Ectocarpus siliculosus* (Le Bail *et al.*, 2010). However, it is sometimes difficult to determine whether the compounds are indeed produced by the alga or provided externally by bacteria (Bradley, 1991). Based on *in silico* analyses of the *Ca. P. ectocarpi* genome obtained during sequencing of the *Ectocarpus* genome (Cock *et al.*, 2010), both cytokine and auxin can be produced in algal-bacterial co-cultures, when allowing for exchanges of intermediates between the bacterium and the alga (Dittami *et al.*, 2014).

In green macroalgae, morphological changes in the algal host have been linked to bacterial associations (Spoerner *et al.*, 2012a) and the production of Thallusin, a morphogenetic compound excreted by *Cytophaga* sp. (Matsuo *et al.*, 2005). Morphogenetic activity was shown to be a shared characteristic among *Ulva*-associated bacteria, supporting the idea that bacteria from other seaweeds, such as *Ectocarpus*, may behave in a similar way.



## Macroalgal holobionts in a changing environment

Currently, marine environments are changing, mainly due to human-induced climate change. Abiotic stressors, such as elevated temperatures or changing salinities, are known to significantly alter microbial community composition (Stratil *et al.*, 2014; Dittami *et al.*, 2016; Minich *et al.*, 2018). Several studies have shown that dysbiosis caused by environmental disturbance can impact the macroalgal holobiont (Egan *et al.*, 2013). For example, pathogenic invasion of the red alga *Delisea pulchra* by a Rhodobacteraceae was shown to be temperature dependent (Campbell *et al.*, 2011; Case *et al.*, 2011). Similarly, in *Fucus vesiculosus*, the relative abundance of Rhodobacteraceae was increased during temperature stress, although this was not linked to decreased algal performance (Stratil *et al.*, 2013; Saha *et al.*, 2014). In *Macrocystis pyrifera*, a temperature rise led to a proliferation of alginate-degrading bacteria on the algal surface which may make the alga more susceptible to decomposition and subsequent colonization by opportunistic pathogens (Minich *et al.*, 2018). Nevertheless, some detrimental processes are an inherent part of the host life cycle, such as the degradation of algal cell walls, which contributes to the carbon and nutrient cycle in the ocean via the microbial loop. It is thus difficult to predict the outcome of stress-induced changes in the holobiont.

The environmental change that is studied here is the change from high to low salinity. The marine environment contains over ~2000 known species of brown (macro)algae (Guiry and Guiry, 2018), of which only eight species are known to also occur in freshwater (Dittami *et al.*, 2017). The freshwater strain (FWS) of *Ectocarpus subulatus* (West and Kraft, 1996; Peters *et al.*, 2015), represents one of those species and is currently the only publicly available freshwater strain publicly available. *E. subulatus* FWS grows equally well in seawater and fresh water (Dittami *et al.*, 2012), and cultures can be transferred back and forth without any problems. Others strains of the same species are also known for their particularly high tolerance to abiotic stressors, such as temperature (Bolton, 1983), and salinity (Bolton, 1983; Peters *et al.*, 2015). These processes, and in particular algal growth in fresh water, have been shown to depend on interactions with symbiotic bacteria (Dittami *et al.*, 2016). Therefore, *Ectocarpus subulatus* FWS is developed as a model for brown algal adaptation and acclimation. *Ectocarpus* is easily cultivable in vitro, has a short life cycle and a relatively small genome which has been sequenced (Peters *et al.*, 2004; Cock *et al.*, 2010; Dittami *et al.*, 2018). How the algal holobiont responds to and mediates these salinity changes, and the role of the algal microbiome herein is the subject of my thesis.

## **Thesis subject: The response of *Ectocarpus* holobionts to changing salinity**

The main question I aim to answer during my thesis is: How do *E. subulatus* FWS and bacteria interact during acclimation of the holobiont to low salinity?

The microbiome composition of *Ectocarpus subulatus* had been described before the start of my thesis, using 16S rRNA gene metabarcoding techniques (Dittami *et al.*, 2016), and it was shown that the transition from seawater to fresh water, strongly affects the composition of the bacterial community. The aim of my project was to move from descriptive studies (who is there) towards the actual role of bacteria within the holobiont (what do they do).

To enable targeted experiments, it was critical to work with cultivable organisms. The first part of my thesis, therefore, consisted of the cultivation of bacteria living in association with *E. subulatus* FWS. Several cultivation methods were applied in parallel, and I isolated and characterized 388 bacterial isolates, corresponding to 46 different 16S sequences, capturing 33 different genera. These experiments and the results were recently published and are presented in **Chapter 1**.

**Chapter 2** describes the first algal-bacterial co-culture experiments that I carried out using the set of cultured bacteria. The bacteria were tested individually or in mixtures for their effect on growth of *E. subulatus* in fresh water. None of the cultivated bacteria had a strong beneficial effect on algal growth in freshwater (**Chapter 2 – subsection I**). Therefore, a new method to select bacterial communities for *in vitro* testing was implemented and experimentally verified (**Chapter 2 – subsection III**). This work shows that metabolic complementarity between algae and a subset of cultured bacteria, is a promising way to select bacterial communities that are beneficial to the algal, at least in seawater. The collection of cultured bacteria was also tested on a different model system, *i.e.* *Ulva mutabilis*. During my stay at the Friedrich Schiller University in Jena I tested whether bacteria that were cultivated from *E. subulatus* had an effect on the development of *Ulva mutabilis*. The results show that the cultured bacteria, although derived from a distantly related host, have similar beneficial effects on *Ulva* as bacteria derived from *Ulva* itself (**Chapter 2 – subsection II**).

The majority of the *Ectocarpus* microbiome, however, is comprised of uncultured bacterial taxa. To incorporate those interactions, I implemented a metatranscriptomics / metagenomics approach (**Chapter 3**). By comparing the metatranscriptome and the simultaneously obtained

metabolite data of three different holobionts in fresh water and in seawater, I gained insights in how the holobiont as a whole reacts to the change in salinity.

All together these results contribute to a better understanding of how the *Ectocarpus* holobiont responds during abiotic stress and especially how bacteria are involved in this process.

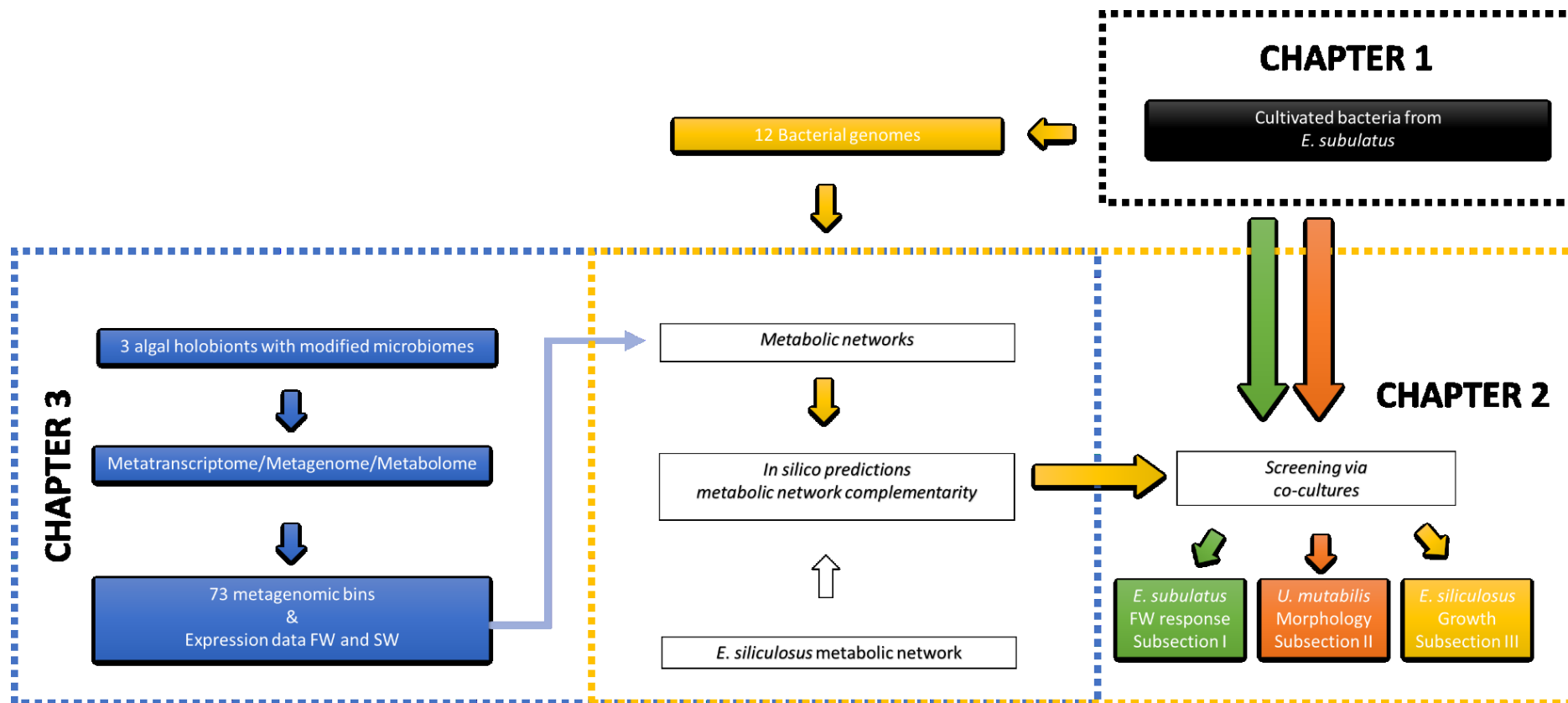


Figure 0-5 Schematic overview of experiments that were carried out during my PhD thesis. Two complementary strategies were carried out in parallel, namely algal-bacterial co-culture experiments (chapter 2) and metatranscriptome/metagenomic analysis of different algal holobionts (Chapter 3).

# Chapter 1.

# Chapter 1. Exploring the cultivable *Ectocarpus* microbiome

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Keywords: *Ectocarpus*, holobiont, bacterial cultivation, brown macroalgae, dilution-to-extinction, metabarcoding

Published: 11 December 2017

KleinJan, H., Jeanthon, C., Boyen, C., and Dittami, S. M. (2017). Exploring the cultivable *Ectocarpus* microbiome. *Front. Microbiol.* 8, 2456. doi:10.3389/fmicb.2017.02456.

## Abstract

Coastal areas form the major habitat of brown macroalgae, photosynthetic multicellular eukaryotes that have great ecological value and industrial potential. Macroalgal growth, development, and physiology are influenced by the microbial community they accommodate. Studying the algal microbiome should thus increase our fundamental understanding of algal biology and may help to improve culturing efforts. Currently, a freshwater strain of the brown macroalga *Ectocarpus subulatus* is being developed as a model organism for brown macroalgal physiology and algal microbiome studies. It can grow in high and low salinities depending on which microbes it hosts. However, the molecular mechanisms involved in this process are still unclear. Cultivation of *Ectocarpus*-associated bacteria is the first step towards the development of a model system for *in vitro* functional studies of brown macroalgal-bacterial interactions during abiotic stress. The main aim of the present study is thus to provide an extensive collection of cultivable *E. subulatus*-associated bacteria.

To meet the variety of metabolic demands of *Ectocarpus*-associated bacteria, several isolation techniques were applied, *i.e.* direct plating and dilution-to-extinction cultivation techniques, each with chemically defined and undefined bacterial growth media. Algal tissue and algal growth media were directly used as inoculum, or they were pretreated with antibiotics, by filtration, or by digestion of algal cell walls. In total, 388 isolates were identified falling into 33 genera (46 distinct strains), of which *Halomonas* (*Gammaproteobacteria*), *Bosea* (*Alphaproteobacteria*), and *Limnobacter* (*Betaproteobacteria*) were the most abundant. Comparisons with 16S rRNA gene metabarcoding data showed that culturability in this study was remarkably high (~50%), although several cultivable strains were not detected or only present in extremely low abundance in the libraries. These undetected bacteria could be considered as part of the rare biosphere and they may form the basis for the temporal changes in the *Ectocarpus* microbiome.

## Introduction

Coastal areas form the major habitat of brown macroalgae, photosynthetic eukaryotic organisms that are important primary producers and form biodiversity hotspots for other marine (macro)organisms by providing them with food and shelter (Thornber, Jones and Thomsen, 2016). The seaweed surface is a highly attractive substrate for the settlement of marine microorganisms, due to the fact that they actively excrete carbohydrates and other organic or growth-promoting substances (Salaün *et al.*, 2012; Goecke, Thiel, *et al.*, 2013) that can be rapidly utilized by bacteria. Several stable relationships exist that have been shown to benefit brown macroalgal hosts (Goecke *et al.*, 2010; Hollants *et al.*, 2013; Singh and Reddy, 2016). Algae-associated (symbiotic) microbes can, for example, communicate on a chemical level through the provision of growth hormones (Pedersén, 1973), vitamins (Pedersén, 1969; Croft *et al.*, 2005), or morphogens (Tapia *et al.*, 2016), and some algal-bacterial interactions are known to affect biofouling and pathogenic invasion by other microorganisms (Singh and Reddy, 2014). Due to the tight relationships and functional co-dependencies between algae and their associated microbiomes, both can be seen as one functional entity or “holobiont” (Zilber-Rosenberg and Rosenberg, 2008; Egan *et al.*, 2013).

Elucidating the functions and molecular mechanisms that shape the algal holobiont is of crucial importance, not only for the fundamental understanding of macroalgal functioning in marine ecosystems, but also to improve macroalgal culturing, an industry that has increased intensively over the last decade due to the growing interest in algae as a source for nutrients, chemicals and bioactive compounds (Wells *et al.*, 2017). *In vitro* studies of the commercially valuable and environmentally most relevant brown macroalgae (kelps, order *Laminariales*) remain challenging due to their size and complex life cycles (Peters *et al.*, 2004). Model organisms, such as the filamentous brown alga *Ectocarpus* are therefore an essential tool to enable functional studies on algal-bacterial interactions in the laboratory. *Ectocarpus* is easily cultivable *in vitro*, has a short life cycle and a relatively small genome which has been sequenced several years ago (Peters *et al.*, 2004; Cock *et al.*, 2010).

Here we study the microbiome of a freshwater strain of *Ectocarpus subulatus* (West and Kraft, 1996). The transition to fresh water is a rare event in brown algae that occurred in only a few species (Dittami *et al.*, 2017). The examined strain is currently the only publicly available freshwater isolate within the Ectocarpales, and it is still able to grow in both seawater and



freshwater (Dittami *et al.*, 2012). This and other isolates of the same species are known for their particularly high tolerance to abiotic stressors (Bolton, 1983; Peters *et al.*, 2015) and are being developed as a model to study brown algal adaptation and acclimation. These processes, and in particular algal growth in fresh water, have been shown to depend on interactions with symbiotic bacteria (Dittami *et al.*, 2016).

The aim of the present study is to develop an extensive collection of cultivable *E. subulatus*-associated bacteria that can be used to study the functions of bacterial symbionts during abiotic stress in controllable and reproducible experimental settings, using the freshwater strain of *E. subulatus* as a model. Different bacterial isolation techniques were applied in parallel to increase the number and diversity of cultivable strains, *i.e.* direct plating and dilution-to-extinction cultivation techniques, each with chemically defined and undefined bacterial growth media. Algal tissue and algal growth media were directly used as inoculum, or they were pretreated with antibiotics, by filtration, or by digestion of algal cell walls. Our data show an overall high culturability of *Ectocarpus*-associated bacteria including a high number of low abundance taxa.

## **Material and methods**

### **Cultivation of algae - starting material for isolation of bacterial symbionts**

All experiments were carried out using sporophytes of the *Ectocarpus subulatus* freshwater strain (EC371, accession CCAP 1310/196, West & Kraft 1996). This culture was obtained from Bezhin Rosko (Santec, France) in 2007 and maintained in our laboratory under the following conditions since then: cultures of EC371 were grown in Petri dishes (90 mm Ø) in natural seawater (NSW; collected in Roscoff 48°46'40"N, 3°56'15"W, 0.45 µm filtered, autoclaved at 120°C for 20 min), or in diluted seawater-based medium (DNSW; by twenty-fold dilution of natural seawater with distilled water). Both media were enriched with Provasoli nutrients (Starr and Zeikus, 1993) and cultures kept at 13°C with a 12h dark-light cycle (photon flux density 20 µmol m<sup>-2</sup>·s<sup>-1</sup>).

### **Isolation and characterization of algae-associated bacteria**

A range of cultivation strategies as well as bacterial growth media was exploited. The starting material for bacterial isolation was EC371 grown with its full microbial flora (direct plating and

dilution-to-extinction cultivation) and EC371 with a reduced microbial flora (size-fractionation and antibiotic treatment), both originating from the same algal culture. Algal subcultures were sampled 5-10 days after the last change in medium. Algal growth medium and ground algal tissue, in NSW and DNSW were used. The isolation experiments took place from November 2013 to September 2016. Three selected cultivation experiments (dilution-to-extinction cultivation, direct plating with antibiotics; direct plating without pretreatment) were repeated under identical conditions after six months, one year, or three years, respectively, to assess the reproducibility of the results over time. An overview of the isolation methods and cultivation strategies is provided in Figure 1-1.

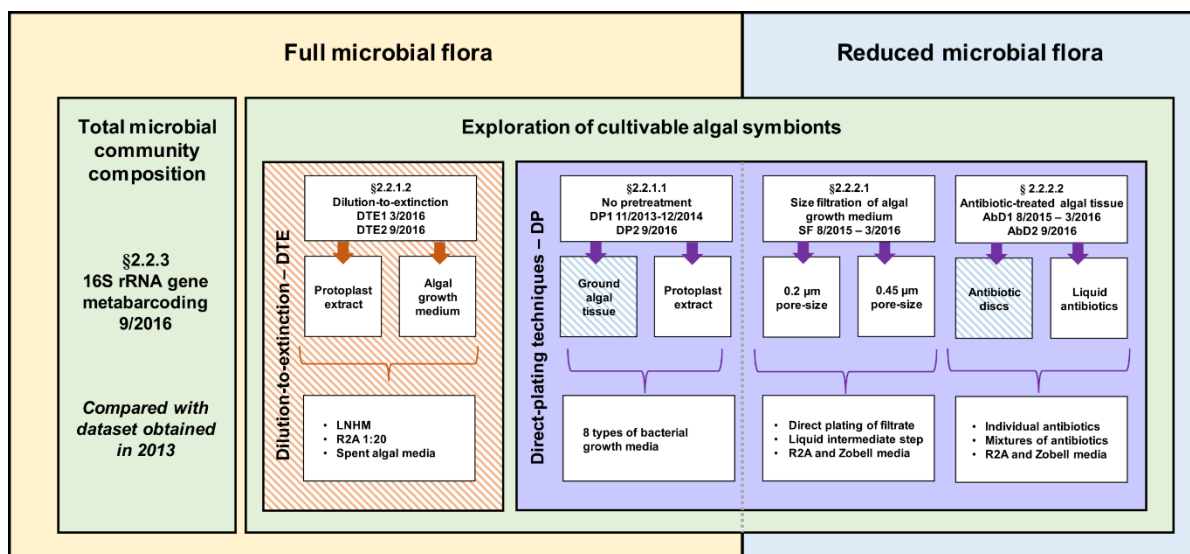


Figure 1-1 Overview of the methodology and cultivation strategies used to cultivate algae-associated bacteria. On one hand, direct inoculation with algal tissue and/or algal growth medium was used (yellow), while on the other hand, the microbial community was reduced before inoculation (blue). Additionally, a distinction can be made between direct plating (DP, purple) with and without pretreatment (§2.2.1.1-2.2.2.2), and dilution-to-extinction cultivation (§2.2.1.2; DTE, orange). 16S rRNA gene metabarcoding of the total prokaryotic community was carried out in parallel. Striped boxes indicate experiments that have been repeated twice within a six months interval for DTE1-DTE2, a one-year interval for AbD1-AbD2, and a three-year interval for DP1-DP2 and META13-META16 (Dittami *et al.*, 2016).

## Isolation of bacteria from algae with their full microbial flora

### Direct plating techniques

To isolate bacteria, algal growth media, ground algal tissue, and algal protoplast digest product of EC371 grown in DNSW or NSW were directly plated (DP) on eight different growth media solidified with 1.5% agar. The eight bacterial growth media were: R2A prepared in distilled

water (adapted from Reasoner & Geldreich 1985); R2A prepared in natural seawater instead of distilled water; Zobell marine agar (Zobell 1941); Zobell marine agar with 16-fold reduced salinity; *Ectocarpus*-based medium (ground *E. subulatus* 5 g DW·L<sup>-1</sup>; Peptone 0.5 g·L<sup>-1</sup>, Provasoli nutrients 10 ml·L<sup>-1</sup>, 5% NSW); Peptone Yeast Glucose (PYG) agar (Peptone 0.5 g·L<sup>-1</sup>; Yeast Extract 0.5 g·L<sup>-1</sup>; Glucose 0.5 g·L<sup>-1</sup>); PYG with glucose replaced by mannitol (5 g·L<sup>-1</sup>) and Provasoli nutrients 10 ml·L<sup>-1</sup>; and LB with NaCl (2 g·L<sup>-1</sup>). In some cases, a liquid intermediate step was applied, and in all cases, non-inoculated media and plates were included as negative controls. The exact recipes of the media can be found in Supplementary table 1-1 and the detailed experimental treatments in Supplementary table 1-3. The algal protoplast digest product (used in dilution-to-extinction cultivation as well) was produced using the protocol from Coelho et al. (2012) with an additional 2.0 µm size-filtration after complete cell wall digestion (step 5) and the filtrate was used for direct plating. After incubation for up to 45 days at either 4 °C, 13 °C, 30 °C, or room temperature (RT), 1-10 single colonies were picked randomly. Furthermore, any colonies that differed with regard to their shape, size or color were also included. The colonies were grown in liquid growth media and identified by sequencing their 16S rRNA gene via Sanger sequencing (as described below). Direct plating of ground EC371 tissue grown in NSW was repeated after three years. Due to the variety of experiments colony counts were variable, ranging between one and several hundred per plate.

### **Dilution-to-extinction cultivation**

As a strategy to reduce nutrient competition between the cultivable members of the EC371 microbiome, the high throughput dilution-to-extinction (DTE) cultivation approach was used as originally described by Cannon & Giovannoni (2002): microbial communities from either algal growth medium or algal protoplast digest product (see the previous section) were 0.6 µm-filtered to remove microbial and carbohydrate aggregates, diluted to a predefined cell number and distributed into 96-well deep well plates with low-nutrient media. Algal tissue may harbor cell-wall attached bacteria whose numbers cannot be determined with flow cytometry. In addition, the algal fragments block the flow cytometer which also prevents correct cell counting. Therefore, algal tissue could not be directly used in dilution-to-extinction experiments. Four liquid bacterial growth media were used to cultivate bacteria: 20-fold diluted R2A prepared in DNSW with starch replaced by alginate (0.025 g L<sup>-1</sup>); Low Nutrient Heterotrophic Medium (LNHM) with 0.001 g L<sup>-1</sup> mannitol (adapted from Cho and Giovannoni, 2004; Stingl et al., 2008; Jimenez-Infante et al., 2014; Carini et al., 2012; Stingl et al., 2007) and 2 and 7 weeks old spent EC371 growth medium (5% NSW). Recipes can be found in

Supplementary table 1-2. For R2A and Zobell media, stock solutions of the individual components were prepared and autoclaved separately and the final bacterial growth medium was prepared on the day of the experiment. For LNHM, stock solutions were 0.2 µm filter-sterilized but not autoclaved. On the day of the experiment, individual components were mixed, the pH was adjusted to 7.3, and the bacterial growth media was filter-sterilized (0.1 µm) and divided into 96-well deep well plates before inoculation (0.5 ml/well). Preliminary tests of inoculations with 3, 1, and 0.5 cells/well, showed that 0.5 cells/well was the optimal inoculation density to limit the occurrence of bacterial mixtures and to obtain pure bacterial clones. Non-inoculated bacterial growth medium was used as a negative control. The experiment was performed twice within a six-month interval (DTE1 in March 2016 and DTE2 in September 2016). Flow cytometry was used to obtain both the bacterial cell counts of the inocula and to monitor bacterial growth. After 4 weeks of incubation (16 °C, 12:12h dark:light cycle, 27 µmol s<sup>-1</sup>·m<sup>-2</sup>), bacterial growth was screened by flow cytometry using a BD Accuri C6 cytometer (BD Biosciences): 100 µl of the cultures were fixed with glutaraldehyde (0.25%, final concentration) and stained with Sybr Green (Life Technologies) as described by Marie et al. (1997). Wells with cell densities of 10<sup>4</sup> cells/ml and higher were considered positive. The number of cultivable bacteria  $n_{\text{cult}}$  in the original inoculum was estimated based on the proportion of negative wells ( $p_{\text{neg}}$ ) according to a Poisson distribution using the formula  $n_{\text{cult}} = \ln(1/p_{\text{neg}}) \cdot w$ , where  $w$  is the total number of wells inoculated (Button *et al.*, 1993). This allowed for the calculation of the ratio of cultivable to total bacteria (the latter determined via flow cytometry) in these experiments (estimated culturability).

## **Isolation of bacteria from algae with a reduced microbial flora**

### **Size-fractionation of algal growth media**

As a second strategy to reduce bacterial cell numbers before plating, size-fractionation (SF) was used to facilitate the growth of smaller and less abundant bacterial strains. EC371 culture medium was filtered with 0.2 (SF0.2), 0.45 (SF0.45), or 40 (SF40) µm pore-size, and 50 µl filtrate were directly plated on R2A or Zobell agar. At the same time, 100 µl filtrate were used to inoculate liquid R2A or Zobell as an intermediate to enhance bacterial growth before plating. After five to eight days of incubation at RT, 50 µl of the liquid culture were plated on solidified R2A and/or Zobell. In both cases, plates were incubated until single colonies were visible (3-20d) and the latter identified with 16S rRNA amplicon sequencing as described below.

### **Antibiotic-treatments of algal tissue and growth media**

Antibiotics were used to reduce the abundance of dominant bacterial strains from the algal tissue and/or growth media, in our case especially *Halomonas* sp., and to facilitate the growth of other less abundant or slower-growing bacteria. Algal growth media and/or ground algal tissue was spread on R2A agar plates and incubated with two antibiotic discs (AbD1 and AbD2; Antimicrobial Susceptibility Disks, Bio-Rad Laboratories, Inc, France) for five days at RT, using antibiotics that were shown to be effective against *Ectocarpus*-derived *Halomonas*. Alternatively, algal subcultures of the same strain were treated with liquid antibiotics (AbL) for 3 days before plating on R2A or Zobell, whereafter 50 µl were plated on solidified R2A or Zobell. An overview of the antibiotics (discs and liquid) and their concentration can be found in Supplementary table 1-2. Plates were incubated (3-20d) until single colonies were visible and the latter identified with 16S rRNA amplicon sequencing as described below. Two experiments using antibiotic-treated algal tissue (AbD2 with chloramphenicol and erythromycin) were repeated after one year.

### **Bacterial identification by 16S rRNA gene sequencing**

To identify bacterial isolates, single colonies were grown in the corresponding liquid growth media until maximal density was reached. Approximately 50-100 µl of cultures were heated for 15 minutes at 95 °C. Then the 16S rRNA gene was amplified using universal primers (8F 5' AGAGTTTGATCCTGGCTCAG and 1492R 5' GGTTACCTTGTTACGACTT from Weisenburg et al. (1991) and the GoTaq polymerase in a PCR reaction with the following amplification conditions: 2 min. 95 °C; [1 min 95 °C; 30 sec. 53 °C; 3 min 72 °C] 30 cycles; 5 min 72 °C. In some cases, a commercial kit was used to extract DNA (NucleoSpin® Tissue, Machery-Nagel; support protocol for bacteria). The PCR products were purified using ExoSAP (Affymetrix Inc, Thermo Fisher Scientific) and sequenced with Sanger technology (BigDye Xterminator v3.1 cycle sequencing kit, Applied Biosystems®, Thermo Fisher Scientific). Only the forward primer 8F was used for the sequencing reaction. For classification and analyses of the sequences, RDP classifier (Wang *et al.*, 2007) and BLAST<sup>1</sup> against the NCBI nr and 16S rRNA gene databases were used and sequences classified at the genus level if possible. Sequences were aligned and checked manually to verify mismatches and to identify distinct strains within a genus (>99% identity). The 16S rRNA gene sequences from each distinct

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<sup>1</sup> <https://blast.ncbi.nlm.nih.gov/Blast.cgi>

cultivable strain were aligned using MAFFT<sup>2</sup> version 7 (Kato *et al.*, 2002) and the G-INS-i algorithm. Only well-aligned positions with less than 5% alignment gaps (492 positions) were retained. Phylogenetic trees were reconstructed using the Maximum-Likelihood method implemented in MEGA6.06 (Tamura *et al.*, 2013), and the GTR+G+I model. Five hundred bootstrap replicates were tested to assess the robustness of the tree. The unique 16S rRNA gene sequences were submitted to the European Molecular Biology Laboratory (EMBL) database and are available under project accession number PRJEB22665. Stocks of bacterial isolates were preserved in 40% glycerol at -80°C. The numbers of sequences obtained per taxa were normalized against the total number of sequences obtained within the complete cultivation study. To assess cultivation biases statistically, the absolute abundances of cultivable isolates were analyzed in R (RStudio Team, 2016) with the Fisher-exact test and Bonferroni post hoc correction for multiple testing ( $\alpha=0.05$ , significant if  $p<0.0011$ ).

### **16S rRNA gene metabarcoding**

To estimate the proportion of cultivable bacteria in our algal cultures, the collection of bacterial isolates was compared with 16S rRNA gene libraries from the same algal culture used for our isolation experiments. These libraries served as a reference to assess the total microbiome, including cultivated and non-cultivated bacteria; they were not used to infer diversity *per se*. EC371 cultures were grown for 9 weeks in seawater-based culture medium (changed on a monthly basis, last one week prior to sampling). Algal tissue was filtered with sterile coffee filters, dried on a paper towel, and snap-frozen in liquid nitrogen. Four technical replicates were pooled two by two. Total DNA was isolated (NucleoSpin® Plant II, Machery-Nagel; standard protocol) and purified with Clontech CHROMA SPIN™-1000+DEPC-H<sub>2</sub>O Columns. The V3-V4 region of the 16S rRNA gene was amplified and sequenced with Illumina MiSeq technology by MWG Eurofins Biotech (Ebersberg, Germany) using their proprietary protocol. The first preliminary quality control was done with FastQC<sup>3</sup>, and fastq\_quality\_trimmer from the FASTX Toolkit<sup>4</sup> was used to quality-trim and filter the 568,100 reads (quality threshold 25; minimum read length 200). The resulting 553,896 sequences (2.5 % removed) were analyzed with Mothur (V.1.38.0) according to the MiSeq Standard Operating Procedures<sup>5</sup> (Kozich *et al.*, 2013). Filtered reads were assembled into 270,522 contigs, preclustered (allowing for 4

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<sup>2</sup> <http://mafft.cbrc.jp/alignment/software>

<sup>3</sup> <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

<sup>4</sup> [http://hannonlab.cshl.edu/fastx\\_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)

<sup>5</sup> [https://www.mothur.org/wiki/MiSeq\\_SOP](https://www.mothur.org/wiki/MiSeq_SOP)

mismatches), and aligned with the non-redundant Silva SSU reference database version 123 (Quast *et al.*, 2013). Chimeric sequences were removed using the Uchime algorithm (Edgar *et al.*, 2011) implemented in Mothur, and the remaining sequences classified taxonomically using the method of Wang *et al.* (2007). Non-bacterial sequences were removed. The sequences were then clustered into Operational Taxonomic Units (OTUs) at a 97% identity level and each OTU was classified taxonomically. All OTUs with  $n \leq 5$  sequences were removed (0.02%) resulting in a final data matrix with 217,923 sequences. The sequences obtained from cultivable isolates were compared with the 16S rRNA gene metabarcoding data using BLASTn searches against raw reads (99% identity) and consensus OTU sequences (97% identity). In addition, the current dataset (META2016-NSW) was compared to previous datasets (META2013-NSW, META2013-DNSW) obtained from the same algal strain three years earlier (Dittami *et al.*, 2016). All counts for each individual OTU were normalized against the total number of sequences in the corresponding dataset. Raw Illumina reads were deposited at the European Nucleotide Archive under project accession number PRJEB22665. To compare the cultivable sequences and their abundance in the 16S metabarcoding data (META13-NSW and META16-NSW) a heat map was created using the iTOL web application<sup>6</sup> (Letunic and Bork, 2016). Log(x+1)-transformed data was used for OTU sequence counts and cultivation abundances. All datasets (three for metabarcoding, 10 for cultivation) were grouped by hierarchical clustering using Euclidean distance calculations and the average linkage method implemented in the pvclust R-package<sup>7</sup> (Suzuki and Shimodaira, 2006). The resulting tree was tested using bootstrap analysis (500 replications). OTUs that did not correspond to cultivable strains are not shown in the graphical representation. In this manuscript, “isolate” refers to every bacterial culture for which a 16S rRNA sequence was obtained. All isolates with identical 16S rRNA sequences are considered to belong to the same “strain”.

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<sup>6</sup> <http://itol.embl.de>, version 3.5.3

<sup>7</sup> <http://stat.sys.i.kyoto-u.ac.jp/prog/pvclust/>

## Results

### Isolation and characterization of algae-associated bacteria

#### Global taxonomic distribution of cultivable bacteria

16S rRNA gene sequences were obtained for 388 bacterial isolates and they were distributed among four phyla, 15 bacterial orders, 34 genera, and 46 taxonomically unique strains. Five genera encompassed more than one distinct strain (*i.e.* at least one verified mismatch in the 16S rRNA sequence): *Limnobacter* sp. (2), *Moraxella* sp. (2), *Sphingomonas* sp. (3), *Bacillus* sp. (8) and *Roseovarius* sp. (2). The most abundant phylum among the cultivable isolates was *Proteobacteria*, with 89% of all isolates and 26 unique strains belonging to this group. Within *Proteobacteria*, *Alpha*- and *Betaproteobacteria* accounted for 34% and 32% of the isolates, respectively. However, *Betaproteobacteria* comprised three unique strains, while *Alphaproteobacteria* comprised 16 unique strains in our experiments. 23% of proteobacterial isolates belonged to *Gammaproteobacteria*, covering seven unique strains. *Bacteroidetes* (4% of isolates), *Firmicutes* (4%), and *Actinobacteria* (3%) were cultivated less frequently compared to *Proteobacteria*. Despite their lower abundance, the three groups contribute considerably to the cultivable diversity, accounting for 20 out of 46 unique strains. The most abundant cultivable bacterial genera were *Limnobacter* (27% of all isolates), *Halomonas* (20%), and *Bosea* (9%). 80% of *Limnobacter* isolates were obtained from dilution-to-extinction cultivation experiments. *Halomonas* strains were predominantly cultivated using direct plating techniques and algae with full flora (84% of *Halomonas* isolates). For *Bosea*, most isolates (83%) originated from antibiotic-treated algae. An overview of all bacterial isolates characterized and their corresponding sequence abundances can be found in Figure 1-2 and Supplementary table 1-3.



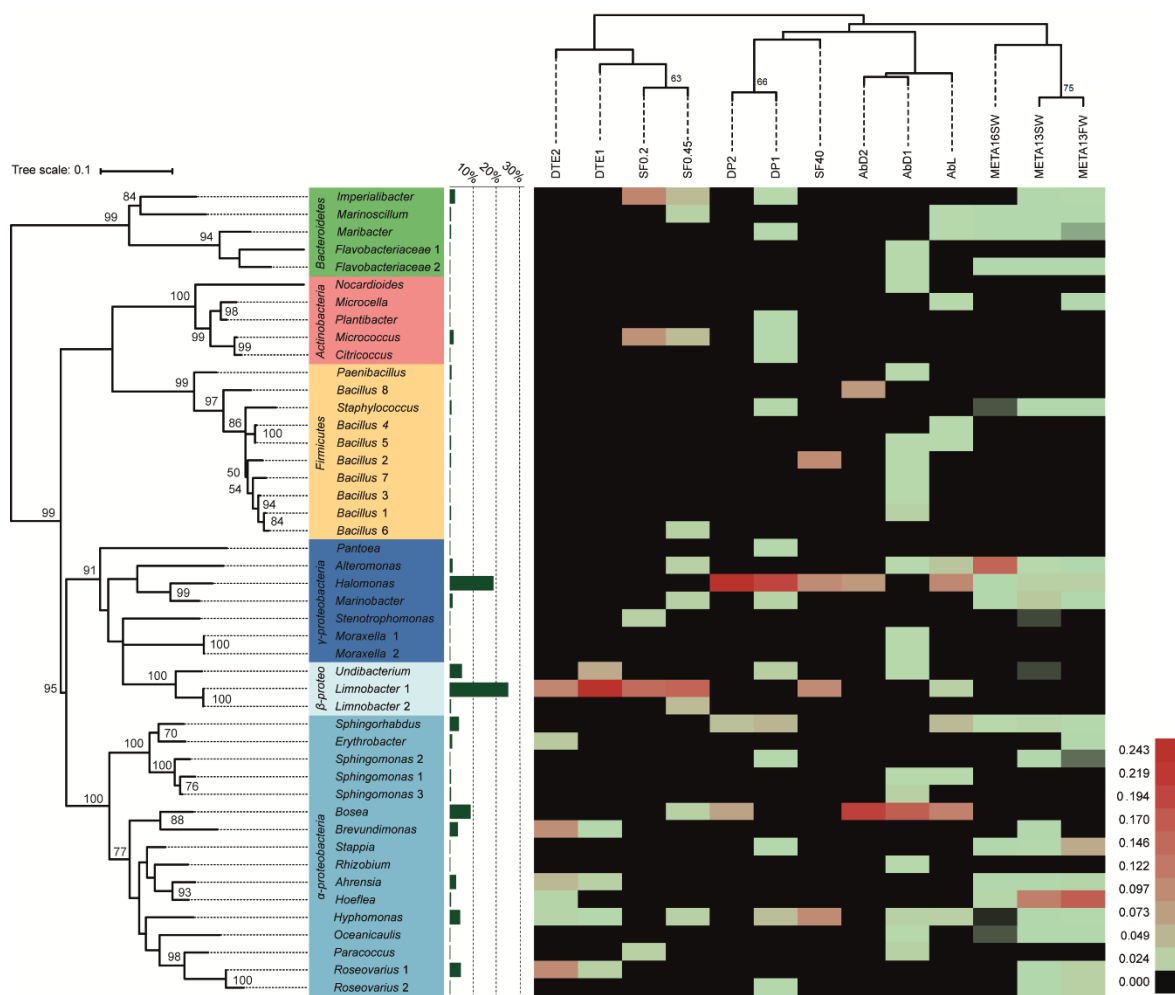


Figure 1-2 Heat-map of cultivation and metabarcoding data. The number of sequences was normalized and  $\log(x+1)$ -transformed for each unique cultivable strain and each experimental treatment (DP: direct plating without pretreatment, AbD: DP with pretreatment with antibiotic discs, Abl: DP with pretreatment with liquid antibiotics, SF: DP with pretreatment by size-fractionation, DTE: dilution-to-extinction cultivation). A comparison is made with molecular data from 16S rRNA metabarcoding (META16-NSW = this study; META13-NSW and META13-DNSW = previous study by Dittami et al. (2016); uncultured OTUs not shown). Red colors indicate high abundance, while green corresponds to relatively low abundance. Black color indicates taxa/strains that were not retrieved/isolated. Experimental treatments are grouped (top dendrogram) using hierarchical clustering (Euclidean distance, average linkage method) and the phylogenetic tree (left) was calculated using the Maximum-Likelihood method and the GTR+G+I model. Bootstrap analysis for both trees was done using 500 replications. Only bootstrap values  $\geq 50$  are shown. The bar graph (green) shows the proportion of unique strains obtained in the whole cultivation dataset.

## **Isolation of bacteria from algae with their full microbial flora**

### **Direct plating of ground algae and algal protoplast extract (DP)**

Direct plating of ground algal tissue and protoplast digest resulted in the isolation and characterization of 110 isolates corresponding to 17 strains of which seven were uniquely isolated with this method. The most frequently isolated strain was *Halomonas* sp., a gammaproteobacterium that makes up for 58% of isolates obtained with this method. Isolates of this strain originated predominantly from ground algal tissue rather than algal growth medium ( $p = 1.92E-13$ ). After *Halomonas*, *Sphingopyxis* (10%) and *Hyphomonas* (8%) were the most frequently isolated taxa. Four isolates originated from protoplast extracts: *Imperialibacter* sp. (two isolates), *Sphingomonas* sp. (one isolate) and *Plantibacter* sp. (one isolate). Direct plating of algal tissue was repeated after three years and *Halomonas* sp. was again the most frequently isolated strain (nine out of 12 isolates). *Sphingomonas* 2, and *Plantibacter* sp. were two protoplast-specific strains obtained using DP, but they were only isolated once in the experiment.

### **Dilution-to-extinction cultivation (DTE)**

One hundred and fifty isolates were identified and 16S rRNA gene sequences revealed eight unique strains. There were no isolates that were specific for the origin of the starting material used (protoplast extract or spent algal growth medium). The most abundant isolates belonged to the genus *Limnobacter* (55% of isolates), suggesting that they were the most abundant cultivable bacterium in the original algal cultures. Furthermore, five unique strains (*Brevundimonas*, *Erythrobacter*, *Hoeflea*, *Ahrensia*, and *Roseovarius* 1) were exclusively found with dilution-to-extinction cultivation. *Brevundimonas* sp. was significantly more isolated from algal growth media ( $p = 0.00045$ ) compared to algal tissue/protoplast extract (). In addition, some *Hyphomonas* sp. and *Undibacterium* sp. strains were isolated but they were not exclusive for this method. Experiments were performed twice in a 6-month interval (DTE1 and DTE2), and *Limnobacter* was, in both experiments, the most frequently isolated taxon. The ratio of cultivable to total bacteria (estimated culturability) in the experiment varied from 44 to 68%, with different culturability dependent on the type of bacterial growth medium applied. DTE statistics and the Poisson calculations can be found in Table 1-1.

Table 1-1. Estimation of the ratio of cultivable to total bacteria in the dilution-to-extinction cultivation experiments based on a Poisson distribution:  $n_{cult} = \ln(1/p_{neg}) * w$ . P = protoplast digest product; M = low salinity algal growth medium; DTE1 = March 2016; DTE2 = September 2016; ECM = spent low salinity algal growth medium from 2 weeks (2W) or 7 weeks (7W) old cultures.

Experiment	Type of inoculation	bacterial growth medium	# bacterial cells inoculated ( $n_{total}$ )	# inoculated wells (w)	# negative wells ( $P_{neg}$ )	theoretical # of cultivable cells ( $n_{cult}$ )	Estimated culturability ( $n_{cult}/n_{total}$ )
DTE1	P	ECM 2W	52	104	74	35.39	68%
DTE1	P	ECM 7W	52	104	83	23.46	45%
DTE1	M	ECM 7W	48	96	73	26.29	55%
DTE1	M	ECM 2W	48	96	77	21.17	44%
DTE1	M	LNHM	52	104	81	25.99	50%
DTE1	M	1:20 R2A	52	104	79	28.59	55%
DTE2	M	1:20 R2A	140	280	201	92.82	66%

## Isolation of bacteria from algae with reduced flora

### Antibiotic-treated algae

The 16S rRNA gene sequences from 80 isolates revealed 27 unique strains, 16 of which were obtained only with this cultivation method. *Bosea* was the most abundant (44% of isolates) followed by *Halomonas* with 38%. Most others were only isolated once or twice. Unique strains isolated with this method were *Sphingomonas* sp. (strains 1, 3), *Bacillus* sp. (strains 1, 3-5, 7), *Nocardioides* sp., *Microcella* sp., *Moraxella* sp. (strains 1, 2), *Pantoea* sp., *Rhizobium* sp., two unclassified members of the *Flavobacteriaceae*, and *Oceanicaulis* sp. These were all (except *Microcella*) isolated from algal tissue that was exposed to 10 different antibiotics (1-2). The cultivation of bacteria from antibiotic-treated algae was performed twice within a one-year interval and in both experiments, *Bosea* was the most frequently isolated taxon.

### Size-fractionation

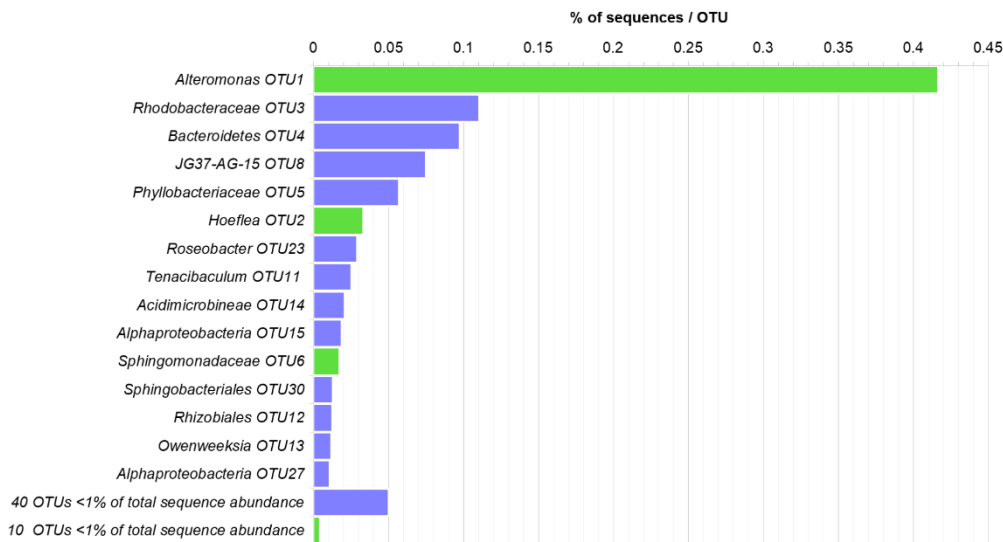
The 16S rRNA gene sequences of the 43 isolates from this experiment cover 14 unique strains. Three of them were uniquely found using this method: *Bacillus* strain 6 (1 isolate), *Limnobacter* strain 2, (2 isolates), and *Stenotrophomonas* sp. (1 isolate). Among the other strains cultivated, the most abundant one was *Limnobacter* strain 1 (40% of isolates), followed by *Imperialibacter* sp. (16% of isolates).

## Estimating the proportion of cultivable bacteria in *Ectocarpus* cultures

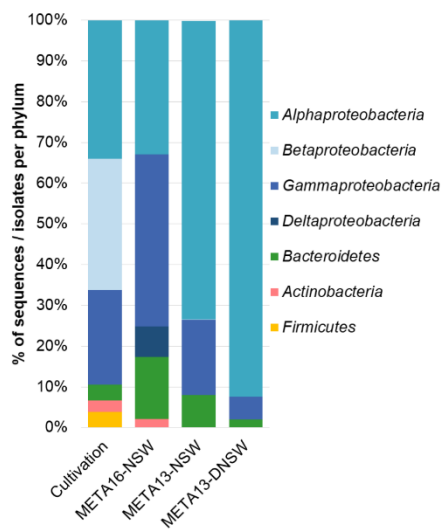
16S metabarcoding experiments were carried out with the same algal culture also used for the isolation of bacteria and the libraries were used as a reference for the cultivated and non-cultivated microbiome as a whole. After cleaning and filtering of the data, the sequences were clustered into 48 OTUs. The most abundant OTU belonged to the genus *Alteromonas* (OTU1) and accounted for 41.6% of the reads, which makes *Gammaproteobacteria* the most abundant class (42.3%). Other abundant OTUs corresponded to an unclassified *Rhodobacteraceae* (OTU3, 11%) and an unclassified *Bacteroidetes* (OTU4, 10%). Together these three OTUs correspond to 62% of all sequences (Figure 1-3A). *Alphaproteobacteria* make up 32.8% of the sequences and *Bacteroidetes* 15.3% (Figure 1-3B). Other phyla identified are *Actinobacteria* (2.1%) and *Deltaproteobacteria* (7.5%). Of the 48 OTUs, 10 corresponded to strains cultivated in our experiments. These 10 OTUs accounted for 47% of the reads in the metabarcoding data. Purely based on absence/presence of OTUs the culturability was 21%. Three additional cultivable strains corresponded to OTUs with sequence abundance below the threshold ( $n \leq 5$ , *Staphylococcus*, *Hyphomonas*, *Oceanicaulis*; Figure 1-3). Furthermore, taking into account all 16S rRNA gene libraries and rare reads, 22 of the 46 cultivable strains were detected. Among the 24 undetected strains that were not found in any of the barcoding libraries, 11 were isolated exclusively from DNSW and 8 exclusively from NSW. In the same vein, 11 strains were cultured exclusively from algal medium, and 7 only from algal tissue (Supplementary table 1-4).

The 16S rRNA gene metabarcoding data obtained in this study (META2016-NSW) differed strongly from that obtained three years earlier (META2013-NSW) from the same *Ectocarpus* strain. Several OTUs that were present in the 2013 samples were no longer present in 2016 or declined in abundance below the detection limit. However, there were still 50 OTUs (corresponding to 90% of the sequences) shared between the 2013 and 2016 samples. The most abundant OTU in 2013 belonged to the genus *Hoeflea* (29% of reads) while the most abundant OTU in 2016 (*Alteromonas* sp.; 42% of reads) accounted for only 2.4% of the reads in 2013.

**A**



**B**



**C**

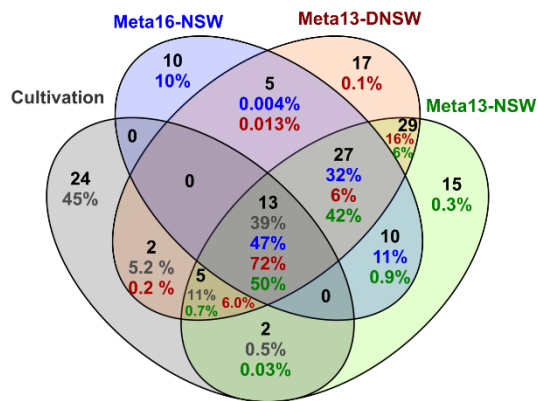


Figure 1-3 Overview of metabarcoding data and comparison with cultivable isolates. Panel A shows the distribution of OTUs in the metabarcoding experiment (META16-NSW). OTUs with >1% of total sequence abundance are displayed separately: bars in green display OTUs that correspond to cultivable strains obtained in this study, while purple bars correspond to OTUs that were not cultivated; OTUs with <1% of total sequence abundance are combined and the sum of sequences is displayed. Panel B shows the distribution of 16S rRNA gene metabarcoding sequences per phylum compared to data obtained from the cultivation study. Panel C shows a Venn-diagram of the OTUs that are shared between the 2 metabarcoding datasets from 2013 and 2016 and the cultivable isolates. Numbers in blue correspond to META16-NSW, numbers in red correspond to META2013-NSW, numbers in green to META2013-DNSW, and numbers in grey correspond to the proportion of sequences for cultivable isolates.

## Discussion

### Global taxonomic distribution of cultivable bacteria

The main aim of this study was to establish a diverse collection of cultivable *Ectocarpus*-associated bacteria that can be used to perform functional studies of brown macroalgal-bacterial interactions in this model organism. We applied different cultivation strategies to facilitate the growth of less abundant or slow-growing bacteria and thus increased the variety of cultivable bacteria.

Among the cultivable taxa frequently found on brown macroalgal surfaces (such as *Laminaria*, *Saccharina*, *Fucus*, *Ascophyllum*) are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, where the latter three phyla are generally less abundant (Ivanova *et al.*, 2002; Wang *et al.*, 2008; Wiese *et al.*, 2009; Salaün *et al.*, 2010; Dong *et al.*, 2012; Goecke, Labes, *et al.*, 2013; Goecke, Thiel, *et al.*, 2013; Hollants *et al.*, 2013; Martin *et al.*, 2015). Two cultivation studies in *Ectocarpus* species showed the presence of *Gammaproteobacteria*, *Actinobacteria*, and *Flavobacteria* (Kong and Kwong-yu, 1979; Tapia *et al.*, 2016). The results of our study, with *Proteobacteria* being most abundant followed by *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, largely agree with these findings, except that *Alphaproteobacteria* were the most abundant proteobacteria in this study (Figure 1-2 and Supplementary table 1-3), compared to *Gammaproteobacteria* in the previous studies.

The three dominant genera obtained were *Limnobacter*, *Bosea*, and *Halomonas* (Figure 1-2 and Supplementary table 1-3). *Limnobacter* sp. are oligotrophic freshwater sulfur-oxidizing bacteria (Spring, Kämpfer and Schleifer, 2001) that occur naturally in aquatic environments (Lu *et al.*, 2011; Zhang *et al.*, 2014) and drinking water reservoirs (Wu *et al.*, 2014) and are generally considered rare in marine settings (Staufenberger *et al.*, 2008; Wiese *et al.*, 2009). The majority of the isolation experiments in this study were indeed carried out with low-salinity culture media, and the algal source also came from fresh water (West and Kraft, 1996), providing two possible explanations for the presence of *Limnobacter* in our experiments. As these experiments were always complemented with negative controls (*i.e.* non-inoculated bacterial growth media), a contamination with *Limnobacter* from the water source used to prepare the bacterial growth media is unlikely.

Members of the genus *Bosea* are known to be (multi)drug-resistant (Falcone-Dias, Vaz-Moreira and Manaia, 2012; Zothanpuia *et al.*, 2016). The results from our study agree with these

observations since 92% of the *Bosea* isolates came from antibiotic-treated algae (Figure 1-2 and Supplementary table 1-3). In addition, several other strains were uniquely isolated from antibiotic-treated algal tissue suggesting that part of the algae-associated microbiome is (multi)drug-resistant, or otherwise protected by the algal cell wall / inside the cell, where drug concentrations may be too low to be effective. In addition, some antibiotics employed in this study, such as chloramphenicol and erythromycin, may have had only temporal bacteriostatic effects.

The genus *Halomonas* comprises cultivable isolates from various saline environments, (Eilers *et al.*, 2000; Donachie *et al.*, 2004; Arahal and Ventosa, 2006; Poli *et al.*, 2009), including microalgal (Keshtacher-Liebson, Hadar and Chen, 1995; Croft *et al.*, 2005; Baggesen, Gjermansen and Brandt, 2014) and macroalgal surfaces (Ivanova *et al.*, 2002; Wang *et al.*, 2008; Hollants *et al.*, 2013; Tapia *et al.*, 2016). *Halomonas*-algae associations are potentially beneficial for the alga, since the bacteria may provide vitamins (Croft *et al.*, 2005), release siderophores (Keshtacher-Liebson, Hadar and Chen, 1995; Baggesen, Gjermansen and Brandt, 2014), or excrete morphogenetic compounds (Spoerner *et al.*, 2012a; Tapia *et al.*, 2016) that are essential for algal growth. Symbiotic associations with algae may be linked to the capacity of *Halomonas* to degrade algal excreted polysaccharides and/or the presence of alginate lyases (Wong, Preston and Schiller, 2000; Tang *et al.*, 2008; Wang *et al.*, 2008; Goecke *et al.*, 2012) and indeed, bacterial cells can be closely attached to algal cell walls (Croft *et al.*, 2005; Tapia *et al.*, 2016). In this study, *Halomonas* was the most abundant isolate obtained with direct plating techniques without pretreatment (DP1 and DP2). More isolates were derived from tissue/protoplasts compared to algal growth medium ( $p = 1.92E-13$ ), suggesting a close association between *Ectocarpus* and the *Halomonas* sp.

In summary, each cultivation strategy resulted in the cultivation of unique strains that were not cultivated with any of the other methods. For example, the application of antibiotics to eliminate *Halomonas* sp. reduced the competitive pressure between antibiotic-resistant bacteria and led to the cultivation of 16 additional bacterial strains. Similar observations have been made in sponges (Sipkema *et al.*, 2011; Lavy *et al.*, 2014), lichens (Parrot *et al.*, 2015), and tap water (Vaz-Moreira *et al.*, 2013). Interestingly, direct plating without pretreatment, although dominated by *Halomonas* sp., also resulted in the isolation of 7 unique strains.

## The cultivated vs. uncultivated microbiome

Marine pelagic bacteria often have complex growth and nutrient requirements (Stewart, 2012; Zengler, 2013). In addition, they are generally considered to be oligotrophs, since they inhabit a nutrient-poor environment and grow only very slowly, which might also compromise the cultivation process (Amann, Ludwig and Schleifer, 1995; Keller and Zengler, 2004; Zengler, 2013). Hence, a large part of the marine environmental microbiome has been considered non-cultivable using standard cultivation techniques (Amann, Ludwig and Schleifer, 1995; Morris *et al.*, 2002; Giovannoni and Stingl, 2005).

Here, we aimed to cultivate bacteria that were associated with the algae/algal cell-walls, a carbohydrate-rich environment due to the accumulation of algal (poly)saccharides *i.e.*, alginates, fucans, and mannitol (Michel *et al.*, 2010a; Popper *et al.*, 2011). The divergence in community structure between pelagic and algae-associated microbiomes is well-established (Kong and Kwong-yu, 1979; Staufenberg *et al.*, 2008; Bengtsson, Sjøtun and Øvreås, 2010; Burke, Thomas, *et al.*, 2011; Wahl *et al.*, 2012; Goecke, Thiel, *et al.*, 2013; Mancuso *et al.*, 2016), and several algae-associated bacteria are able to digest/decompose algal cell material (Rieper-Kirchner, 1989; Goecke, Thiel, *et al.*, 2013; Groisillier *et al.*, 2015; Martin *et al.*, 2015) *e.g.* via the production of alginate lyases (Sawabe, Ohtsuka and Ezura, 1997; Dong *et al.*, 2012) and glycoside hydrolases/fucanases (Ficko-Blean, Hervé and Michel, 2015). It is thus possible that algae-associated bacteria, contrary to pelagic bacteria, are well adapted to grow on the laboratory cultivation media provided, resulting in relatively high numbers of cultivable bacteria.

Our data support this hypothesis since culturability was between 44 and 68% based on the dilution-to-extinction experiments (Table 1-1). For pelagic studies, culturability is usually below 15%, with some observation going as low as 0.05% (Connon and Giovannoni, 2002; Page, Connon and Giovannoni, 2004; Stingl, Tripp and Giovannoni, 2007; Stingl *et al.*, 2008; Yang, Kang and Cho, 2016). In the same vein, dilution-to-extinction cultivation studies on pelagic bacteria generally apply between 1-25 bacterial cells/well as inoculum (Connon and Giovannoni, 2002; Stingl, Tripp and Giovannoni, 2007). In our study, however, concentrations as low as 0.5 cells/well were required to obtain pure cultures, further demonstrating that a relatively large part of the algae-associated microbiome is cultivable compared to pelagic bacteria.

Culturability was also assessed by comparing the distribution and abundance of taxa obtained in the cultivation study with taxa inferred from 16S rRNA gene libraries. In a previous study on



*Ectocarpus*, this type of comparison demonstrated an overall ratio of culturability of 11% based on the presence/absence of OTUs (Tapia *et al.*, 2016). In the present study, this number was further increased with 21% of the OTUs and 47% of all 16S rRNA sequences corresponding to cultivable strains (Figure 1-3C).

To our knowledge, this is the first study to apply dilution-to-extinction cultivation to macroalgae-associated bacteria, and the standardized cultivation method (Connon and Giovannoni, 2002) was amended by adding the algal metabolites alginate and/or mannitol to the culture media. We assume that it was, therefore, well-adapted to the metabolic needs of the majority of *Ectocarpus*-associated bacteria, and indeed several bacteria known to be potential cell-wall digesters have representatives in our culture collection, e.g. *Alteromonas* (Sawabe, Ohtsuka and Ezura, 1997), *Flavobacteriia* (Groisillier *et al.*, 2015), *Maribacter* (Martin *et al.*, 2015), *Erythrobacter* (Goecke, Thiel, *et al.*, 2013), and *Halomonas* (Wong, Preston and Schiller, 2000). Together these results validate the combination of cultivation approaches chosen to increase culturability in our system.

### **Cultivable bacteria not detected by metabarcoding**

Of the 46 unique strains that were isolated in this study, 16 were isolated at least once from algal tissue grown in NSW, and could thus be directly compared to META2016-NSW metabarcoding data set generated in this study. Seven of them (44%) were represented in this gene library. To be able to compare also strains isolated only from low salinities with metabarcoding data, we included two further data sets obtained for the same strain in 2013. All data sets taken together, 22 of the 46 (48%) strains were found at least in one of the libraries, while 24 were undetectable or below the detection limit. Whether a strain was isolated directly from algal tissue or from the algal culture medium did not have a strong impact on these numbers (Supplementary table 1-4).

There are several hypotheses to explain this observation. First, methodological flaws or biases including the inadequacy to extract DNA from certain bacterial cells due to species-specific characteristics (*e.g.* gram-positive are generally more difficult to extract than gram-negative cells), primers specificity, or PCR conditions (Suzuki and Giovannoni, 1996; Donachie *et al.*, 2004; Donachie, Foster and Brown, 2007). This may explain biases but, is unlikely to account for the complete absence of a taxon, because all cultivable taxa were detectable with standard primers and extraction methods in our cultures. A second explanation is that some “rare”

microbes may be laboratory- or human-derived contaminants, e.g. *Staphylococcus*, sp., and *Bacillus* sp. All measures to avoid bacterial contamination of our algal/bacterial samples were taken and the controls were included in all cultivation experiments and generally negative for growth. Nevertheless, it is plausible that some of these “rare” bacteria were acquired during the monthly transfers of the algal cultures or during the bacteria cultivation procedures and growth of these bacteria might have been facilitated by the experimental treatments that were applied.

A third explanation is that the sequencing depth or the number of time points examined (one for DNSW two for NSW) may have been too low to identify members of the microbiome that are “rare” (Zilber-Rosenberg and Rosenberg, 2008; Skopina *et al.*, 2016). Bacteria might be present in low abundance in the natural environment but they can amplify rapidly under specific environmental conditions (Epstein, 2009; Buerger *et al.*, 2012; Lindh *et al.*, 2015). Rare bacteria might thus serve as a “seed bank” (Pedrós-Alió, 2012) that contributes to the microbial richness and may form the basis for temporal instability of the microbiome (Sogin *et al.*, 2006; Shade and Gilbert, 2015; Jousset *et al.*, 2017). Recently, it has been suggested that in particular marine macro-organisms, and possibly *Ectocarpus* as well, might serve as incubators for rare bacteria (Troussellier *et al.*, 2017), since surface-associated microbiomes generally exhibit higher OTU diversity and harbor many rare OTUs compared to the surrounding seawater. In this scenario, the removal of competing microbes via the antibiotic treatments and our other measures to reduce competition during our cultivation experiments, have probably allowed them to increase in abundance. This explanation is supported by the variability of the microbiome observed in this study compared with the previous study of the same strain under the same conditions (Dittami *et al.*, 2016), and by the fact that 4 of 46 cultured OTUs were found only among the rare ( $n \leq 5$ ) reads in the available barcoding data.

Similar observations of cultivable isolates not being detected in corresponding gene libraries have been made in human stool samples (Lagier *et al.*, 2012), sponges (Sipkema *et al.*, 2011; Esteves *et al.*, 2016), seawater (Eilers *et al.*, 2000), and soil (Shade *et al.*, 2012); more examples are discussed by Donachie and colleagues (2007). We put forward the hypothesis that, in analogy to “uncultivable” microbes that become cultivable by improving cultivation conditions, at least part of the undetected strains may, therefore, become “barcodable” merely by significantly increasing sequencing depths (Pedrós-Alió, 2012) and/or the temporal resolution of the study.

## **Perspectives: (meta)genome-guided cultivation and inference of metabolic networks**

In this study, we show that a remarkably high number of bacterial cells (~50%) associated with *Ectocarpus* was cultivable using a range of cultivation techniques. Each cultivation strategy resulted in another dominant genus or weed-species (*Bosea* for antibiotic-treated algae, *Limnobacter* for dilution-to-extinction cultivation) and each strategy also led to the cultivation of unique isolates that were not found with any other cultivation method. Our results thus emphasize the need to use samples from different environmental/abiotic conditions to obtain rare taxa and thus increase the overall cultivable diversity. To further improve these numbers, a metagenomics approach may be used to predict the specific cultivation requirements of yet uncultured taxa (Garza and Dutilh, 2015). One successful example of this approach is the cultivation of members of the SAR11 clade, for which genomic analysis revealed their requirement for exogenous reduced sulfur (Tripp *et al.*, 2008). Such metagenomic analyses of the *Ectocarpus* holobiont are currently ongoing.

Regarding the cultivable isolates, genomic data currently in preparation for several strains may be used to predict their metabolic capacities and to generate hypotheses on how they may complement the metabolism of the alga (Dittami, Eveillard and Tonon, 2014). Because the bacteria are cultivable it will be possible to experimentally verify the hypothesis generated using this approach. Sixty-two bacterial isolates and 12 artificial bacterial communities have already been experimentally tested in preliminary algal-bacterial co-culture experiments. They showed interactions ranging from weak beneficial effects on survival of *E. subulatus* in diluted natural seawater (29 isolates, 15 unique strains; three communities) to growth-inhibition (data not shown). These strains may serve as the first candidates to study the role of algal-bacterial interactions under abiotic stress.

The present bacterial culture collection constitutes a valuable tool to study the *Ectocarpus* holobiont *in vitro* and complements the genomic tools available for the model *Ectocarpus*. Together, they can be used to address fundamental questions regarding the functions of brown macroalgal holobionts during exposure to abiotic stressors, for instance during the acclimation to low salinity in *E. subulatus*.

## **Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## **Author Contributions**

All authors conceived the study; HK, CJ, and SMD carried out experiments; HK wrote the manuscript with help from SMD, CB, and CJ; all authors approved of the final manuscript.

## **Funding**

This work has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement number 624575 (ALFF) and ANR project IDEALG (ANR-10-BTBR-04) 'Investissements d'avenir, Biotechnologies-Bioressources'.

## **Acknowledgments**

We would like to thank Bernard Billoud for help with statistical analyses; Laurence Darteville for help with algal culturing; Akira Peters for providing the algal cultures; Dominique Marie for help with the flow cytometry; the Genomer platform (Station Biologique de Roscoff) for access to their sequencing equipment; the ABIMS Platform (Station Biologique de Roscoff) for technical support and server time; Laetitia Mest, Lolita Lecompte, and Maeva Harrivel for their participation in early isolation experiments; Christophe Destombe and Thomas Wichard for helpful discussions; and Angélique Gobet for critical reading of the manuscript.

# Chapter 2.

## Chapter 2. Cultivable bacteria from the *Ectocarpus* surface – applications

### Introduction

The freshwater strain (FWS) of *Ectocarpus subulatus* depends on its associated bacteria for growth in fresh water, which stresses the significance of algal-bacterial interactions in acclimation and/or adaptation processes (Dittami *et al.*, 2016). Yet, it is unknown which mechanisms/interactions underlie this symbiotic relationship. 16S rRNA gene meta-barcoding of *E. subulatus* FSW cultures has shown that the transition from seawater to fresh water, strongly affects microbiome composition (Dittami *et al.*, 2016). However, molecular barcoding does not provide any information on a functional level (genes, proteins), thus to get closer to answering the question we needed an experimental system to study the *Ectocarpus* holobiont *in vitro*.

Targeted co-culture experiments are a valuable tool to perform functional studies and in this case, could lead to the identification of those bacteria responsible for freshwater tolerance in *E. subulatus*. For that, cultivable organisms are required. The first part of my thesis was therefore focused on the cultivation of bacteria. A set of 46 unique bacterial strains was cultivated from the surface of *E. subulatus* FWS, and they were shown to belong to 33 different genera (Chapter 1; KleinJan *et al.*, 2017). The next step, and subject of subsection I of this chapter (**Figure 2-1**) is to test these bacteria for their effect on freshwater tolerance in *E. subulatus*. This chapter thus covers the results of the first algal-bacterial co-incubation experiments. Eventually, the aim of this work was to establish a co-culture system that can be used to elucidate the effects of the bacterial strains on *Ectocarpus* physiology on a molecular level, *e.g.* via differential gene expression analysis.

In parallel to the experiments with *E. subulatus*, the isolated strains were also tested with a different host, the green alga *Ulva mutabilis*. Bacteria are often functionally redundant, and we thus wanted to test if brown alga-derived bacteria may have an impact on distantly related species, such as algae from the green lineage. The work on *U. mutabilis* was executed at the Institute for Inorganic and Analytical Chemistry (Friedrich Schiller University Jena, Jena, Germany; collaborators: Thomas Wichard & Gianmaria Califano) and is summarized in subsection II of this chapter (Figure 2-1).

Finally, based on the results of both *Ectocarpus* and *Ulva* bacterial co-cultivation experiments, a selection of 12 bacteria was subjected to genome sequencing. Their genomes served as the starting point for comparative genomics and metabolic network analysis. This led to the *in silico* prediction of potentially beneficial cross-talk between *Ectocarpus* and these bacterial partners and eventually, potentially beneficial bacterial communities were designed based on metabolic complementarity with the *Ectocarpus* metabolic network. The computational work was carried out by collaborators from the Dyliss team (IRISA, Rennes, France; collaborators Clémence Frioux, Enora Fremy, Meziane Aite, Anne Siegel). The experimental validation of those predictions was implemented in a master student project (carried out by Bertille Burgunter-Delamare), and the results are summarized in subsection III.

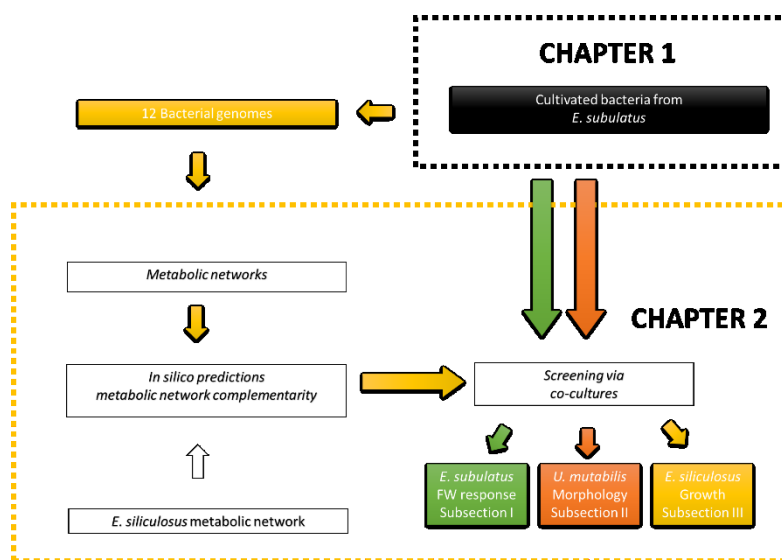


Figure 2-1 Schematic overview of the experiments described in chapter 2 of my thesis, comprising three strategies to explore algal-bacterial interactions. First, cultivated bacteria were tested in co-culture with *E. subulatus* (subsection I – green route) and *Ulva* (subsection II; orange route). Metabolic complementarity analysis is described in subsection III.

## **I. The impact of cultivable bacterial symbionts on the freshwater response in *Ectocarpus subulatus* freshwater strain**

To examine the effect of bacteria on the freshwater response in *Ectocarpus subulatus* FWS, sterilized algae were grown in co-culture with different bacterial isolates or communities for several weeks and the effects on reproduction and algal growth in fresh water were observed during this time.

### **Materials and methods**

#### **Preparation of antibiotic-treated *Ectocarpus* cultures**

Cultures of *Ectocarpus subulatus* FWS (accession CCAP 1310/196, West and Kraft, 1996) were exposed to ten different types of antibiotic discs (Table 2-1 & Figure 2-2) on Zobell agar for four successive weeks under standard algal growth conditions (13 °C., 12h light cycle), according to the protocol described in Müller et al. (2008). Then, the antibiotic-treated algae were transferred to Petri dishes (90 mm) with natural seawater (NSW) to recover. Removal of bacterial cells was confirmed with light microscopy (Olympus BX60, phase contrast, 800x magnification; no/few bacteria visible), and plating of the tissue on Zobell and/or R2A medium (no growth). If the efficiency of the treatment was confirmed, the algae were further cultured in Provasoli enriched natural seawater (NSW-PE) until the start of the experiment. All manipulations were carried out in a sterile environment under a laminar flow hood to prevent contamination.



Table 2-1 Different types of antibiotics used to sterilize the algal filaments according to Müller et al. (2008)

Antibiotic	Concentration per disc
Ampicillin (Amp)	10 µg
Ciprofloxacin (Cip)	5 µg
Chloramphenicol (Chl)	30 µg
Erythromycin (Ery)	15 µg
Kanamycin (Kam)	30 µg
Penicillin (Pen)	6 µg
Polymyxin B (PolB)	50 µg
Rifampicin (Ram)	30 µg
Streptomycin (Strep)	10 µg
Tetracycline (Tet)	30µg

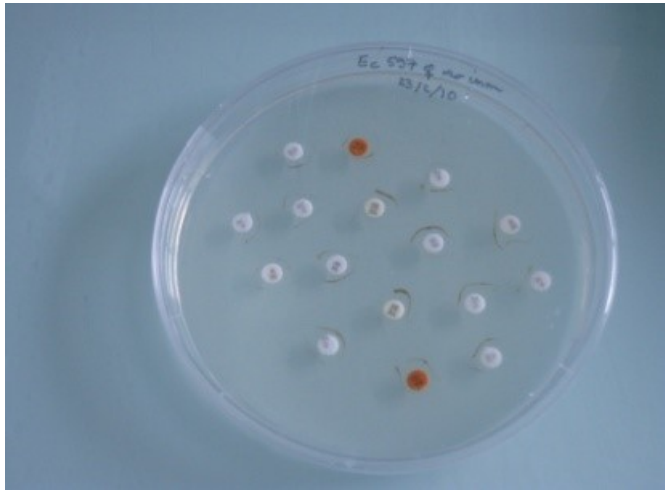


Figure 2-2 An example of *Ectocarpus* algal filaments positioned around the antibiotic disc in order to sterilize them. Source: “Protocol N° 15 – Antibiogrammes”; courtesy of L. Dartevelle.

### Co-culture experiments

Bacteria were grown in liquid Zobell and/or diluted R2A (depending on which medium they originally were isolated with) until sufficient density (~3 days at room temperature; RT). Before the start of the experiment, the OD600 was measured for each culture, and concentrations were adjusted to the least dense culture (OD = 0.1-0.3, depending on the experiment). Antibiotic-treated algae (see above), still growing in NSW-PE, were inoculated with individual bacterial cultures or with a mixture of strains. For both, the final inoculum was 0.1% (v/v), *i.e.* 30 µl per 30 ml in one Petri dish. After one week of adaptation in NSW-PE, the filaments were either transferred to 20-fold diluted NSW enriched with Provasoli nutrients (5% NSW) or transferred to fresh NSW-PE (100% NSW). Each experiment was carried out with biological triplicates. However, they all originated from one starter culture. The effects on spore release and algal

growth in fresh water were observed by eye and pictures (camera and microscopy) were taken at the start of the experiments as well as after 14 and 24 days to record the results. Controls were run in parallel and created by inoculation of antibiotic-treated algae with 200 µl of non-antibiotic treated algal growth medium (positive control), or antibiotic-treated algae were not inoculated at all (negative control). An overview of all tests can be found in Table 2-2.

## Results & discussion

### Selection of bacterial communities

Both individuals as well as artificial bacterial communities were tested for their effects on algal growth in low salinity. The number of distinct bacterial communities that can be established based on 46 distinct bacterial strains is almost infinite. Therefore, bacterial strains for assembly of artificial communities needed to be selected. This was done based on observations made during the cultivation study (dominant in the cultivation study; isolated from the cell wall/protoplast), and co-occurrence of OTUs across algal cultures (Dittami *et al.*, 2016). For example, the gammaproteobacterium, *Halomonas* sp. frequently dominates *Ectocarpus* cultures and was abundant in the bacterial cultivation study. Therefore, this strain was tested in combination with several other strains. Communities comprised of members of the *Rhizobiales* (*Bosea*, *Ahrensia*, *Hoeflea*, *Rhizobium*) were tested because bacteria from this order were known to be beneficial to plant growth. Communities comprised of *Sphingomonas* sp. were also tested because previous work showed that a *Sphingomonas* OTU was more often found in association with the alga, compared to the algal growth medium (Dittami *et al.*, 2016). Finally, communities comprised of all bacteria tested in the experiments were also included.

### Co-culture experiments

A total of 42 bacterial isolates and 12 artificial bacterial communities have been tested in co-culture with *Ectocarpus subulatus* FWS. Those results are summarized in Table 2-3 and a complete overview is given in Supplementary table 2-1. Two examples are shown in Figure 2-3. None of the bacterial strains had a strong beneficial effect on algal growth in fresh water (5% NSW) and none led to spore release. However, a weak differentiation could be made between inoculations that:

- Had no or only a slightly positive effect on survival in 5% NSW, but did not lead to sustainable growth; Categorized as “No or slightly positive effect in low salinity”; 15 isolates and 4 mixtures

- Inhibited algal growth in 5% NSW; Categorized as “Negative effects in low salinity”; 23 isolates and 4 mixtures
- Inhibited algal growth in 5% NSW and 100% NSW; Categorized as “Algicidal”; 4 isolates and 4 mixtures

Among the inocula that had a negative effect in 5% NSW, eight strains also inhibited growth in seawater. Interestingly, *Halomonas* sp. (isolate 58) was included in five of these inocula. Also, for most inocula that inhibited growth in seawater, the algal filaments were covered with a biofilm (Figure 2-4)

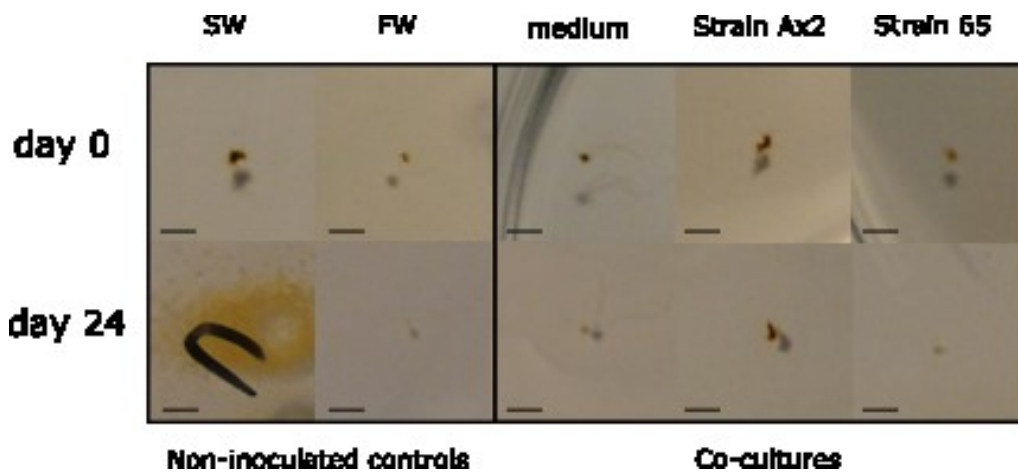


Figure 2-3 Examples of antibiotic-treated algal cultures that were inoculated with individual bacterial strains (Ax2, 65) or 200  $\mu$ l non-sterile algal growth medium, at the start of the experiment (day 0) and after 24 days of co-cultivation (day 24). Scale bar: 4 mm.

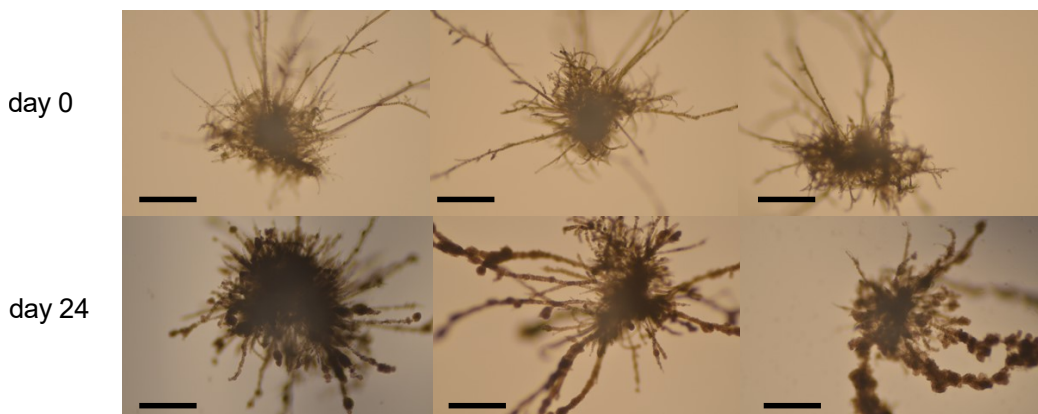


Figure 2-4 Example of algal filaments covered in biofilm after 24 days of co-culture in 5% NSW with *Alteromonas* sp. (left); *Marinobacter* (middle); Mix of *Halomonas* sp. & *Moraxella* sp. (right); scale bar: 1 mm.

Table 2-2 Classification of bacteria based on their effects on *E. subulatus* grown in co-culture with bacteria in 5% and 100% NSW.

Category	Strain id	Taxonomy
No or slightly positive effect in low salinity	47.2	<i>Limnobacter</i> sp.
	50	<i>Sphingorhabdus</i> sp.
	87	<i>Imperialibacter</i> sp.
	83	<i>Imperialibacter</i> sp.
	107	<i>Marinoscillum luteum</i>
	109	<i>Sphingorhabdus</i> sp.
	Ax1	<i>Moraxella</i> sp.
	Ax2	<i>Sphingomonas hunanensis</i>
	Ax3	<i>Hyphomonas</i> sp.
	Ax4	<i>Hyphomonas</i> sp.
	Q8	<i>Undibacterium</i> sp.
	97	<i>Bosea</i> sp. sp.
	123	<i>Bacillus megaterium</i>
	152	<i>Limnobacter</i> sp. sp.
	13a	<i>Staphylococcus</i> sp.
	Mix_4_2015	<i>Sphingorhabdus</i> sp. (50), <i>Imperialibacter</i> sp. (83), <i>Bosea</i> sp. (65)
	Mix_5_2015	<i>Sphingorhabdus</i> sp. (50), <i>Hyphomonas</i> sp. (Ax3), <i>Hyphomonas</i> sp. (Ax4)
Mix_3_2016	<i>B. mycoides</i> (71), <i>B. megaterium</i> (123), <i>B. aerius</i> (29b), <i>Bacillus</i> sp. (33b), <i>B. idriensis</i> (5a), <i>B. subtilis</i> (94b)	
Mix_4_2016	<i>Rhizobium</i> (ax2bis2), <i>Microcella</i> sp. (Z68), <i>Moraxella</i> sp. (17a), <i>Micrococcus</i> sp. (74), <i>Sphingomonas</i> sp (25a), <i>Sphingophyxis</i> (T1)	
Negative effect in low salinity	A4	<i>Ahrensia</i> sp.
	Ax6	<i>Sphingomonas hunanensis</i>
	65	<i>Bosea</i> sp. sp.
	Ax5	<i>Moraxella</i> sp.
	111	<i>Pantoea</i> sp.
	71	<i>Bacillus mycoides</i>
	74	<i>Micrococcus aloeverae</i>
	77	<i>Paenibacillus</i> sp.
	121	<i>Limnobacter</i> sp.
	130	<i>Limnobacter</i> sp.
	117b2a	<i>Pantoea</i> sp.
	17a	<i>Moraxella osloensis</i>
	25a	<i>Sphingomonas</i> sp.
	29b	<i>Bacillus aerius</i>
	33b	<i>Bacillus</i> sp.
	5a	<i>Bacillus idriensis</i>
	76B	<i>Limnobacter</i> sp. sp.

	94b	<i>Bacillus subtilis</i>
	ax2bis1	<i>Alteromonas sp.</i>
	ax2bis2	<i>Rhizobium sp.</i>
	T1	<i>Sphingopyxis sp.</i>
	T3	<i>Plantibacter sp.</i>
	Z68	<i>Microcella sp.</i>
	A4	<i>Ahrensia sp.</i>
	Mix_6_2015	<i>Hyphomonas sp. (Ax3) + Hyphomonas sp. (Ax4) + Sphingorhabdus sp. (50) + Imperialibacter sp. (83) + Bosea sp. sp. (65) + Marinoscillum sp. (107)</i>
	mix_2016	<i>A mix of 22 strains used in exp 2016.</i>
	Mix_2_2016	<i>Bosea sp. (97) + Limnobacter sp. (121) + Limnobacter sp. (76b) + Limnobacter sp. (152) + Rhizobium sp. (ax2bis2)</i>
	Mix_5_2016	<i>Plantibacter sp. (T3) + Peanibacillus sp. (77) + Staphylococcus sp. (13a) + Pantoea sp (117b2a)</i>
<b>Algicidal</b>	38	<i>Altermonas sp.</i>
	39	<i>Marinobacter sp.</i>
	I3	<i>Roseovarius sp.</i>
	58	<i>Halomonas sp.</i>
	Mix_2_2015	<i>Halomonas sp. (58), Moraxalla sp. (ax1)</i>
	Mix_3_2015	<i>Halomonas sp. (58), Bosea sp. sp. (65)</i>
	Mix_all_2015	<i>A mix of 20 strains tested in Exp1</i>
	Mix_1_2015	<i>Halomonas sp. (58), Sphingorhabdus sp. (50)</i>

The absence of bacterial-induced algal growth in fresh water can be explained in different ways. First, the collection of cultivated bacteria is not extensive, *i.e.* the majority of *Ectocarpus*-associated bacteria have not yet been cultivated (KleinJan et al., 2017; Tapia et al., 2016; 79%-89% of OTUs were not cultivated; Chapter 1). It is thus possible that the bacteria responsible for the algal fresh water phenotype are not yet part of the culture collection and therefore not tested. However, in the positive control experiments (inoculation with full microbiome), fresh water tolerance was also absent. Therefore, it cannot be excluded that the absence of fresh water tolerance is due to a delayed direct effect of the antibiotics treatments rather than an effect of their impact on the microbiome. Inoculation with the full microbiome has been shown to restore fresh water tolerance previously (Dittami *et al.*, 2016), and several attempts have been made to re-establish this phenotype. For instance, a fresh culture of the FWS obtained from BEZHIN ROSKO<sup>8</sup> and tested in the co-culture experiments, but this did not change the outcome of the experiments. Furthermore, different algal sterilization protocols were tested, aiming to obtain new axenic algal cultures. The freshwater response of those newly created axenic algae was verified by co-incubation with algal growth medium (also derived from EC371 in various growth conditions), but none resulted in a working positive control.

Moreover, most of the sterilization protocols tested were equally and/or less efficient in removal of bacteria from the algal tissue and they were therefore not advantageous over the pre-existing method. Thus, obtaining axenic *Ectocarpus* remains a challenge, due to the low viability of sterilized filaments, and the increase in antibiotic-resistance among the algal associated bacteria. Yet, creating axenic algae is a crucial step towards reliable and reproducible algal-bacterial co-cultivation experiments, because even the smallest bacterial contamination, may interfere with the outcome of the experiment (Wichard, 2015).

## Conclusion

These preliminary experiments confirm the existence of bacterial interactions with *E. subulatus* FWS: although the bacterial strains did not restore the fresh water phenotype, they did have an impact on algal growth in seawater and fresh water. The results also suggest a potential role of the uncultivated microbiome in conferring fresh water tolerance to the alga. Surely, these *Ectocarpus* co-culture experiments require optimization, primarily by improving the method for sterilization of algal tissue. Alternative strategies to obtain sterile algal cultures could involve more complex algal growth medium so that the alga is less dependent on interactions

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<sup>8</sup> <http://www.bezhinrosko.com/>

with the microbiome; furthermore, the alga could be cultured with one/some selected antibiotic-resistant symbionts that allow algal growth, but prevent the proliferation of other (opportunistic) bacteria. Other improvements incorporated in following up co-culture experiments were a quantitative method to measure algal growth; flow cytometry counting of the bacterial cells in the inoculum; and verification of the presence of the inoculated strain over the time of the experiment (16S rRNA gene metabarcoding). Moreover, an alternative approach to select bacteria and assembled artificial communities should be implemented, to narrow down the number of strain to test. One example of such an approach is the construction of metabolic networks from bacterial genomes, and to select beneficial strains based on metabolic network complementary with the algal host. The first attempts to apply such an approach are presented in subsection III.

The work on *E. subulatus* FWS was continued using an alternative strategy for functional studies, *i.e.* a metatranscriptomics/metagenomics approach, using mild-antibiotic treated algae with modified microbiomes, each responding differently to fresh water. By comparing the metatranscriptome of the different algal holobionts in fresh water and in seawater, we hope to gain insights in how bacteria contribute to the response of *E. subulatus* to fresh water. This work is described in Chapter 3 of the thesis.

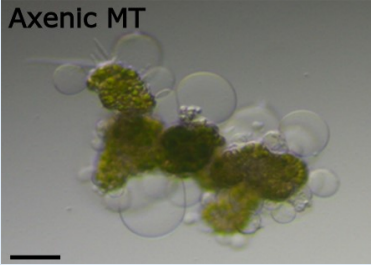

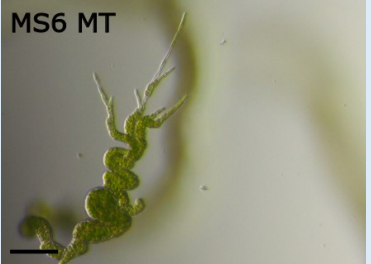

## II. Specificity of cross-lineage cross-talk: do *Ectocarpus*-derived bacteria interact with *Ulva* gametes?

### Introduction

*Ulva*, also called “sea lettuce”, is a small green macroalga with foliaceous morphology, that has been well-described regarding its response to the presence of bacterial symbionts (Wichard, 2015). Gametes growing under axenic conditions develop into callus-like structures and have striking cell wall deformations (Table 2-3 – Axenic morphotype). However, a combination of two bacteria isolated from *Ulva mutabilis* was shown to recover the complete morphogenesis of the alga: *Roseovarius* strain MS2 was shown to induce cell division (Table 2-3 – MS2 morphotype), while *Maribacter* strain MS6 was shown to induce rhizoid formation (Table 2-3 – MS6 morphotype), and both bacteria combined induce the normal thallus-like morphotype (Table 2-3 – complete morphotype). The activity of those two bacteria is not exclusive, and a range of bacteria from different taxonomic groups can induce the same phenotype (Grueneberg *et al.*, 2016). Thus, I wanted to test if bacteria derived from brown algae (Chapter 1; KleinJan *et al.*, 2017), can be morphogenetically active towards axenic *Ulva* gametes. To that aim, I tested 45 *Ectocarpus*- and 12 *Laminaria*-derived (Salaün *et al.*, 2012) bacteria on their own, in combination with *Ulva*-derived *Roseovarius* (strain MS2), and in combination with *Ulva*-derived *Cytophaga* (MS6).



Table 2-3 The different morphotypes (MT) observed when axenic gametes of *Ulva mutabilis* are grown without bacteria (axenic MT), in the presence of an MS2-like bacteria (MS2 MT), in the presence of an MS6-like bacteria (MS6 MT), or with both MS2 and MS6-like bacteria (complete MT). Scale bar = 2 mm. Pictures were taken after 4 weeks of co-culture.

	<b>Rhizoid formation</b>	<b>Cell wall protrusions</b>	<b>Cell division and longitudinal growth</b>	<b>Morphology</b>
<b>None</b>	-	+	-	<p>Axenic MT</p> 
<b>MS2</b>	-	+	+	<p>MS2 MT</p> 
<b>MS6</b>	+	-	-	<p>MS6 MT</p> 
<b>Both</b>	++	-	++	<p>Complete MT</p> 

## Materials and methods

### Experimental set-up

Based on the tripartite system, *i.e.* *Ulva* as algal host and the two bacterial symbionts (strain MS2 and MS6), a bioassay has been developed, which facilitates fast and reliable screening of bacterial cultures for morphogenetic activity under controlled conditions (Wichard, 2015; Grueneberg *et al.*, 2016). This bioassay utilizes axenic gametes of the developmental mutant slender, a mutant that, compared to the wild type, exhibits faster growth, a shorter lifecycle, and develops into tube-like structures, with only primary rhizoids (Spoerner *et al.*, 2012b; Wichard, 2015). Axenic gametes were inoculated with the bacteria of interest, the bacteria of interest plus *Roseovarius* (MS2 activity), and the bacteria of interest plus *Maribacter* MS6 (Figure 2-5A). Based on the morphogenetic activity, the bacteria were classified as MS2-like, MS6-like bacteria, bacteria with MS2 and MS6 activity (inducers of complete morphotype), or bacteria with no activity (Figure 2-5B). Intermediate phenotypes were observed in some cases, and these bacteria were treated based on the effect the bacteria alone had on algal morphology (without MS2 or MS6).

B1	B1 + MS2	B1+ MS6
B2	B2 + MS2	B2+ MS6
B3	B3 + MS2	B3+ MS6
None	MS2	MS6

Figure 2-5 A. Simplified schematic overview of the experimental set-up used to test the morphogenetic activity of cultured bacteria. Experiments were carried out in 96-wells plates, but here only 3 columns are shown. Axenic gametes were inoculated with the bacteria to test alone (column 1), the bacteria to test plus strain MS2 (column 2), and the bacteria to test plus MS6 (column 3). Inoculations without the bacteria to test were included as a control (row H). MS2: *Ulva*-derived *Roseovarius* strain; MS6: *Ulva*-derived *Maribacter* strain; MT: Morphotype. B. Classification of bacteria that were tested was based on their effect on algal

morphology when tested alone, in combination with MS2, or in combination with MS6. Intermediate phenotypes are classified based on the effect of the bacteria alone.

### **Production of axenic algae**

Axenic gametes were produced according to Califano and Wichard (2018). Three days after gametogenesis was induced (via fragmentation of the tissue), the gametes were released and purified. Purification relies on the photo-tactic activity of the gametes, and the fact that the gametes move faster towards the light than associated microorganism (Spoerner et al., 2012). Purification was carried out in capillary glass tubes and repeated three times to ensure sterility. The absence of bacteria was confirmed via 16S rRNA gene amplification on the centrifuged supernatant of the purified gametes (DNA extracted via heating), with the primers 27F and 1392R and the following amplification conditions: 5 min 95°C; [30 sec. at 95 °C; 30 sec. at 58 °C; 1.5 min at 72°C] 30 cycles; 7 min 72°C. A detailed description of the established protocol can be found in chapter 9 of “Protocols for Macroalgae Research” (Califano and Wichard, 2018).

### **Co-culture experiments**

All bacterial strains including the originally described *Ulva*-derived strains MS2 and MS6 were grown in liquid growth medium (Marine broth or R2A) at room temperature (RT) until the start of the experiment. Gametes were inoculated from dense liquid bacterial cultures using sterilized toothpicks, thus the number of bacteria was not controlled in this experiment. Also, these experiments were not replicated. However, the purpose of this experiment was merely to screen a high variety and number of bacteria to see if there are any that act in a similar way as the *Ulva*-derived MS2 and MS6 strain. In a possible follow-up experiment and based on the preliminary data discussed here, a subset of promising candidate bacteria could be tested in more detail and then replicates must be included. Non-inoculated axenic gametes were run in parallel as a negative control. After inoculation, the plates were covered with a permeable seal (Breathe-Easy ®) and grown at 20°C at a 17:7h light-dark regime. The development of the gametes was observed after 2 and 4 weeks using an inverted microscope (Leica, Wetzlar, Germany), and scores were given for the shape of the tissue, the presence/absence of cell wall deformations and the presence/absence of rhizoids. For practical reasons, pictures were only taken after 4 weeks. Based on those scores the bacteria were categorized as inducers of the “MS2 morphotype”, the “MS6 morphotype”, the “complete morphotype, or the “axenic

morphotype” (Table 2-3). The 16S rRNA gene of each bacterial culture used in the co-culture experiment was re-sequenced afterward to confirm their taxonomic classification. For each bacterial culture, 50 µl was heated (95 °C, 15 min) and the 16S rRNA gene was amplified with primers 8F and 1492R with following amplification conditions: 2 min 95°C; 30 cycles [1 min. 95 °C; 30 sec. 53 °C; 3 min 72°C]; 5 min 72°C. Purified PCR products were sequenced with Sanger Technology. A detailed description of this method is given in chapter 1 of the thesis (pg. 36).

## Results and discussion

A total of 57 bacterial strains was tested, 45 of which originated from the *E. subulatus* FSW and 12 from *L. digitata* (Salaün *et al.*, 2012). Based on the observations, the bacteria were grouped as described in Table 2-3, Table 2-5 & Figure 2-5B. 43 strains were categorized as MS2-like (including 17 “intermediates”), while two were categorized as MS6-like (both “intermediates”; *Cobetia* Ld15, *Sphingomonas* Ax6; Table 2-4 & Table 2-5). This is in agreement with the literature, showing that MS2 activity is a characteristic that is shared among taxonomically different bacteria, while MS6 appears less commonly and is taxa-specific (Grueneberg *et al.*, 2016). For four strains the gametes developed into the typical axenic morphotype. However, for three of those, the complete morphotype was restored in combination with MS6 (*Brevundimonas*\_323, *Bosea*\_46, *Hoeflea*\_135; Table 2-4). This could indicate that these particular strains induce cell division via a different mechanism than the original MS2 strain, or that their activity depends on the presence of MS6.

Seven bacteria were algicidal (Table 2-5; *Agrococcus* Ld12, *Pseudoalteromonas* Ld20, *Limnobacter* 312, *Citricoccus* K5, *Bosea* L3, *Stenotrophomonas* 78, *Cobetia* Ld13) because the gametes died when exposed to the bacterium. In some cases, the algicidal effect disappeared when cultured in combination with MS2 (for *Stenotrophomonas*, *Cobetia*, and *Limnobacter* 312) or MS6 (*Agrococcus*, Strain\_Ld20). Interestingly, Grueneberg and co-workers (2016) also discovered six bacteria with algicidal effects on *Ulva*, and two were classified as *Pseudoaltermonas*. One strain induced the complete morphotype on its own (Complete MT; *Marinobacter* 39; Table 2-4). The only other two bacteria described so far with FMT activity, *i.e.* *Algoriphagus* sp. and *Polaribacter* sp., belong to the *Bacteroidetes*. Interestingly, it has also been shown that *Marinobacter* sp. (a gammaproteobacterium) has a morphogenetic effect on *E. siliculosus* (Tapia *et al.*, 2016; and the results in subsection III of this chapter).

In some cases, remarkable phenotypes were observed (Figure 2-6 and Table 2-5), e.g., algae with extreme curvature (*Ahrensia\_33*; Figure 2-6A), and algae with extreme elongation (Figure 2-6B). Some bacteria formed a biofilm on the algal surface (Figure 2-6C). In addition, 19 of the bacteria tested induced an intermediate algal morphotype (Table 2-4 & Table 2-5), e.g. bacteria with MS2 activity did not always restore the complete morphotype when combined with MS6 (e.g. *Cellulophaga* Ld17). There may thus be more ways to affect morphogenesis in *Ulva* than the activity described for MS2 and MS6 so far.

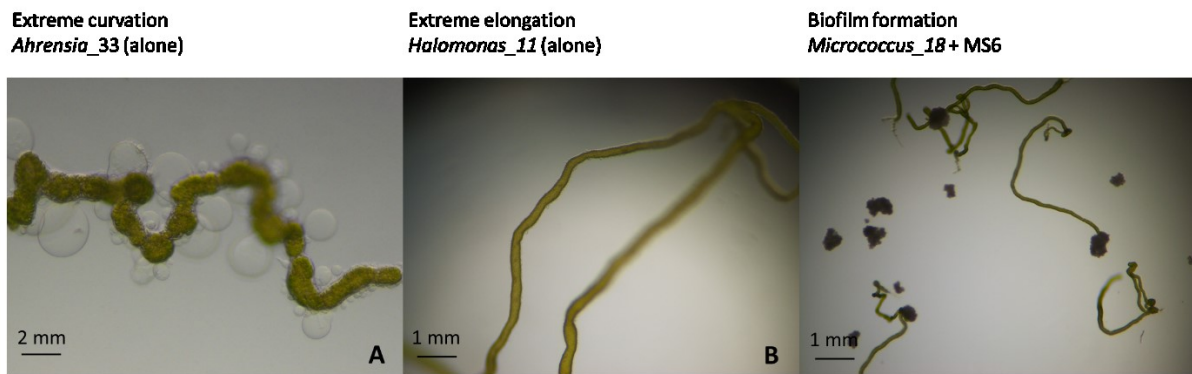


Figure 2-6 Some examples of notable phenotypes observed during the co-culture experiments.

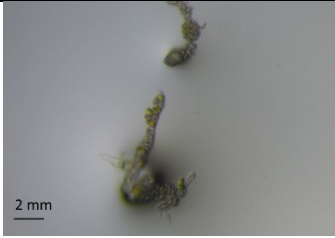

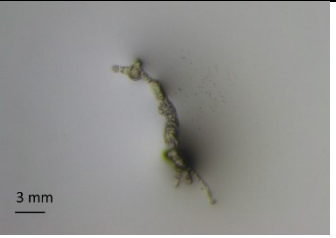






Overall, the screening showed that 46 strains of the 57 cultivated brown algal derived bacteria had a morphogenetic effect (80%), compared to 13 of the 57 *Ulva*-derived bacteria (22%) that were tested previously in the study carried out by Grueneberg and co-workers (2016). However, because most bacteria remain uncultivated, these numbers are not a reliable representation of the full morphogenetic capacity of the microbiome.

## Conclusion

This preliminary study aimed to investigate the host-specificity of bacterial morphogenetic activity between taxonomically distinct algal hosts. The data suggest that brown alga-derived bacteria have similar effects on morphology as bacteria derived from *Ulva* and may, therefore, exert similar functions. Interesting candidates could be tested in more detail, for example, the *Marinobacter* isolate, which induced complete morphogenesis. Follow-up experiments should incorporate biological replicates of bacterial cultures. Also, an additional control inoculating MS2 and MS6 together should be included to confirm that the complete morphotype can still

be induced by the original strains. Additionally, bacterial cultures should be freshly prepared and measured by flow cytometry, to know the exact number of bacteria in the inoculum before the start of the experiment. Only then quantitative analysis can be done and reliable conclusions can be drawn from the results that are obtained.

Table 2-4 Some examples of algal morphologies observed when axenic *Ulva* gametes were inoculated with the bacteria alone, bacteria + MS2, and bacteria + MS6. Pictures were taken after 4 weeks of inoculation.

	Alone	+ MS2	+ MS6	Categorized as:
<i>Cobetia Ld15</i>				MS6
<i>Sphingomonas Ax6</i>				MS6 – intermediate; no FMT with MS2
<i>Marinobacter 39</i>				Complete MT

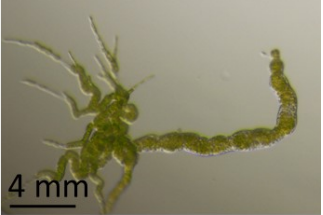
<p><i>Sphingomonas</i> 5a</p>				<p>MS2</p>
<p><i>Cellulophaga</i> Ld17</p>				<p>MS2 – intermediate; no FMT with MS6</p>
<p><i>Hoeflea</i> 135</p>				<p>Axenic, but FMT with MS6.</p>



Table 2-5 Overview of the classification of bacteria based on the *Ulva*-bioassay screening.

\* = inducing biofilm formation; \*\* Inducing an intermediate phenotype;

Category	Origin bacterial isolate	
	<i>Laminaria digitata</i>	<i>Ectocarpus subulatus</i>
MS2 (43)	<i>Paracoccus</i> (Ld14)	Microcella (Z68)
	<i>Agrococcus</i> (Ld7) *	<i>Pantoea</i> (111)
	<i>Arthrobacter</i> (Ld9)	<i>Bacillus</i> (348-401)
	<i>Microbacterium</i> (Ld19)**	<i>Bacillus</i> (348dte)
	<i>Pseudomonas</i> (Ld10) **	<i>Roseovarius</i> (134)
	<i>Agrococcus</i> (Ld11) **	<i>Ahrensia</i> (346)
	<i>Cellulophaga</i> (Ld17) **	<i>Bacillus</i> (123)
		<i>Limnobacter</i> (94B)
		<i>Alteromonas</i> (Ax2bis)*
		<i>Sphingorhabdus</i> (109)
		<i>Alteromonas</i> (154-2)
		<i>Microbacterium</i> (38)
		<i>Paenibacillus</i> (11a)
		<i>Flavobacterium</i> (350)*
		<i>Sphingomonas</i> (25a)
		<i>Paenibacillus</i> (130)
		<i>Ahrensia</i> (287)
		<i>Bacillus</i> (136)
		<i>Moraxella</i> (17a)
		<i>Rhizobium</i> (Ax2bis2)
		<i>Micrococcus</i> (74)
		<i>Bacillus</i> (349)
		<i>Halomonas</i> (3B)
		<i>Hyphomonas</i> (110a)**
		<i>Marinoscillum</i> (107) * / **
		<i>Bosea</i> (29b) **
		<i>Bacillus</i> (71) **
		<i>Bosea</i> (33B) **
		<i>Bosea</i> (125) **
		<i>Moraxella</i> (Ax1) **
		<i>Plantibacter</i> (T3) **
		<i>Brevundimonas</i> (G8) **
	<i>Cryptococcus</i> (T2) **	
	<i>Sphingomonas</i> (T1) **	
	<i>Halomonas</i> (100) **	
	<i>Bosea</i> (5a) **	
MS6 (2)	<i>Cobetia</i> (Ld15) **	<i>Sphingomonas</i> (Ax6) **
FMT (1)		<i>Marinobacter</i> (39)
Algicidal (7)	<i>Cobetia</i> (Ld13)	<i>Stenotrophomonas</i> (78)
	<i>Agrococcus</i> (Ld12)	<i>Limnobacter</i> 312
	<i>Pseudoalteromonas</i> (Ld20)	<i>Citricoccus</i> (K5)
		<i>Bosea</i> (L3)
Axenic (4)	<i>Microbacterium</i> (Ld8)	<i>Brevundimonas</i> (325)
		<i>Bosea</i> (46)
		<i>Hoeflea</i> (135)
<b>Total</b>	12	45

### **III. Metabolic complementarity between *Ectocarpus* and associated cultivable bacteria: experimental verification of *in silico* predicted beneficial communities**

#### **Introduction**

As explained in the introduction of the thesis, microbial symbionts are omnipresent in nature and important for the development and functioning of multicellular eukaryotes. Elucidating the interactions within microbial communities and how this affects host physiology is a complex task because it requires the understanding of the dynamics within the microbiome, host metabolism, as well possible inter-species interaction and/or metabolic exchanges that could occur between the two partners.

One way to dissect those interactions is via targeted co-culture experiments using cultured bacteria. This strategy was applied on *E. subulatus* to identify bacteria that had a possible beneficial effect on algal growth at low salinity (discussed in subsection I). These experiments were unsuccessful in that the bacterial strains and artificial communities that I selected were not able to restore algal growth in fresh water. This outcome may be, in part, attributed to the way the bacterial consortia were selected. Because, even from a small set of cultured bacteria, there are countless combinations to test experimentally, it is possible that it is merely the combinations I chose that were not functional. Hence, the main question that is addressed in this subchapter is how to select those bacteria that are most interesting, *i.e.* contributing to the host in a beneficial way?

One approach to improve the selection of beneficial bacterial interactions is via metabolic network analysis (van der Ark *et al.*, 2017). Metabolism is the sum of all biochemical reactions catalyzed by enzymes in an organism<sup>9</sup>. Individual reactions combined form metabolic pathways, that together form a metabolic network *i.e.* a representation of an organism's metabolism (Chalancon, Kruse and Babu, 2013). One can assess the functionality of or completeness of the network by testing whether it is able to produce specific target compounds known to be present in the biomass (e.g. via physiological measurements, metabolite and/or transcriptome data) of the organism it was based on (Feist *et al.*, 2009). By adding reactions to

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<sup>9</sup> <https://www.ebi.ac.uk/training/online/course/introduction-metabolomics/what-metabolomics>

the network that contribute to the *in silico* production of those target compounds, one can manually curate the network, *i.e.* fill the gaps, and improve the completeness and functionality of the metabolic model (Prigent *et al.*, 2017). Metabolic complementarity analysis builds further on this feature and tests whether some of the gaps in the host metabolic network can be resolved by allowing for metabolite exchanges with associated bacteria in the models. If so, this could indicate that the exchange of compounds between both partners may be beneficial for the algal holobiont (Dittami *et al.*, 2014; Dittami, Eveillard and Tonon, 2014; Prigent *et al.*, 2014). A new pipeline to carry out metabolic network complementarity analysis has recently become available (Frioux *et al.*, 2018; in press).

The main objective of this work was to provide a first experimental assessment (proof of concept) of how well such *in silico* predictions based of metabolic complementarity can estimate actual cooperation between algae and bacteria grown in co-culture. To reach this aim, ten bacteria were selected from the culture collection and their genomes were sequenced. The bacterial genomes served as the starting point for metabolic network reconstruction, followed by the prediction of metabolic complementarity between *Ectocarpus* and the cultured bacteria. Based on this analysis, six bacterial communities were identified *in silico* that were predicted to have the strongest beneficial effect on the alga, *i.e.* they would complete the algal metabolic network and increase the number of producible target compounds in the algal host. Three bacterial communities were tested experimentally via algal-bacterial co-culture experiments. The selection and experimental verification of those three communities were implemented in the master project of Bertille Burgunter-Delamare.

In the previous co-culture experiments with *E. subulatus* (as described in subsection I), I encountered some difficulties, and therefore some improvements were made in this experiment to prevent those issues from happening again. First, the focus was exclusively on growth in seawater, to eliminate negative effects on the alga simply due to the change in salinity. Experiments were carried out using *Ectocarpus siliculosus* (Ec32) instead of *E. subulatus* FWS, because of the robustness of the model system and the fact that it is easier to produce reproducible axenic cultures. *E. siliculosus* in seawater without bacteria has a ball-like morphology, while associated with its full microbiome it has a branched morphology (Tapia *et al.*, 2016, see also introduction 'Morphogenetic compounds, pg. 16). Thus, we observed the effect of bacteria on algal morphology and algal growth in seawater. Algal growth was measured semi-quantitatively by microscopy measurements. Experiments were performed in

artificial seawater and not in natural seawater, to control precisely the amount of nutrients and organic compounds including potential hormones provided. Moreover, the density of the bacterial inocula was measured by flow cytometry, rather than optical density which gives a better estimate of bacterial cell counts. Also, bacterial density was measured several times during the experiment to observe cell proliferation. With 16S metabarcoding in the algal tissue at the end of the experiment, the absence/presence of the inoculated strains in the co-culture could be confirmed. Finally, targeted metabolomics was carried out to identify the compounds that were predicted *in silico* to become producible by the alga as a consequence of the potential exchange of reaction intermediates between the alga and its microbiome.

## **Materials and methods**

### **Selection of bacterial strains for genome sequencing**

A culture collection of 46 different bacterial strains is available (Chapter 1), obtained from *Ectocarpus subulatus*. Among these, eleven strains were selected based on their potential interest for further studies: potentially new species, the effects they had on brown alga (*Ectocarpus* – Chapter 2 subsection I) and green algal (*Ulva mutabilis* – Chapter 2 subsection II) growth and development, and on co-occurrence patterns across a range of samples indicating possible inter-bacterial interactions (Dittami *et al.*, 2016). Additionally, one strain from a culture collection, *Imperialibacter roseus* P4(T) KCTC 32399 (Wang *et al.*, 2013) was sequenced because no reference genome was available for this genus. The bacterial genomes served as the starting point for metabolic network analysis. An overview of the genome sequenced bacterial strains is given in Table 2-7.

### **Bacterial genome sequencing and assembly**

Bacterial cultures were inoculated from glycerol stocks and grown in liquid Zobell medium at RT until maximum density was reached. DNA was extracted using the UltraClean® Microbial DNA isolation kit (MoBio Laboratories, Inc.) and 50 µl elution buffer. Quality of DNA was verified by NanoDrop and gel electrophoresis. Library preparation was done at the sequencing platform in Roscoff (Platform de Séquencage-Génotypage GENOMER, FR2424) using one pair-end run of an Illumina Miseq (V3 chemistry; 2x300bp). After cleaning (Trimmomatic, default parameters; Bolger *et al.*, 2014), the paired-end reads were assembled using SPADES v3.7.0 (Bankevich *et al.*, 2012; default parameters for long reads) by the ABIMS platform (Platform ABiMS, FR2424, Roscoff). Statistics of the assembled genomes are given in (Table

2-7) and assembly quality (11-72 scaffolds per genome) and coverage (79X-326X) were sufficient for all downstream analyses. The RAST/SEED server<sup>10</sup> (Aziz *et al.*, 2008) was used for gene annotation, and sequences were later also incorporated into the MAGE platform<sup>11</sup>.

### ***In silico* predictions of metabolic interactions and selection of communities**

Bacterial metabolic networks were constructed using pathway tools (Karp *et al.*, 2016, version 20.5) and the version 2 of the *Ectocarpus siliculosus* Ec32 (Aite *et al.*, 2018) was used as host metabolic network. The Miscoto tool (Frioux *et al.*, 2018 - in press) was used to compute all potentially beneficial metabolic exchanges that may occur between *Ectocarpus* and any of the ten target bacteria (*Imperialibacter* strain R9 and *Imperialibacter roseus* P4 were excluded from network construction). This resulted in a list of 160 compounds that became producible by the algae through metabolic exchanges with the bacteria, *i.e.* these compounds were not producible by the alga alone and thus the exchanges could potentially be beneficial to the alga. Three bacterial communities were tested experimentally via algal-bacterial co-culture experiments. In addition to the three communities, all bacterial strains were tested individually, and two additional strains were tested, which were predicted to be less beneficial to the alga, *i.e.* *Sphingomonas* and *Erythrobacter*.

### **Experimental validation**

#### **Preparation of antibiotic-treated algae**

*Ectocarpus siliculosus* (strain 32; accession CCAP 1310/4, origin San Juan de Marcona, Peru) was cultured under standard conditions (13 °C; 12h light regime) in Provasoli-enriched natural seawater (NSW-PE) until the start of antibiotic treatment. Algal sterilization was done using a mixture of the following liquid antibiotics: 45 µg/ml Penicillin G, 22.5 µg/ml streptomycin, and 4.5 µg/ml chloramphenicol dissolved in in Provasoli-enriched artificial seawater (450 mM Na<sup>+</sup>, 532 mM Cl<sup>-</sup>, 10 mM K<sup>+</sup>, 6 mM Ca<sup>2+</sup>, 46 mM Mg<sup>2+</sup>, 16 mM SO<sub>4</sub><sup>2-</sup>). Filaments were exposed to the 25 ml of this solution for 3 days and then placed in Provasoli-enriched artificial seawater for 3 days to recover. Sterility was confirmed by microscopy using phase contrast (Olympus BX60, 1.3- PH3 immersion objective, 800x magnification).

#### **Preparation of bacterial cultures**

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<sup>10</sup> <http://rast.nmpdr.org/>

<sup>11</sup> <http://www.genoscope.cns.fr/agc/microscope/mage/index.php>

Bacteria were grown in liquid Zobell and/or diluted R2A until sufficient density (~3 days at room temperature RT). For each bacterial culture, 50 µl were heated (95 °C, 15 min), spun down, and 1 µl of supernatant was used to amplify the 16S rRNA gene with the primers 8F and 1492R with following amplification conditions: 2 min 95°C; 30 cycles [1 min. 95 °C; 30 sec. 53 °C; 3 min 72°C]; 5 min 72°C. Purified PCR products were sequenced with Sanger Technology. A detailed description of this method is given in chapter 1 of the thesis (pg. 36).

### **Co-culture experiments**

The co-culture experiments were performed as described in subsection I, with the important difference that the bacterial inoculation density was based on flow cytometry rather than optical density, which gives a better estimate of bacterial cell density. This was done using a BD FACS Canto™ II flow cytometer (BD Bioscience, San Jose, CA; fixed in Tris-EDTA) before the start of the experiment and algae were finally incubated with  $2.3 \cdot 10^5$  bacterial cells per strain and per ml medium. Co-cultures were incubated for four weeks under standard algal growth conditions. Controls were run in parallel, *i.e.* a non-antibiotic treated positive control (POS), and an antibiotic-treated non-inoculated alga as a negative control (NEG). The following measures were taken to quantify the effect of bacteria:

- Algal growth was quantified by measuring the filament length of the algae each week, using the binocular microscope (three measurements per replicate).
- Algal morphology was observed via microscopy (LEICA DMI8) at day 0 and day 28 of the experiment; attention was paid to the structure of filaments (branching pattern, filament length), and the general look of the alga (ball-like structure vs. filamentous appearance)
- Bacteria in the algal growth medium were counted using flow cytometry (described above) and bacteria attached to algal cell walls were counted by microscopy (5x 10 µm long filaments observed per biological replicate, 800x magnification in phase contrast)

### **Analysis of bacterial community composition**

A metabarcoding approach was implemented to investigate the composition of the bacterial community, and to confirm the occurrence of the inoculated bacterial strain inoculated, after 4 weeks of co-culture. For each co-culture, 20 mg ground freeze-dried tissue (TissueLyserII Qiagen, Hilden, Germany; 2x45sec, 30 Hz) was used for DNA extraction (DNeasy Plant Mini Kit, Qiagen; standard protocol). Nucleotide concentrations were verified with Nanodrop ONE (Thermofisher Scientific). A mock community comprised of DNA from 32 bacterial strains (covering a variety of taxa) was included in each of the subsequent steps (Supplementary table

2-2). Libraries were prepared according to the standard protocol for metabarcoding with Illumina MiSeq technology<sup>12</sup> and the V3–V4 region of the 16S rRNA gene was amplified. Libraries were quantified with Qubit (High-Sensitivity dsDNA Assay; Life Technologies, Australia) and library sizes were verified with the Agilent Bioanalyzer (DNA 1000 kit; Agilent Technologies, Inc., CA, USA). The final pool for sequencing contained 5 µl of each library (4nM final concentration in 10 nM Tris-pH 8.5) and was further diluted to 5 pM after denaturation in 4nM NaOH before sequencing using Illumina MiSeq Technology (2x300 bp, pair-end reads; MiSeq Reagent v3 kit; Platform de Séquencage-Génotypage GENOMER, FR2424, Roscoff). The raw sequences (7,354,164 read pairs) were trimmed using the `fastq_quality_trimmer` from the FASTX Toolkit<sup>4</sup> (quality threshold 30; minimum read length 200), and assembled into 6,804,772 contigs using PandaSeq<sup>13</sup> (Masella *et al.*, 2012). Finally, the data were analyzed with Mothur (V.1.40.3) according to the MiSeq Standard Operating Procedures<sup>5</sup> (Kozich *et al.*, 2013). Contigs were preclustered (allowing for four mismatches), and aligned to the Silva\_SEED 132b database for sequence classification. Chimeric sequences were removed (`vsearch`) and the remaining sequences classified taxonomically (Wang *et al.*, 2007). Non-bacterial sequences were removed. The sequences were then clustered into operational taxonomic units (OTUs) at a 97% identity level and each OTU was classified taxonomically. All OTUs with  $n \leq 10$  sequences were removed resulting in a final data matrix with 1,834,992 sequences. The OTU matrix was subsampled to have the same number of sequences per sample for downstream analyses. Sequences obtained from the isolates tested in co-cultures were compared with the 16S rRNA gene metabarcoding, using BLASTn searches against consensus sequences of each OTU.

### Targeted metabolomics

Of the 160 compounds identified as potentially newly producible by *Ectocarpus* based on exchanges of intermediates with bacteria, a subset of 8 metabolites selected for targeted metabolomics based on literature research and after manual verification of automatic predictions of corresponding pathways in the algal and bacterial networks: L-histidine, putrescine, β-alanine, nicotinic acid, folic acid, auxin, spermidine, and preQ1. Metabolites were extracted from ground freeze-dried tissue using 2 ml of methanol:chloroform:water (6:4:1) solvent. The remaining pellet was extracted a 2<sup>nd</sup> time with 1 ml of chloroform:methanol (1:1);

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<sup>12</sup>[https://support.illumina.com/downloads/16s\\_metagenomic\\_sequencing\\_library\\_preparation.html](https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html)

<sup>13</sup> <https://github.com/neufeld/pandaseq>

Finally, a 3<sup>rd</sup> extraction was done Using H<sub>2</sub>O as a solvent. The supernatants of each extraction were pooled before measurement. The solvents were evaporated under a stream of nitrogen and metabolites were resuspended in 100 µl methanol:water (1:1). Finally, data liquid chromatography was performed on a Viridis BEH column (3x100 mm, 1.7 µm), using an ACQUITY UPLC system (Waters<sup>®</sup>, Millford, USA). A linear gradient of two solvents was used to separate peaks: supercritical carbon dioxide (Solvent A), and methanol spiked with 0.1% formic acid (Solvent B). The gradient ran from 5% to 25% of solvent B (35% for spermidine and nicotinic acid) during 2 minutes, was kept at this level for another 2 minutes and then gradually reduced back to 5% during 3 minutes. The UPLC system was coupled to a Xevo G2 Q-ToF mass spectrometer (Waters<sup>®</sup>, Millford, USA), operating in positive ESI ion mode (m/z 20–500). Standards of all 8 compounds were run in parallel to samples. Analyses were performed at METABOMER platform (FR2424, Station Biologique de Roscoff).

### **Statistical analysis**

Data were tested for normality using the Shapiro test (Rstudio v1.0.44; RStudio Team, 2016). Significant differences between all treatments after four weeks of co-culture (day 28) were calculated with ANOVA and Tukey post-hoc testing with a significance level  $\alpha$  0.05 using the PAST software<sup>14</sup> (version 3.20; Hammer et al., 2001).

## **Results & discussion**

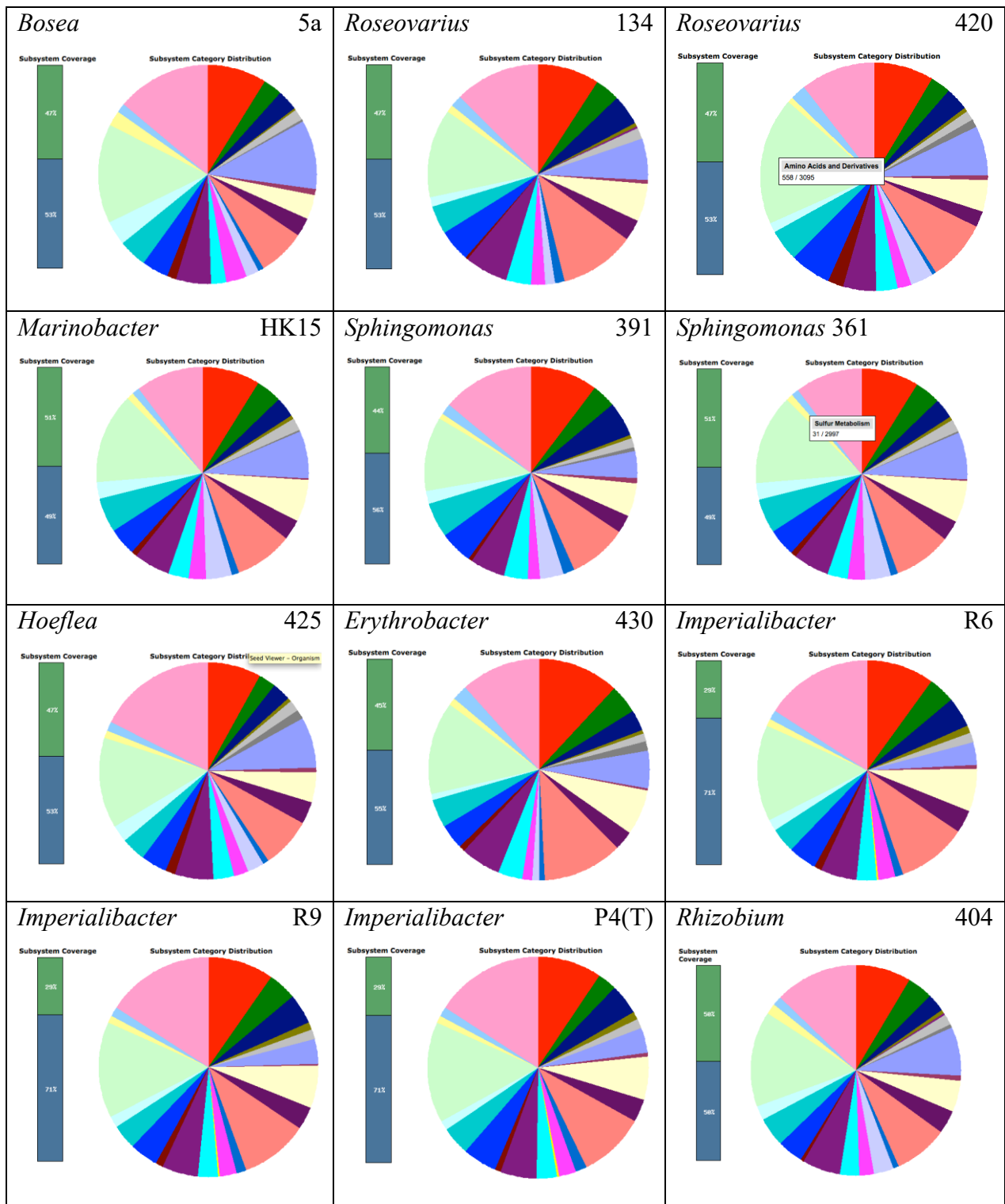
### **Bacterial genome sequencing**

Bacterial genome sequences were successfully assembled for all twelve bacterial strains and the assembly statistics can be found in Table 2-7. 27 functional categories (*i.e.* “subsystems”) that were identified by the RAST server (Figure 2-7). The subsystems ‘carbohydrates’, ‘amino acids and derivatives’, ‘Cofactors/vitamins’, ‘membrane transport’ and protein metabolism were the most abundant for each bacterium. No strong differentiation was observed between the different isolates at this level of comparison. The main aim of the bacterial genome assembly was to construct the metabolic network for the *Ectocarpus*-derived strains, and use those networks to predict metabolic exchange with the host. Hence, the bacterial genomes were not investigated in more detail in the context of my thesis.

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<sup>14</sup> <https://folk.uio.no/ohammer/past/>





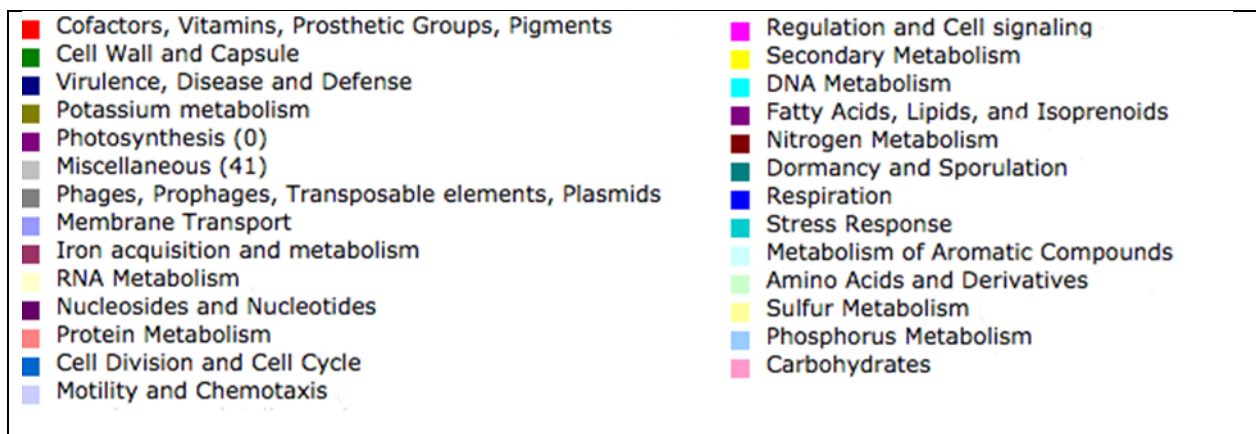


Figure 2-7 Overview of functional categories (subsystems) identified in the ten bacterial genomes by de RAST server.

### Selection of beneficial bacterial consortia

Metabolic networks were reconstructed for ten of the bacterial genome sequenced strains. Strain *Imperialibacter roseus* P4 was excluded from network construction because it was not an alga-associated bacterium and not isolated from *Ectocarpus*. *Imperialibacter sp.* strains R6 and R9 were highly similar based on genomic comparison (average nucleotide identity 99.75%) and only R6 was used for network reconstruction. On average 261 pathways were predicted per bacterium, corresponding to 1,714 reactions, 111 transport reactions, and 1,405 metabolites (Table 2-7). Based on metabolic complementarity analysis, a total of six bacterial consortia were predicted that allowed for the production of 160 algal compounds (Table 2-6). Of these, three communities were chosen for further testing in algal-bacterial co-cultures. The communities not tested were composed of the same genera as the tested ones but comprised different strains.

Table 2-6 Predicted bacterial consortia that enabled the production of 160 algal compounds.

Bacterial consortium		Experimentally verified (y/n)
Solution 1	<i>Marinobacter</i> HK15, <i>Roseovarius</i> 420, <i>Hoeflea</i> 425 (MRH)	Yes
Solution 2	<i>Roseovarius</i> 420, <i>Imperialibacter</i> R6, <i>Hoeflea</i> 425 (RIH)	Yes
Solution 3	<i>Marinobacter</i> HK15, <i>Hoeflea</i> , <i>Roseovarius</i> 134	No
Solution 4	<i>Imperialibacter</i> R6, <i>Hoeflea</i> , <i>Roseovarius</i> 134	No
Solution 5	<i>Marinobacter</i> HK15, <i>Bosea</i> , <i>Roseovarius</i> 420 (MBR)	Yes
Solution 6	<i>Marinobacter</i> HK15, <i>Bosea</i> , <i>Roseovarius</i> 134	No

Table 2-7 Overview of bacterial isolates that were selected for genome sequencing (A) and the corresponding assembly statistics (B) of the annotated genomes. Selection criteria are explained in more detail in the text. Isolates indicated with an asterisk (\*) were used in the algal co-culture experiments. *Imperialibacter roseus* P4(T) served merely as a reference because no genome was available for a described strain of this genus. Predicted metabolic pathways, reactions, and metabolites from metabolic networks are given in table C.

Taxonomic affiliation & strain number		A. Selection criteria for genome sequencing					B. Assembly statistics of annotated genomes						C. Network reconstruction			
		Effect on <i>Ulva</i> gametes	Effect on Ec32	Probable new species	Correlation	High sequence abundance in 5% NSW	total raw reads	# scaffolds	genome size (mbp)	N50 (mbp)	Coverage (X)	mapped reads	Pathways	Reactions	Transport reactions	metabolites
<i>Bosea</i> sp. *	5A	X					1 863 417	26	6.34	0.98	133	99.91%	298	1892	153	1557
<i>Erythrobacter</i> sp. *	430					X	1 065 278	11	3.14	0.44	157	99.93%	218	1532	63	1247
<i>Hoeflea</i> sp. *	425					X	3 734 649	41	5.22	1.26	326	99.94%	315	1920	129	1558
<i>Imperialibacter</i> sp.	R6			X			1 553 981	65	6.8	0.21	111	99.94%	239	1711	100	1425
<i>Imperialibacter</i> sp. *	R9			X			2 125 399	62	6.8	0.22	152	99.94%	na	na	na	na
<i>Imperialibacter roseus</i>	P4(T)			X			1 442 853	35	6.69	0.67	105	99.94%	na	na	na	na

<i>Marinobacter</i> sp. *	HK15	X	X		X		1 587 675	14	4.39		172	99.93%	249	1679	128	1364
<i>Rhizobium</i> sp.	404	X		X			1 332 560	27	4.2	0.45	148	99.93%	289	1814	125	1462
<i>Roseovarius</i> sp.	134	X		X			987 463	73	4.68	0.18	150	99.92%	263	1703	125	1418
<i>Roseovarius</i> sp. *	420	X		X			803 175	85	4.68	0.12	79	99.89%	263	1701	125	1418
<i>Sphingomonas</i> sp. *	361	X		X			1 111 277	25	3.28	0.29	150	99.87%	224	1519	69	1239
<i>Sphingomonas</i> sp.	391	X			X		1 150 343	74	4.6	0.16	113	99.91%	254	1671	92	1358

## Co-cultures

The presence of bacterial strains enhanced algal growth by a factor of 3 to 5 compared to the non-inoculated negative control (Figure 2-9A). Interestingly, the mixes did not stimulate growth more than the individual strains, indicating that the tested bacteria may be functionally redundant with respect to the beneficial effects they have on algal growth. Likewise, effects on morphology were seen in all co-cultures (Figure 2-8), and all inocula tested were able to induce the branched morphology. The negative control (NEG) displayed a ball-like morphology (Figure 2-8). Those results are similar to the observations made by Tapia and co-authors (2016). However, differences could be seen between the co-cultures, as they each induced different branching patterns. For example, *Marinobacter-Roseovarius-Hoeflea* (MRH) induced long ramifications, *Marinobacter-Bosea-Roseovarius* (MBR) short ramifications, and *Roseovarius-Imperialibacter-Hoeflea* (RIH) long but very few ramifications. Moreover, some inocula induced atypical filament shapes and ramifications (e.g. horse tail shaped ramifications in the MBR co-culture). *Imperialibacter* induced aggregation of individuals, while in all other co-cultures individuals remained more or less separated.

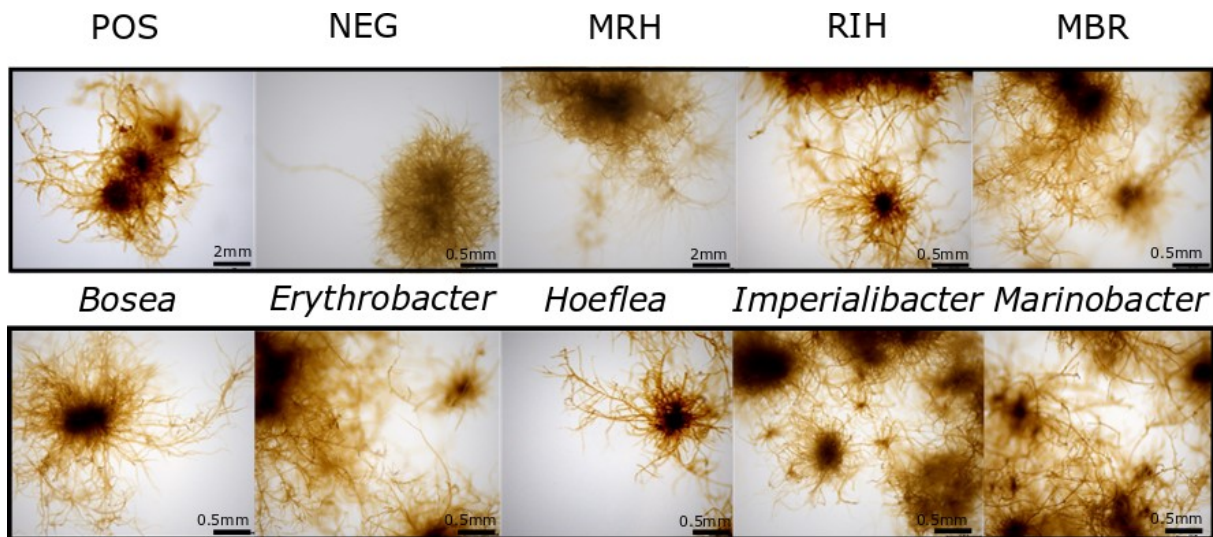


Figure 2-8 Morphological effect on *E. siliculosus* after 4 weeks of co-culturing. MRH: *Marinobacter-Roseovarius-Hoeflea*; RIH: *Roseovarius-Imperialibacter-Hoeflea*; MBR: *Marinobacter-Bosea-Roseovarius*; POS: non-antibiotic treated non-inoculated control; NEG: antibiotic-treated non-inoculated control.

The effects of co-culture on bacterial density were also observed (Figure 2-9 B&C). Flow cytometry analysis shows an increase in bacterial density in the medium after the 4 weeks of

co-culture in *Marinobacter-Roseovarius-Hoeflea* (MRH), *Hoeflea* and *Roseovarius-Imperialibacter-Hoeflea* (RIH). Bacteria were also detected in the medium of the negative control, an alga that had not been inoculated with bacteria (NEG), although the concentration was lower than in the co-cultures (Figure 2-9B). The negative control also showed a strong proliferation of bacteria on the algal surface ( $>150$  cells/ $\mu\text{m}$ ), a phenomenon not seen in any of the other co-cultures (Figure 2-9C). This indicates that the filaments were not sterile at the start of the experiment.

Finally, the bacterial composition was analyzed with the aim of measuring the abundance of the original isolate(s) in the co-culture after 4 weeks of incubation (Table 2-8). Generally, strains used as inoculum could be recovered after 4 weeks of co-cultivation in the correct experimental condition (*Sphingomonas*, *Bosea*, *Marinobacter*, and *Roseovarius*). However, *Imperialibacter* and *Erythrobacter* were not found in any of the gene libraries. *Hoeflea* was highly abundant in every co-culture experiment, even in those where *Hoeflea* was not used as an inoculum. The latter suggests that *Hoeflea* is antibiotic-resistant, or it is not accessible to antibiotics because it is embedded in the algal cell wall, where the drug concentrations may be too low to be effective.

### **Metabolite profiling:**

Eight key metabolites, each of them predicted to depend on metabolite exchanges with bacteria to become producible, were quantified with UHPLC after four weeks of co-culture (Table 2-8). In the negative control, *i.e.* antibiotic-treated algae that were not inoculated with bacteria, only one compound could be identified (preQ1). In contrast, in all co-cultures  $\geq 1$  target compounds were identified. Each compound became producible in the presence of bacteria in at least one of co-cultures. Thus, these results confirm the hypothesis of metabolic complementarity and metabolic exchange between alga and associated bacteria. However, none of the co-cultures displayed all target metabolites, which should have been the case based on the predictions that were made. This observation may indicate that the reactions that were predicted are influenced by the community members in the algal holobiont. The pathways involved in the metabolic exchange may have been affected leading to altered interactions with the host. Alternatively, the compounds may have been further metabolized in certain holobionts, without allowing an accumulation to reach the detection limit.

Table 2-8 “Metabarcoding” (upper half of the table): Observed abundance of target OTUs after four weeks of co-culture. The left column indicates the consensus OTUs closest to the sequence of the inoculated bacterial strain. “Targeted metabolomics” (lower half of the table): Compounds identified by UPC<sup>2</sup>-QTOF after 4 weeks of co-culture. (-): metabolite was not detected (+): metabolite was detected. *Bosea* samples could not be analyzed; Each of the columns corresponds to one specific co-culture experiment: MRH: *Marinobacter-Roseovarius-Hoeflea*; RIH: *Roseovarius-Imperialibacter-Hoeflea*; MBR: *Marinobacter-Bosea-Hoeflea*; Sphingo: *Sphingomonas*; Imperi: *Imperialibacter*; Marino: *Marinobacter*; Roseo: *Roseovarius*; NEG: negative control, *i.e.* non-inoculated alga.

		Co-culture tested											
		Mixes			Individual strains								Control
		MBR	MRH	RIH	Sphingo	Erythro	Bosea	Hoeflea	Imperi	Marino	Roseo	NEG	
Metabarcoding	<i>Hoeflea</i> - OTU00001	12688	10265	7644	10216	16390	11104	15321	13426	4483	15635	8899	
	<i>Marinobacter</i> - OTU00030	39	82	0	38	0	0	0	1103	0	0	0	
	<i>Imperialibacter</i> - OTU00044	0	0	0	0	0	0	0	0	0	0	0	
	<i>Roseovarius</i> - OTU00055	45	8	11	0	0	0	1	0	1	41	0	
	<i>Sphingomonas</i> - OTU00097	0	0	0	4	0	0	0	1	0	0	0	
	<i>Bosea</i> - OTU00100	43	0	0	0	0	26	0	0	0	0	0	
	<i>Erythrobacter</i> - no OTU	0	0	0	0	0	0	0	0	0	0	0	
	Other OTUs	31470	39403	23458	29066	19791	25653	34190	38076	26223	36374	42323	
Targeted metabolomics	Spermidine	-	-	-	-	-	Na	+	-	+	-	-	
	Putrescine	-	-	+	-	+	Na	+	-	-	+	-	
	Nicotinic acid	+	-	+	-	-	Na	-	+	+	-	-	
	Folic acid	+	-	-	+	-	Na	-	-	-	-	-	
	Auxine	-	+	+	+	-	Na	+	+	-	-	-	
	L-Histidine	-	-	-	+	-	Na	+	+	-	-	-	
	β-Alanine	-	-	+	-	-	Na	+	-	-	-	-	
	preQ1	+	+	+	+	+	Na	+	+	+		+	

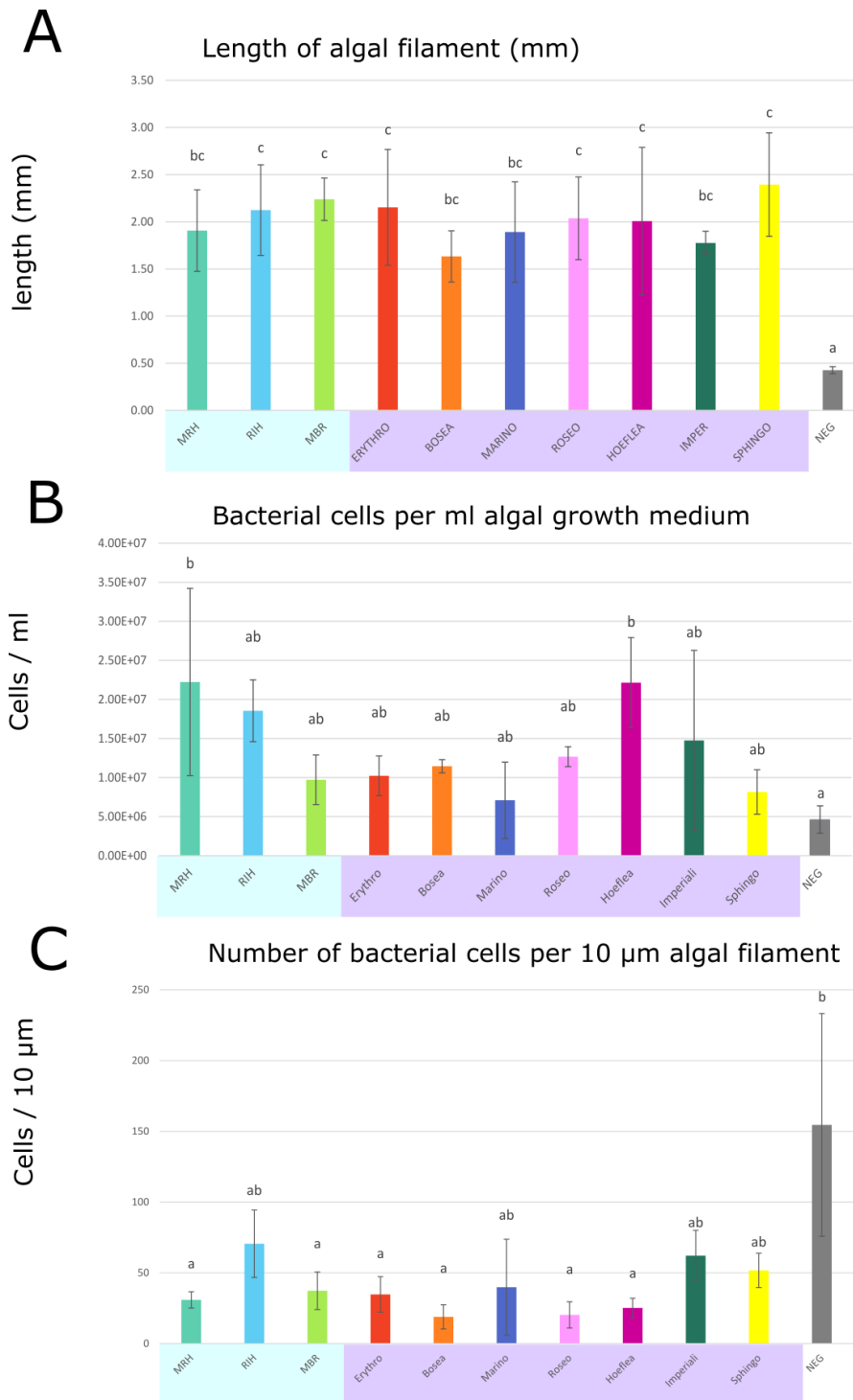


Figure 2-9 The average algal filament length (A), number of bacterial cells in the algal growth medium (B), and number of bacterial cells attached to the cell wall (C) after four weeks of co-culture, based on three replicate cultures. Significance was calculated with ANOVA, Tukey post-hoc testing, and  $\alpha = 0.05$ . NEG: negative control, not inoculated with bacteria; MRH: *Marinobacter-Roseovarius-Hoeflea*; RIH: *Roseovarius-Imperialibacter-Hoeflea*; MBR: *Marinobacter-Bosea-Roseovarius*; Erythro: *Erythrobacter*; Marino: *Marinobacter*; Roseo: *Roseovarius*; Imperiali: *Imperialibacter*; Sphingo: *Sphingomonas*;



## Conclusion

The aim of this study was to provide a first experimental verification of *in silico* predictions of potentially beneficial algal-bacterial interactions based on metabolic complementarity. We can conclude that all of the bacterial communities tested, had a beneficial effect on algal growth in seawater, and also impacted algal morphology. The metabolites that became producible by the alga upon interaction with bacteria were identified in the growth medium only when bacteria were present. Thus, the predicted bacterial consortia were accurate regarding those two criteria. To further elucidate the chemical ecology of the beneficial interactions in the holobiont, follow-up experiments could include the measuring of compounds that should be exchanged in order for the alga to produce the target compound. Furthermore, targeted metabolomics on antibiotic-treated algae without bacteria but with the exchangeable compound added to the culture could be compared with co-cultures in which bacteria provide the exchangeable compound.

Nevertheless, not all of the observations fit the predictions. Notably, all bacterial communities, even the individual strains including *Sphingomonas* and *Erythrobacter*, which were predicted to be 'less' beneficial, exhibited a growth-inducing effect. This may be related to the fact that the algae were not 100% sterile, and thus the final outcome of the predicted communities may have been different due to additional interactions with bacteria that are still present in the microbiome after antibiotic treatment. Still, despite those limitations, the method described here is advantageous over other criteria used to select bacterial communities. It can help narrow down the number of communities to test starting from a large set of cultured bacteria. Because it provides a list of target compounds and exchangeable intermediates, the predictions that were made have the added value of providing some first hypothesis/clues on the actual interactions that may occur in co-culture.

# Chapter 3.

## **Chapter 3. Omics approaches to explore the role of the *Ectocarpus* microbiome during acclimation to fresh water**

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Article in preparation

## Introduction

The freshwater strain (FWS) of *Ectocarpus subulatus* can grow in seawater and fresh water, yet, growth at lower salinities is only possible when the alga is associated with the right microbiome. Algae deprived of their microbiomes, *i.e.* antibiotic-treated algae, do not acclimate to fresh water (Dittami *et al.*, 2016). To identify the bacteria responsible for the freshwater response in *E. subulatus*, targeted co-cultures were carried out with a subset of bacteria that were cultivated from the algal surface and/or algal growth medium (Cultivation study: Chapter 1, pg. 27; Co-culture experiments: Chapter 2, subsection I). None of the bacterial strains alone, nor any of the bacterial communities that were tested so far had a clear beneficial effect on algal growth in fresh water, suggesting a possible role for the uncultured microbiome during acclimation. To incorporate interactions between alga and uncultured bacteria in this study, a new strategy was put in place. Rather than reconstructing the microbiome from scratch using the collection of cultured bacteria, I worked with algal holobionts that were treated with different combinations of antibiotics. The antibiotics that I applied were sufficient to modify the algal microbiome, but many of them did not eliminate the freshwater tolerance of the alga.

Like this, I created three algal holobionts, each with different microbiomes and different response to fresh water. The metatranscriptome and metabolome of the three holobionts were compared during the change in salinity, in order to generate hypotheses about the interactions that occur between the alga and its microbiome during acclimation to fresh water (Figure 3-1).

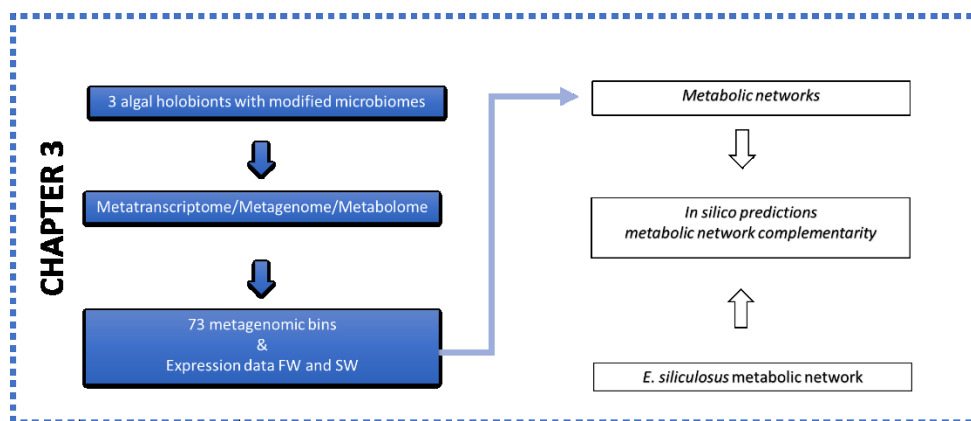


Figure 3-1 Schematic overview of the analysis described in Chapter 3.

## **Materials and methods**

### **Preparation of biological material**

All experiments were carried out using sporophytes of the *Ectocarpus subulatus* freshwater strain (EC371, accession CCAP 1310/196, West & Kraft, 1996). Algae were grown in natural seawater (NSW; collected in Roscoff 48°46'40"N, 3°56'15"W, 0.45 µm filtered, autoclaved at 120°C for 20 min), enriched with Provasoli nutrients (NSW-PE; Starr and Zeikus, 1993) and kept at 13°C with a 12h dark-light cycle (photon flux density 20 µmol m<sup>-2</sup>·s<sup>-1</sup>). Algal growth medium was changed on a weekly basis.

### **Acclimation experiments and implementation of metatranscriptomics approach**

As described in the introduction of this chapter, the aim was to work with algal holobionts with modified microbiomes and to compare the metabolome and metatranscriptome of those holobionts during the acclimation of the alga to low salinity. To this aim, preliminary tests were done to determine if changes in microbiome composition, as a result of mild antibiotic treatment, could alter the freshwater response in the alga. The following antibiotics were tested: penicillin, rifampicin, chloramphenicol, streptomycin, ampicillin, kanamycin, erythromycin, ciprofloxacin, neomycin, and polymyxin B. In addition, four mixtures were tested (Supplementary table 3-1). Antibiotics were added to the algal growth medium (100% NSW) and algae were incubated for three consecutive days (protected from light). After three days, the algae were transferred to 20-fold diluted natural seawater (5% NSW) without antibiotics. The effects of the antibiotic treatments were evaluated by observing the induction of spore release and/or the capacity of algae to grow in 5% NSW, over a time span of four weeks. Cultures transferred to new 100% NSW medium were included as a positive control. Interestingly, most antibiotic-treated algae could still grow in 5% NSW, only four cultures could not (Amp, Pen100, Mix3, Mix5; Supplementary table 3-1). Some antibiotics had also a negative effect on algal growth in seawater (Amp, Cip, Strep). Based on these results, two treatments were chosen for further experiments (mix 1 and mix 4; Table 3-1). These two freshwater-tolerant holobionts (Holobiont 1 and 2) were compared with an alga that has lost the capacity to grow in fresh water (treated with antibiotic mix 5; holobiont 3). The next paragraphs of the material & methods section will thus focus only on these three holobionts (H1, H2, and H3).

Table 3-1 Different algal holobionts used for the metatranscriptomics/metabolomics experiment; Holobiont 1 (H1): treated with rifampicin, penicillin and neomycin (each 100 µg/ml); Holobiont 2 (H2): idem, plus streptomycin (25 µg/ml) and chloramphenicol (5 µg/ml); Holobiont 3 (H3): treated with penicillin (12000UI), Chloramphenicol (0.75 µg/ml), Polymyxin B (0.75 µg/ml), Neomycin (0.9 µg/ml). The untreated holobiont (H0) was only used for 16S rRNA gene metabarcoding, and not for metatranscriptomics/metabolomics.

	Full flora	Modified microbiomes		
<b>Abbreviation</b>	<b>H0</b>	<b>H1</b>	<b>H2</b>	<b>H3</b>
<b>Antibiotic treatment</b>	0	3	5	4
<b>Exposure time</b>	n/a	3 days	3 days	5 weeks
<b>Adaptation time after antibiotic treatment</b>	n/a	2 months	2 weeks	7 months
<b>Bacterial growth on Zobell agar</b>	Yes	No	No	No
<b>Survival in 100% NSW</b>	Yes	Yes	Yes	Yes
<b>Survival in 5% NSW</b>	Yes	Yes	Yes	No

### **Creation of antibiotic-treated algal holobionts with modified microbiomes**

For Holobiont 1 (H1), algae were grown in 100% NSW-PE in Petri dishes (90 mm Ø) and exposed to liquid antibiotics for three days (Table 3-1). Then, the algae were transferred to medium without antibiotics. After three weeks of cultivation, the algae were transferred to 10L flasks to maximize growth before the start of the experiment (3 weeks). For holobiont 2 (H2), algal biomass in 100% NSW-PE was produced in a 10L flask for four weeks, before adding liquid antibiotics to the algal culture. After three days of antibiotic exposure, the medium was exchanged for medium without antibiotics. For holobiont 3 (H3), algal filaments were grown on Zobell media infused with liquid antibiotics for five weeks, where after the filaments were transferred and grown in 100% NSW for another five weeks, and subsequently in 100% NSW-PE for 16 weeks. Then, the algae were transferred to 10L flasks to grow biomass (9 weeks). Before the start of the experiment, the algae (each growing in 10L flasks now) were divided and further grown in 2L flasks for one week to create ten replicates with the same microbiome composition. Five replicates were transferred to 15% NSW-PE and grown at low-salinity for one week and the other five replicates were transferred to new 100% NSW-PE as a control. Salinity levels were reduced to only to 15% NSW, the minimal salinity I determined in preliminary experiments to avoid lethal effects on the fresh water-intolerant holobiont (H3). Additional data on the transfer of alga to a range of salinities can be found in supplementary data supplementary figures 3-1 and 3-2. Algal tissue was harvested using UV-sterilized coffee filters. Excess water was removed by dipping the algal tissue on clean paper towel. Samples

were split in three (one aliquot for metabolomics, one for meta-transcriptomics and metagenomics, and one spare sample), snap-frozen in liquid nitrogen, and stored at -80 °C until further processing.

### **16S RNA gene metabarcoding**

To confirm the divergence between the bacterial community composition before the start of the metatranscriptomics experiment, 16S rRNA gene libraries were created for each of the holobionts in 100% NSW (H1S, H2S, H3S) and for a non-antibiotic treated alga, that served as a reference sample (H0S; algae grown in 100% NSW-PE for 9 weeks). The aim of this experiment was merely to ensure that the three holobionts selected for acclimation experiments did indeed differ in terms of microbiome composition, before performing costly RNA-Seq experiments. Therefore, we chose to analyze only technical replicates of the actual starter cultures used for the experiment rather than independent cultures. Technical replicates (4 of each holobiont) were pooled two by two, and finally, eight libraries were created (2 per holobiont; H0S, H1S, H2S, H3S). Total DNA was isolated (NucleoSpin Plant II, Machery-Nagel; standard protocol) from the snap-frozen tissue, and purified with Clontech CHROMA SPINTM-1000+DEPC-H<sub>2</sub>O Columns. The V3–V4 region of the 16S rRNA gene was amplified and sequenced with Illumina MiSeq technology by MWG Eurofins Biotech (Ebersberg, Germany) using their proprietary protocol. The first quality control was done with FastQC<sup>3</sup>, and fastq\_quality\_trimmer from the FASTX Toolkit<sup>4</sup> was used to quality-trim and filter the 1,859,076 reads (quality threshold 25; minimum read length 200). The resulting 1,778,369 sequences (4.34% removed) were analyzed with Mothur (V.1.38.0) according to the MiSeq Standard Operating Procedures<sup>5</sup> (Kozich *et al.*, 2013). Filtered reads were assembled into 852,323 contigs, preclustered (allowing four mismatches), and aligned with the non-redundant Silva SSU reference database version 123 (Quast *et al.*, 2013). Chimeric sequences were removed using the Uchime algorithm (Edgar *et al.*, 2011) implemented in Mothur, and the remaining sequences classified taxonomically (Wang *et al.*, 2007). Non-bacterial sequences were removed. The sequences were then clustered into operational taxonomic units (OTUs) at a 97% identity level and each OTU was classified taxonomically. All OTUs with  $n \leq 5$  sequences were removed resulting in a final data matrix with 578,076 contigs. To describe the dissimilarity between each algal-bacterial holobiont, a distance matrix was calculated using the Bray-Curtis index as a quantifier of compositional dissimilarity. The resulting distance matrix was used as input for a non-metric multidimensional scaling (NMDS).

## **RNA extraction and sequencing**

16S rRNA gene metabarcoding analysis demonstrated that the three algal cultures were associated with different bacterial communities, which allowed us to continue with the next steps, *i.e.* processing of the samples from the acclimation experiment for metagenomics, metatranscriptomics, and metabolomics. About 50 mg (fresh weight) of algal tissue were ground in liquid nitrogen and sterilized sand using pestle and mortar. Nucleic acids (DNA and RNA) were extracted with Tris (100 mM pH 7.5), cetyltrimethylammonium bromide (CTAB; 2%), sodium chloride (NaCl; 1.5 M), ethylenediaminetetraacetic acid (EDTA; 50 mM, pH 8.0) and dithiothreitol (DTT; 50 mM). They were further purified with a mixture of chloroform and isoamyl alcohol (IAA; 24:1), and RNA was precipitated and incubated overnight (O/N) with lithium chloride (LiCl, 12 M) at -20 °C. After centrifugation (30 min, 4 °C, 20.000 g), the supernatant containing the DNA was stored at -80 °C until further processing (“Metagenome library preparation and sequencing”, pg. 96). The pellet containing the RNA was extracted once more with phenol:chloroform (1:1, pH 4.3) and precipitated with 3M sodium acetate (NaAC) and 3 volumes of 100% ethanol for 3h at -80 °C. The final RNA was washed in 70% cold ethanol and re-suspended in 15 µl RNase free water before measuring with NanoDrop.

Ribosomal rRNA molecules were removed using the RiboMinus™ Plant Kit protocol for RNA-Seq (ThermoFisher Scientific) according to the manufacturer’s instructions. This allowed to selectively remove abundant nuclear, mitochondrial, and chloroplast rRNAs of the host. In addition, 1 µl of bacterial probe (RiboMinus™ Transcriptome Isolation Kit for Yeast and Bacteria, ThermoFisher Scientific) was added in the last five minutes of hybridization to remove bacterial ribosomal RNAs as well. Removal of ribosomal RNA was confirmed by gel electrophoresis. The RNA was concentrated (RiboMinus™ Concentration module, ThermoFisher Scientific) before checking the quality with the bio-analyzer (Plateforme de Séquencage-Génotypage GENOMER, FR2424, Roscoff) and subsequent library preparation (TruSeq Stranded mRNA Library Prep kit, Illumina) and sequencing (five lanes; Illumina HiSeq 3000) at the GeT PlaGe platform (Genotoul, Auzeville).

## **Cleaning and pre-processing of transcriptome data**

Ribosomal RNA reads that were not removed by the RiboMinus treatment, were removed *in silico* by alignment to the Silva databases (16s-id90, bac-23s-id98.fasta, euk-18s-id95.fasta, euk-28s-id98.fasta; 6.09E+08 reads; 18.87% remaining) using the SortMeRNA tool (version



2.1, Kopylova et al., 2012). After quality trimming (Trimmomatic 0.36, requiring a minimal Phred score of 20 and a minimum remaining read length of 36 nucleotides) the reads ( $5.70E+08$ , 17.6% of total reads remaining) were aligned to the *E. subulatus* Bft15b reference nuclear and organellar genomes (Dittami et al., 2018 - preprint) (STAR aligner version 020201, Dobin et al., 2013) and counted with the internal “quantMode GeneCounts” function. Reads that mapped to the *E. subulatus* genome ( $2.07E+08$  nuclear, 6.39% of total reads;  $2.65E+08$ , 8.16% organelles) were further used for differential gene expression analysis. The reads that did not map were considered to be of bacterial origin ( $9.85E+07$  reads; 3.01%). They were mapped against the bacterial metagenome (see “Mapping of bacterial transcriptome”, pg. 98).

### **Algal differential gene expression analysis**

The DESeq2 R package (Love, Huber and Anders, 2014) was used to detect significantly differentially expressed genes (DEGs) between the experimental conditions. Principal component analysis was carried out on rlog-transformed count data. This method is based on log<sub>2</sub> transformation but corrects genes with low counts more strongly than genes with high counts. Plots were created with R-Studio and the ggplot package (Wickham, 2009; RStudio Team, 2016). The following differential gene expression analyses were made (Figure 3-2):

- Holobiont freshwater (FW) response: each of the three holobionts (H1, H2, H3) was analyzed individually to determine genes that were differentially expressed in 15% NSW water compared to 100% NSW.
- Microbiome effect in seawater: H1S and H2S were treated as one group (*i.e.* algae that do acclimate to low salinity) and compared with H3S (*i.e.* alga that does not acclimate to low salinity).
- Interaction microbiome:FW-response: To incorporate the holobiont specific FW-response, an interaction term was added to the statistical design. These results represent the difference in the FW-response of H3 compared to that of H1+H2.

For each analysis, the significantly differentially expressed genes were selected based on the log<sub>2</sub>-fold change (LFC > 0.585) and adjusted p-values ( $P < 0.05$ ). Gene set enrichment analysis was performed using the Fisher’s exact module within Blast2Go (Version 4.1.9; 2-tailed test; FDR < 0.05; Götz et al., 2008). Automatic protein annotations were based on Blast hits (e-value cutoff  $1e-5$ ) with the NCBI nr protein database as described by (Dittami et al., 2018 - preprint)

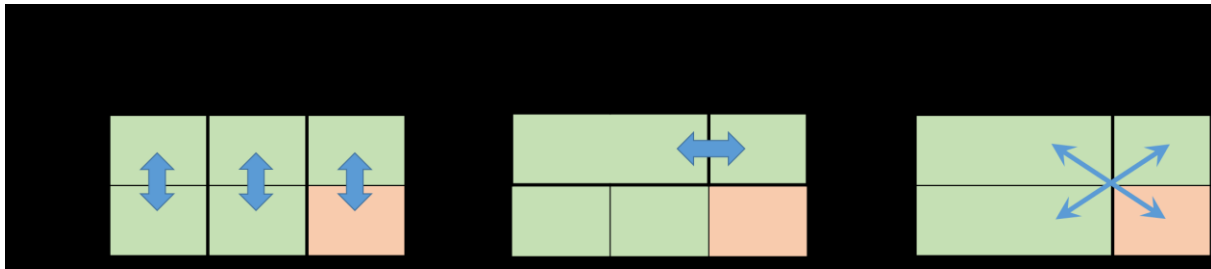


Figure 3-2 Different statistical analyses that were carried out to identify differentially expressed genes among the three algal holobionts.

### **Bacterial differential gene expression analysis**

### **Metagenome library preparation and sequencing**

The first steps of the DNA extractions are described in paragraph “RNA extraction and sequencing” (pg. 94). The supernatant of the LiCl-precipitation containing DNA was precipitated with one volume of isopropanol, resuspended in 300  $\mu$ l of DNase free water, purified once with one volume of Phenol:Chloroform:isoamyl alcohol (25:24:1; pH 8) and then twice with one volume of chloroform. The aqueous phase was precipitated once more with 3 volumes of 100% ethanol and 0.1 of 3M sodium acetate and the pellet resuspended in 50  $\mu$ L of molecular biology grade water before measuring sample concentrations using a NanoDrop one. The samples were pooled (same concentration of each sample), and the pooled DNA was purified using cesium chloride gradient centrifugation according to the protocol described by Le Bail et al. (2008). After library preparation (TruSeq DNA Nano, Illumina), the library was sequenced on 4 Illumina HiSeq 3000 lanes (GeT PlaGe Genotoul, Auzeville).

### **Cleaning and pre-processing of metagenome data**

The raw reads were quality trimmed with Trimmomatic (version 0.36, minimal Phred score: 20, minimal read length: 36 nt) and the remaining reads ( $2.7521E+09$ ; 93.92%), were aligned to the *E. subulatus* Bft15b reference nuclear genome (STAR aligner version 020201, Dobin et al., 2013). The  $1.43E+09$  bacterial reads (48.64% of total), *i.e.* the reads that did not align to the algal genome, were assembled using MetaSPAdes (Nurk *et al.*, 2017). The resulting contigs (983,772 contigs; 739 Mbp) were filtered by length (>500 nt; 210,262 contigs kept, 670 Mbp) using Prinseq<sup>15</sup> (version 0.20.4; Schmieder and Edwards, 2011) and 65,204 algal contigs (218

<sup>15</sup> <http://prinseq.sourceforge.net/faq.html>

Mbp; 31%) were removed with Taxoblast (version 1.21; Dittami and Corre, 2017). Then, the raw reads were mapped back on the 145,058 assembled bacterial contigs with BWA-MEM<sup>16</sup> (Version 0.7.15-r1140; Li and Durbin, 2010).

Initially, the metagenomic binning was carried out using CONCOCT (Alneberg *et al.*, 2014), following the tutorial “Complete Example V0.4”<sup>17</sup>. In brief, this involved: fragmentation of the assembly (10 kb); mapping of the raw reads to the fragmented metagenome; removal of PCR duplicates; coverage calculation; and finally binning with CONCOCT. The evaluation (Supplementary figure 3-3) was based on single-copy core gene (SCGs; *i.e.* a set of COG ids) and made clear that some of the bins contained multiple copies of the SCGs, and these bins were thus likely to contain a mix of genomes. Other bins were missing some SCGs or did not have any SCGs at all and these bins were likely to be from algal origin or to correspond to incomplete bacterial genomes. Thus, the results were not satisfactory and the assembled metagenome was instead processed according to the “Anvi’o User Tutorial for Metagenomic Workflow”<sup>18</sup> (Eren *et al.*, 2015). In brief, these were the steps: First a contigs database was created and HMM profiles were imported. Raw metagenome reads were mapped against the contigs database using BWA-MEM (version 0.7.15, Li and Durbin, 2010). Taxonomy was assigned to the contigs with help of Centrifuge<sup>19</sup> (Kim *et al.*, 2016) and the NCBI nucleotide non-redundant database (nt\_2018\_3\_3). Finally, the Anvi’o interactive interface was used for manual curation of the bins, and contigs were grouped based on GC-content, coverage, and redundancy of SCGs. This resulted in 73 bacterial bins with variable coverage and quality and one algal bin that was manually removed (Bin 33). The fasta sequences were extracted for each bin and used for protein annotation with Prokka<sup>20</sup> (Seemann, 2014). The assembled metagenome served as a backbone to map the bacterial transcripts and it thus allows to combine the gene expression profiles with taxonomic information.

### **Differential expression of metabolic reactions**

Metabolic networks were reconstructed for each of the 73 bacterial bins using the AuReMe pipeline (Aite *et al.*, 2018). For each holobiont, the expression data for all genes of all bins that

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<sup>16</sup> <http://bio-bwa.sourceforge.net/>

<sup>17</sup> [http://concoct.readthedocs.io/en/latest/complete\\_example.html](http://concoct.readthedocs.io/en/latest/complete_example.html)

<sup>18</sup> <http://merenlab.org/2016/06/22/anvio-tutorial-v2/>

<sup>19</sup> <https://ccb.jhu.edu/software/centrifuge/>

<sup>20</sup> <https://github.com/tseemann/prokka>

are associated with the same metabolic reaction were combined. The resulting expression data per reaction per holobiont was subjected to differential expression analysis, using the same DESeq protocol as outlined in “Algal differential gene expression analysis (pg. 95). A WIKI page<sup>21</sup> has been created that allows to explore reactions and pathways for each of the analysis that was carried out.

## Mapping of bacterial transcriptome

Bacterial transcriptomes were mapped against the 73 bacterial bins with STAR aligner version 020201 (Dobin et al., 2013) and counted with the internal `--quantMode GeneCounts` function. This resulted in a final data matrix containing the number of mapped reads per gene, per bin, and per experimental condition. To obtain the overall “transcriptomic activity” of each bin in each condition, read pair counts were summarized for all genes of the same bin and normalized by the total number of mapping read pairs per sample (Table 3-2); The resulting matrix was used as input for hierarchical clustering (distance: correlation; method:average) with ClustVis<sup>22</sup> (Metsalu and Vilo, 2015). Considering the low final number of bacterial read pairs mapping to the metagenomics bins, differential expression analysis was not carried out on a gene to gene basis for each bin.

Table 3-2 Simplified overview (two bacterial bins each with four genes; two replicates) of the normalization that was carried out on the read count data after mapping of the bacterial transcriptomes to the 73 genomic bins to determine the “transcriptomic activity”.

Bins	Genes	Read counts were summed up per bin		Normalization	
		Rep1	Rep2	Rep1	Rep2
Bin 1.	Gene a	$\sum$ reads bin 1	$\sum$ reads bin 1	$\frac{\sum \text{reads bin 1}}{\sum \text{reads Rep1}}$	$\frac{\sum \text{reads bin 1}}{\sum \text{reads Rep2}}$
	Gene b				
	Gene c				
	Gene d				
Bin 2.	Gene e	$\sum$ reads bin 2	$\sum$ reads bin 2	$\frac{\sum \text{reads bin 2}}{\sum \text{reads Rep1}}$	$\frac{\sum \text{reads bin 2}}{\sum \text{reads Rep2}}$
	Gene f				
	Gene g				
	Gene h				
		$\sum$ reads Rep1	$\sum$ reads Rep2		

<sup>21</sup> [http://gem-aureme.irisa.fr/test/index.php/Main\\_Page](http://gem-aureme.irisa.fr/test/index.php/Main_Page)

<sup>22</sup> <https://biit.cs.ut.ee/clustvis/>

## **Metabolite profiling**

### **Optimization of metabolite extraction protocol**

The aim of this analysis was to identify algal biomarkers that are involved in the control and/or regulation of algal growth in fresh water, and that may give clues on which metabolic interactions occur between the alga and its microbiome during acclimation. Algal endometabolome profiles were obtained via GC-MS and LC-MS (positive and negative mode), as both methods target different compound classes. Before the start of the experiment, three solvents with different degrees of polarity were tested and compared for their efficacy to extract metabolites. Each of the solvents was tested on 40 mg of freeze-dried *Ectocarpus* tissue obtained from Holobiont 0 (H0). The following solvents were tested:

1. Methanol: Ethanol: Chloroform (2:6:2), optimized for extraction from *Ulva*, according to Kuhlisch et al., 2018
2. Methanol (100%), according to Dittami et al., 2012
3. Methanol: H<sub>2</sub>O (8:2), according to Ritter et al., 2014.

Extraction was performed as described in the paragraphs for GC-MS. Efficacy of the solvent was quantified by evaluating the number of features extracted from the GC-MS data (AMDIS/MET-IDEA – Vidoudez and Pohnert, 2012). The solvent with the highest total number of metabolites and/or the highest number of unique compounds was used in the final experiment, which was 100% methanol.

### **Sample preparation & data acquisition**

Ten mg of freeze-dried ground tissue was lysed (TissueLyser, 2\*30s, 30 Mhz, Qiagen, Hilden, Germany) and subsequently used for extraction of metabolites with 1 ml of 100% methanol. After vortexing, 5 µl of ribitol (4 mM in H<sub>2</sub>O, >99%, Sigma-Aldrich, München, Germany) was added to each sample as an internal standard before sonication (10 min, at room temperature; RT). After 15 minutes of centrifugation (30,000 g, 4 °C) the top layer was transferred to glass inserts (250 µl) and stored at -20 °C until measurement. Before data acquisition, the samples were centrifuged again (6 minutes, 2,500 rpm) to remove any remaining particles.

Liquid chromatography was performed on C18 column (Thermo Scientific™; Accucore™, 2,6 µm x 2,1 mm x 100 mm) kept at 25°C using an UltiMate HPG-3400 UHPLC system (Thermo

Scientific, Bremen, Germany). The injection was set to 1  $\mu$ L and the elution was carried out with a gradient shown in Figure 3-3. Solvent A contained water with 2% acetonitrile and 0.1% formic acid (v:v), while solvent B contained acetonitrile with 0.1% formic acid (v:v). The UHPLC system was coupled to a Thermo Scientific™ QExactive plus™ hybrid quadrupole-Orbitrap mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ESI ion mode with a scan range of 100 to 1,500  $m/z$  and a resolution of 35,000 FWHM. To avoid overloading the detector and to reduce the source of contamination a solvent delay was set at the beginning (0.5 min) and at the end (1 min) of the chromatographic run.

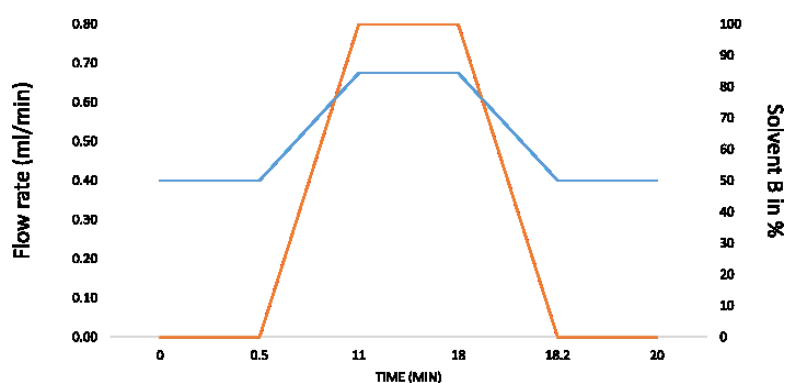


Figure 3-3 Gradient used for LC-MS chromatography.

For GC-MS, the bottom layer was further processed by evaporation under vacuum (O/N), derivatisation in 50  $\mu$ l methoximation solution (20 mg/ml in pyridine), and incubation at 60  $^{\circ}$ C for 1 hour and afterward at RT (O/N). The samples were silylated for 1h at 40 $^{\circ}$ C in 50  $\mu$ l MSTFA (1 ml + 40  $\mu$ l retention index mix) and centrifuged (6 min, 2500 rpm) to pellet the precipitate. The supernatant was used for gas chromatography analyses. The instrument used, a 6890N GC, was equipped with a 7683B autosampler (Agilent), a glass liner (Agilent, 4  $\times$  6.3  $\times$  78.5 mm) and a DB-5MS column (Agilent, 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m), coupled to a Micromass GCT Premier™ (Waters®) mass spectrometer. The gas chromatograph is operated with helium as mobile phase, split 10, and 250 $^{\circ}$ C injector temperature. The initial oven temperature was 60 $^{\circ}$ C ramping to 310 $^{\circ}$ C at a rate of 15 $^{\circ}$ C per min. The mass spectrometer was used with source temperature at 300 $^{\circ}$ C and dynamic range extension mode. The resolution was >6,000 FWHM at  $m/z$  501.97. After randomization, samples were measured twice to obtain 2 technical replicates. Solvent blanks were prepared in parallel.

## LC-MS – Data pre-processing and statistical analysis with W4M

After data acquisition, the data files were converted into mzXML format using the MSconvert tool within ProteoWizard<sup>23</sup> (Kessner et al., 2008). The data was split into positive and negative mode using the command line version of MSconvert using and the tcltk R package. The data was further processed within the workflow4metabolomics infrastructure (Giacomini *et al.*, 2015). A detailed description of the parameters settings is provided in Supplementary table 3.3. Briefly they involved: peak identification with the centWave algorithm for MS data in centroid mode; peak grouping based on retention time and m/z of the ions; retention time correction with the loess method (non-linear alignment) followed by a second peak grouping; peak filling to integrate areas of missing peaks (method chrom); and annotation with CAMERA. The resulting variableMetadata file provides a list of identified metabolites (m/z, rt) annotations (isotopes, adducts, pcgroup), t-test or ANOVA statistics, fold-changes, and the peak areas per sample. The data matrix (20,224 features in LC-MS positive mode; 9,425 features in LC-MS negative mode) was downloaded and further processed in Excel. Metabolite intensities were corrected if a peak was detected in the blanks: to be certain not to have any contaminants in the final matrix, the maximal value among all blanks was multiplied by three and subtracted from the samples. Variables with less than two samples with intensities above zero were removed, as well as variables with negative t-statistics. Redundant ions (isotopes) were removed. The filtered datasets (13,034 features in LC-MS positive mode; 1,989 features in LC-MS negative mode) were re-imported into W4M galaxy for further statistical analysis. Batch correction was not applied, because it did not improve clustering of samples. Quality assessment of the data confirmed that there were no outliers and there was no signal drift. Several normalization methods were tested, *i.e.* dry weight, dry weight + log10-scaling, and total ion content. In the end normalization by dry weight followed by log10 scaling was chosen, as it performed best in separating H3, the only holobiont with poor growth at low salinity, from the other two holobionts. The student t-test was used to detect metabolites that were significantly (FDR < 0.01) different in each holobiont during the freshwater response, between H1/H2 and H3 in seawater. The FDR for LC-MS was set to 0.01 because of the high number of significant features otherwise. A selection of significant features will be further analyzed in the near future

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<sup>23</sup> <http://proteowizard.sourceforge.net/tools.shtml>

with MS/MS analysis, which should enable the identification of several of the biomarkers. The selection criteria for MS/MS candidates from LC-MS positive mode data were:

- Highly significant feature, FDR < 0.01
- High absolute intensity value
- Annotated with Massbank
- Retention time between 3.5 and 16.5 minutes
- Retention time in peak table confirmed in raw chromatograms
- MZ confirmed in mass spectra.

### **GC-MS – Data pre-processing and statistical analysis with W4M**

The workflow4metabolomics pipeline was used to process the GC-MS chromatograms. After data acquisition files the raw data files were directly converted into netCDF format using the DataBridge tool within MassLynx software (Waters, version 4.1). The chromatograms were then processed with the function metaMS.runGC (Galaxy version 1.0) provided within the workflow4metabolomics pipeline. The metaMS package (Wehrens, Weingart and Mattivi, 2014) identifies chromatographic peaks with the standard functions provided by XCMS. Then, the CAMERA package was used to cluster masses with similar retention times (Kuhl, Tautenhahn and Neumann, 2016). These co-eluting masses or ‘pseudospectra’ were summarized into a final compounds table in MSP format, a format that can be used to search in spectral databases. An overview of settings can be found in Supplementary table 3-4. The resulting list of 689 compounds (pseudospectra) was exported into Excel and processed similarly to the LC-MS (*i.e.* removal of blanks, no batch correction, quality check, normalization by dry weight, log10-scaling). A Student’s t-test was used to detect metabolites that were significantly (FDR < 0.05) different in each holobiont during the freshwater response, between H1/H2 and H3 in seawater. The spectra of each significant feature were compared to reference databases using NIST MS Search (version 2.0) using GOLM libraries<sup>24</sup> and an in-house library (Vidoudez and Pohnert, 2012). The quality of the comparison is given by reversed match value (R match value) which gives an indication of spectral similarity based on peaks that are present in both the library and the query spectrum. Query spectra were annotated according to this value.

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<sup>24</sup> <http://gmd.mpimp-golm.mpg.de/download/>



## Results

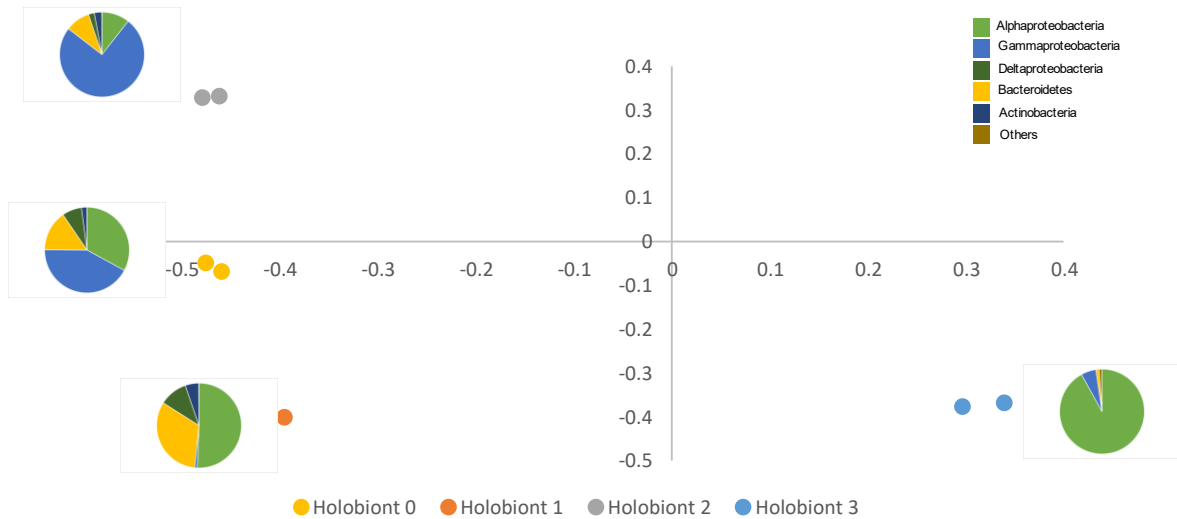
### Antibiotic-treated algal holobionts have different bacterial community composition

To confirm the divergence between the bacterial community composition, 16S rRNA gene libraries were created for each of the holobionts. Bacterial communities of the antibiotic-treated holobionts were compared also compared with a reference sample, a non-antibiotic treated alga (“Holobiont 0”). For each treatment, we observed the number of shared OTUs among treatments, the dominant genus and phyla/class in each treatment. After filtering and cleaning the data, sequences were clustered into operational taxonomic units (OTUs). The number of OTUs varied between 42 and 59 (Supplementary table 3-5). The reference sample had 58 OTUs. Several OTUs were found to be specific to some of the holobionts (Figure 3-3) and even for the core OTUs relative abundances, varied between the holobionts. In holobiont 1, the most abundant OTU was an unclassified Bacteroidetes (OTU04; 20%); in Holobiont 2 an unclassified Alteromonas (OTU01; 64%), and in Holobiont 3 an unclassified Rhodobacteraceae (OTU03; 34%). The divergence can also be seen in the NMDS plot (Figure 3-4A). Here, the holobionts are separated from the reference samples (H0). H1 and H2 group closer to each other than to H3 (Figure 3-4A). This separation also reflects the fact that H3 has 17 unique OTUs (Figure 3-4B). Holobiont 1 showed an increased abundance of *Bacteroidetes* and *Alphaproteobacteria* at the expense of *Gammaproteobacteria*, while in Holobiont 2 a strong proliferation of *Gammaproteobacteria* was seen. Holobiont 3 was dominated by *Alphaproteobacteria*, while the abundance of all other taxa was strongly reduced. In comparison to Holobiont 0 (the control sample with full flora), the microbiomes of the antibiotic-treated holobionts were each shifted in another direction. Thus, treatment with mixtures of antibiotics did result in the establishment of different microbial communities.

The shift in microbiome composition is likely linked to the different types of antibiotic treatments that were applied in each holobiont. However, this effect is not easily explainable, because each of the antibiotic mixtures contained compounds with activity against Gram-negative and Gram-positive bacteria, and *Proteobacteria* (Gram-negative) are the dominant phylum in each of the holobionts. Holobiont 3, treated with polymyxin B which is a strong inhibitor of Gram-negative bacteria, did not show a strong proliferation of Gram-positive bacteria, such as *Actinobacteria* or *Firmicutes*. But those two phyla are usually present in low abundance in algal microbiomes (Hollants *et al.*, 2013). Direct negative effects of the antibiotics

on algal growth are unlikely because each holobiont was able to acclimate to seawater without antibiotics for several weeks before the transfer to fresh water was made.

**A**



**B**

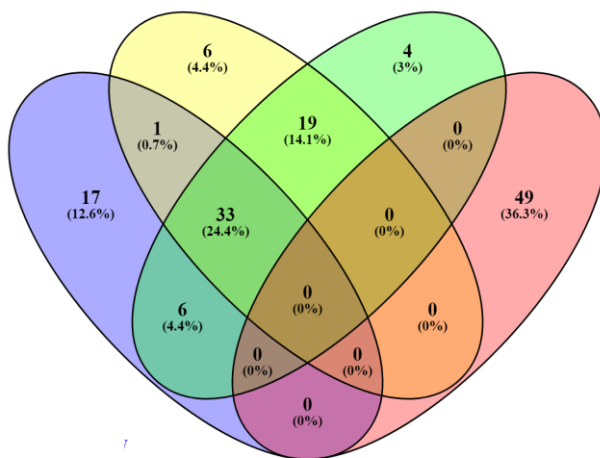


Figure 3-4 A. Non-metric dimensional scaling of metabarcoding data based on the Bray-Curtis dissimilarity shows the differences in bacterial communities between the three holobionts (H1, H2, H3) and the reference sample (H0). Pie graphs show the most dominant OTUs in each holobiont. The group ‘Others’ comprises 75 OTUs). B.) The Venn diagram shows the number of shared OTUs.

## Transcriptomics analysis of algal holobionts during acclimation

### Quality assessment of data

Sequencing of the five Illumina HiSeq libraries resulted in  $3.24 \times 10^9$  sequences, with an average of  $2.7 \times 10^6$  per sample. Eighty-one percent of the total reads were categorized as ribosomal RNA, and 18% as mRNA derived from *Ectocarpus*: three to 11% percent of the total reads mapped with the nuclear genes of the alga, while 3 to 21% mapped with organellar genomes. Between 0.4 to 12% of the total reads were categorized as bacterial mRNA. Results of the cleaning and mapping can be found in Figure 3-5 & supplementary table 3-2 & Figure 3-6.

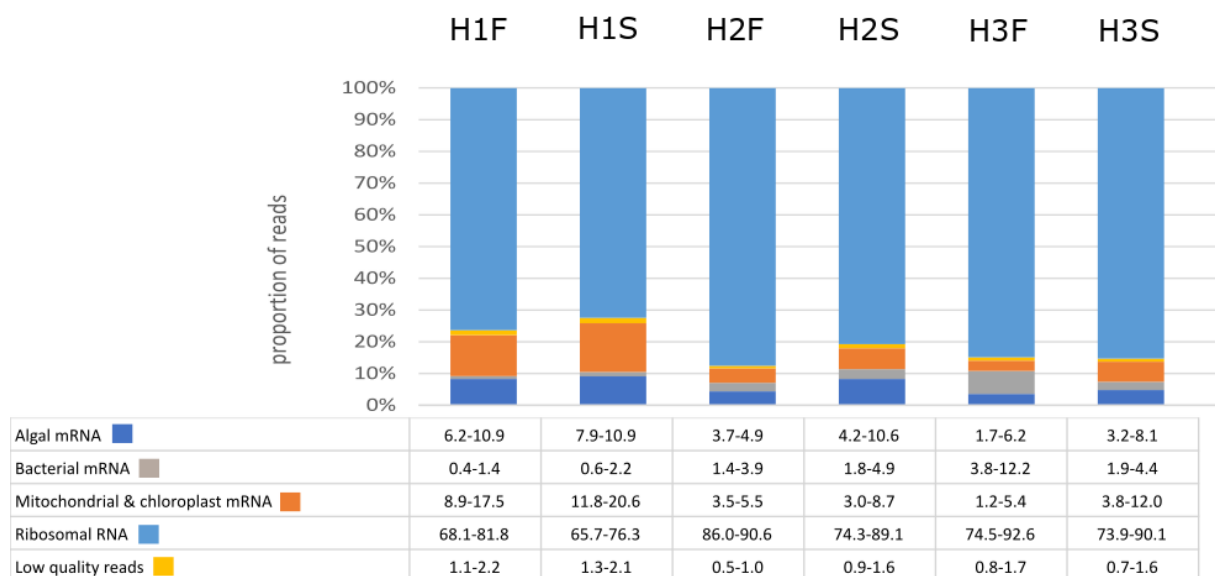


Figure 3-5 Results of the cleaning and mapping procedure per holobiont, each sampled four times in 100% NSW and four times in 15% NSW conditions. The variability is depicted by the range (min % - max %) shown in the data table under the graph. The numbers shown were calculated as percentage of the total raw reads.

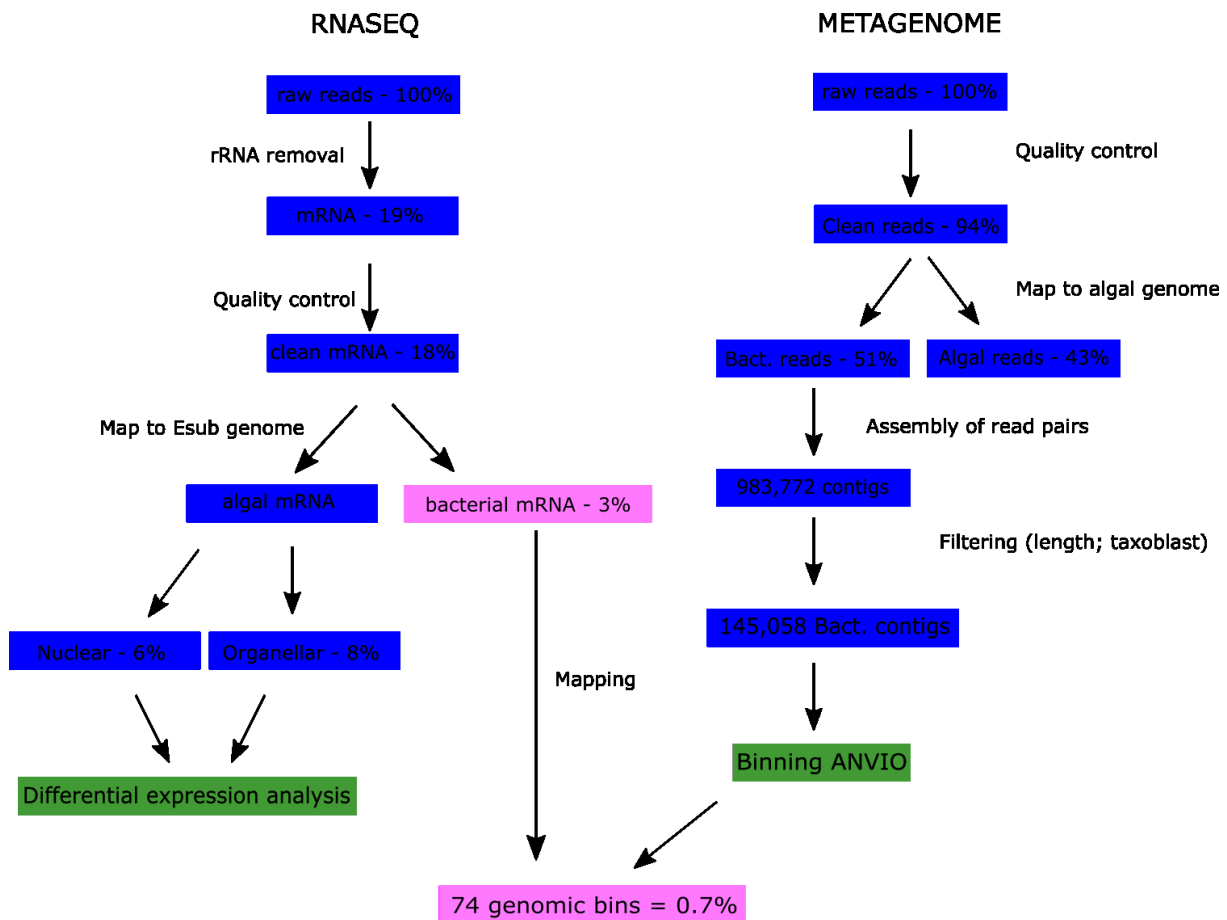


Figure 3-6 Schematic overview of the different types of analyses that were performed on the three holobionts and the results of the cleaning and mapping procedure. The numbers shown were calculated as percentage of the total raw reads.

### Algal transcriptome

Although each holobiont was capable of growing in seawater, their expression profiles were clearly different based on principal component analysis (Figure 3-7 and supplementary table 3-6). The PCA plot shows that H1 and H2 cluster closely together, and that they are separated from H3. H3F formed a unique cluster, distant from all other groups, including H3S. The aim of DEG analysis was to define the genes that are specifically regulated in H3 (*i.e.* fresh water-intolerant alga), compared to H1 and H2 (fresh water-tolerant algae). Thus, because H1 and H2 were reacting similarly, I chose to treat them as one group (*i.e.* fresh water-tolerant algae) and jointly compared them with H3S (fresh water-intolerant alga). Similarly, for the difference in the FW-response (interaction term), H1F and H2F were treated as one group and jointly compared to H3-F.

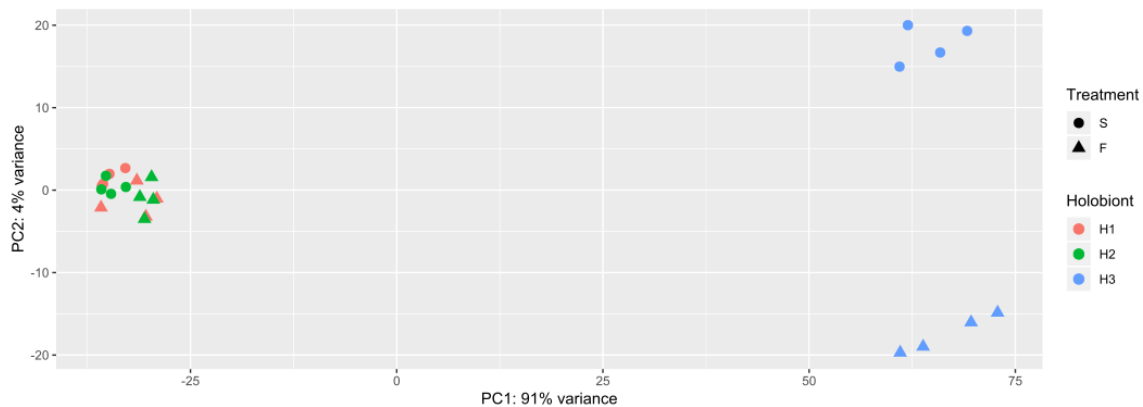


Figure 3-7 Principal component analysis of algal transcriptomes on rlog-transformed data. S: 100% NSW; F: 15% NSW. H1: holobiont 1; H2: holobiont 2; H3: holobiont 3.

### Holobiont freshwater response

Each of the three holobionts (H1, H2, H3) was analyzed separately for differentially expressed genes (DEGs) in 15% NSW compared to 100% NSW. The extent to which the microbiome was modified was positively correlated with the number of differentially expressed genes but inversely related to fresh water tolerance: six genes in Holobiont 1 (fresh water-tolerant), 92 in Holobiont 2 (fresh water-tolerant), and 2355 in Holobiont 3 (fresh water-intolerant). Correspondingly, in the gene set enrichment analysis, we found the lowest number of overrepresented GO terms in H1 (none), more in H2 (35), and the most in H3 (100) (Figure 3-8A). A summary of the most important processes that were regulated in each of the holobionts, is given in Table 3-4.

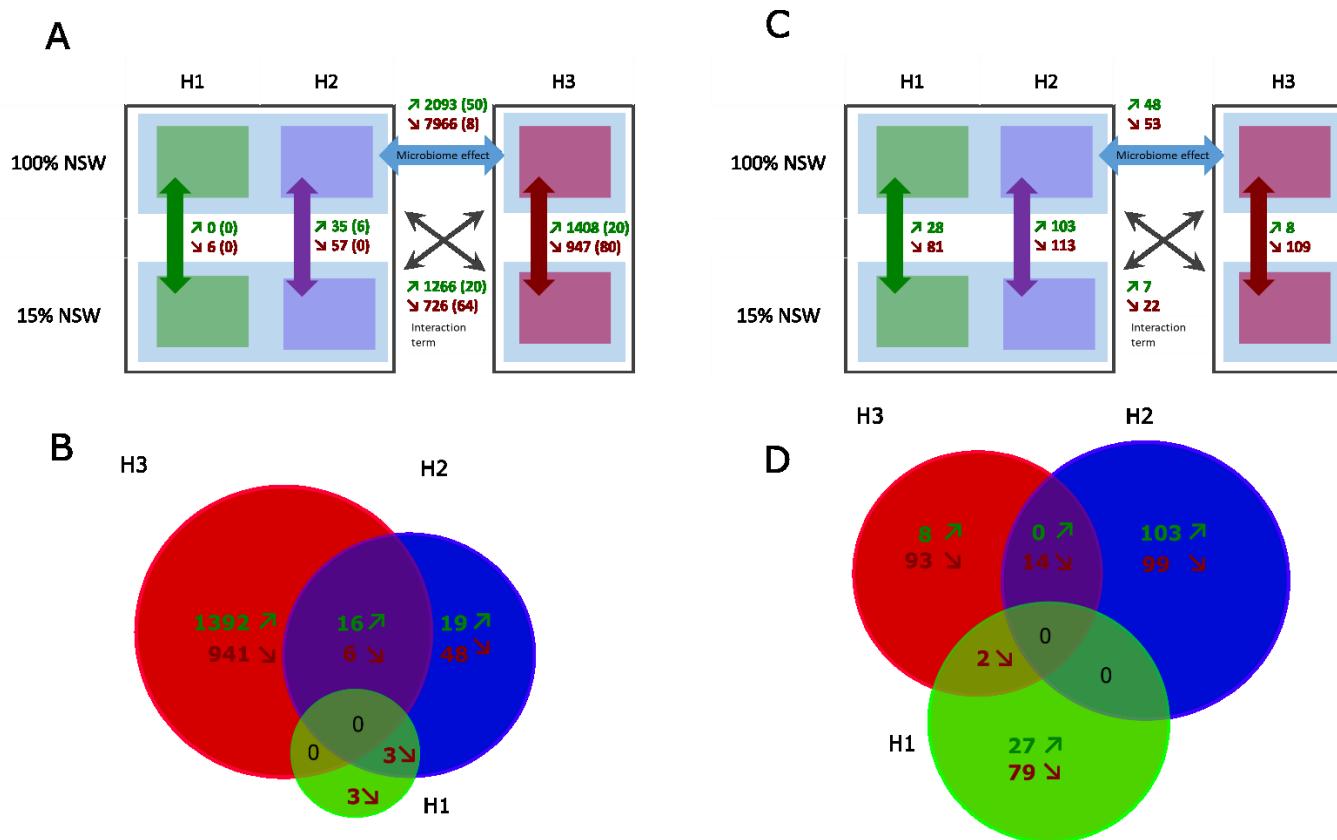


Figure 3-8 A) Number of differentially expressed ALGAL genes in each of the holobionts in 15% NSW compared to 100% NSW (H1, H2, and H3); H1 + H2 jointly compared with H3 in 100% NSW (microbiome effect); and the difference in the FW-response of H3 compared to that of H1+H2 (interaction term); Numbers in brackets correspond to overrepresented GO terms associated with the differentially regulated genes. B) Venn diagram of differentially expressed algal genes shared between the three holobionts. In green the number of up-regulated (↗) genes, and in red the number of down-regulated (↘) genes. C & D: Similar analysis as for A and B but on the BACTERIAL transcriptome; analysis was based on differentially expressed metabolic reactions and not on genes in this case.

## **Holobiont 1**

Among the six DEGs in holobiont 1 were a I SAM-dependent methyltransferase gene (EsuBft1865\_3), a heat shock protein 70 (EsuBft527\_7), a dynein heavy chain protein (EsuBft360\_3), and 3 not annotated/unknown proteins. Three of the DEGs were shared with holobiont 2 (Figure 3-8B).

## **Holobiont 2**

For H2, significantly enriched GO categories are listed in supplementary (Table 3-3) and were related to ribosome activity; translation, and photosynthesis/light harvesting. Only up-regulated genes were enriched in GO terms. Significantly up-regulated genes were mostly derived from the chloroplast, and encoding Ribosomal Protein Large subunit proteins (RPL 3, 4, 5, 6, 14, 16, 21, and 23) and Ribosomal Protein Small subunit proteins (RPS 3, 8, and 19). Other up-regulated genes derived from the chloroplast were annotated as Photosystem II cytochrome c550 (EsuBft\_cp\_81) and cytochrome b6-f complex subunit V (EsuBft\_cp\_81). Three nuclear genes, encoding light-harvesting complex proteins of the LHCR/LHCF family were also up-regulated (EsuBft1455\_9, EsuBft1455\_9, EsuBft637\_17). Other automatically annotated nuclear genes, not related to photosynthesis, comprised a tyrosinase (EsuBft95\_12), a Malate/L-lactate dehydrogenase-like protein (EsuBft586\_6), an ARC and TPR repeat-containing protein (EsuBft720\_2), a high-CO<sub>2</sub>-inducible periplasmic protein (EsuBft72\_32), an unknown protein kinase (EsuBft2023\_6), and a PAPP-A2 protein (EsuBft1649\_2). Twelve genes were not annotated and/or unknown. Holobiont 2 shares 3 DEGs with holobiont 1 and 22 with holobiont 3 (Figure 3-8B & Supplementary table 3-6).

Gene enrichment of the down-regulated genes in holobiont 2 did not show any significantly overrepresented processes, functions, or cellular compartments. Manual examination of the automatic annotations generated for those genes showed functions related to osmoregulation (sarcosine dimethylglycine methyltransferase), isoleucine turnover (isoleucine patch superfamily), the cell wall (C5-epimerase), transcription (zinc finger motif as well as bHLH140), as well as phosphatases (polyphosphate 5 phosphatase, dual-specificity phosphatase), an FAD-dependent oxidoreductase, a heat shock protein 70, Ankyrin repeat domain proteins, Leucine-rich repeat domain proteins, transposons, a glucose 6-phosphatase isomerase, and a phosphoenolpyruvate/phosphate translocator (chloroplast).

Table 3-3 Over-represented GO terms identified in holobiont 2, among the transcripts of significantly up-regulated genes in 15% NSW compared to 100% NSW.

GO ID	GO Category	GO Name	FDR
GO:0003735	Molecular_function	structural constituent of ribosome	1.39E-12
GO:0042254	Biological_process	ribosome biogenesis	1.94E-11
GO:0006412	Biological_process	translation	8.52E-11
GO:0009765	Biological_process	photosynthesis, light harvesting	6.48E-03
GO:0019843	Molecular_function	rRNA binding	3.33E-02
GO:0015935	Cellular_component	small ribosomal subunit	4.94E-02

### Holobiont 3

In holobiont 3, 2,355 genes were differentially expressed in response to a change to low salinity (Figure 3-8B). The 1,408 up-regulated genes in were significantly enriched in GO terms that were, among others, categorized as being involved in nitrogen metabolism (transaminase activity), lipid metabolism (cellular lipid catabolic process), transmembrane transport (ammonium transmembrane transport, ammonium transmembrane transporter activity), carbohydrate synthesis (GDP-mannose biosynthetic process; glyoxylate cycle; mannose-6-phosphate isomerase activity), sulfur metabolism (sulfuric ester hydrolase activity), defense/oxidation (peroxidase activity, oxidoreductase activity, cell redox homeostasis). Among the 947 down-regulated genes in H3 the following functions were overrepresented: photosynthesis (e.g. light harvesting), amino acid biosynthesis (e.g. isoleucine biosynthetic process), transcription/translation (e.g. sigma factor activity), nitrogen metabolism (e.g. nitrate assimilation), defense/oxidation-reduction, sulphur metabolism (e.g. sulphur compound metabolic process), carbohydrate synthesis (e.g. gluconeogenesis), transporters (inorganic phosphate transmembrane transporter activity), and vitamin biosynthesis (e.g. water-soluble vitamin biosynthetic process). An overview of the gene enrichment analysis is shown in Figure 3-9 and in Supplementary table 3-7).



Table 3-4 Summary of the differentially regulated processes during the response to low salinity in each holobiont. Arrows pointing upwards indicate GO terms that are overrepresented among upregulated genes in 15% NSW compared to 100% NSW in each of the holobionts. Idem, arrows pointing downwards indicate GO terms overrepresented among downregulated genes.

Holobiont 1	Holobiont 2	Holobiont 3
	<u>Photosynthesis</u> ↑ LHCR/LHCF ↑ Cytochrome / PSII (cp) ↑ Ribosomal proteins (cp) ↑ Transcription / Translation	<u>Stress response</u> ↑ Ammonium transmembrane transport ↑ Mannose synthesis
↓ heat shock protein ↓ SAM-dependent methyl transferase ↓ dynein heavy chain protein	↓ C5-epimerase ↓ heat shock protein 70	↓ Nitrate assimilation ↓ Photosynthesis ↓ Amino acid metabolism ↓ Vitamin biosynthesis ↓ Lipid metabolism

### Microbiome effect in seawater

The focus of the next analysis was on the comparison between the three holobionts in seawater, two (H1 and H2) that are able to acclimate low salinity and one that cannot (H3). Hence, the aim was to elucidate how the different starting points, *i.e.* different microbiomes, can result in such a strong difference when exposed to low salinity.

10,059 genes were differentially expressed between the H1+H2 and H3 (Figure 3-8A); 7,966 were down-regulated and 2,093 were up-regulated in H3S. Overrepresented GO terms associated with up-regulated genes in H3 were, amongst others, related to transmembrane transport (inorganic cation transmembrane transporter activity), transcription/translation (e.g. RNA polymerase II transcription factor activity, translation initiation factor activity), photosynthesis (e.g. chlorophyll metabolic process, magnesium chelatase activity), nitrogen metabolism (e.g. cellular biogenic amine biosynthetic process, glutamate-ammonia ligase activity), amino acid metabolism (methionine metabolic process, glutamine biosynthetic process, glutamate biosynthetic process), lipids (cellular lipid metabolic process, lipid biosynthetic process), carbohydrate metabolism (pentose-phosphate shunt, carbon utilization, one-carbon metabolic process), oxidoreductase activity. Overrepresented GO terms in down-regulated genes were categorized as microtubule motor activity, microtubule binding, cilium assembly, cilium movement and calcium ion binding. An overview of the gene enrichment analysis is shown in Figure 3-10, Supplementary table 3-7, and Table 3-5.

## Interaction term

The freshwater response in H3 differed from that in H1+H2 (interaction term) regarding the regulation of 1,992 genes (Figure 3-8). Overrepresented GO terms for genes specifically up-regulated (1,266) in H3 in 15% NSW were related to transmembrane transport (e.g. ion transmembrane transporter activity), alpha-amino acid catabolic process, lipid break down (cellular lipid catabolic process, acetyl-CoA carboxylase complex), oxidation-reduction processes, motor activity, polysaccharide nucleotide-sugar biosynthetic process, and nitrogen transaminase activity. GO terms associated with down-regulated genes were, amongst others, related to photosynthesis (e.g. violaxanthin de-epoxidase activity, heme binding, light harvesting), oxidation-reduction process, transcription/translation (sigma factor activity, transcription factor activity), vitamins (phosphopantetheine binding, pyridoxal phosphate binding), amino acid metabolism (e.g. Isoleucine biosynthetic process, L-serine metabolic process), transmembrane transport (e.g. inorganic phosphate transmembrane transporter activity, ion transmembrane transporter activity), lipids (e.g. fatty acid biosynthetic process), carbohydrate metabolism (glycolytic process, pentose-phosphate shunt), and Sulphur metabolism (e.g. disulfide oxidoreductase activity). An overview of the gene enrichment analysis is shown in Figure 3-11, Supplementary table 3-7, and Table 3-5.

Table 3-5 The predominant changes observed between the holobionts in 100% NSW (left) and regarding the response to low salinity in H3 compared to H1/H2 (right). Arrows pointing upwards indicate GO terms that are overrepresented among upregulated genes in H3S compared to H1/H2S (left, microbiome effect), or specifically upregulated in 15% NSW compared to 100% NSW in holobiont 3. Arrows pointing downwards indicate GO terms overrepresented among downregulated genes in the corresponding categories.

<b>H1/H2 vs H3 in 100% NSW (Microbiome effect)</b>	<b>H1/H2 vs H3 - FW-response (Interaction term)</b>
<ul style="list-style-type: none"> <li>↑ Photosynthesis</li> <li>↑ Transmembrane transport</li> <li>↑ Transcription / translation</li> <li>↑ Amino acid metabolism</li> <li>↑ Lipid metabolism</li> <li>↑ Carbohydrate metabolism</li> <li>↑ Nitrogen metabolism</li> </ul>	<ul style="list-style-type: none"> <li>↑ Transmembrane transport</li> </ul>
<ul style="list-style-type: none"> <li>↓ Cytoskeleton</li> </ul>	<ul style="list-style-type: none"> <li>↓ Amino acid metabolism</li> <li>↓ Lipid metabolism</li> <li>↓ Photosynthesis</li> <li>↓ Vitamins</li> <li>↓ Carbohydrate metabolism</li> </ul>

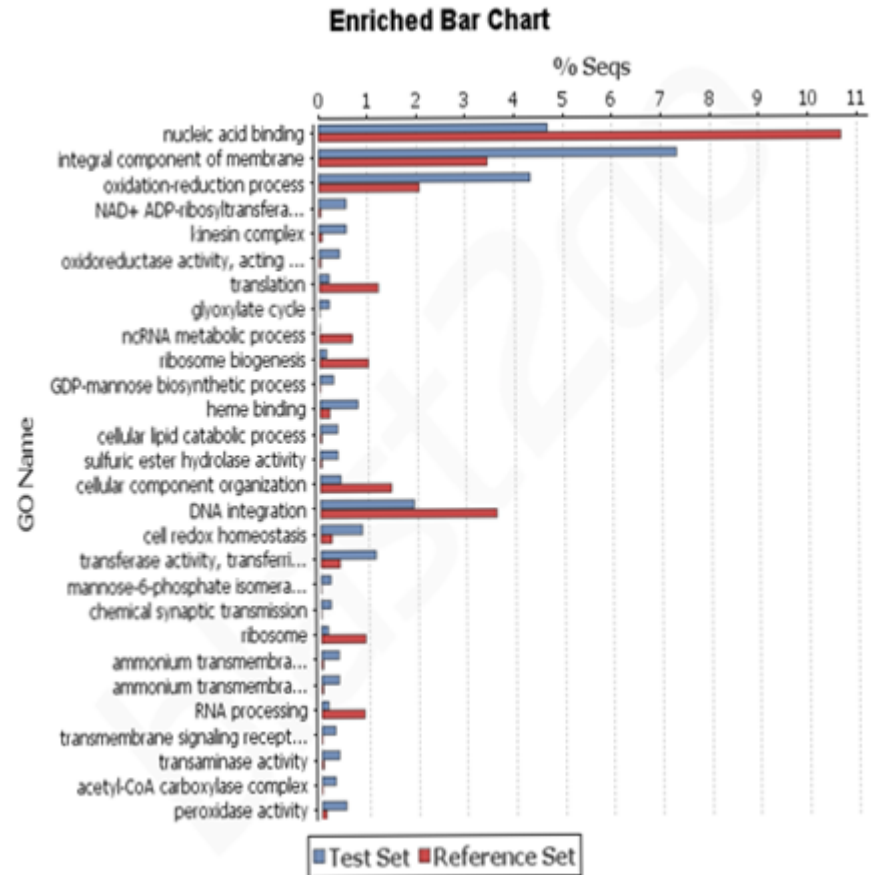
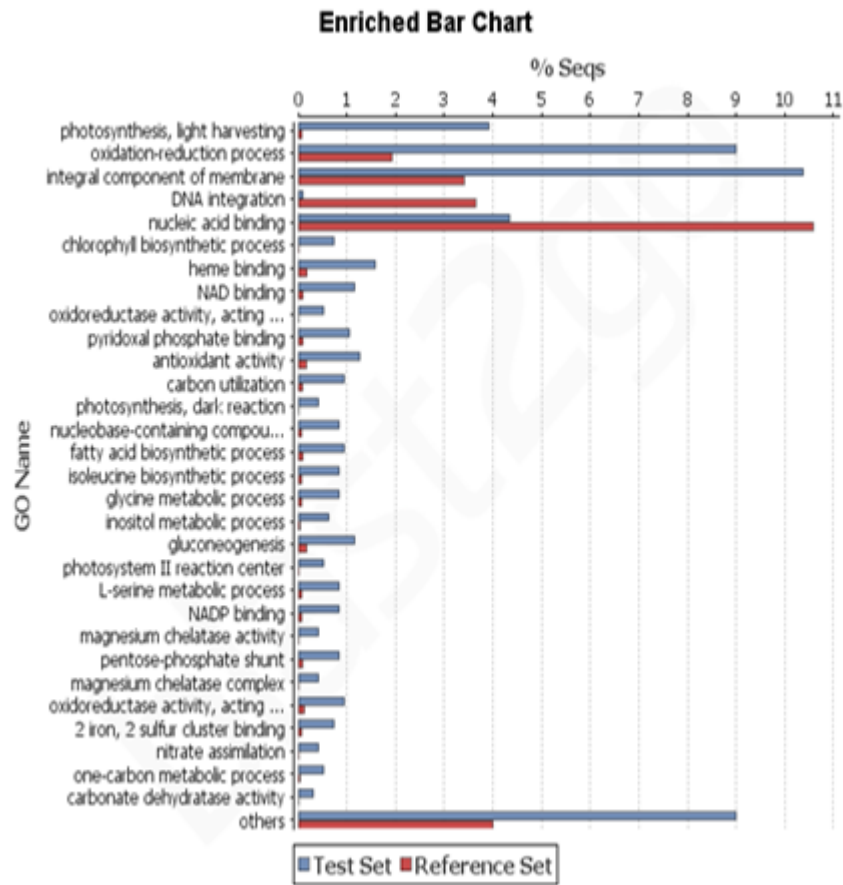


Figure 3-9 Gene enrichment analysis (Fisher's exact test; FDR < 0.05) of differentially expressed genes in Holobiont 3 in response to 15% NSW. Left: GO categories associated with down-regulated genes; right: GO categories associated with up-regulated genes.

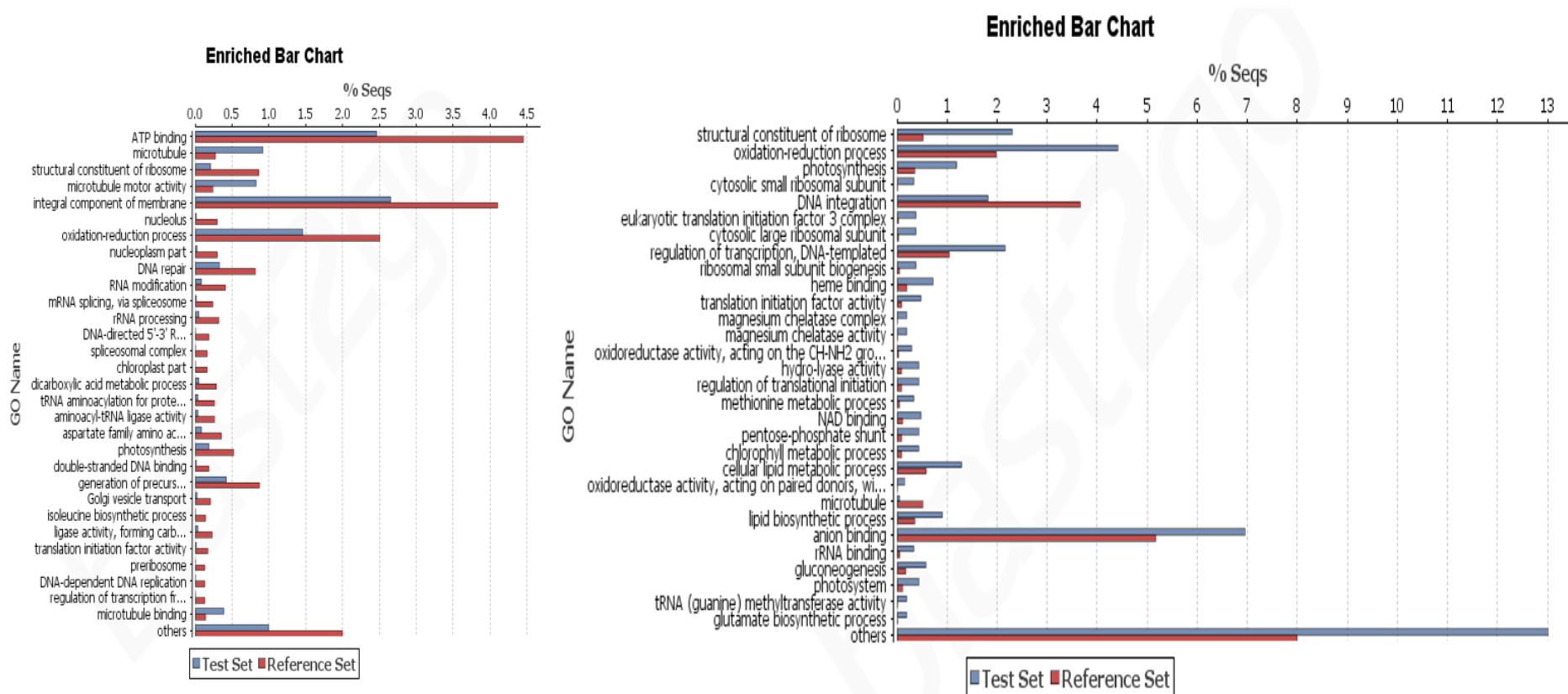


Figure 3-10 Gene enrichment analysis of differentially expressed genes in H3S compared to H1/2-S (Microbiome effect in SW). Left: GO categories associated with down-regulated genes in H3S; right: GO categories associated with up-regulated genes in H3S.

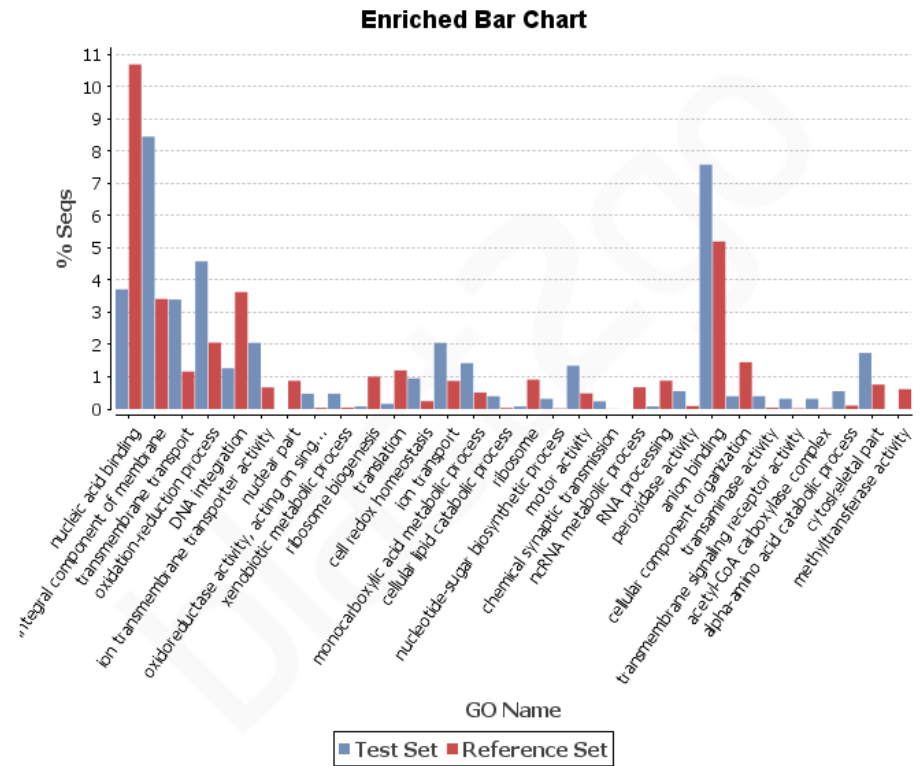
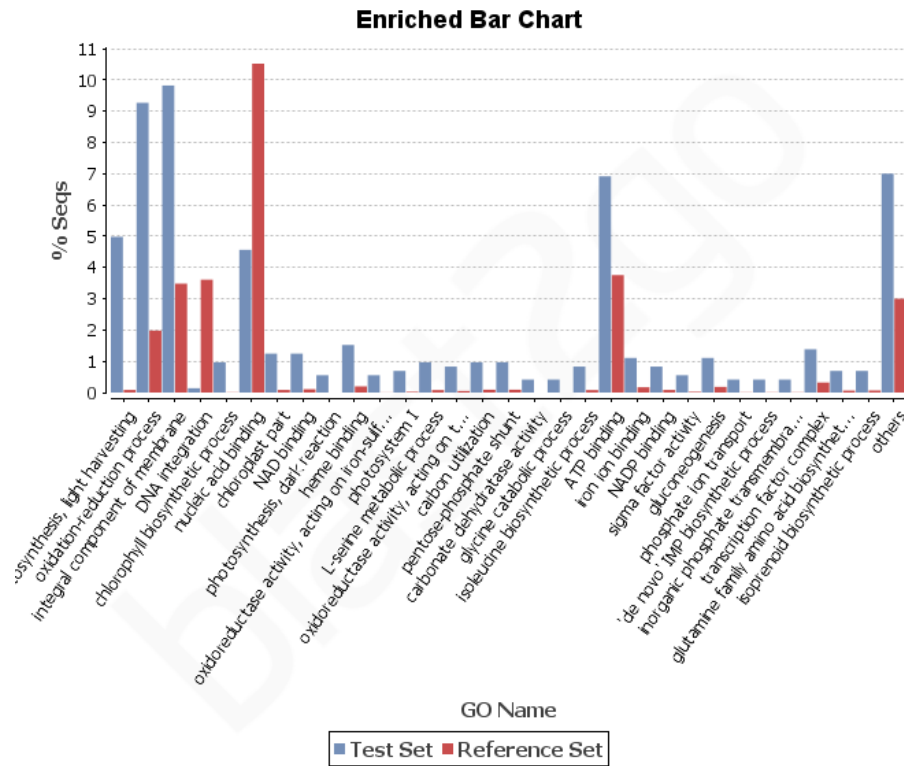


Figure 3-11 Gene enrichment analysis of differentially expressed genes in H3F compared to H1/2F, interaction term. Left: GO categories associated with down-regulated genes; right: GO categories associated with up-regulated genes.

## Bacterial metagenome

Approximately 2.93 billion metagenomics reads were generated from 4 lanes of Illumina HiSeq sequencing 3000, which after cleaning and removal of algal reads, resulted in 1.5 billion bacterial sequences (Figure 3-6). These were assembled and clustered into 73 bacterial metagenomic bins using the Anvi'o pipeline (Figure 3-12). The genomes were classified based on the redundancy of single-copy core genes and completeness of the set of SCG. Bin 33 had the lowest % of completeness (1.23%) and contained contigs of algal origin that had been missed in previous cleaning steps, which was confirmed by blasting the contigs against the *E. subulatus* genome once more: 3083 (95%) of the contigs mapped.

The quality of the metagenomes was assessed based on the presence/abundance of single-copy core genes (SCGs; set of 139 used here; Campbell et al., 2013). Thirty-five metagenomic bins had a completeness  $\geq 90\%$  (categorized as “full”). Four of those were  $\geq 10\%$  redundant (Bin 29: 18%, Bin 61: 18%, Bin 74: 16%, Bin 42: 21%). 38 bins were  $\leq 90\%$  complete and categorized as “partial”. 56 bacterial bins were taxonomically assigned to *Alphaproteobacteria* (41), followed by *Bacteroidetes* (11) and *Gammaproteobacteria* (9). Within the *Alphaproteobacteria*, *Rhizobiales* (12) and *Rhodobacterales* (15) were the most abundant, followed by *Sphingomonadales* (5). *Gammaproteobacteria* were dominated by *Alteromonadales* (4), and *Oceanispirallis* (2). *Bacteroidetes* were comprised of 6 *Flavobacteria* and 1 *Cytophaga* (Table 3-6). A complete overview of the bacterial bins and assembly statistics can be found in Supplementary table 3-8.

Table 3-6 Overview of the number of metagenomic bins obtained with Anvi'o, divided per taxa.

<b>Taxonomic affiliation</b>	<b>Number of metagenomic bins</b>
<i>Actinobacteria</i>	3
<i>Alphaproteobacteria</i>	41
<i>Gammaproteobacteria</i>	9
<i>Deltaproteobacteria</i>	1
<i>Unclassified Proteobacteria</i>	2
<i>Bacteroidetes</i>	11
<i>Planctomycetes</i>	3
<i>Unclassified bacterium</i>	2
<i>Unclassified organism</i>	1
<i>Ectocarpus</i>	1
Total number of bins	74

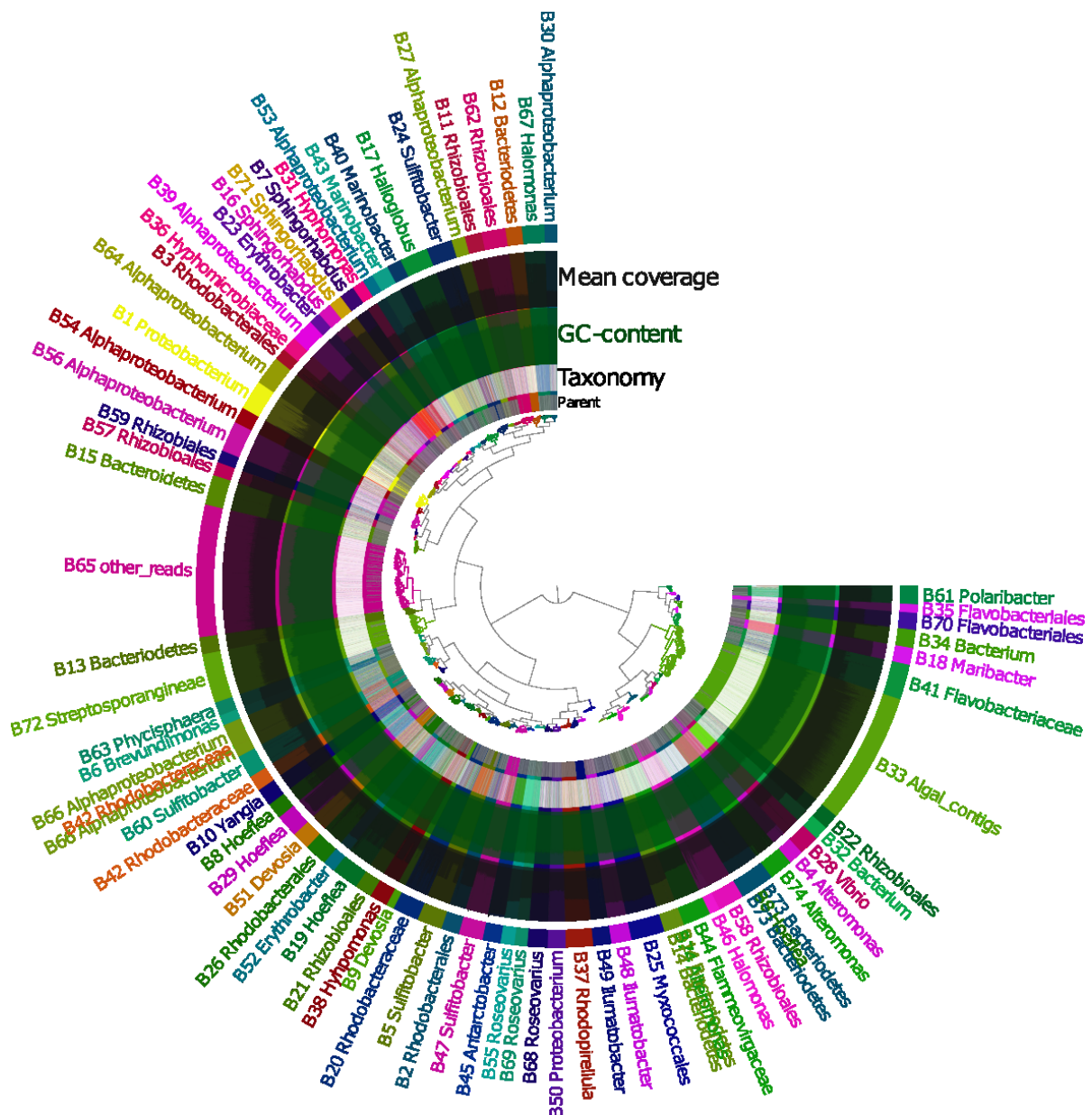


Figure 3-12 Result of the manual binning of metagenomic data using the Anvi'o pipeline, resulting in 74 metagenomic bins. Clustering was based on Euclidean distance and Ward linkage. Bin 33 was classified as algal-derived, and bin 65 was considered a 'garbage' bin comprised of all bacterial sequences of low quality or that could not be clearly assigned to a bin.

### Bacterial transcriptome

For each of the 73 metagenomic bins, the sequences were annotated and used to map the bacterial transcriptomic reads. The normalized "transcriptional activity" per bin is visualized in a heat map (Figure 3-13). Regarding the experimental treatment (column clustering), each of the holobionts in SW clustered together with the corresponding FW group; in addition, Holobiont 1 and 2 grouped closer together than to Holobiont 3, similar to what was already

described for the algal transcriptome. In holobiont 1, 26 bins were more active compared to the other 2 holobionts; 12 bins were activated in holobiont 2, and 35 in Holobiont 3.

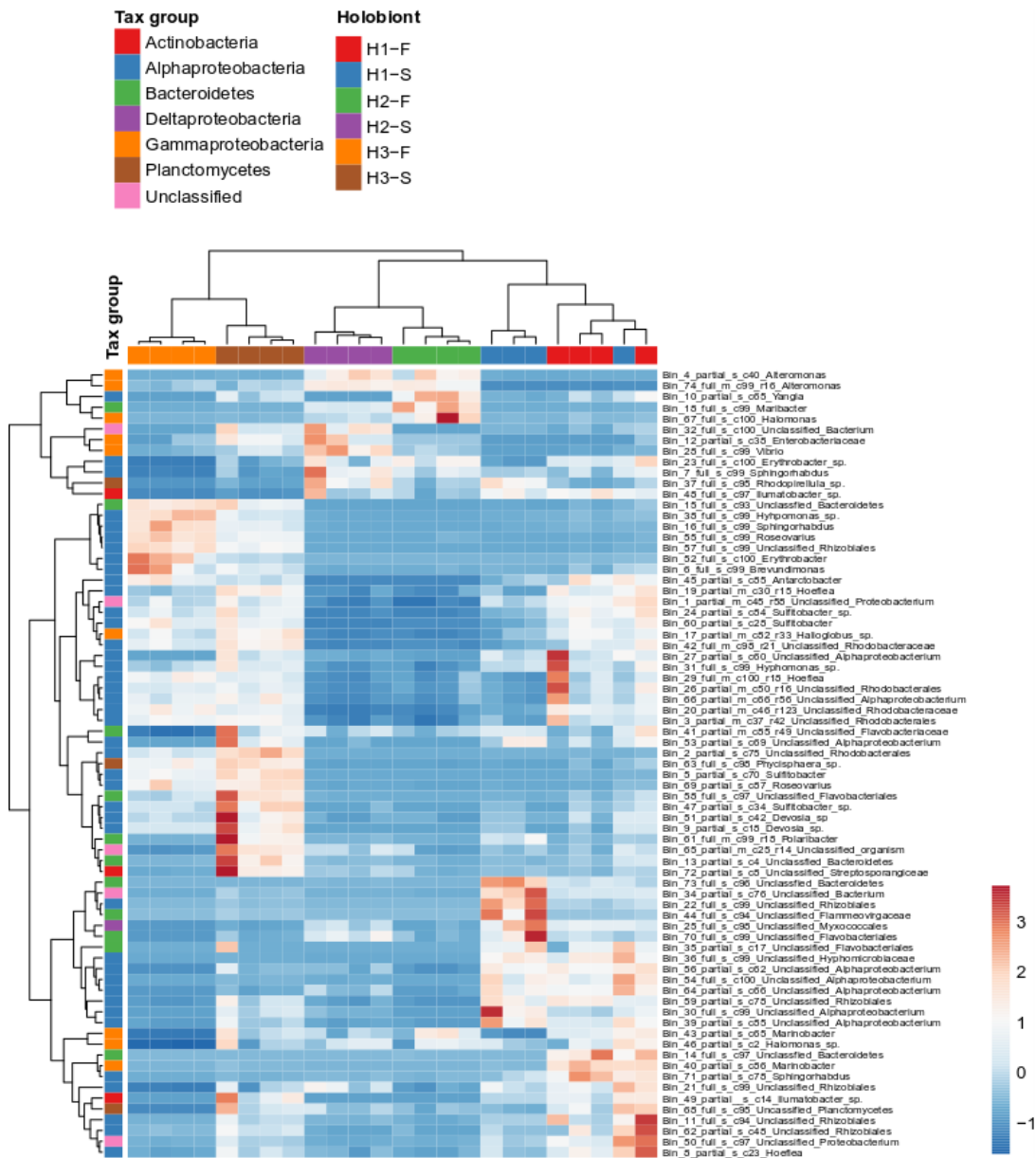


Figure 3-13 Heat map based on hierarchical clustering of bacterial gene expression profiles in each holobiont (Pearson correlation coefficient; clustering method: average linkage; unit variance scaling of row data).



## **Metabolic networks**

A first attempt was made to perform differential gene expression analysis on a bin per bin basis, but read counts were too low and gene numbers too high to allow statistical testing. Therefore, differential expression analysis was carried on a microbiome level, which also is more in line with the holobiont concept. The starting point for this analysis were the metabolic reactions that occur in each microbiome. The expression data for all genes of all bins that contribute to a specific metabolic reaction were combined and the resulting expression data per reaction per holobiont was subjected to differential expression analysis.

A total of 3,957 metabolic reactions was subjected to differential expression analysis. Hundred nine reactions were identified in holobiont 1, 226 in holobiont 2, and 117 in holobiont 3. Hundred and one reactions were significant in the analysis H3S compared with H1/2S (microbiome effect). The interaction term identified 29 significant reactions. Fourteen reactions were shared between H2 and H3; Two between H1 and H3, and none between H1 and H2. In-depth analysis of these results and the corresponding pathways is ongoing. (Figure 3-8C&D).

## **Metabolite profiling of algal holobionts**

### **Comparison of metabolite extraction solvents**

A comparison of the three tested solvents (methanol:ethanol:CHCl<sub>3</sub>, methanol 100%, and methanol:H<sub>2</sub>O) revealed that extraction with 100% methanol gave the highest number of features detected by GCMS/AMDIS (Figure 3-14). The number of unique features, however, was higher with MeOH:H<sub>2</sub>O. Differences between the tested solvents became visible already via differences in the color of the extracts (Figure 3-14B). Finally, I chose to continue the experiment using 100 % MeOH as a solvent. This solvent was also successfully applied before on the freshwater strain of *Ectocarpus* (Dittami *et al.*, 2012).

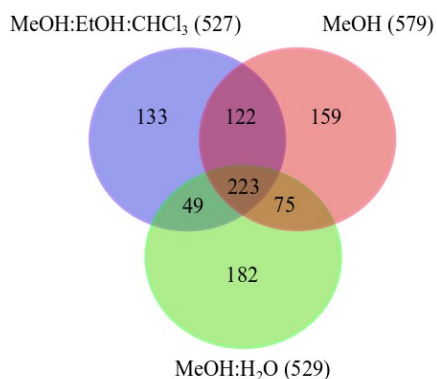
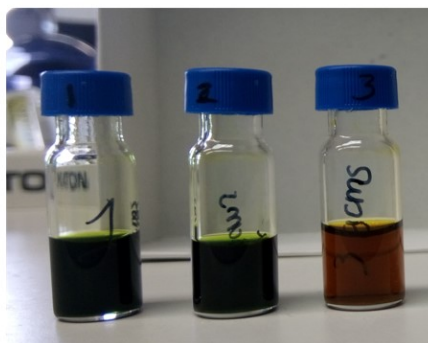
**A****B**MeOH:EtOH:CHCl<sub>3</sub>    MeOH    MeOH:H<sub>2</sub>O

Figure 3-14 A) Venn diagram representing the number of features identified with the AMDIS software and NIST libraries. B) Solvents after extraction of metabolites.

### LCMS results

LMCS chromatograms were processed with the workflow4metabomics pipeline. Data were separated in positive and negative mode and analyzed separately. The t-test (FDR < 0.01) on each of the holobionts in FW compared to SW revealed no significant features in H1, 2 in H2 and 880 in H3 for the positive mode data. For negative mode, these numbers were 0, 11, and 339 respectively. These results are summarized in Table 3-7. Preliminary annotations using the internal Massbank annotator within W4M are given in Supplementary table 3-9. Those annotations are useful to get a first impression of type of metabolites that were identified and significant in the treatment, but additional analysis is required to say with more certainty what compounds we are dealing with. For illustration, the Massbank annotated compounds for holobiont 2 are given in Table 3-8. A selection of significant biomarkers for all of the conditions is currently being analyzed with MS/MS analysis.

Table 3-7 Significant features detected in LC-MS data analyzed with the W4M workflow. Testing with FDR < 0.01.

	Positive mode				Negative mode			
	H1F vs H1S	H2F vs H2S	H3F vs H3S	[H1S+H2S] vs H3S	H1F vs H1S	H2F vs H2S	H3F vs H3S	[H1S+H2S] vs H3S
↑ in FW or in H3S	0	2	470	1014	0	10	205	297
↓ in FW or in H3S	0	0	410	307	0	1	134	98
<b>Total</b>	0	2	880	1321	0	11	339	395
<b>Preliminary annotation</b>	0	2	119	135	0	11	135	70

Table 3-8 Significant features detected in LC-MS data (negative and positive mode) analyzed with the W4M workflow for holobiont 2 only. All other data is given supplementary table Supplementary table 3-9; lcmsneg: negative mode data; lcmspos: positive mode data;

mode	Up (↑) or down (↓)	MZ/R T pair	FDR	MassBank annotation
lcmsneg	↑ in 15% NSW	M638T 668	0.0021	N2-Isobutyryl-5'-O-(4,4'-dimethoxytrityl)- 2'-deoxyguanosine
lcmsneg	↑ in 15% NSW	M527T 645	0.0094	Bromadiolone
lcmsneg	↑ in 15% NSW	M626T 623	0.0038	Sakuranetin
lcmsneg	↑ in 15% NSW	M800T 597	0.0030	Phosphatidylcholine 20:0-18:1
lcmsneg	↑ in 15% NSW	M683T 554	0.0038	Methyllycaconitine
lcmsneg	↑ in 15% NSW	M500T 520	0.0094	Phosphatidylethanolamine 20:4-22:6
lcmsneg	↑ in 15% NSW	M197T 144	0.0078	DL-4-Hydroxy-3-methoxymandelic acid
lcmsneg	↑ in 15% NSW	M342T 134	0.0071	Pentamidine
lcmsneg	↓ in 15% NSW	M182T 133	0.0003	Na,Na-Dimethylhistidine
lcmsneg	↑ in 15% NSW	M386T 132	0.0021	Buspirone
lcmsneg	↑ in 15% NSW	M514T 132	0.0094	Taurocholic acid
lcmspos	↑ in 15% NSW	M328T 133	0.0054	3',5'-Cyclic AMP
lcmspos	↑ in 15% NSW	M320T 129	0.0054	Norfloxacin

## GMCS results

The final dataset contained 609 features that were (after normalization) used for statistical testing. The numbers of significant features found with each statistical test are summarized in Table 3-9 and a complete overview of annotated features can be found in Supplementary table 3-9. In total, 73 compounds were shown to be significant among all statistical tests performed.

Table 3-9 Significant features detected in GC-MS data analyzed with the W4M workflow. Testing with FDR < 0.05.

	H1-F vs H1-S	H2-F vs H2-S	H3-F vs H3-S	[H1-S+H2-S] vs H3-S
↑ in FW or H3	0	1	4	29
↓ in FW or H3	1	0	35	18
<b>Total</b>	<b>1</b>	<b>1</b>	<b>39</b>	<b>47</b>

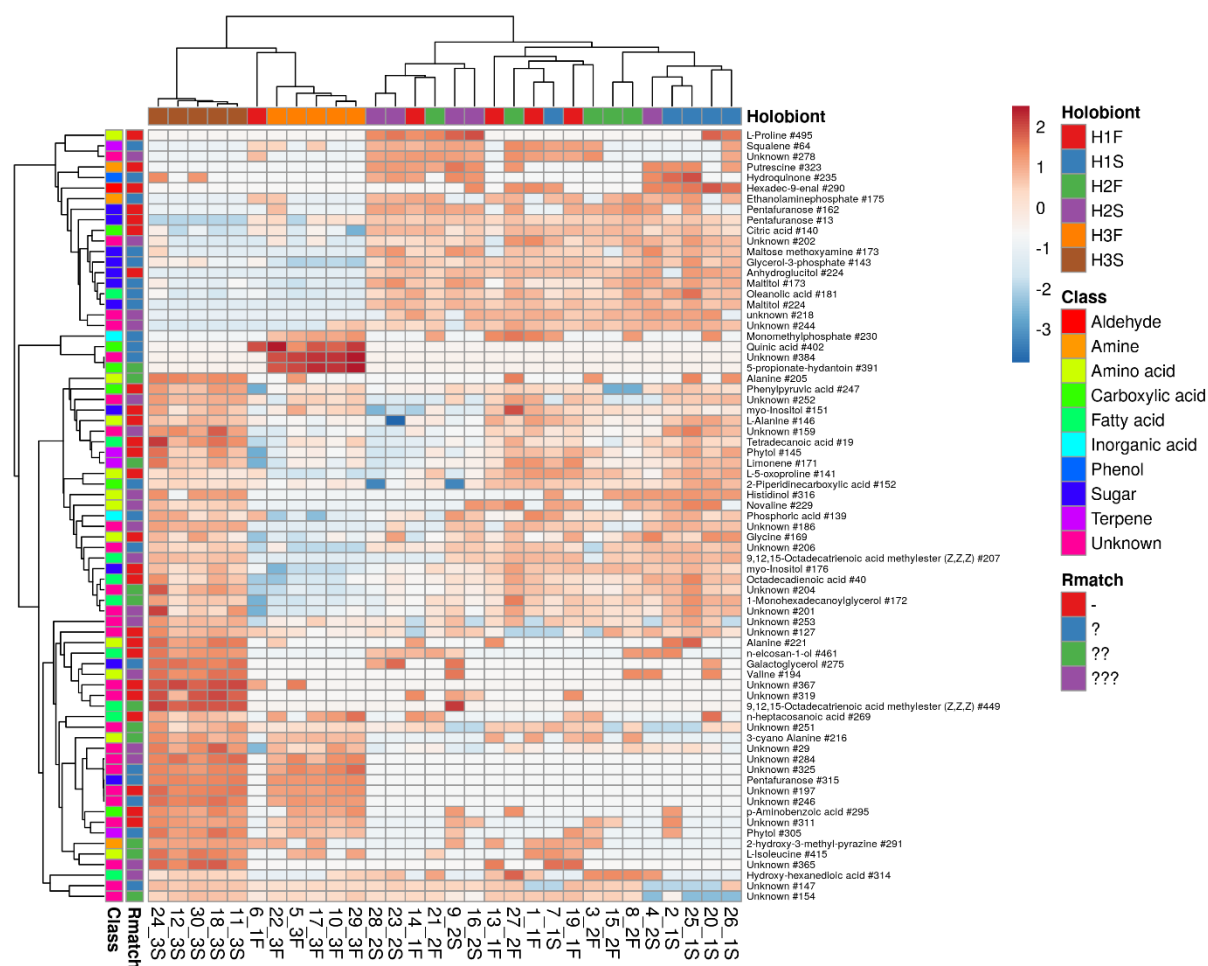


Figure 3-15 Heat map based on hierarchical clustering of the 86 significant features obtained with GC-MS analysis (distance: Pearson correlation coefficient; clustering method: average linkage; unit variance scaling of row data). R match refers to the quality of the annotation, and

a value > 800 (noted as “-” in the figure) is considered a reliable annotation; a value between 700-800 (noted as “?” in the figure) is reliable but needs additional verification using a standard. Values < 700 (“??” and “?”) are only tentatively annotated.

## Discussion

The main aim of this work is to elucidate bacteria and bacterial functions that enable *E. subulatus* holobionts to successfully acclimate to low salinity. To that aim, three algal-bacterial holobionts were created that differed in their capacity to grow in low salinities. Our data shows that the variation in microbiome has an impact on several aspects of the alga, notably gene expression profiles, and metabolomic features. The more the microbiome was disturbed (stronger antibiotic treatment) the more the holobionts exhibited a transcriptomic/metabolomic response to low salinities.

### Algal response to low salinity

**Holobiont 1.** was the least affected by the transfer to low salinities. Only 6 algal genes were differentially expressed in 15% NSW (Figure 3-8A), and changes in the metabolome were also minimal (one significant feature with GC-MS; no significant features with LC-MS). Moreover, gene expression of a heat shock protein 70 previously reported as stress-induced (Dittami *et al.*, 2009; Ritter *et al.*, 2014) was down-regulated in this holobiont. Thus, the alga can cope well with the change in salinity. In contrast, the transcriptomic profile of the microbiome shows a clear distinction between activated bacterial bins in holobiont 1 in 15% NSW compared to 100% NSW (Figure 3-13). Thus, there was a shift in microbiome activity, despite the absence of a response in the algal host.

**Holobiont 2.** The metabolomic and algal transcriptomic regulation of holobiont 2 in response to low salinity was stronger compared to holobiont 1, *i.e.* 92 DEGs genes were identified (Figure 3-8A) and one metabolic feature with GC-MS (tentatively annotated as Hydroquinone) and 13 with LC-MS. Gene set enrichment analysis showed that a high proportion of genes up-regulated in low salinity were involved in processes related to photosynthesis/light harvesting, ribosome biogenesis, and translation. A detailed analysis of the genes involved in specifically those processes showed that for ribosome biogenesis and translation, the genes were all chloroplast-derived, and encoded ribosomal proteins of the large (rpl 3, 4, 5, 6, 14, 16, 21, 23) and small (rps 3, 8, 19) subunit. Ribosomal proteins form and stabilize the ribosomal complex and are essential for protein synthesis. Plastid translation itself is essential for cell survival

because most of the plastid-encoded gene products are required for photosynthesis (Rogalski *et al.*, 2008). Indeed, we found an induced expression of two additional chloroplast genes encoding Photosystem II cytochrome c550 (EsuBft\_cp\_81) and cytochrome b6-f complex subunit V (EsuBft\_cp\_81). The activation of PSII may be related to the increased expression of plastid-derived ribosomal proteins.

Genes up-regulated in holobiont two in response to low salinity and involved in photosynthesis/light harvesting were derived from the nucleus and characterized as light-harvesting complex (LHC) proteins of the LHCF/R family. LHCs are chlorophyll binding proteins classically involved in light harvesting processes, however, some LHC proteins have different functions in algae such as non-photochemical quenching (Peers *et al.*, 2009). The expression of several chlorophyll binding proteins including the LHCF/R family has been shown to be stress-induced in brown algae (Dittami *et al.*, 2009; Ritter *et al.*, 2014), and diatoms (Nymark *et al.*, 2009; Park *et al.*, 2010). Recently it was also shown that in a brackish water strain of *E. subulatus* (strain Bft15b; a close relative of *E. subulatus* FWS), the LHCF/LHCF family of chlorophyll binding proteins was expanded in comparison with the marine strain *E. siliculosus* (Dittami, 2018; Preprint), but the exact function of the genes/proteins remains to be elucidated.

A C5-epimerase (EsuBft\_1053\_3) was among the down-regulated genes in holobiont 2 in low salinity. C5-epimerases are cell wall modifying enzymes involved in remodeling of the cell wall of brown algae (Fischl *et al.*, 2016). Cell wall regulatory processes may play an important role in the acclimation of *E. subulatus* to lower salinities (Dittami *et al.*, 2012). However, here only one C5-epimerase was regulated, while previously six were shown to be regulated by changing salinities, four which were reduced in low salinity (Dittami *et al.*, 2012).

In summary, Holobiont 2 also shows only a few signs in response to the transfer from 100% to 15% NSW and the number of regulated genes and metabolites are relatively low (Figure 3-8A, Table 3-7). Previous work on the *E. subulatus* FWS showed differential regulation of 3004 genes (27%) in freshwater (Dittami *et al.*, 2012; same FDR < 0.05). However, in the former study, the salinity level was reduced to 5% NSW, and the algal holobiont was still functional and able to acclimate to low salinity. In this study, salinity levels were reduced to only to 15% NSW to avoid lethal effects on the fresh water-intolerant holobiont 3. This may be one explanation as to why fewer genes were regulated during acclimation.

Despite the few changes observed in the alga during the response to low salinity, the activity of the microbiome was strongly affected: similar to what was described for holobiont 1, there was a shift in microbiome activity, despite the comparatively weak response in the algal host (Figure 3-13).

**Holobiont 3.** showed a much stronger response to low salinity compared to holobiont 1 and 2, both regarding the transcriptomic regulation (2355 DEGs; Figure 3-8A) and metabolomic features (1219 with LC-MS; 39 in GC-MS). The regulation can be described as a ‘classical’ stress response, including the breakdown of amino acids, lipids and sugars, reduced photosynthetic activity, and reduced protein synthesis at 15%-NSW.

Some of those observations were supported by GC-MS analysis. For example, several amino-acids were reduced at 15% NSW compared to 100% NSW, in particular, Serine, Alanine, Glycine, and Proline. An increase in alanine was previously observed in *E. subulatus* grown in 5% NSW (Dittami *et al.*, 2012) and may be linked to higher nitrogen assimilation (Gravot *et al.*, 2010). Here, alanine was decreased in low salinities, and the algal transcriptome confirms that nitrate assimilation was negatively regulated in H3F. These findings are also in contrast to what we see in H3S compared to H1/2 in 100% NSW, namely, an increase in nitrogen assimilation via transcriptional activation of glutamate-ammonia ligase activity (GO:0004356), and production of glutamine (GO:0006542) and glutamate (GO:0006537). It seems that holobiont 3 in seawater needs to put more energy towards nitrogen assimilation, yet it can still cope. As soon as the transfer to low salinity is made, primary metabolism is impaired leading to reduced growth and eventually a collapse of the system. Interestingly, an increase in ammonium transmembrane transport and mannose biosynthesis GO terms were found among up-regulated genes in holobiont 3 in low salinity, which may suggest that remaining nitrogen assimilation in holobiont 3 in low salinity is more likely done via ammonium transport than nitrate. Previously, it was shown that the transfer to low salinities led to an increase in intracellular ammonium contents in *E. subulatus* that could successfully acclimate to low salinity (Dittami *et al.*, 2012).

For the regulation of fatty acids, we observed a similar pattern. Metabolite levels of several fatty acids (Myristic acid, Arachidyl alcohol, linolenic acid methyl ester) were increased in holobiont 3 in high salinity compared to H1/2. Yet, in low salinity, holobiont 3 showed a strong

reduction of those fatty acids, as well as two additional ones (Linoleic acid, Glyceryl palmitate). In *Laminaria digitata*, some fatty acids (e.g. myristic acid, linoleic acid, linolenic acid), were increased after exposure to bacteria-derived lipopolysaccharides (defense reaction). In *Ectocarpus*, fatty acids (e.g. linoleic acid) were induced during copper stress (Ritter *et al.*, 2008, 2014) and regulated during a change in salinity (e.g. Linoleic acid was increased in low salinity; Dittami *et al.*, 2012). Other compounds regulated in response to low salinity in H3 have previously been shown to be involved in chloroplast functioning in plants and algae (Phytol; de Souza and Nes, 1969; Ischebeck *et al.*, 2006), histidine turnover (5-proprionate hydantoine<sup>25</sup>), and programmed cell death in plants (Myo-inositol; Donahue *et al.*, 2010), but the significance of these (and other) observations in the *Ectocarpus* holobiont remains to be elucidated.

Generally, in H3S, primary metabolic processes were activated, such as synthesis of amino acids and lipids, photosynthesis, carbohydrate metabolism, transcription/translation, while in low salinity we see the inverse. The activation of these processes in seawater may be an indirect compensatory mechanism resulting from missing and/or changed bacterial contributions. The only biological process that was negatively regulated in Holobiont 3S was categorized as “microtubule activity” (the only overrepresented GO category among all 7966 down-regulated genes), which may also be related the differences observed in morphology between (ball-like vs more filamentous shape; see chapter 2 – subsection III).

The phenotype observed in low salinity is in strong contrast with previous studies of *E. subulatus* with its full flora and also in contrast with holobionts 1 and 2, which were able to acclimate to low salinity, without detrimental effects on photosynthesis and primary metabolism. Here, the alga is able to cope with the absence of bacterial functions as long as it is growing in 100% NSW, however, it can no longer meet the metabolic demand when exposed to 15% NSW.

### **Microbiome effect in seawater**

The aim of this study is to investigate how the microbiome contributes to algal acclimation to low salinity. Holobiont 3 displayed the strongest metabolomic and transcriptomic regulation of the alga in low salinity and we need to assume that this was caused by differences in the microbiome, as this was the only factor that changed between the three holobionts in seawater.

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<sup>25</sup> <https://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=HISHP-PWY>



Thus, the next steps are aimed at the integration of the algal response with that of the microbiome and finding possible points of interactions between the two that could explain why holobiont 3 is no longer able to acclimate to low salinity.

With that aim in mind, different strategies can be used to explore the data. A first attempt was made to perform differential gene expression analysis on a bin per bin basis, but read counts were too low and gene numbers too high to allow statistical testing. Alternatively, a global approach was implemented, combining the expression values of all genes predicted in each bin. This provided us with the activity map (Figure 3-13), that gives the transcriptomic activity per bin in each holobiont. However, that does not necessarily help with finding clues on how the bacteria interact with the host, and, considering that the microbiome is an interacting unit (with cooperation/exchange between bacteria as well), we also chose to look at the combined bacterial potential, *i.e.* the microbiome as a whole, rather than individual bins.

To perform differential expression analysis on a microbiome level, a method has been put in place, that takes as a starting point metabolic reactions that may occur in the microbiome of a particular holobiont. For each holobiont, expression data for all genes of all bins that contribute to a specific metabolic reaction were combined and the resulting expression data per reaction per holobiont was subjected to differential expression analysis. Reactions were identified that were significantly different in each holobiont. Preliminary results showed that in contrast to the algal transcriptome, holobiont 2 had the highest number of differentially expressed reactions (216 reactions), followed by holobiont 3 (117 reactions) and holobiont 1 (109 reactions) (Figure 3-8C&D). Thus, the microbiomes of holobiont 1, 2, and 3 exhibited a metabolic response to low salinity.

A first look at the differentially expressed reactions in the microbiome of holobiont 3 in low salinity (interaction term), showed that the eight reactions that were up-regulated all correspond to pathways involved in the production of Acyl-homoserine lactones (AHLs). AHLs are quorum sensing molecules involved in virulence, symbiosis, and biofilm formation. The reactions could be traced back to three bacterial bins, notably *Roseovarius* (bins 69 and 55) and *Hoeflea* (Bin 29) and *Sulfitobacter* (Bin 5). Interestingly, *Roseovarius* and *Hoeflea* were among the bacterial strains that had a positive effect on algal growth in seawater (*E. siliculosus*; Chapter 2 – subsection III). Hence, we can speculate that in holobiont 3 (alga that does not acclimate to low salinity), virulence is induced in otherwise beneficial bacteria when the alga

is transferred to low salinity. Induction in virulence may go in parallel with a reduction of algal defense mechanisms (e.g. production of halogens, phenols, proline). However, further in-depth analyses of the corresponding pathways and experimental verification will be required to test this.

Similarly, among the most down-regulated pathways in the microbiome of H3 compared to holobiont 1 and 2 in seawater, was nitrogen reduction, indicating that less ammonium may be provided by the microbiome. Indeed, regarding the algal transcriptomic response, we found an activation of nitrogen reduction. Currently, we do not know if or how this would impact the alga when transferred to low salinity, but as stated above, nitrogen assimilation was one of the key features previously observed during the successful acclimation of *E. subulatus* to fresh water fresh water (Dittami et al. 2012).

Thus, to conclude, all data has become available, and are ready to explore in more depth. Several strategies have been put in place that enables a targeted approach to explore bacterial contributions during acclimation of holobionts to low salinity. In-depth analysis is ongoing, but first indications point towards a change in the microbiome regarding nitrogen assimilation and virulence. In parallel, metabolic networks have been created for each of the bacterial bins which will be followed by analysis of metabolic complementarity between the bacterial bins and the algal host in a similar way as described in Chapter 2 – subsection III for *E. siliculosus* and cultured bacteria.

# **Final Conclusions**

## Final conclusions & perspectives

### How does *Ectocarpus subulatus* interact with bacteria during acclimation to fresh water?

The main aim of my PhD thesis was to generate hypotheses about the interactions that occur in the *Ectocarpus* holobiont during acclimation to fresh water. This objective was built on the idea that the interactions within the holobiont can be dissected into its individual parts and that, by combining those parts again from scratch, one could eventually find “THE” bacteria or “THE” bacterial processes that are central to holobiont functioning.

My attempts to incorporate such a reductionist approach were unsuccessful at first, *i.e.* antibiotic-treated cultures of *E. subulatus* did not show a strong response to any of the cultured bacteria tested in co-cultures in fresh water (Chapter 2, section I). However, in a simpler setting where the response of the alga was tested in seawater, the alga did show a strong response to the presence of bacteria (Chapter 2, section III).

These results emphasize the importance of how beneficial bacterial communities are selected. For targeted co-culture systems, the number of communities that can be tested is, restricted by the number of bacteria present in the culture collection. The cultured bacteria isolated from *E. subulatus* covered only 21% of the bacterial OTU diversity in the holobiont (Chapter 1). Thus, it remains questionable whether that what is observed in co-cultures, is comparable to what happens in a holobiont comprised of all members.

Results of the co-culture experiments illustrate that *Ectocarpus* functions as a “true” holobiont system, *i.e.* algal growth is strongly reduced when bacteria are absent (or reduced), and metabolic interactions with a subset of only ten cultured bacteria, enhanced the metabolic capacity of the alga (Chapter 2 – subsection III). This brings forward the paradox of establishing a simplified co-culture system to study *Ectocarpus* holobionts: completely sterilized algae are a prerequisite for the establishment of such systems, yet they do not exist or they do but they won't grow.

High-depth whole genome shotgun (WGS) sequencing of the whole holobiont is a promising complementary tool to study macroalgal holobionts, that avoids the issue of sterilizing algae and cultivation biases because it captures the complete bacterial variety present in the holobiont. This strategy was successfully applied to three *E. subulatus* holobionts with different

microbiomes and different response to fresh water. The first analyses gave indications of changes that occurred in the microbiome during acclimation (e.g. virulence, and nitrogen assimilation) and in-depth analysis is currently ongoing.

For me, an exciting following step would be to use metabolic complementarity analysis (chapter 2 – subsection III) on *E. subulatus* FWS and apply this to the more complex system involving abiotic stress. Beneficial communities could be predicted based on the 73 metagenomes that were assembled. This can provide clues on the taxa important for the algal response to freshwater, and it can be used to predict compounds that are exchangeable within the holobiont as a whole and that may be involved in the acclimation of the alga to low salinity.

Experimental validation is an absolute requirement for functional studies to confirm hypotheses based on transcriptome or metagenome data. Here, at least twenty of the bacterial genomic bins that I obtained, had a cultured representative on genus level, making it more likely that some of the proposed hypotheses can be validated. Alternatively, the metagenomes could be used to predict the specific cultivation requirements of thus far uncultured taxa. However, even without a cultured representative, simply the predicted compounds could be tested for their effect on axenic algae.

I believe that the “sequencing” all strategy is the way to go when studying holobionts, especially when one wants to put results in the bigger context of environmental change. Not only because of the inherent complexity of the microbiota (cooperation, metabolic exchange) that cannot be grasped by cultivation-dependent methods, but also because it resembles better the situation outside the laboratory. Holobionts don't have boundaries and are continuously changing. Ultimately, the purpose of this work is to go beyond the lab and transfer the knowledge to the field. For example, the drastic effect of the environmental perturbation on marine holobionts is well illustrated by bleaching event in corals. Similar, but perhaps less obvious shifts may occur in macroalgal holobionts during environmental stress and affect their well-being. With the tools presented here, we have taken the first steps towards the holistic view that is required to fully understand holobionts or any other biological system in their natural environment.

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# **Annex**

# Annex 1 Supplementary data

## Supplementary data Chapter 1

Supplementary table 1-1 Bacterial growth media recipes for LB, R2A, Zobell, PYG, EC-based, and LNHM. Data available via link:

[https://www.frontiersin.org/articles/file/downloadfile/312517\\_supplementary-materials\\_tables\\_1\\_xlsx/octet-stream/Table%201.XLSX/1/312517](https://www.frontiersin.org/articles/file/downloadfile/312517_supplementary-materials_tables_1_xlsx/octet-stream/Table%201.XLSX/1/312517)

Supplementary table 1-2 Antibiotics used to reduce the abundance of *Halomonas* sp. Data available via link:

[https://www.frontiersin.org/articles/file/downloadfile/312517\\_supplementary-materials\\_tables\\_2\\_xlsx/octet-stream/Table%202.XLSX/1/312517](https://www.frontiersin.org/articles/file/downloadfile/312517_supplementary-materials_tables_2_xlsx/octet-stream/Table%202.XLSX/1/312517)

Supplementary table 1-3 Number of bacterial isolates obtained per experimental treatment. Data available via link:

[https://www.frontiersin.org/articles/file/downloadfile/312517\\_supplementary-materials\\_tables\\_3\\_xlsx/octet-stream/Table%203.XLSX/1/312517](https://www.frontiersin.org/articles/file/downloadfile/312517_supplementary-materials_tables_3_xlsx/octet-stream/Table%203.XLSX/1/312517)

Supplementary table 1-4 Comparisons were based on 1) the salinity of the algal growth medium and 2) on sample type. The three examined 16S rRNA gene libraries were META16-NSW: natural seawater from 2016; META13-NSW: natural seawater from 2013; META13-DNSW: diluted natural seawater from 2013.  $\cup$  = union, *i.e.*; strains that have a representative OTU in at least one of the gene libraries;  $\cap$  = intersection = cultivable strains were isolated from both conditions. These data include strains found only among the rare reads. For instance, among the 13 strains cultivated only from natural seawater-based samples, 4 were found in the 2016 metabarcoding data set, and a total of 5 were found in the union of all metabarcoding libraries. 8 strains were not found in any of the barcoding data sets. Data available via link:

[https://www.frontiersin.org/articles/file/downloadfile/312517\\_supplementary-materials\\_tables\\_4\\_xlsx/octet-stream/Table%204.XLSX/1/312517](https://www.frontiersin.org/articles/file/downloadfile/312517_supplementary-materials_tables_4_xlsx/octet-stream/Table%204.XLSX/1/312517)

## Supplementary data Chapter 2

Supplementary Table 2-1 Overview of the cultured bacteria obtained that were tested in co-culture with *E. subulatus* FWS for their effect on algal growth in 5% natural seawater and 100% natural seawater. ++ algal growth; - no algal grow.

Stock number	Strain	final OD for inoculation	SW Response	FW response
A4	<i>Ahrensia</i> sp.	0.1	++	-
38	<i>Alteromonas</i> sp.	0.1	-	-
Mix_2_2015	<i>Halomonas</i> sp. (58) + <i>Moraxalla</i> sp. (ax1)	0.1	-	-
Mix_3_2015	<i>Halomonas</i> sp. (58) + <i>Bosea</i> sp. (65)	0.1	-	-
Mix_all_2015	mix 20 strains tested in Exp1 (2015)	0.1	-	-
Mix_1_2015	<i>Halomonas</i> sp. (58) + <i>Sphingorhabdus</i> sp. (50)	0.1	-	-
Mix_5_2015	<i>Sphingorhabdus</i> sp. (50) + <i>Hyphomonas</i> sp. (Ax3) + <i>Hyphomonas</i> sp. (Ax4)	0.1	+	+
65	<i>Bosea</i> sp.	0.1	++	-
58	<i>Halomonas</i> sp.	0.1	-	-
Ax3	<i>Hyphomonas</i> sp.	0.1	++	+
Ax4	<i>Hyphomonas</i> sp.	0.1	++	+
87	<i>Imperialibacter</i> sp.	0.1	++	+
83	<i>Imperialibacter</i> sp.	0.1	++	+
47.2	<i>Limnobacter</i> sp.	0.1	++	+
39	<i>Marinobacter</i> sp.	0.1	-	-
107	<i>Marinoscillum luteum</i>	0.1	++	+
Ax1	<i>Moraxella</i> sp.	0.1	++	+
Ax5	<i>Moraxella</i> sp.	0.1	++	-
111	<i>Pantoea</i> sp.	0.1	++	-
Mix_6_2015	<i>Hyphomonas</i> sp. (Ax3) + <i>Hyphomonas</i> sp. (Ax4) + <i>Sphingorhabdus</i> sp. (50) + <i>Imperialibacter</i> sp. (83) + <i>Bosea</i> sp. (65) + <i>Marinoscillum</i> sp. (107)	0.1	+	-
I3	<i>Roseovarius</i> sp.	0.1	-	-
Ax6	<i>Sphingomonas hunanensis</i>	0.1	++	-
Ax2	<i>Sphingomonas hunanensis</i>	0.1	++	+
50	<i>Sphingorhabdus</i> sp.	0.1	++	+
109	<i>Sphingorhabdus</i> sp.	0.1	++	+
Mix_4_2015	<i>Sphingorhabdus</i> sp. (50) + <i>Imperialibacter</i> sp. (83) + <i>Bosea</i> sp. (65)	0.1	++	+
Q8	<i>Undibacterium</i> sp.	0.1	++	+
ax2bis1	<i>Alteromonas</i> sp.	0.2	++	-
29b	<i>Bacillus aerius</i>	0.2	++	-
5a	<i>Bacillus idriensis</i>	0.2	++	-
123	<i>Bacillus megaterium</i>	0.3	++	+
71	<i>Bacillus mycoides</i>	0.2	++	-

<b>33b</b>	<i>Bacillus</i> sp.	0.2	++	-
<b>94b</b>	<i>Bacillus subtilis</i>	0.2	++	-
<b>97</b>	<i>Bosea</i> sp.	0.3	++	+
<b>121</b>	<i>Limnobacter</i> sp	0.3	++	-
<b>130</b>	<i>Limnobacter</i> sp.	0.1	++	-
<b>76B</b>	<i>Limnobacter</i> sp	0.2	++	-
<b>152</b>	<i>Limnobacter</i> sp	0.2	++	+
<b>Z68</b>	<i>Microcella</i> sp.	0.1	++	-
<b>74</b>	<i>Micrococcus aloeverae</i>	0.2	++	-
<b>17a</b>	<i>Moraxella osloensis</i>	0.2	++	-
<b>77</b>	<i>Paenibacillus</i> sp.	0.2	++	-
<b>117b2a</b>	<i>Pantoea</i> sp.	0.2	++	-
<b>T3</b>	<i>Plantibacter</i> sp.	0.3	++	-
<b>ax2bis2</b>	<i>Rhizobium</i> sp.	0.2	++	-
<b>25a</b>	<i>Sphingomonas</i> sp.	0.2	++	-
<b>T1</b>	<i>Sphingopyxis</i> sp.	0.2	++	-
<b>13a</b>	<i>Staphylococcus</i> sp.	0.2	++	+
<b>mix_2016</b>	Mix of 22 strains used in exp. 2016.	0.3	++	-
<b>Mix_2_2016</b>	<i>Bosea</i> sp. (97) + <i>Limnobacter</i> sp. (121) + <i>Limnobacter</i> sp. (76b) + <i>Limnobacter</i> sp. (152) + <i>Rhizobium</i> sp. (ax2bis2)	0.3	++	-
<b>Mix_5_2016</b>	<i>Plantibacter</i> sp. (T3) + <i>Paenibacillus</i> sp. (77) + <i>Staphylococcus</i> sp. (13a) + <i>Pantoea</i> sp (117b2a)	0.3	++	-
<b>Mix_3_2016</b>	<i>B. mycoides</i> (71) + <i>B. megaterium</i> (123) + <i>B. aerius</i> (29b) + <i>Bacillus</i> sp. (33b) + <i>B. idriensis</i> (5a) + <i>B. subtilis</i> (94b)	0.3	++	+
<b>Mix_4_2016</b>	<i>Rhizobium</i> (ax2bis2) + <i>Microcella</i> sp. (z68) + <i>Moraxella</i> sp. (17a) + <i>Micrococcus</i> sp. (74) + <i>Sphingomonas</i> sp (25a) + <i>Sphingopyxis</i> (T1)	0.3	++	+



Supplementary table 2.2 Mock community used for 16S rRNA gene metabarcoding

Taxon	Quantity in Mock (ng)	Nr of copies 16S rRNA gene	Genome size (Mb)	Concentration 16S rRNA	Relative concentration of 16S RNA (%)
<i>Zobellia amurskyensis</i>	2.4	4	5.2	1.85947E-06	0.05
<i>Zobellia galactanivorans</i>	12	2	5.5	4.34648E-06	0.12
<i>Zobellia laminariae</i>	60	2	5.1	2.33225E-05	0.64
<i>Zobellia russellii</i>	300	2	4.9	0.000121702	3.32
<i>Zobellia uliginosa</i>	300	2	5.3	0.000112939	3.08
<i>Formosa agariphila</i>	300	5	4.2	0.000354748	9.68
<i>Maribacter forsetii</i>	300	3	4.5	0.000199364	5.44
<i>Maribacter orientalis</i>	300	2	4.1	0.000144214	3.93
<i>Mariniflexile fucanivorans</i>	300	1	4.7	6.34688E-05	1.73
<i>Nonlabens Ulvanivorans</i>	300	2	3.7	0.000183177	5.00
<i>Polaribacter</i>	300	1	3.8	7.82967E-05	2.14
<i>Dokdonia</i>	300	1	3.5	8.58257E-05	2.34
<i>Cellulophaga</i>	300	1	4.7	6.44952E-05	1.76
<i>Winogradskyella</i>	300	1	4.3	6.95005E-05	1.90
<i>Imperialibacter roseus</i>	300	1	6.7	4.48113E-05	1.22
<i>Dinoroseobacter shibae</i>	47.25	2	3.8	2.49368E-05	0.68
<i>Roseobacter denitrificans</i>	300	1	4.3	6.92643E-05	1.89
<i>Roseovarius mucosus</i>	300	1	4.2	7.06261E-05	1.93
<i>Paracoccus</i> LD14	300	1	3.6	8.41942E-05	2.30
<i>Sphingomonas</i> sp.	300	1	3.3	9.14964E-05	2.50
<i>Hoeflea</i> sp.	300	1	5.2	5.73787E-05	1.57
<i>Bosea</i> sp.	300	1	6.3	4.73374E-05	1.29
<i>Alteromonas fortis</i>	300	3	4.7	0.00019296	5.26
<i>V. crassostreae</i>	300	8	5.8	0.000414674	11.31
<i>V. splendidus</i>	300	8	5.0	0.00048243	13.16
<i>MO2_Pseudoalteromonas</i>	300	1	4.8	6.24146E-05	1.70
<i>Psychrobacter</i>	300	1	2.7	0.000113178	3.09
<i>Cobetia</i> LD12	300	1	4.2	7.05889E-05	1.93
<i>Agrococcus</i> LD11	300	1	3.0	9.97967E-05	2.72
<i>Arthrobacter</i> LD09	300	1	3.4	8.82288E-05	2.41
<i>Microbacterium</i> LD19	300	1	2.9	0.000101774	2.78
<i>Rhodopirellula</i>	300	1	7.1	4.1984E-05	1.15
Total	8521.65			0.003665331	100.00

## Supplementary data Chapter 3

Supplementary Table 3-1 Overview of antibiotics used to modify algal holobionts and measure how this affects the algal response fresh water. The three holobionts used in subsequent experiments are marked with stars.

Antibiotic	Abbreviation	Concent ration (µg/ml)	Activity spectrum	Antibiotic class		Mechanism of action <sup>26 27</sup>	5% NSW	100% NSW
<b>Polymyxin B</b>	PolB	200	G-	Bacteriocidal	Polypeptides	Disrupts cell membrane integrity	++	++
<b>Penicillin</b>	Pen100	100	G+	Bacteriocidal	Beta-lactams	Inhibits cell wall synthesis	-	++
	Pen50	50					++	++
<b>Erythromycin</b>	Ery	50	G+, G	Bacteriostatic	Macrolide	Inhibition of protein synthesis (50S)	++	++
<b>Ampicillin</b>	Amp	50	G+, G-	Bacteriocidal	Beta-lactams	Inhibits cell wall synthesis	-	-
<b>Chloramphenicol</b>	Chl	5	G+, G-	Bacteriostatic	Unique compound	Inhibition of protein synthesis (50S)	++	++
<b>Ciprofloxacin</b>	Cip	50	G+, G-	Bacteriocidal	Quinolones	Interfere with DNA replication and transcription	++	-
<b>Kanamycin</b>	Kam25	25	G+, G-	Bacteriocidal	Aminoglycoside	Inhibition of protein synthesis (30S)	++	++
	Kam50	50					++	++
<b>Neomycin</b>	Neo	100	G+, G-	Bacteriocidal	Aminoglycoside	Inhibition of protein synthesis (30S)	++	++
<b>Rifampicin</b>	Ram100	100	G+, G-	Bacteriocidal	Ansamycins	Inhibits RNA synthesis	++	++
	Ram50	50	G+, G-				++	++
<b>Streptomycin</b>	Strep	25	G+, G-	Bacteriocidal	Aminoglycoside	Inhibition of protein synthesis (30S)	++	-
<b>Rifampicin, Penicillin, Neomycin</b>	Mix 1*	100 + 100 + 100					++	++

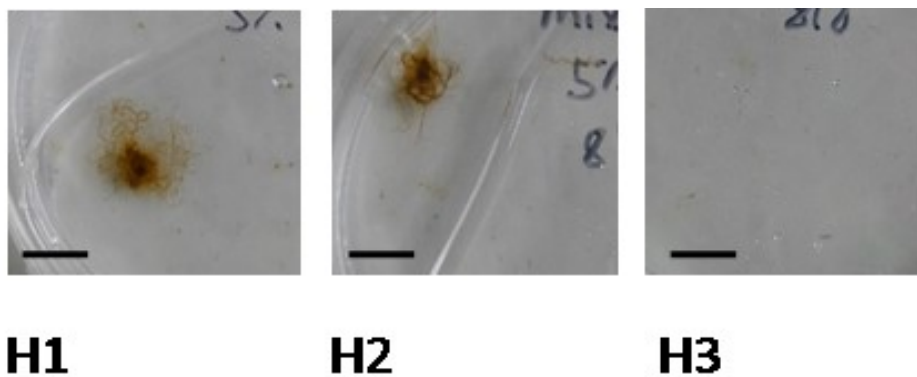
<sup>26</sup> <http://www.compoundchem.com/2014/09/08/antibiotics/>

<sup>27</sup> <https://www.drugbank.ca/drugs/DB00199>

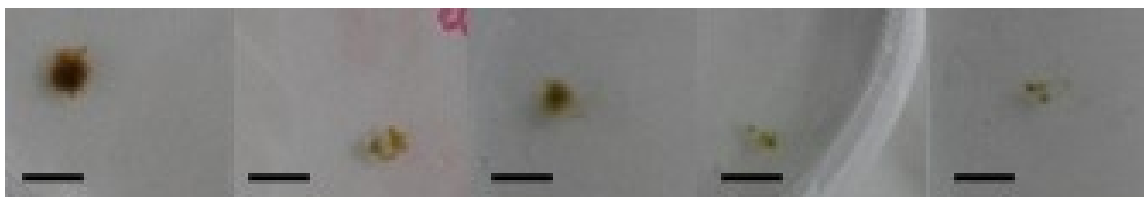
<b>Penicillin, Streptomycin, Chloramphenicol</b>	Mix 2	50 + 25 + 5		++	++
<b>Penicillin, Streptomycin, Neomycin</b>	Mix 3	100 + 25 + 100		-	++
<b>Rifampicin, Penicillin, Neomycin, Streptomycin, Chloramphenicol</b>	Mix 4*	100 + 100 + 100 + 25 + 5		++	++
<b>Penicillin, Chloramphenicol, Polymyxin B, Neomycin</b>	Mix 5*	12000UI + 0.75 + 0.75 + 0.9		-	++
<b>No antibiotics (control)</b>	Na	Na		++	++

## Verification of freshwater response in algal holobionts

The FW-response for each holobiont was verified before start of the experiment by transferring a small part of the tissue growing in 100% NSW to 5% NSW. H1 and H2 were able to grow successfully in low salinity, but H3 did not (Supplementary figure 3-1). To determine the salinity threshold that still allows growth of Holobiont 3, the corresponding culture was exposed to a range of salinities (Supplementary figure 3-2). The threshold for holobiont 3 that still allowed acclimation was 15% NSW and this concentration was thus used to perform the experiment.



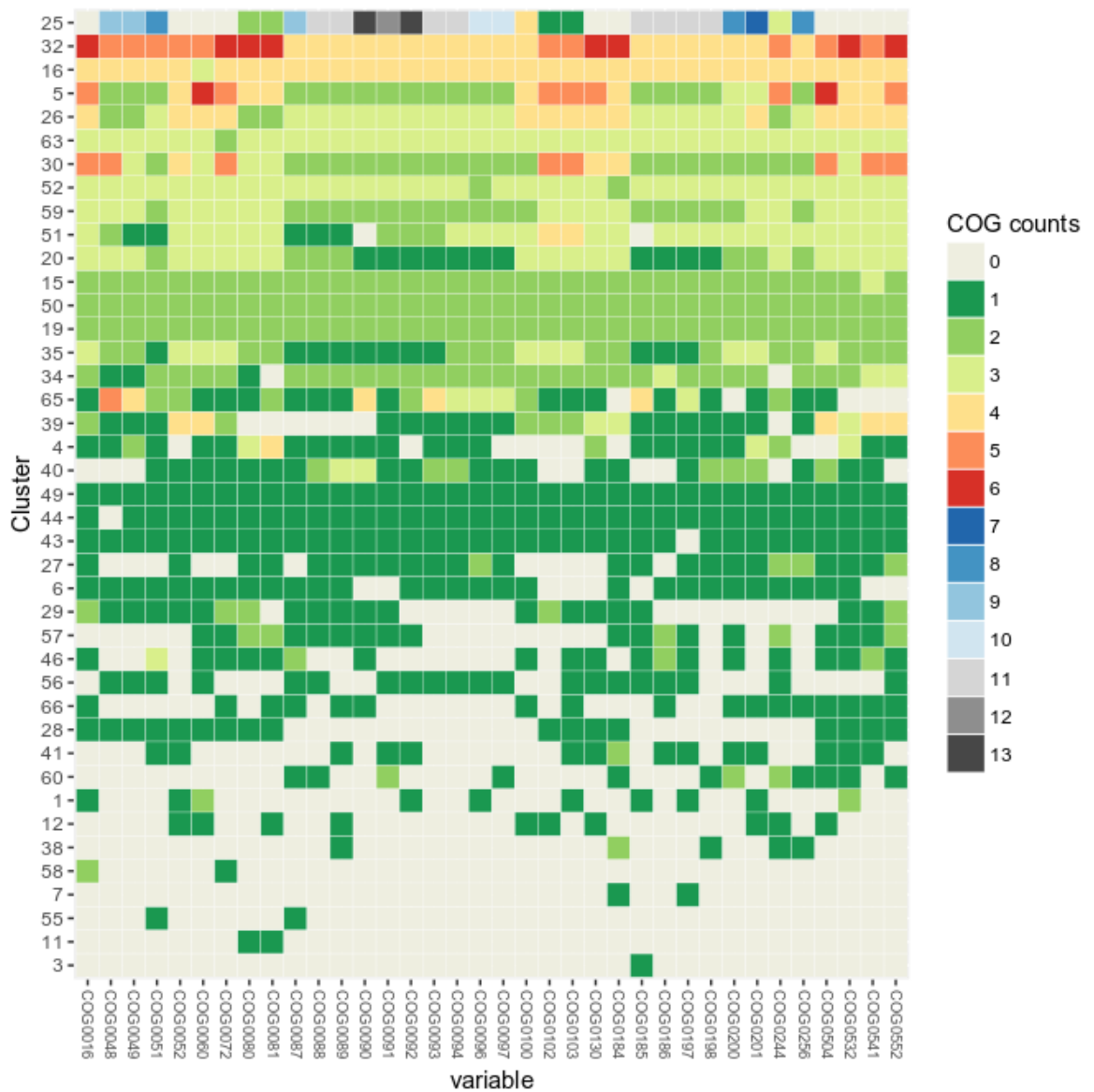
Supplementary figure 3-1 Algal holobionts growing in 5% NSW-PE. Pictures taken after 3 weeks after transfer from 100% NSW to 5% NSW. Scale bar = 1 cm; H1: holobiont 1; H2, holobiont 2; H3: holobiont 3. Pictures were taken 3 weeks after the transfer from 100% NSW to 5% NSW.



Supplementary figure 3-2 Holobiont 3 exposed to different levels of salinities (from left to right): 100% NSW, 25% NSW, 15% NSW, 10% NSW, and 5% NWS. Scale bar = 1 cm. Pictures were taken 1 week after the transfer from 100% NSW to salinity level tested. One of two replicates shown.

Supplementary table 3-2 Cleaning statistics of RNA data cleaning; separated per replicate.

	Algal mRNA	Bacterial mRNA	Mitochondrial & chloroplast mRNA	Ribosomal RNA (algal, bacteria, chloroplast, mitochondria)	Junk
1-1F	7.42%	0.56%	8.87%	81.70%	1.45%
13-1F	8.54%	1.44%	14.69%	73.76%	1.57%
19-1F	6.22%	0.44%	10.46%	81.81%	1.07%
6-1F	10.86%	1.31%	17.54%	68.07%	2.23%
20-1S	9.02%	1.53%	11.79%	76.31%	1.34%
25-1S	8.38%	2.24%	14.32%	73.40%	1.65%
26-1S	7.95%	1.19%	14.69%	74.28%	1.90%
7-1S	10.91%	0.62%	20.63%	65.70%	2.15%
15-2F	4.77%	2.82%	5.47%	85.95%	0.99%
21-2F	4.92%	2.78%	5.42%	86.17%	0.71%
27-2F	4.11%	3.90%	3.48%	87.66%	0.85%
3-2F	3.68%	1.44%	3.74%	90.62%	0.52%
16-2S	4.20%	2.77%	2.99%	89.13%	0.90%
22-2S	9.67%	3.05%	8.35%	77.53%	1.40%
28-2S	8.69%	1.79%	6.28%	82.08%	1.16%
4-2S	10.62%	4.86%	8.69%	74.26%	1.58%
10-3F	6.23%	12.17%	5.38%	74.52%	1.71%
23-3F	3.90%	6.25%	4.09%	84.84%	0.92%
29-3F	2.48%	6.75%	2.40%	87.57%	0.80%
5-3F	1.72%	3.78%	1.15%	92.59%	0.76%
12-3S	3.17%	1.87%	4.20%	90.07%	0.68%
18-3S	3.82%	2.00%	3.77%	89.71%	0.69%
24-3S	3.99%	2.18%	5.47%	87.59%	0.77%
30-3S	8.10%	4.44%	11.96%	73.92%	1.57%



Supplementary figure 3-3 Heatmap visualization of the 36 different single-copy core genes (horizontal) in each of the by concoct defined metagenomic bins (vertical). The figure was produced using the COGplot.r script which is integrated in the concoct pipeline.

Supplementary table 3-3 Parameter settings for the LC-MS pre-processing within the workflow4metabolomics galaxy environment.

<b>function</b>	<b>what it does</b>	<b>argument</b>	<b>value</b>
<b>xcmsSet</b>	Filtration and Peak Identification	nSlaves	4
		method	centWave
		ppm	5
		peakwidth	520
		mzdiff	0.05
		snthresh	6
		integrate	1
		noise	25000
		prefilter	310000
<b>xcms.group</b>	Group peaks together across samples	method	density
		minfrac	0.1
		bw	5
		mzwid	0.01
		sleep	0.001
		max	10
<b>xcms.retcor</b>	Retention Time Correction	method	peakgroups
		smooth	loess
		extra	1
		missing	1
		span	0.2
		family	gaussian
		plottype	deviation
<b>xcms.group</b>	Group peaks together across samples	method	density
		minfrac	0.1
		bw	3
		mzwid	0.01
		sleep	0.001
		max	10
<b>xcms.fillPeaks</b>	Integrate a sample's signal in regions where peak groups are not represented to create new peaks in missing areas		chrom
<b>CAMERA.annotate</b>	Annotation of isotope peaks, adducts and fragments		

Supplementary table 3-4 Parameter settings for the GC-MS pre-processing within the workflow4metabolomics galaxy environment.

<b>Input Parameter</b>	<b>Value</b>
Multiplier of the standard deviation	6
Percentage of FWHM width	0.6
General ppm error	5
General absolut error in m/z	0.015
Max. ion charge	3
Max. number of expected isotopes	4
The percentage number of samples, which must satisfy the C12/C13 rule for isotope annotation	0.5
Mode	FALSE
groupCorr: correlation threshold (0..1)	0.75
groupCorr: Method selection for grouping peaks after correlation analysis into pseudospectra	hcs
groupCorr: significant correlation threshold	0.05
groupCorr: Use correlation inside samples for peak grouping	True
groupCorr: Use isotopic relationship for peak grouping	False
groupCorr: Use correlation across samples for peak grouping	False
Which polarity mode was used for measuring of the ms sample	positive
How much peaks will be calculated in every thread using the parallel mode	100
Use a personal ruleset file	FALSE
If no ruleset is provided, calculate ruleset with max. number n of [nM+x] clusterions	3
Number of condition	show
Number of the most significantly different analytes to create EICs for	0
Width (in seconds) of EICs produced	200
Intensity values to be used for the diffreport	into
Numeric variable for the height of the eic and boxplots that are printed out	480
Numeric variable for the width of the eic and boxplots print out made	640
Number of decimal places of title m/z values in the eic plot	2
logical indicating whether the reports should be sorted by p-value	False
Convert retention time (seconds) into minutes	False
Number of decimal places for mass values reported in ions' identifiers.	4
Number of decimal places for retention time values reported in ions' identifiers.	0
General used intensity value	into



Supplementary table 3-5 Read processing statistics of 16S rRNA gene metabarcoding that was carried out on the three algal holobionts before the implementation of metatranscriptome/metagenome approach.

Replicate	Sample name	Raw sequences	Clean reads	% of reads kept	Number of contigs	Nr of OTUs
<b>H0_SW_a</b>	H0_SW_a_fw	151266	148214	98.0%	144152	52
	H0_SW_a_rv	151266	146852	97.1%		
<b>H0_SW_b</b>	H0_SW_b_fw	132784	130160	98.0%	126370	53
	H0_SW_b_rv	132784	128670	96.9%		
<b>H1_SW_a</b>	H1_SW_a_fw	138692	135851	98.0%	131769	59
	H1_SW_a_rv	138692	134288	96.8%		
<b>H2_SW_a</b>	H2_SW_a_fw	122017	119259	97.7%	109827	51
	H2_SW_a_rv	122017	111974	91.8%		
<b>H2_SW_b</b>	H2_SW_b_fw	118288	115758	97.9%	113071	59
	H2_SW_b_rv	118288	115320	97.5%		
<b>H3_SW_a</b>	H3_SW_a_fw	116547	113440	97.3%	83082	42
	H3_SW_a_rv	116547	84937	72.9%		
<b>H3_SW_b</b>	H3_SW_b_fw	149944	146022	97.4%	144052	56

Supplementary table 3-6 List of differentially expressed genes in holobiont1, 2, and 3 in 15% NSW compared to 100% NSW; in H1H2S compared to H3S (microbiome effect), and in H1H2F compared to H3F (interaction term).

Supplementary table 3-7 Complete overview of gene enrichment analysis of down-regulated genes (LFC < 0) and up-regulated genes (LFC > 0) in H1H2S compared to H3S (microbiome effect), in H1H2FvsH3F (interaction term), in H3F compared to H3S, and in H2F compared to H2S. Diff: difference in intensity values between the two treatments.

Supplementary table 3-8 Overview of the bacterial bins that were obtained via metagenome assembly and manual binning.

<b>Bins</b>	<b>Taxon</b>	<b>Length (kb)</b>	<b>Contigs</b>	<b>N50</b>	<b>GC-content</b>	<b>% completion</b>	<b>% redundancy</b>
<b>Bin_48_full_s_c97</b>	<i>Ilumatobacter</i> sp.	5165927	26	330188	63.08	97.12	3.60
<b>Bin_49_partial_s_c14</b>	<i>Ilumatobacter</i> sp.	1303659	453	2848	64.80	14.39	-
<b>Bin_35_partial_s_c17</b>	<i>Unclassified</i> <i>Flavobacteriales</i>	573786	214	2594	36.13	16.55	-
<b>Bin_18_full_s_c99</b>	<i>Maribacter</i>	4335843	19	381789	35.82	98.56	0.72
<b>Bin_44_full_s_c94</b>	<i>Unclassified</i> <i>Flammeovirgaceae</i>	7233000	29	484497	45.28	94.24	2.88
<b>Bin_70_full_s_c99</b>	<i>Unclassified</i> <i>Flavobacteriales</i>	4319737	16	509936	38.34	99.28	0.72
<b>Bin_73_full_s_c96</b>	<i>Unclassified</i> <i>Bacteroidetes</i>	8749299	38	451152	44.36	96.40	0.72
<b>Bin_14_full_s_c97</b>	<i>Unclassified</i> <i>Bacteroidetes</i>	5154297	12	618067	45.72	97.12	0.72
<b>Bin_13_partial_s_c4</b>	<i>Unclassified</i> <i>Bacteroidetes</i>	1487596	424	3657	60.77	3.60	-
<b>Bin_69_partial_s_c87</b>	<i>Roseovarius</i>	3284045	27	220361	61.26	87.05	-
<b>Bin_45_partial_s_c85</b>	<i>Antarctobacter</i>	4647251	26	293882	61.80	84.89	-
<b>Bin_58_full_s_c97</b>	<i>Unclassified</i> <i>Flavobacteriales</i>	6740106	58	214397	46.59	97.12	1.44
<b>Bin_61_full_m_c99_r18</b>	<i>Polaribacter</i>	4315843	70	469891	30.01	98.56	17.99
<b>Bin_15_full_s_c93</b>	<i>Unclassified</i> <i>Bacteroidetes</i>	8070005	203	61529	52.94	92.81	2.16
<b>Bin_37_full_s_c95</b>	<i>Rhodopirellula</i> sp.	7466849	130	89089	59.51	94.96	2.88
<b>Bin_68_full_s_c95</b>	<i>Unclassified</i> <i>Planctomycetes</i>	5201868	49	199999	61.78	94.96	7.19
<b>Bin_63_full_s_c98</b>	<i>Phycisphaera</i> sp.	3445259	4	1109408	67.20	97.84	2.16
<b>Bin_46_partial_s_c2</b>	<i>Halomonas</i> sp.	1313842	249	7061	46.27	2.16	-
<b>Bin_74_full_m_c99_r16</b>	<i>Alteromonas</i>	5823541	121	449994	43.73	98.56	15.83
<b>Bin_34_partial_s_c76</b>	<i>Unclassified</i> <i>Bacterium</i>	4346962	60	828154	36.77	75.54	-
<b>Bin_22_full_s_c99</b>	<i>Unclassified</i> <i>Rhizobiales</i>	3867490	8	551257	45.01	98.56	-
<b>Bin_11_full_s_c94</b>	<i>Unclassified</i> <i>Rhizobiales</i>	4314944	30	334579	56.19	93.53	3.60

<b>Bin_21_full_s_c99</b>	<i>Unclassified Rhizobiales</i>	4412484	33	181600	58.90	99.28	-
<b>Bin_4_partial_s_c40</b>	<i>Alteromonas</i>	3386756	244	24944	43.16	40.29	0.72
<b>Bin_62_partial_s_c48</b>	<i>Unclassified Rhizobiales</i>	2421120	601	4135	57.65	48.20	0.72
<b>Bin_53_partial_s_c69</b>	<i>Unclassified Alphaproteobacterium</i>	2957606	133	33089	55.40	69.06	0.72
<b>Bin_30_full_s_c99</b>	<i>Unclassified Alphaproteobacterium</i>	3367437	92	74864	55.03	98.56	0.72
<b>Bin_36_full_s_c99</b>	<i>Unclassified Hyphomicrobiaceae</i>	3786868	17	322945	57.60	98.56	2.16
<b>Bin_9_partial_s_c18</b>	<i>Devosia</i> sp.	1088585	193	7274	60.42	17.99	0.72
<b>Bin_54_full_s_c100</b>	<i>Unclassified Alphaproteobacterium</i>	4075657	12	642168	49.89	100.00	1.44
<b>Bin_27_partial_s_c60</b>	<i>Unclassified Alphaproteobacterium</i>	3441462	27	317410	56.93	60.43	0.72
<b>Bin_10_partial_s_c65</b>	<i>Yangia</i>	4646955	52	120478	67.21	64.75	0.72
<b>Bin_12_partial_s_c38</b>	<i>Unclassified Enterobacteriaceae</i>	1218105	408	2924	52.57	38.12	1.43
<b>Bin_59_partial_s_c78</b>	<i>Unclassified Rhizobiales</i>	3141578	21	322993	49.48	77.70	1.44
<b>Bin_51_partial_s_c42</b>	<i>Devosia</i> sp	2411216	438	6319	62.63	42.45	1.44
<b>Bin_40_partial_s_c86</b>	<i>Marinobacter</i>	3661616	33	274759	59.31	85.61	1.44
<b>Bin_71_partial_s_c78</b>	<i>Sphingorhabdus</i>	3337733	10	751824	58.12	78.42	2.16
<b>Bin_2_partial_s_c75</b>	<i>Unclassified Rhodobacterales</i>	4249049	116	154097	59.72	74.82	2.16
<b>Bin_39_partial_s_c85</b>	<i>Unclassified Alphaproteobacterium</i>	6231921	136	70285	57.71	84.89	2.88
<b>Bin_64_partial_s_c66</b>	<i>Unclassified Alphaproteobacterium</i>	7437506	69	171398	55.96	66.19	2.88
<b>Bin_43_partial_s_c65</b>	<i>Marinobacter</i>	4391377	36	487078	57.20	64.75	2.88
<b>Bin_72_partial_s_c8</b>	<i>Unclassified Streptosporangiceae</i>	6068071	1201	5901	66.28	8.02	3.70
<b>Bin_47_partial_s_c34</b>	<i>Sulfitobacter</i> sp.	2652926	596	5148	61.25	33.81	4.32
<b>Bin_60_partial_s_c28</b>	<i>Sulfitobacter</i>	2651458	524	5436	64.10	28.06	4.32
<b>Bin_56_partial_s_c62</b>	<i>Unclassified Alphaproteobacterium</i>	4989316	705	10227	52.59	61.87	5.76
<b>Bin_29_full_m_c100_r18</b>	<i>Hoeflea</i>	4935401	137	2774898	61.74	100.00	17.99

<b>Bin_5_partial_s_c70</b>	<i>Sulfitobacter</i>	4841471	287	134708	60.20	69.78	6.47
<b>Bin_31_full_s_c99</b>	<i>Hyphomonas</i> sp.	3060655	23	307362	54.01	99.28	0.72
<b>Bin_42_full_m_c98_r21</b>	<i>Unclassified</i> <i>Rhodobacteraceae</i>	4045944	90	100637	64.40	97.84	20.86
<b>Bin_8_partial_s_c23</b>	<i>Hoeflea</i>	3110191	331	14308	60.79	23.02	7.19
<b>Bin_24_partial_s_c84</b>	<i>Sulfitobacter</i> sp.	5662040	387	35614	57.71	83.45	7.91
<b>Bin_65_partial_m_c25_r14</b>	<i>Unclassified organism</i>	18994926	3194	7351	59.47	24.46	14.39
<b>Bin_16_full_s_c99</b>	<i>Sphingorhabdus</i>	3567444	9	816851	56.67	99.28	1.44
<b>Bin_38_full_s_c99</b>	<i>Hyphomonas</i> sp.	3927588	108	430158	59.64	99.28	4.32
<b>Bin_52_full_s_c100</b>	<i>Erythrobacter</i>	3137991	5	1387267	62.82	100.00	-
<b>Bin_55_full_s_c99</b>	<i>Roseovarius</i>	3584632	26	219210	61.04	99.28	-
<b>Bin_57_full_s_c99</b>	<i>Unclassified</i> <i>Rhizobiales</i>	3393346	2	3122496	49.81	99.28	-
<b>Bin_6_full_s_c99</b>	<i>Brevundimonas</i>	2608479	13	416070	66.93	99.28	-
<b>Bin_25_full_s_c98</b>	<i>Unclassified</i> <i>Myxococcales</i>	8711421	78	189923	63.28	97.12	1.44
<b>Bin_23_full_s_c100</b>	<i>Erythrobacter</i> sp.	2943928	8	535557	57.04	100.00	-
<b>Bin_67_full_s_c100</b>	<i>Halomonas</i>	5778650	76	214372	54.60	100.00	0.72
<b>Bin_19_partial_m_c30_r15</b>	<i>Hoeflea</i>	6110204	414	28767	58.94	30.22	15.11
<b>Bin_7_full_s_c99</b>	<i>Sphingorhabdus</i>	3853638	16	376496	54.04	99.28	-
<b>Bin_28_full_s_c99</b>	<i>Vibrio</i>	5214155	63	159036	44.21	99.28	3.60
<b>Bin_26_partial_m_c50_r16</b>	<i>Unclassified</i> <i>Rhodobacterales</i>	2330028	542	4047	63.51	52.52	15.83
<b>Bin_17_partial_m_c82_r33</b>	<i>Halioglobus</i> sp.	6694539	403	34916	61.38	82.01	33.09
<b>Bin_3_partial_m_c37_r42</b>	<i>Unclassified</i> <i>Rhodobacterales</i>	2378758	217	20975	55.83	37.41	41.73
<b>Bin_41_partial_m_c85_r49</b>	<i>Unclassified</i> <i>Flavobacteriaceae</i>	7639462	543	29866	37.08	84.17	48.92
<b>Bin_50_full_s_c97</b>	<i>Unclassified</i> <i>Proteobacterium</i>	4470076	57	189623	62.46	97.12	0.72
<b>Bin_66_partial_m_c66_r56</b>	<i>Unclassified</i> <i>Alphaproteobacterium</i>	8974827	345	46368	66.15	66.19	56.12
<b>Bin_32_full_s_c100</b>	<i>Unclassified Bacterium</i>	3535771	35	385799	41.96	100.00	-
<b>Bin_1_partial_m_c45_r58</b>	<i>Unclassified</i> <i>Proteobacterium</i>	5165325	539	25621	52.85	45.32	58.27
<b>Bin_20_partial_m_c46_r123</b>	<i>Unclassified</i> <i>Rhodobacteraceae</i>	5431513	383	44778	59.83	46.04	123.02

Supplementary table 3-9 Overview of significant metabolites in the LC-MS and GC-MS data and their annotations. Diff: difference in intensity values between the two treatments. LCpos: LC data in positive mode; LCneg: LC data in negative mode; RT: retention time;

## **Annex 2 The visualization and localization of bacteria on the surface of *Ectocarpus subulatus* FWS using FISH and SEM techniques.**

### **Introduction**

The aim of this work is to visualize and localize bacteria on the *Ectocarpus* surface and monitor changes during the acclimation process, and complement some of the results described in previous chapters, where we gathered information on the microbiome of *Ectocarpus* and its role of bacteria within the holobiont. For example, after the sterilization procedure, we do not see any viable bacteria with microscopy, nor was there bacterial growth on plates. However, 16S rRNA gene metabarcoding showed that a large number of OTUs is still present in the algal culture, despite these observations. Especially *Hoeflea* sp. was shown to be very abundant in all antibiotic treated algal cultures (Chapter 2 – subsection III). Bacteria that are not completely removed by the antibiotic treatment, could be resistant or tolerant to the antibiotics that were applied (but we should be able to see the remaining bacteria in that case); bacteria may remain viable but in low abundance after the antibiotic treatment and thus difficult to detect by eye; alternatively, the bacteria may find protection by “hiding” in the cell wall, or intracellularly. One strategy to find out what could be the exact underlying cause, is via Fluorescent In Situ Hybridization (FISH). FISH is a technique used to visualize and identify cells in their natural environment using 16S rRNA oligonucleotide probes and it combines spatial information with phylogeny/taxonomy.

FISH requires the design of oligonucleotide probes and the development of a hybridization method that is specific enough to let FISH probes bind to target sequences, but limit aspecific binding to sequences of other microorganisms in the environment. This section of my thesis describes the first steps of those two processes, *i.e.* probes design and optimization of the hybridization protocol. The FISH experiments were complemented with scanning electron microscopy (SEM) of the *Ectocarpus* surface. Both experiments were performed at Marine Scotland Science (Aberdeen; Collaborator Eileen Bresnan) and Aberdeen University (Kevin MacKenzie).

## Fluorescent *in situ* hybridization (FISH)

### Selection of targets

Genus specific probes were designed for *Hoeflea*, *Marinobacter*, and *Imperialibacter*. All three had a strong effect on morphology of Ec32 (Chapter 2 – subsection III). Pre-existing probes were ordered that target *Betaproteobacteria* (BET42a; Manz et al., 1992), *Gammaproteobacteria* (GAM42a), *Alphaproteobacteria* (ALF968), *Bacteroidetes* (CFB563; Weller et al., 2000), *Rhizobiales* (RHIZ1244; Thayanukul et al., 2010), the *Roseobacter* clade (ROS537; Eilers et al., 2000), and all eubacteria (EUB338; Amann et al., 1990). For an overview, see Supplementary Table 4-1.

### Probe design

Probes were designed using the PROBE\_DESIGN tool of the ARB software package (Ludwig *et al.*, 2004), according to the protocol described by Hugenholtz et al. (2002; probe length 18 bp; Temperature 55-100; GC-content 50-100; Max Non Group Hits 10; Min Group Hits 50%). They targeted hypervariable regions of the bacterial 16S sequence. Specificity of the probe was verified by blast searches and the Silva test-probe utility<sup>28</sup>. Manual verification of specificity was done by aligning (using MAFFT; Katoh et al., 2002) the probe sequences with 16S gene sequences obtained from target and non-target species that were present in the *E. subulatus* 16S metabarcoding data and/or *E. subulatus* bacterial culture collection (Dittami *et al.*, 2016; KleinJan *et al.*, 2017). In this way, probes were selected that were specific towards sequences found in association with *Ectocarpus*, and not necessarily specific for the genus as a whole. Probes were checked regarding self-complementarity, formation of hairpins/dimers, and melting temperature using OligoAnalyzer 3.1<sup>29</sup>. The optimal formamide concentration was estimated using the ProbeMelt<sup>30</sup> function of the Decipher software package (Wright *et al.*, 2014). Results of those calculations are plotted in Figure 4-1. Probes were ordered from Eurofins (Ebersberg, Germany) and 5' end-labeled with the cy3, fluorescein, or Texas Red fluorochromes. Those fluorophores were chosen based on their excitation spectra, as they minimize the overlap with the excitation caused by auto fluorescence of the chloroplasts/pigments which is between 640 - 780 nm (personal communication; D. Scornet). I

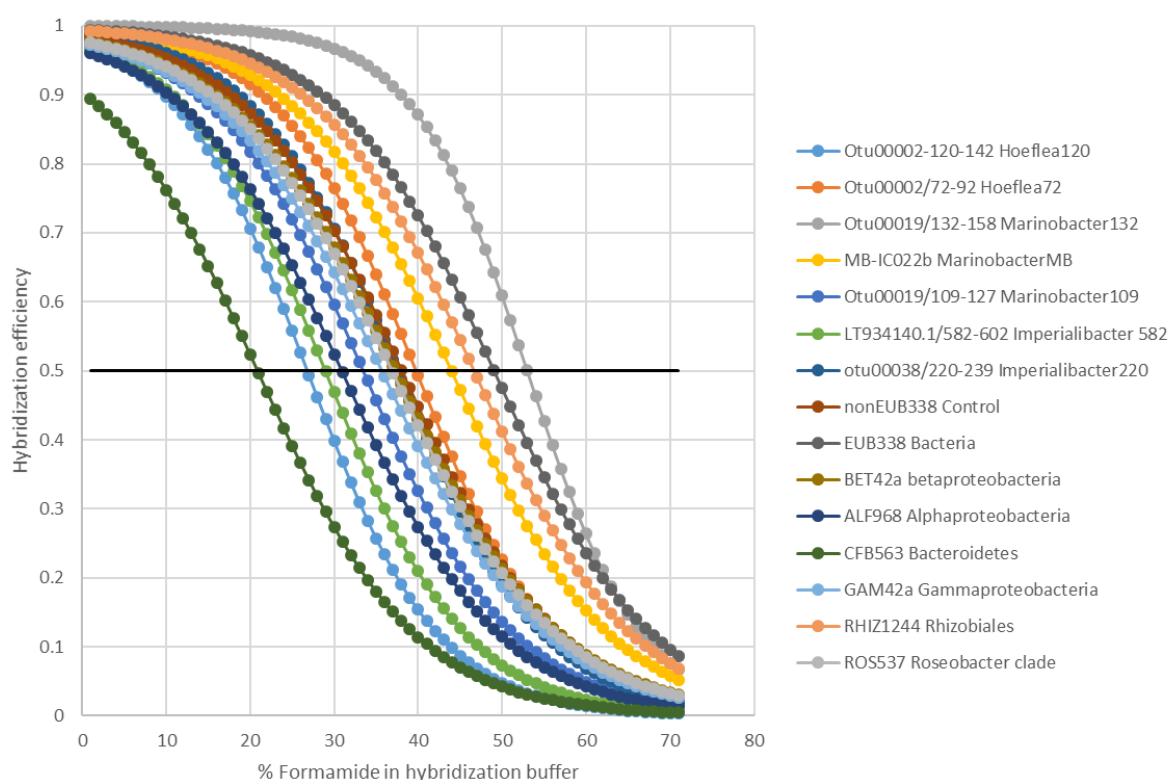
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<sup>28</sup> <https://www.arb-silva.de/?id=650>

<sup>29</sup> <https://eu.idtdna.com/calc/analyzer>

<sup>30</sup> <http://www2.decipher.codes/ProbeMelt.html>

chose three different fluorophores, so that communities of three bacteria could be visualized at the same time. Probes were delivered lyophilized and resuspended in 10 mM TrisHCl 1 mM EDTA. Working solution of 50 ng/μl probe (diluted with TrisHCl – no EDTA) were aliquoted and stored at -20 C to avoid excessive freezing-thawing of the probe stocks. An overview of all probes can be found in Supplementary Table 4-1.



Supplementary Figure 4-1 predicted hybridization efficiency of the probe/target pair at 0% to 70% (v/v) formamide and 46°C.

Supplementary Table 4-1 Oligonucleotideprobes that were used in the study.

Name	fluorophore	Target group	Probe sequence (5-3)	Formamide %	Target site	Reference
Otu00002-120-142	cy3	<i>Hoeflea</i>	CTCAAGATCGCCA GTATGAAAGG	to be determined	V3-V4	PhD thesis
Otu00002-72-92	cy3	<i>Hoeflea</i>	CACCCCTCACTTAA CGATCCG	to be determined	V3-V4	PhD thesis
Otu00019/132-158	fitc	<i>Marinobacter</i>	ACACTCCTCTACCA TACTCTAGCCTGA	to be determined	V3-V4	PhD thesis
MB-IC022b	fitc	<i>Marinobacter</i> <i>sp. strain IC022</i> <i>group</i>	GTTTCCGCCCGACT TGCA	25	V1	Brinkmeyer et al., 2003



Otu00019 109-127	fitc	<i>Marinobacter</i>	TCGAAATGCCGTTC CCAGG	to determined	be V3- V4	PhD thesis
LT934140. 1 582-602	fitc	<i>Imperialibacter</i>	CGCTTACCTCAACC AAACTCA	to determined	be V3- V4	PhD thesis
otu00038 220-239	fitc	<i>Imperialibacter</i>	TCAGTATCGGCCCA GTAAGC	to determined	be V3- V4	PhD thesis
nonEUB33 8	cy3	control complementary to EUB338	ACTCCTACGGGAG GCAGC	20	V2- V3	Wallner et al., 1993
EUB338	cy3	most bacteria	GCTGCCTCCCGTAG GAGT	20	V2- V3	Amann et al., 1990
BET42a	cy3	<i>Betaproteobacte ria</i>	GCCTTCCCACCTCG TTT	35	23s	Manz et al., 1992
ALF968	cy3	<i>Alphaproteobact eria</i>	GGTAAGGTTCTGC GCGTT	20	V5	Glöckner et al., 1999
CFB563	cy3	<i>Bacteroidetes</i>	GGACCCTTTAAACC CAAT	20	V3	Weller et al., 2000
GAM42a	cy3	<i>gammaproteoba cteria</i>	GCCTTCCCACATCG TTT	35	23s	Manz et al., 1992
RHIZ1244	cy3	<i>Order Rhizobiales</i>	TCGCTGCCCACTGT CACC	50	V7	Thayanuku l et al., 2010
ROS537	texas red	<i>Roseobacter clade</i>	CAACGCTAACCCC CTCC	35	V3- V4	Eilers et al., 2000

### Specificity testing with bacterial cultures and algal filaments

A homemade filtration system made from 15 ml tubes with a cell strainer unit at one end was used to filter the bacterial cultures. The 15 ml tubes were attached to a manifold/vacuum system and all washing steps were done using this system.

A volume of 20 to 100 µl of liquid bacterial cultures and/or one *Ectocarpus* filament (washed first) were transferred to 1.2 µm filters and remaining liquid was removed using the vacuum pump. Bacterial cultures and/or algal tissue was fixed in 4% paraformaldehyde (PFA) in 67% natural seawater over night at 4 °C in parafilm-sealed tubes.

PFA was removed and filters were washed in 2 ml of 100% ethanol:PBS (1:1) solution. Filters were incubated for 2 minutes in 100% ethanol to bleach the cells. After removal of the ethanol, filters were air-dried to remove any remaining liquid. Filters were incubated with 400 µl of hybridization buffer (Supplementary Table 4-2) for 2 minutes after which 4 µl of probe (50 ng/µl working solution) were added to the hybridization solution. This and all following steps were carried under protection from light. Hybridization took place at 46 °C (hybridization oven) in the dark for 90 minutes, in closed tubes to prevent dehydration. After hybridization, the filters

were incubated in pre-warmed washing buffer (Table 4-2) at 46 °C for 15 minutes. Filters were washed in water once more before letting them air-dry.

The filters were stained with 25 µl of dapi solution (5 ug/ml) for 5 minutes at room temperature (RT), washed in 2 ml distilled H<sub>2</sub>O (d H<sub>2</sub>O), washed in 2 ml of 80% ethanol, and finally air-dried before mounting them to a glass slide and adding a coverslip. SlowFade™ Gold Antifade (ThermoFisher Scientific) was added to each filter to maintain the fluorescence. Samples were observed either with the EVOS®FL digital inverted fluorescent microscope (ThermoFisher Scientific) with GFP for probes tagged with FITC; RFP for probes tagged with Cy3 and Texas red, at 400x magnification, or with the Axio imager M2 confocal microscope (ThermoFisher Scientific) with DsRed (Cy3 and Texax red tagged probes) or ALEXA fluor 489 (FITC tagged probes), at x630 magnification.

Supplementary Table 4-2 Hybridization and washing buffer used to hybridize the oligonucleotide probes. Optimal formamide concentrations vary between probes, thus the solutions are given dependent on the amount of formamide added. Volumes are given in milliliters.

	Hybridization buffer			
	formamide 20%	formamide 25%	formamide 35%	formamide 50%
	(non)EUB388 ALF968 CFB563	MB-IC022b	BET42a GAM42a ROS537	RHIZ1244
5M NaCl	1.8	0.9	1.8	0.9
1M Tris-HCL pH 7.4	0.2	0.1	0.2	0.1
Formamide (99.5%)	2	1.25	3.5	2.5
SDS 10%	0.01	0.005	0.01	0.005
EDTA 0.5 M	not added	not added	not added	not added
Sterile water	5.99	2.745	4.49	1.495
total volume	10	5	10	5
	Washing buffer			
	formamide 20%	formamide 25%	formamide 35%	formamide 50%
5M NaCl	2.15	1.49	0.7	0.18
1M Tris-HCL pH 7.4	1	1	1	1
Formamide (99.5%)	0	0	0	0
SDS 10%	0.05	0.05	0.05	0.05
EDTA 0.5 M	0.5	0.5	0.5	0.5
Sterile water	46.3	46.96	47.75	48.27
total volume	50	50	50	50

## Results & conclusions

All pre-existing probes were tested for the specificity by incubating them with both a target and a non-target bacterial strain. First trials made clear that the auto fluorescence of the filters was high. Nevertheless, it was possible to visualize the bacterial cells and it gave a first confirmation that the hybridization protocol worked on the liquid bacterial cultures (Supplementary Table 4-

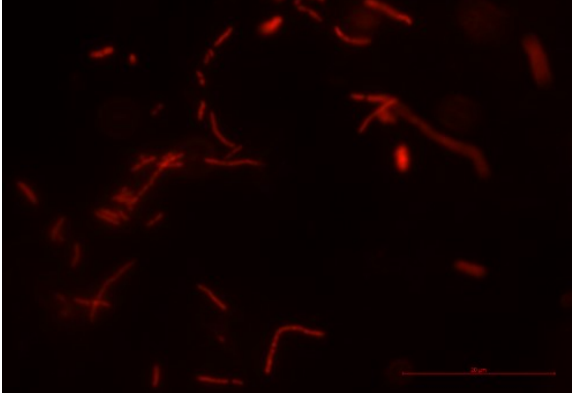
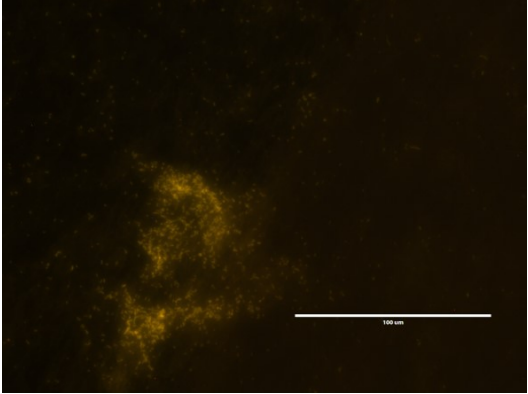
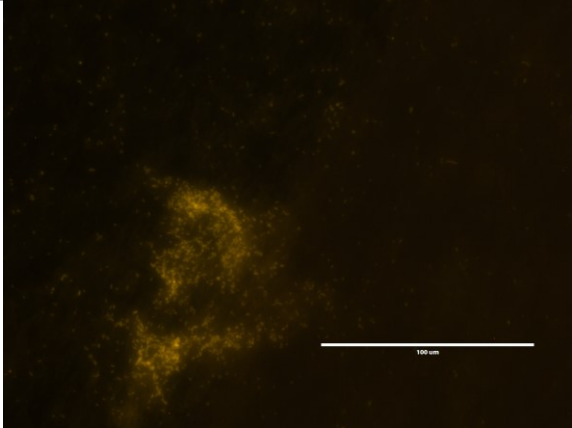
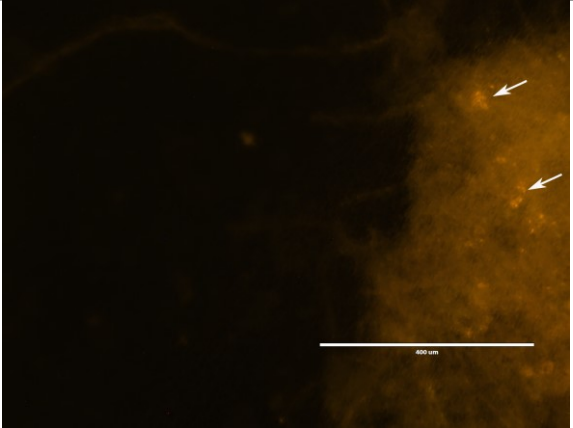
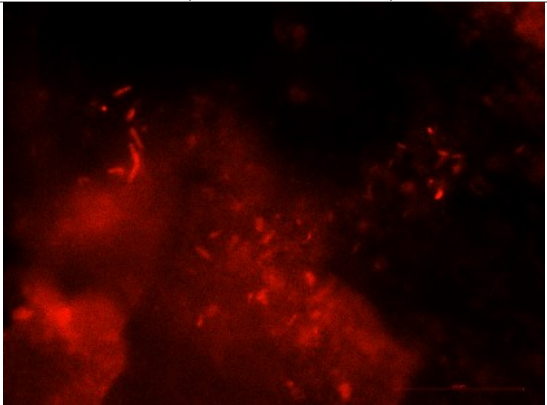
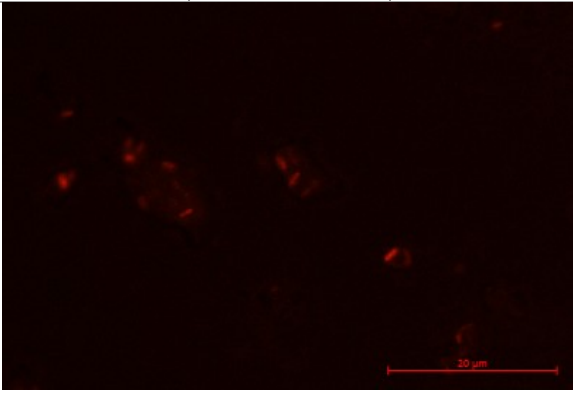
3 and 4-4). It was not possible to nicely visualize nicely the *Ectocarpus* filament. The amount of tissue was too high and it was highly auto fluorescent. Probes that did not work in the experiment were CFB563, MB-IC022b, and ROS537. One reason may be that the bacterial cultures used to test the probes were too dense/too old, or contained too many aggregates. The aggregates may block the filter which may negatively affect the washing and hybridization steps, and reduce the fluorescent signal. This may be the case for strain 420 in combination with probe ROS537, as the washing steps took much longer than for the other tested strains. This hypothesis could be in the future verified by testing targeting the bacterial culture with the general EUB338 probe in parallel to the taxa specific probe to separate culture dependent effects from probe specificity effects. Another reason for the high fluorescence signal may be that a too high concentration of probe solution was added to the filter. The autofluorescence of the filter may then overrule the signal exerted by the bacteria. This was the case for probe MB-IC022b plus 377, and for probe CFB 563 plus R9.

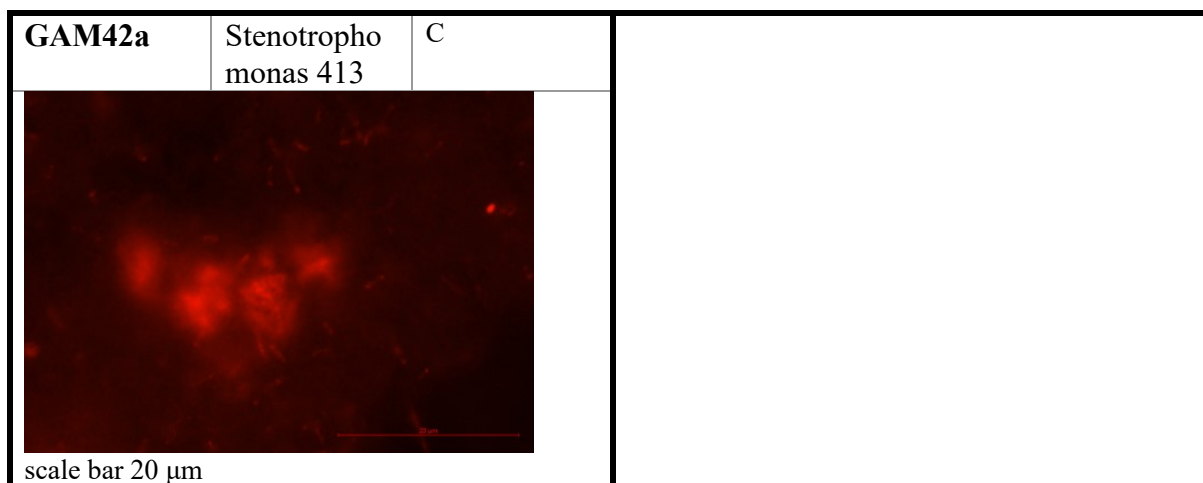
These few tests were the very first steps towards the development of FISH staining techniques on bacteria associated with the brown alga *Ectocarpus*. These data are clearly incomplete and require much more work before the method can be used to stain bacteria on *Ectocarpus* filaments.

Supplementary Table 4-3 Results of the specificity test on target and non-target strains; probes were tested on bacteria from within the target genus and outside target genus. NEGATIVE=no signal observed; POSITIVE=fluorescence signal was observed.

		Positive control		Negative control	
		Strain tested	Score	Negative target strain	Score
<b>EUB388</b>	Confocal	R9	POSITIVE	Sphingomonas	POSITIVE
<b>EUB388</b>	EVOS_FL	Not observed		Sphingomonas	POSITIVE
<b>ALF968</b>	EVOS_FL	<i>Bosea</i>	NEGATIVE	<i>Stenotrophomonas</i>	NEGATIVE
<b>ALF968</b>	Confocal	<i>Bosea</i>	POSITIVE	<i>Stenotrophomonas</i>	Not taken
<b>BET42a</b>	EVOS_FL	<i>Bosea</i>	POSITIVE	<i>Limnobacter</i>	NEGATIVE
<b>BET42a</b>	Confocal	<i>Bosea</i>	POSITIVE	<i>Limnobacter</i>	NEGATIVE
<b>CFB563</b>	EVOS_FL	<i>Imperialibacter</i>	NEGATIVE	<i>Stenotrophomonas</i>	NEGATIVE
<b>GAM42a</b>	EVOS_FL	<i>Stenotrophomonas</i>	POSITIVE	<i>Bosea</i>	NEGATIVE
<b>GAM42a</b>	Confocal	<i>Stenotrophomonas</i>	POSITIVE	Not observed	
<b>MB-IC022b</b>	EVOS_FL	<i>Marinobacter</i>	NEGATIVE	<i>Alteromonas</i>	NEGATIVE
<b>MB-IC022b</b>	Confocal	<i>Marinobacter</i>	NEGATIVE	<i>Alteromonas</i>	NEGATIVE
<b>RHIZ1244</b>	EVOS_FL	<i>Bosea</i>	NEGATIVE	<i>Roseovarius</i>	NEGATIVE
<b>RHIZ1244</b>	Confocal	<i>Bosea</i>	Not taken	<i>Roseovarius</i>	POSITIVE
<b>ROS537</b>	EVOS_FL	<i>Roseovarius</i>	NEGATIVE	<i>Bosea</i>	NEGATIVE
<b>ROS537</b>	Confocal	<i>Roseovarius</i>	Not taken	<i>Bosea</i>	NEGATIVE

Supplementary Table 4-4 Overview of probes that were tested. C = picture taken with the confocal microscope; E: picture taken with Evos FL microscope.

Probe fluorophore	+	Target strain	Microscope	Probe fluorophore	+	Target strain	Microscope
EUB338-Cy3		Imperialibacter R9	C	EUB338-Cy3		Sphingomonas 391	E
							
scale bar 20 μm				scale bar 100 μm			
EUB338-Cy3		Sphingomonas 391	E	EUB338-Cy3		<i>Ectocarpus</i> (100% NSW)	E
							
scale bar 100 μm				scale bar 400 μm; arrows possible staining of bacteria			
ALF968		Bosea 5a	C	BET42a		Limnobacter 130	C
							
scale bar 20 μm				scale bar 20 μm			



### Scanning electron microscopy

In parallel to the FISH experiment described above, pictures were taken with the scanning electron microscope to visualize bacteria on the *Ectocarpus* surface. Three types of *Ectocarpus* were tested: *Ectocarpus* derived from NSW - full microbiome, *Ectocarpus* 5% NSW associated with full microbiome, and an antibiotic treated *Ectocarpus* grown in 100% NSW (long recovery period in 100% NSW without antibiotics). Culture conditions of *Ectocarpus* were as described in Chapter 3 (Preparation of biological material).

*Ectocarpus* filaments were fixed for 5 days at 4 °C in 1.5 ml 4% PFA. The salinity level of the PFA was adjusted according to the salinity level of the original algal culture. *Ectocarpus* derived from seawater were fixed in high salinity PFA (67% NSW). *Ectocarpus* derived from freshwater was fixed in 4% PFA in 17% NSW. Samples were dehydrated using the following steps (each 10 minutes): dH<sub>2</sub>O, 50% ethanol, 70% ethanol, 90% ethanol, 95% ethanol, 100% ethanol, 100% ethanol. Samples were critical point dried (Baltec 030 Critical Point drier), and coated in gold (Quorum Sputter Coater) before visualization with a Zeiss EVO MA10 Scanning Electron Microscope (Supplementary figures 4-2 to 4-6).

### General observations

The SEM observations show that the surface of the examined *Ectocarpus* samples was densely covered with a variety of bacteria. Most bacteria were oriented perpendicularly to the *Ectocarpus* surface (Figure 4-3), and some almost seemed to move into the cell wall. Filaments were not all equally abundantly colonized (Fig). On some filaments, almost no bacteria can be

seen, while neighbouring filaments were densely covered. The tips of the algal filaments seemed less colonized compared to main branch (Supplementary Figure 4-2 and 4-4).

### **Differences between *E. subulatus* filaments derived from 5% and 100% NSW**

Different shapes of bacteria can were found, e.g. tube-like and spiral-shaped bacteria (Supplementary figure 4-5). The spiral-shaped bacteria were less frequently found in *Ectocarpus* derived from 5% NSW, compared to algae derived from 100% NSW. The spiral-shaped features may have occurred due to fixation artefacts, however, spiral-shaped bacteria do exist and can belong to the *Spirochaetes*, *i.e.* bacteria with internal flagella; the gram negative taxa *Spirillum* (*Betaproteobacteria*), *Campylobacter* (*Epsilonproteobacteria*), or *Helicobacter* (*Epsilonproteobacteria*). Also, some Flavobacteria are known to use the spirals to move (Personal communication; T. Barbeyron; Johnston et al., 2017). Some spiral-shaped bacteria have been described to use the spiral shape to enter host surfaces (*Helicobacter pylori*, Kysela et al., 2016; Sycuro et al., 2010), and/or to promote locomotion of host cells (Flagellates; Cleveland and Grimstone, 1964).

### **Antibiotic-treated *E. subulatus* filaments**

Black spots were visible in antibiotic-treated *Ectocarpus* (Figure 4-6), which could be a technical artefact related to the sample preparation (low salinity in fixative), or it may be a malformation in the cell wall linked to the antibiotics it was treated with.

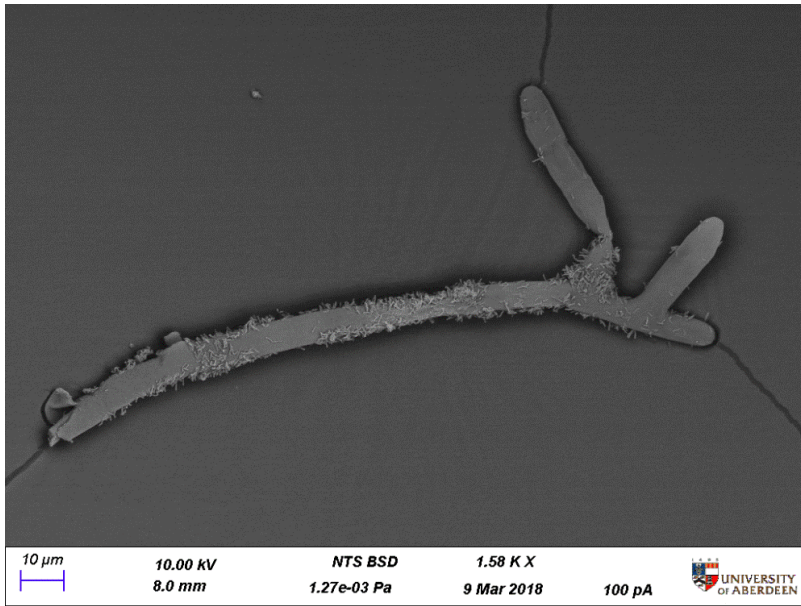


Figure 4-2 Scanning electron microscopy picture showing a global overview of one *E. subulatus* filament originally grown in 5% NSW; The picture shows various levels of bacterial colonization along the length of the filament.

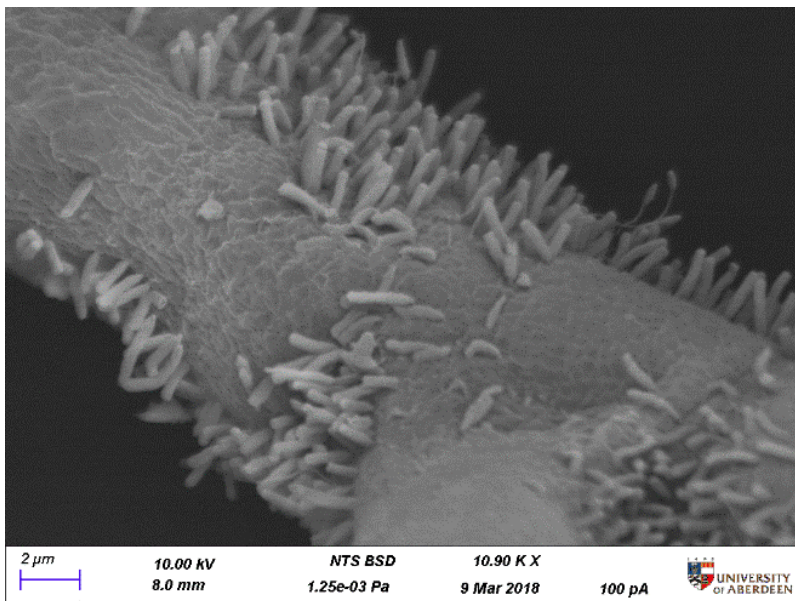


Figure 4-3 Scanning electron microscopy picture of *E. subulatus* filament grown in 5% NSW with various levels of bacterial colonization along the length of the filament. Detail of filament.



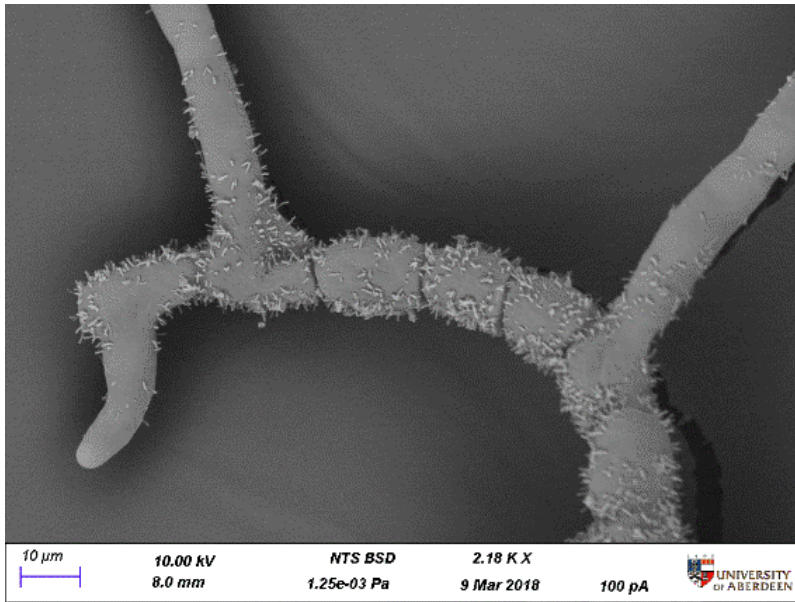


Figure 4-4 Scanning electron microscopy picture of *E. subulatus* filament grown in 100% NSW with various levels of bacterial colonization along the length of the filament.

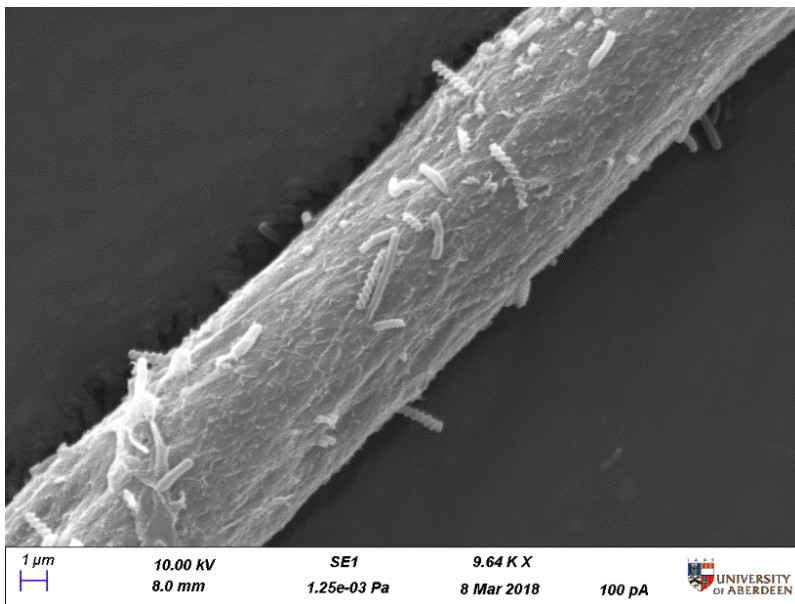


Figure 4-5 Scanning electron microscopy picture of *E. subulatus* filament grown in 100% NSW; detail of filament showing spiral-shaped bacteria as well as rod-shaped bacteria.



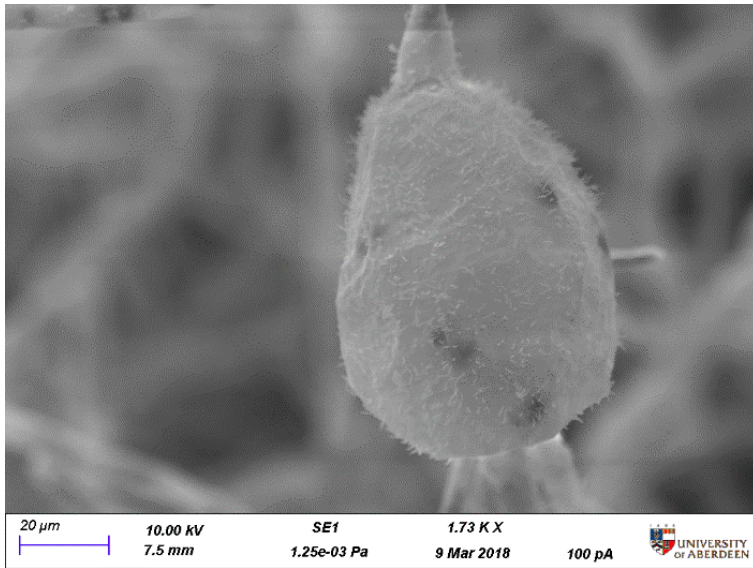


Figure 4-6 Scanning electron microscopy picture of antibiotic-treated *E. subulatus* filament grown in 100% NSW; detail sporangia with black spots.

## Annex 3 The genome of *Ectocarpus subulatus* highlights unique mechanisms for stress tolerance in brown algae

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Contribution: I contributed to this project by manually annotating a set of transporter genes using the Transporter Classification Database<sup>31</sup> as a reference.

### Abstract

Brown algae are multicellular photosynthetic organisms belonging to the stramenopile lineage. They are successful colonizers of marine rocky shores world-wide. The genus *Ectocarpus*, and especially strain Ec32, has been established as a genetic and genomic model for brown algae. A related species, *Ectocarpus subulatus* Kuetzing, is characterized by its high tolerance of abiotic stress. Here we present the genome and metabolic network of a haploid male strain of *E. subulatus*, establishing it as a comparative model to study the genomic bases of stress tolerance in *Ectocarpus*. Our analyses indicate that *E. subulatus* has separated from *Ectocarpus* sp. Ec32 via allopatric speciation. Since this event, its genome has been shaped by the activity of viruses and large retrotransposons, which in the case of chlorophyll-binding proteins, may be related to the expansion of this gene family. We have identified a number of further genes that we suspect to contribute to stress tolerance in *E. subulatus*, including an expanded family of heat shock proteins, the reduction of genes involved in the production of halogenated defense compounds, and the presence of fewer cell wall polysaccharide-modifying enzymes. However, 96% of genes that differed between the two examined *Ectocarpus* species, as well as 90% of genes under positive selection, were found to be lineage-specific and encode proteins of unknown function. This underlines the uniqueness of brown algae with respect to their stress tolerance mechanisms as well as the significance of establishing *E. subulatus* as a comparative model for future functional studies.

<https://doi.org/10.1101/307165>

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<sup>31</sup> <http://www.tcdb.org/>

## Annex 4 Attended conferences & meetings

6th European Phycological Congress; London, UK	24-29 August 2015	Flash talk
Emerging Bioinformatics Approaches in Microbial Ecogenomics; Plouzané, France	24 September 2015	
General assembly of IDEALG consortium; Roscoff, France	3-6 November 2015	Poster presentation
Young Researcher day; Roscoff, France	3 December 2015	Poster presentation
CommOCEAN: 2nd international marine science communication conference; Bruges, Belgium	5-9 December 2016	
La microbiologie dans tous ses états ; Paris, France	7 January 2016	
International Conference on Ecological Sciences; Marseille, France	24-27 October 2016	Oral presentation
General assembly of IDEALG consortium; Lorient, France	7-9 November 2016	
ALFF consortium mid-term meeting; Konstanz, Germany	13-17 February 2017	Oral presentation
International conference on Holobionts; Paris, France	19-21 April 2017	Poster presentation
International Phycological Conference; Szczecin, Poland	13-19 August 2017	Oral presentation
General assembly of IDEALG consortium; Roscoff, France	22-23 November 2017	Oral presentation
ALFF meeting – Oban, UK	15-16 June 2018	
Young Algaeneers Symposium ; Oban, UK	16-18 June 2018	Oral presentation

## Annex 5 Attended courses

Algal culturing: from the field to the lab; Roscoff, France	7-11 September 2015
Commercial Algal Culturing: Technologies, Markets and Business Skills ; Lisboa, Portugal	22-26 February 2016
1th Marine evolutionary and environmental genomics summer school ; Roscoff, France	13-24 June 2016
Linux for beginners & advanced users, Roscoff, France	31 June 2016
Deciphering symbiotic interactions with metabolomics ; Jena, Germany	19-23 September 2016
CommOCEAN Training course on Marine Science Communication Tools; Ostend, Belgium;	5-9 December 2016
Formation ‘Reconstruction et Analyse de Réseaux Métaboliques’; Roscoff, France	28 February 2017
Doctoral course ‘Projet professionnel’; Roscoff, France	10-15 May 2017
Workshop RNA-seq; Roscoff, France	23-24 May 2017
International course ‘Workflow4experimenters’; Paris, France	29 may – 2 June 2017
Systems biology and bioinformatics; Ghent, Belgium;	3-7 July 2017
Deep roots of algal diversity, eukaryotic tree, phylogenomics and endosymbiosis; Ostrava, Czech Republic	20-22 September 2017
ALFF course: Biomonitoring and science communication for policy-making; Oban, UK	14-15 June 2018

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Résumé:

*Ectocarpus* dépend de bactéries associées pour croître en eau douce, ce qui souligne l'importance de l'holobionte lors de stress abiotique. Le but de ma thèse est d'élucider les mécanismes moléculaires qui sous-tendent ce phénomène. Les expériences de co-culture ciblées nécessitent des organismes cultivables. Par conséquent, j'ai caractérisé 388 bactéries associées à *Ectocarpus*, réparties en 33 genres. Aucune des bactéries cultivées testées n'a eu d'effet bénéfique sur la croissance des algues dans l'eau douce. J'ai continué à travailler avec des holobionts, traités aux antibiotiques doux, qui différaient dans leur réponse à l'eau douce. Le métatranscriptome/métabolome de ces holobionts ont été analysés pendant l'acclimatation. L'analyse approfondie est en cours, mais les premières indications indiquent un changement dans le microbiome en ce qui concerne l'assimilation de l'azote et la virulence. Concomitamment et complémentaire à ce qui précède, les interactions algues/bactéries potentiellement bénéfiques ont été prédites *in silico* à l'aide d'une analyse de réseau métabolique et les prédictions ont été vérifiées expérimentalement à l'aide de co-cultures. Ensemble, ces résultats contribuent à mieux comprendre comment l'holobionte *d'Ectocarpus* réagit au stress abiotique et surtout comment les bactéries sont impliquées dans ce processus.

Mots clés: algues brunes, holobionte, *Ectocarpus*, stress abiotique, Métatranscriptomique, Métagénomique

**The influence of bacteria on the adaptation to changing environments in *Ectocarpus*: a systems biology approach**

Abstract:

*Ectocarpus subulatus* depends on its associated bacteria for growth in fresh water, which stresses the significance of the “holobiont” during abiotic stress. The aim of my thesis is to elucidate the molecular mechanisms that underlie this phenomenon. Targeted co-culture experiments require cultivable organisms. Therefore, I have cultivated and characterized 388 *Ectocarpus*-associated bacteria, which belonging to 33 different genera. None of the cultivated bacteria tested had a beneficial effect on algal growth in fresh water. For functional studies, I continued to work with mild antibiotic-treated holobionts that differed in their response to fresh water. The metatranscriptome and metabolome of these holobionts were analyzed during acclimation. In-depth analysis is ongoing, but first indications point towards a change in the microbiome regarding nitrogen assimilation and virulence. In parallel and complementary to the above, potentially beneficial algal-bacterial cross-talk was predicted *in silico* using metabolic network analysis on a subset of cultivated bacteria, and the predictions were experimentally verified using co-culture experiments. Together, these results contribute to a better understanding of how the *Ectocarpus* holobiont responds during abiotic stress and especially how bacteria are involved in this process.

Keywords: brown macroalgae; holobiont; *Ectocarpus*; abiotic stress; metatranscriptomics; metagenomics;