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Microbiomal composition impacts Ulva mutabilis temperature response: the macroalgal holobiont in a warming marine environment

Ruben Algoet
Student number: 01912429

Supervisors: Prof. dr. Olivier De Clerck, Prof. dr. Anne Willems
Counsellors: Prof. dr. Frédérik Leliaert, Luna van der Loos

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Abstract

Macroalgae like the genus *Ulva* provide essential structure and productivity to marine ecosystems around the world. In the last few decades, it has become clear that *Ulva* morphology, metabolism and growth are dependent on a symbiotic relationship with microbial communities of bacteria, archaea, viruses and so on: the *Ulva* individual and its microbiome have been characterised as a distinct functional unit, the holobiont. As climate change causes oceans to warm, the health of macroalgae becomes threatened due to microbiomal breakup, colonisation by pathogens, and induction of virulence in symbiotic partners. However, bacterial symbionts may also prove key mediators of marcoalgal temperature resistance. By co-culturing 43 bacterial strains isolated from wild *Ulva* with lab strain *Ulva mutabilis*, the effect of these strains on the growth rate of *U. mutabilis* germlings was characterised. Additionally, community experiments using these same strains pointed us towards a high importance of intra-microbiomal interactions in defining *U. mutabilis* holobiont heat response.

Introduction

In the marine benthos, sessile photosynthetic eukaryotes known as macroalgae or seaweeds are responsible for the majority of primary productivity (Allen, 1971; Duarte & Cebrián, 1996). These algae are widely varied in their distribution across climates, from tropical to arctic areas (Gibson et al., 2007; Williams & Smith, 2007). Not only do they contribute to their ecosystem in the form of photosynthetic activity, but they are also a key component of the benthic habitat structure, especially in nearshore areas. This shows they are of great importance for higher trophic levels to be sustained in a benthic marine environment (Egan et al., 2013; Mineur et al., 2015; Schiel & Foster, 2006). Aside from their role in biodiversity, they are keystones of ecosystems that provide valuable services in many regions, such as coastline protection (Stiger-Pouvreau & Zubia, 2020), and they also have high economical value in biotechnology (Sfriso, Mistri, Munari, Buosi, & Sfriso, 2020).

Macroalgae live in close association with microbial communities, which assemble epiphytically on the algal surface or, in some cases, associate with their host endophytically. This bond has garnered increased attention over the last few decades and proves to carry vital importance for the host's health (Barott et al., 2011; Mishra & Mohanraju, 2018; Selvarajan et al., 2019). Host-microbial interactions are so essential that a specific name has been coined in the literature to refer to the algal host and its associated microbiome as a functional whole, living in close symbiosis: the holobiont (Bordenstein & Theis, 2015; Egan et al., 2013) Epiphytic bacteria provide algae with nitrogen, carbon dioxide, growth factors and vitamins, at a compensation of structural carbons and oxygen. In addition, the microbiome can perform the role of filtering heavy metals and crude oil, which is also beneficial for the host (Goecke, Labes, Wiese, & Imhoff, 2010; Lage & Graca, 2016). Microbial symbiosis not only benefits the algal host's metabolism and protection against harmful substances: it also plays a pivotal role in the morphological development of some algal species (Marshall, Joint, Callow, & Callow, 2006; Matsuo, Suzuki, Kasai, Shizuri, & Harayama, 2003; Tapia, González, Goulitquer, Potin, & Correa, 2016).

Species of the green alga genus *Ulva* (sea lettuces, from the order of Ulvales, Cholorphyta) normally have a tubular (enteromorphic) or blade-like thallus, with both sometimes occurring in the same species, and a discoid holdfast sporting rhizoids that attaches the alga to a surface. Among *Ulva* is the lab strain species *Ulva mutabilis* Føyn (1958), a key model organism in phycology representing the *Ulva* genus and green macroalgae in a broader sense (Hayden et al., 2003; Steinhagen, Barco, Wichard, & Weinberger, 2019). When cultured without the presence of microbiota that stimulate morphology (i.e. axenic culture conditions), *U. mutabilis* shows a lack of cell differentiation, a lack of rhizoids, a stunted growth and a callus-like form covered by cell wall protrusions (Alsufyani et al., 2020; Spoerner, Wichard, Bachhuber, Stratmann, & Oertel, 2012). However, when reintroduced to natural sea water containing microbiome components, it can be restored to its normal development (Wichard, 2015). The term "axenic" refers to organisms free of association with symbionts or parasites of other species, and in this case, is used to describe *Ulva* cultures or specimens that lack any microbes. *Roseovarius* sp. (MS2) and *Maribacter* sp. (MS6) are two growth-promoting strains that can restore their host's proper morphology when co-cultured with axenic specimens of many *Ulva* species and do so reliably

with *U. mutabilis*. *Roseovarius* is responsible for thallus elongation and cell division, while *Maribacter* promotes cell wall development and induces the formation of rhizoids (Weiss, Costa, & Wichard, 2017). In fact, it is possible for *Ulva*'s morphology to be restored even without physical contact with the microbiome, by means of stimulatory bacterial compounds in the chemosphere (Alsufyani et al., 2020).

Globally, the marine habitats of algae like *Ulva* are experiencing an increase in human impact, which includes climate change-induced temperature anomalies and rising heat. (Halpern et al., 2015). Both components of the algal holobiont have a distinct response to rising temperature: macroalgae can suffer from tissue bleaching under heat wave stress (Straub et al., 2019), and elevated temperature in general cause increased photorespiration and a decrease of photosynthetic productivity (Koch, Bowes, Ross, & Zhang, 2013; Necchi Jr, 2004). Meanwhile marine bacteria are known to respond to stressors like heat by promoting sporulation, biofilm formation, virulence and other mechanisms that improve survivability in adverse conditions (Milewska, Krause, & Szalewska-Pałasz, 2020). The composition of macroalgal microbe communities depends on both the abiotic environment (including but not limited to temperature) and the health of the host, which in itself is also influenced by abiotic factors (Florez, Camus, Hengst, Marchant, & Buschmann, 2019). This makes it clear that the effects of a changing ocean may imbalance the host and its microbiome, leading to holobiont breakup and dysbiosis. Such a disturbance is detrimental for the health of the alga as it allows for infection by bacterial pathogens and secondary invaders. In addition, mutually beneficial interactions may be lost when otherwise helpful bacteria change their behaviour as a response to these conditions (Egan & Gardiner, 2016).

Phenomena of dysbiosis due to anthropogenic effects are already affecting marine ecology to a significant degree (Egan & Gardiner, 2016; Fan, Liu, Simister, Webster, & Thomas, 2013). Over the course of the past years, disease symptoms have become more common in both cultured and natural marine populations (Gattuso et al., 2015; Harvell et al., 2002; Lafferty, Porter, & Ford, 2004). The affected populations span multiple trophic levels, including marine invertebrates, fish, and most importantly for this study, primary producers like algae (Gachon, Sime-Ngando, Strittmatter, Chambouvet, & Kim, 2010). Studying sea sponges, Fan et al. (2013) revealed that holobiont breakup is a strong determining factor for mortality on a large scale, especially when symbionts are highly specialised. They highlight the recruitment of microorganisms with high growth rates and scavenging behaviour during temperature stress, as well as the loss of important symbionts, and these findings are highly corroborated (Pita, Rix, Slaby, Franke, & Hentschel, 2018; Ramsby, Hoogenboom, Whalan, & Webster, 2018; Vargas, Leiva, & Wörheide, 2021). In human studies, a lack of diversity in microbial symbionts lies at the basis of numerous disease syndromes (Egan & Gardiner, 2016).

In the literature surrounding *Ulva* and its holobiont, focus tends to lie on either the algal response to heat stress, ignoring microbiomal effects, or on morphology induction and general developmental effects mediated by the microbiome. The purpose of this thesis was to combine these avenues of study and examine how various (communities of) epiphytic bacteria isolated from wild *Ulva* affect the response of model species *U. mutabilis* to elevated temperatures.

Three experiments have been conducted to achieve this goal. The first sought to characterise the bacteria that assemble on wild *Ulva* species in their capacity to induce morphological traits and stimulate growth. The second quantitatively assesses the effects of individual strains on the

growth capacity of *U. mutabilis* under moderate and elevated temperature conditions, by comparing relative growth of germlings. The final experiment compared varied bacterial communities cocultured with *Ulva*, to determine whether a more diverse microbiome improves the alga's ability to maintain growth under temperature stress. Given the great importance of *U. mutabilis* as a model species in modern algal holobiont research (Dittami et al., 2021; Wichard et al., 2015) the interest of these experiments lies not only in painting a clearer picture of the way higher temperatures may put green algae at risk on a holobiont level, but also in further defining the way *U. mutabilis* behaves as a model holobiont in culture conditions.

Materials and Methods

Basic Reagents and Methods

Ulva Culture Medium (UCM)

Ulva Culture Medium is a growth medium specifically tuned to *Ulva* sp. as used by Califano & Wichard, 2018 and originally proposed by Stratmann, Paputsoglu, & Oertel, 1996. It is a mixture of five base solutions, where 1 L of solution 1 is supplemented with 10 ml of solutions 2-4, and 2 ml of solution 5. The composition of the separate solutions, which were made previously and already available, can be found in both cited papers.

For this paper, UCM was prepared under sterile conditions as axenic germlings were required for many experiments.

Marine Broth and Marine Agar

Any marine broth and agar used refers to Difco™ Marine Agar 2216 and Difco™ Marine Broth 2216. Their ingredients and the process of preparation from dehydrated form are described in the Difco™ & BBL™ Manual, 2nd Edition.

Standard *Ulva* culture conditions

Standard *Ulva* culture conditions refer to storage at 18°C, while exposed to ±100 µmol $m^{-2}s^{-1}$ TL light intensity, as referenced in Califano & Wichard, 2018 and using the available TL light of either the laboratory's growth chamber setup, or one of the available incubators. Light was administered in a 12/12hr light/dark cycle.

Germling photography and measurement

In the *Roseovarius* and *Maribacter* trail experiment, experiment two, and experiment three, germlings were photographed using a binocular microscope equipped with a ToupCam UCMOS 05100KPA camera and the accompanying software ToupView (x64, version 4.11.18081.20201205). Based on these photographs, germling surface area was measured using ImageJ 1.53e (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012).

Data visualisation and analysis

Data obtained during experiments was visualised and processed using Microsoft Excel 2016 and RStudio version 1.2.1335 (a coding environment for R (R Core Team, 2019), which was run in version 3.5.1). Key R packages used include *ggplot2* version 3.3.0 (Wickham, 2016) and *MASS* version 7.3-54 (Venables & Ripley, 2002). R code for important visuals and statistical analyses can be found in the appendix.

Procuring axenic *Ulva* germlings

In order to study the effect of separate bacterial strains on *Ulva mutabilis*, a model species for morphological study on green seaweeds (Wichard et al., 2015), axenic specimens have to be obtained. In order to achieve this a protocol was used that is outlined in Califano & Wichard (2018):

Ulva mutabilis specimens were minced and repeatedly washed using sterile sea water and filtered to remove sporulation inhibitors to induce gametogenesis. *Ulva* fragments were incubated for three days under standard *Ulva* conditions in an UCM-filled culture flask, after which the culture medium was replaced with fresh UCM to remove the swarming inhibitor, and the flask was exposed to bright light to cause gamete release. These gametes were collected in a falcon tube. A dense suspension was obtained by allowing gametes to move to the bottom of the tube through phototaxis, and removing excess UCM.

Once *Ulva* gametes were prepared, they were separated from their accompanying bacteria using their phototactic behaviour. 18.5 cm long glass Pasteur pipettes were filled up sterile UCM, leaving room for 100-150 µl of the gamete suspension. They were positioned horizontally to have their tip face a light source. This resulted in a light gradient along the pipettes strong enough to make gametes move towards the tips. After 20-30 minutes, once a high gamete density could be observed at the tip of the pipette, the gamete suspension was collected. For each batch of gametes, these 20-30 minute runs were repeated a total of three times, to ensure axenicity.

The resulting axenic gamete suspension was used to produce axenic *Ulva* germlings. The suspension was added to culture flasks containing UCM, which were then stored at standard *Ulva* culture conditions to facilitate gamete fusion, resulting in germlings being produced.

***Ulva* culturing: preparatory work**

Before the key experiments were conducted, a number of preparatory steps were carried out to verify the validity of the setup and try out alternative methods of *Ulva* incubation.

Firstly, the efficiency and suitability of different light sources for *Ulva* culturing at 21°C were compared. The lab's existing TL-tube light setup (warm white) was contrasted against a new setup built using LED light strips (cold white). These were arranged in a grid to ensure even spreading of light in an incubator, at a light flux of 100 µmol $m^{-2}s^{-1}$ at the location of the culture flasks and plates holding *Ulva.* This flux was used because it is the optimal light intensity for *Ulva* culturing (Califano & Wichard, 2018), matching the light level of the existing *Ulva* culturing TL light setup (all light measurements are conducted using an Apogee MQ-200 quantum flux meter). In this same preparatory experiment, a third group of *Ulva* germlings were cultured under TL light at 18°C. For each of these light/temperature treatments, separate bottles of *Ulva* with specimens obtained from a short pipette (15 cm) and long pipette (18.5 cm) were used. In additional bottles, a *Roseovarius* and a *Maribacter* bacterial strain (known inducers of Ulva morphology (Wichard, 2015)) were included to represent a non-axenic control.

This experiment thus served three purposes: to study the preferable light conditions for *Ulva* growth, to determine whether 18°C or 21°C is more appropriate as a base temperature condition for *Ulva*, and to confirm the optimal pipette length to use in the axenic protocol.

Secondly, individual germlings were cultured in 6-well plates under different temperature (18°C and 21°C) and bacterial (axenic and non-axenic) conditions for three weeks. They were photographed using chlorophyll fluorometry on a photoacoustic microscopy (PAM) device (Walz IMAGING-PAM *M-Series* MAXI) and the accompanying software (ImagingWin version 2.47).

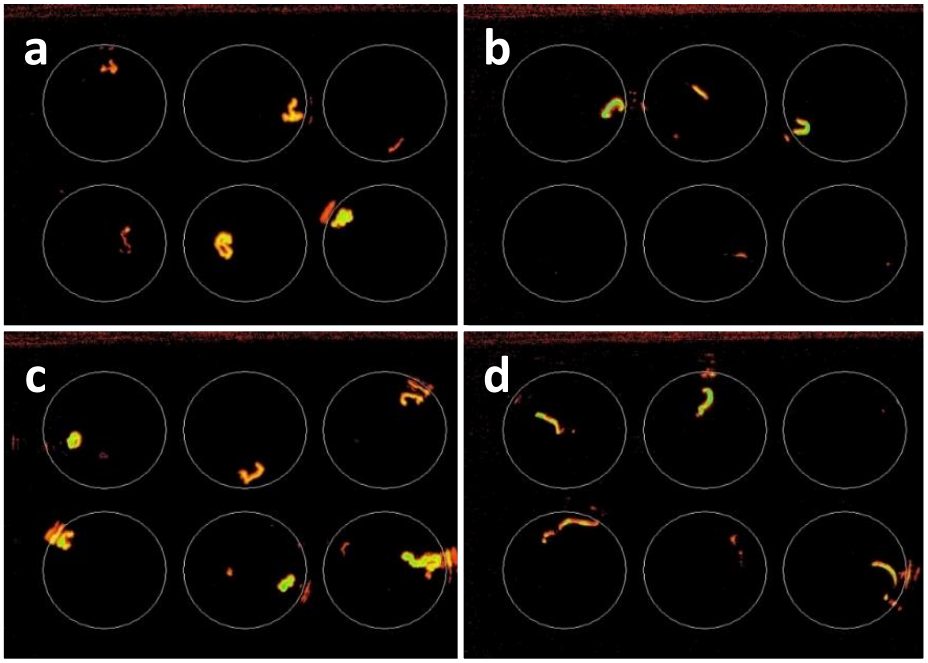

Figure 1: *MAXI-PAM chlorophyll fluorescence imaging of U. mutabilis germlings under the following conditions:* ***a)*** *18°C, axenic.* ***b)*** *18°C, non-axenic.* ***c)*** *21°C, axenic.* ***d)*** *21°C, non-axenic.*

Using ImageJ, the surface area of the germlings was measured based on the area highlighted by chlorophyll fluorescence.

During these preparatory experiments it became clear that caution must be taken with the type of lighting and incubator used. Several issues presented themselves:

The use of TL lights produced a temperature increase of around 3°C (21°C to 24°C) in one of the incubators, due to heating up of the TL tube boxes and poor heat distribution by the incubator. This problem was aggravated by the chrome-coloured interior of the incubator, which was less reflective than the white interior of the others and required additional TL lights to reach the desired light level. An advantage of LED light is that it avoids temperature problems due to light equipment heating up.

During the preparatory experiment using individual germlings, some individuals exhibited signs of bleaching (highly reduced PAM chlorophyll fluorescence compared to germling size). This effect was observed under high intensity (100 µmol $m^{-2}s^{-1}$) LED light, but not TL light. It is possible that the abundance of blue light in the cold LED light was the cause of this, since the effect is not replicated in either TL light (which contains fewer blue wavelengths) or lower intensity LED light (30 µmol/m^2*s). While there is a record of white and even blue LED light improving *Ulva* growth (Le et al., 2018), it is not clear at which intensity of blue light these experiments were conducted. Both Le et al. and further research (Kang, Huang, Lim, Hsu, & Hwang, 2020) note the increase in antioxidant content in *Ulva* sp. Exposed to blue LED light. As Kang et al. indicate, this may be a response to increased photosynthetic stress.

Although LED light may provide some conveniences when used correctly (like improved modularity of the setup and avoidance of heat production), TL lighted climate rooms were used for all following experiments, because they were already available and avoided the heating problems observed in the incubators. Additionally, the construction of a new LED setup encountered too many trials to be ready for use in this study.

Bacterial strains: preparatory work

Bacterial strains were previously isolated from *Ulva* specimens collected in Zeeland, the Netherlands, by crushing the *Ulva* tissue in sterile sea water and streaking the resulting supernatant on marine agar plates. They were stored in Microbank™ cryopreservative vials (product category number PL.170) at -80°C.

The initial strains isolated from natural *Ulva* specimens can be found in Appendix 1. From here on out, bacterial strains will be referred to using their sample label as listed in Appendix 1.

To culture individual strains, beads from the Microbank were streaked out on marine agar plates. These plates were prepared as pure marine agar (MA) , or contained additional sediment (MA+S) or *Ulva australis* tissue (MA+U), according to the culturing needs of the respective strains. After three days of growth in a 20°C incubation chamber, a colony from the first plate was streaked onto a second plate (if colonies were visible), to ensure the plates contained no contamination.

Some strains proved to have difficulty growing on marine agar or did not grow at all, and were therefore eliminated from the line-up. The strains that did grow were transferred to autoclaved liquid marine broth. Table 1 displays the 43 strains were included in the final collection on marine broth used for the experiments.

Table 1: *bacterial strains cultured in marine broth for experimental use.*

Species	**Phylum**	**Sample Nr.**
Alteromonas addita	Proteobacteria	EB001
Polaribacter sp.	Bacteroidetes	EB006
Dokdonia diaphoros	Bacteroidetes	EB007
Pseudoalteromonas translucida	Proteobacteria	EB009
Dokdonia sp.	Bacteroidetes	EB010
Maribacter dokdonensis	Bacteroidetes	EB012
Shewanella electrodiphila	Proteobacteria	EB013
Pseudoalteromonas marina	Proteobacteria	EB015
Maribacter sp.	Bacteroidetes	EB018
Dokdonia aurantiaca	Bacteroidetes	EB019
Arenicella sp.	Proteobacteria	EB023
Epibacterium scottomollicae	Proteobacteria	EB031

Ruegeria meonggei	Proteobacteria	EB037
Alteromonas genovensis	Proteobacteria	EB038
Sulfitobacter undariae	Proteobacteria	EB039
Vibrio sp.	Proteobacteria	EB042
Maribacter forsetii	Bacteroidetes	EB043
Agarivorans aestuarii	Bacteroidetes	EB044
Vibrio atlanticus	Proteobacteria	EB046
Nonlabens ulvanivorans	Bacteroidetes	EB053
Bacillus altitudinis	Firmicutes	EB054
Alteromonas sp.	Proteobacteria	EB055
Sulfitotobacter sp.	Proteobacteria	EB058
Dokdonia genika	Bacteroidetes	EB069
Yoonia sp.	Proteobacteria	EB075
Alteromonadaceae sp.	Proteobacteria	EB077
Aquimarina amphilecti	Bacteroidetes	EB082
Yoonia rosea	Proteobacteria	EB085
Flagellimonas aquimarina	Bacteroidetes	LML002
Maribacter vaceletii	Bacteroidetes	LML003
Polaribacter dokdonensis	Bacteroidetes	LML004
Algibacter pectinivorans	Bacteroidetes	LML005
Maribacter sp.	Bacteroidetes	LML006
Sulfitobacter geojensis	Proteobacteria	LML007
Winogradskyella sp.	Bacteroidetes	LML015
Maribacter litoralis	Bacteroidetes	LML025
Winogradskyella litoriviva	Bacteroidetes	LML031
Maribacter sp.	Bacteroidetes	LML032
Maribacter spongiicola	Bacteroidetes	LML036
Octadecabacter sp.	Proteobacteria	LML037
Lacinutrix sp.	Bacteroidetes	LML038
Erythrobacter longus	Proteobacteria	LML045
Erythrobacter sp.	Proteobacteria	LML047

In addition, the previously mentioned *Roseovarius* and *Maribacter* strain were prepared as well, to function as positive control for the induction of *Ulva* morphology (Wichard, 2015). Optical Densities (OD) of these strains were measured using a Ultrospec spectrophotometer, using a sterile marine broth tube as a baseline. Through serial dilution in sterile UCM, solutions of OD 10^{-4} of each strain were produced.

All handling of bacterial strains was performed under a closed laminar flow hood, using sterile inoculation loops and swabs, to prevent contamination.

Trial experiment using *Roseovarius* and *Maribacter*

A trial morphology and growth experiment validated the effect of bacteria at an OD of 10^{-5} when combined with *U. mutabilis* germlings. 6-well plates were filled with 5.4 ml UCM per well. An *U. mutabilis* germling, previously grown axenically, was added to each well. 0.6 ml of OD 10^{-4} bacterial strain solution was added as well, bringing the total content of a well to 6 ml and the OD to 10^{-5}.

Since this was a trial experiment, only the *Roseovarius* and *Maribacter* strains were used. It was crucial to confirm that these strains were effective at inducing *Ulva* morphology at OD 10^{-5}, because they would be used as positive controls and morphology inducers in the coming experiments.

Germlings were photographed every 3 days for two weeks, and their surface area was measured using ImageJ.

Experiment 1: Strain effect on morphology

The first experiment sought to characterise how the selected strains influence *Ulva* morphology during germling growth. Bacteria like *Roseovarius* and *Maribacter*, influence and can even be necessary for typical *Ulva* morphogenesis (Alsufyani et al., 2020; Ghaderiardakani, Coates, & Wichard, 2017; Wichard, 2015; Wichard et al., 2015). Therefor it was useful to characterise the morphological impact of the bacterial strains that have been isolated, as it would inform the results of following experiments and provide further detail on the role of these bacteria as part of the *U. mutabilis* holobiont.

Two 24-well plates were prepared, and to each well was added 0.9 ml of *Ulva* gamete suspension which had previously been diluted to an OD of 0.05, and 0.1 ml of bacterial strain suspension at OD 10^{-4} (resulting in a bacterial OD of 10^{-5} per well). Wells containing *Roseovarius*, *Maribacter*, both, and no strain (sterile UCM keeping the germling axenic) were also included as positive and negative controls. After three weeks of incubation at standard *Ulva* culture conditions and regular follow-up, germlings in the wells were photographed using a ZEISS Axio Vert.A1 inverted microscope, and the accompanying software Olympus cellSens Entry 2.3 (build 18987).

Germlings traits were described using a Morphological Difference Index (MDI). This index was a number of 0 to 4, evaluating whether 4 characteristics were different from a negative control germling. Each trait that differed from the axenic control added 1 point to the MDI. The following traits were examined:

1. Does the germling have colourless protrusions? (Negative control: yes)
2. Does the germling have rhizoids? (Negative control: no)
3. Is the germling elongated? (Negative control: no)

4. Is the germling shape smooth or irregular, disregarding colourless protrusions? (Negative control: irregular)

Rhizoids are part of the *U. mutabilis* mechanism for surface attachment, typical for *Ulva* morphology (Bråten, 1975; Spoerner et al., 2012). The *Ulva* begins at a dark green stem: the basal cells of this stem produce secondary transparent rhizoid cells through elongation and ultimately separation of a vacuole. As *Ulva* develops these rhizoids form an anchoring network known as the rhizoid pad. The colourless bubble-like protrusions that occur in germlings with lacking morphology originate in the cell wall, with Spoerner et al. (2012) being one of the few to comment on their nature and suspect that they are growths of extracellular matrix material due to unbalanced cell wall synthesis.

It should be noted that the usual recovery of typical morphology by *Roseovarius* and *Maribacter* is not as effective for wild *Ulva* species (such as *Ulva australis* Areschoug (1854) and *Ulva intestinalis* Linnaeus (1753)) as it is for *U. mutabilis*.

Figure 2 gives some examples of these traits, using the photographs of *Ulva* germlings taken for this experiment.

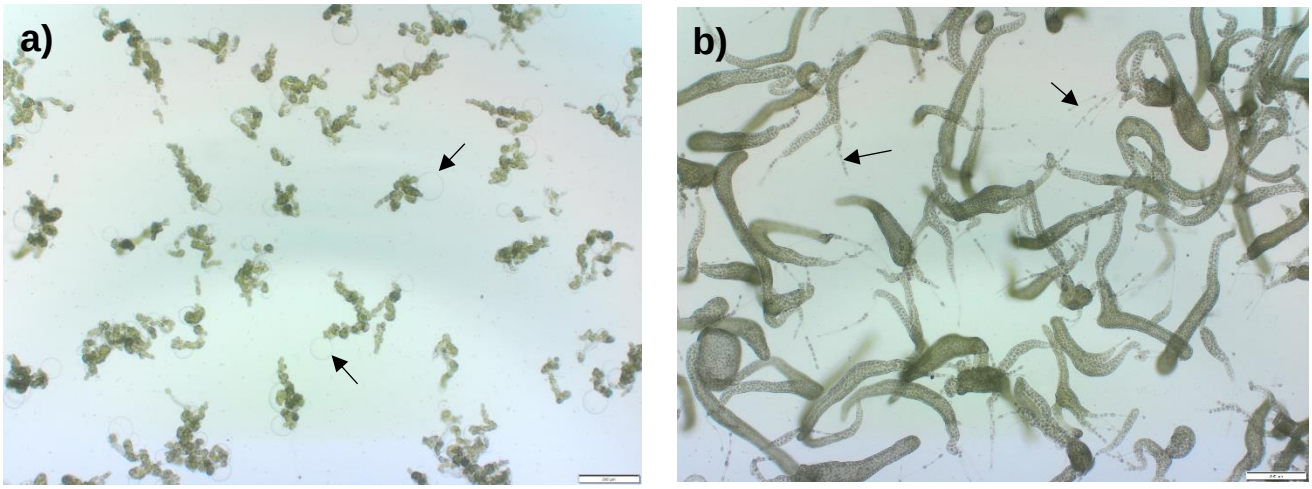

Figure 2: *microscopic photographs of Ulva germlings (200 µm scale at the bottom right) showing different morphological traits.* ***a)*** *Germlings with an irregular shape, no elongation, and round colourless protrusions.* ***b)*** *Germlings with an irregular shape, elongation, and rhizoids.*

As a rudimentary visualisation of the obtained qualitative data (presence/absence of morphological traits like rhizoids, protrusions, etc.), a sscatterplot (figure 3) was made in R using the *ggplot2* package with help from the package *ggrepel* (Slowikowski, 2021) version 0.9.1 to avoid label overlap.

To obtain a more statistically reliable clustering of strains, the R package *vegan* (Oksanen et al., 2020) version 2.5-7 was employed to perform a principal component analysis (PCA) on the morphology data. Instead of only using MDI and size data, presence/absence of each trait was considered separately (size was still taken into account). The package *factoextra* (Kassambara & Mundt, 2020) version 1.0.7 was used to better represent data points and labels. This analysis is visualised as a biplot in figure 4.

Since rhizoid formation and the presence of protrusions were poorly clustered by the biplot resulting from the first PCA analysis, an alternative version was produced, visualised in figure 5. In this version, the numerical value representing presence of these traits was raised to give them additional weight in the analysis: instead of rhizoid and protrusion presence being represented by a value of 1 (as opposed to absence "0"), it was represented by a value of 2. Values signifying size ranged from -3 to 3 in both data representations, -3 representing germlings much smaller than the axenic control, and 3 much larger.

Experiment 2: Strain effect on growth

To measure how the selected strains affected *Ulva* germling growth, the surface area of germlings was monitored over the course of two weeks.

Firstly, 6-well plates were prepared with 5.4 ml of sterile UCM. A germling was added to each well beforehand. These germlings originated from an axenic stock (36 days old), but were supplied with *Roseovarius* and *Maribacter* a week before the start of experiment 2 in order to induce normale morphology and allow for comparability in growth. For the control wells, axenic germlings (originating from a 5 month old stock) were supplied with either only *Roseovarius,* only *Maribacter*, or no bacteria (fully axenic). Attempts at using gametes from a younger batch resulted in their destruction. Axenic gametes were been obtained according to the protocol of Califano & Wichard, 2018 previously outlined, with the following specifics: pipette runs were performed three times in a 18.5cm pipette and then twice in a 15cm one.

Once a germling was placed in each well using a 1ml micropipette, taking only the germling and a minimal amount of medium from the flask, bacterial strains were added. Each strain was combined with a germling in triplicates, resulting in two strains per plate. To a well, the following measures of bacterial suspension were added, each at OD 10^{-4}:

1. 0.2 ml of *Roseovarius* suspension
2. 0.2 ml of *Maribacter* suspension
3. 0.2 ml of the strain of interest

However, when only *Roseovarius* and *Maribacter*, only one of these two, or when no bacterial suspensions were added, the remaining 0.2 ml partitions were substituted with sterile UCM.

Since spare plates with germlings presented themselves at the end of this experiment's preparation, they were used for a preliminary investigation of multiple-strain community effects. These plates contained triplicates for a combination of EB001, EB006, EB007, EB009 and EB010 (community pilot 1) and for a combination of LML002, LML003, LML004, LML005 and LML006 (community pilot 2). In this case, instead of 0.2 ml of one strain, 40 µl of each was added.

The complete set of well plates included triplicates of all strains, two strain combinations, only *Roseovarius and Maribacter*, *Roseovarius and Maribacter* separately, and an axenic control. This set was produced twice: one for growth in standard *Ulva* culture c onditions, and one for growth under an elevated temperature (identical to standard conditions but at 23°C instead of 18°C). They were incubated for two weeks and photographed each week. Surface area per

germling and timepoint was calculated using ImageJ. To describe germling growth over time, relative growth rate (RGR) was calculated using the formula:

$$RGR = \frac{ln\, S_{t1} - ln\, S_{t0}}{t_1 - t_0}$$

Time (t) was measured in number of days, t_0 signifying the start of the experiment and t_1 its end at 14 days of growth. Surface (S) measures were taken in mm^2 at these times. Resulting values of RGR are expressed in units of mm^2 growth per mm^2 per day.

Two-way ANOVA was used to analyse germling relative growth rates, examining the effect of cocultured strains (49 levels including controls and community pilots) and temperature condition (2 levels) as independent variables on the dependent variable of RGR. ANOVA functions for R were supplied by the car and carData packages, version 3.0-2 (Fox & Weisberg, 2011; Fox, Weisberg, & Price, 2018). Residuals of the fitted model followed a normal distribution and no heteroscedacity was observed between groups. To visualise the spread of RGR data per strain and temperature condition, the *ggplot2* package was used to represent data as box plots, which were arranged together using the package *gridExtra* version 2.3 (Auguie, 2017) into figure 6.

With the *emmeans* package version 1.3.4 (Lenth, 2019) additional comparisons were performed between temperature conditions, within each level of the strain variable. P-values were adjusted based on Tukey's HSD. If germlings cocultured with a strain showed significantly different RGRs under the separate temperature conditions, that strain was marked on figure 6 with a * or ** symbol denoting a p-value lower than 0.05 and 0.01, respectively.

Experiment 3: Diversity effect on growth

Finally, an experiment similar to the second was conducted, however, in this case communities of multiple bacterial strains were used, rather than single strains. A broken stick approach (Salles, Poly, Schmid, & Roux, 2009) was used to divide two random assemblages of 40 strains (out of the 43 available) into 4 communities of 20, 8 communities of 10, and 16 communities of 5 strains. The makeup of these communities can be found in table 2. To each community *Roseovarius* and *Maribacter* were added in equal measure to the other individual strains, in order to avoid underestimation of growth due to lacking morphology. Thus, communities consisted in total of 22, 12, or 7 strains (always including *Roseovarius* and *Maribacter*), but are still referred to as 20-, 10-, and 5-strain communities since these were the actual factors of interest that were variable between groups.

Well plates containing UCM and *U. mutabilis* germlings were prepared identically to experiment 2. For each community, three wells (three germlings) were reserved to obtain results in triplicate for statistical analysis. Bacterial suspension in UCM at an OD of 10^{-4} was added to wells in quantities that resulted in a final total bacterial OD of 10^{-5} within wells. This meant an addition of 86 µl of suspension for 5-strain communities, 50 µl for 10-strain, and 27 µl for 20-strain, resulting in a total addition of 600µl of OD 10^{-4} solution, and a 6 ml total volume per well.

For positive and negative control wells (only *Roseovarius* and *Maribacter* or no bacteria at all), UCM was used to substitute the volume of added bacterial strains, like in the previous experiment.

Two sets of plates containing all different communities, a positive control, and a negative control, were produced and assigned to standard culturing conditions with normal (18°C) or elevated (23°C) temperature. Growth of germlings was monitored over the span of 12 days, photographed at the start and end of this period. Their surface area was determined using ImageJ and RGR was calculated identically to experiment two.

Table 2: *Communities obtained using a broken stick model. Each community is named based on the number of strains it is made up of, and a letter to separate different communities of the same size. Larger communities are listed with the smaller communities they share strain contents with.*

5a	*EB018*	*EB043*	*EB075*	*LML025*	*EB069*
5b	*EB055*	*EB054*	*EB026*	*LML038*	*EB012*
5c	*EB037*	*LML005*	*EB019*	*LML004*	*LML006*
5d	*EB058*	*EB031*	*EB023*	*EB085*	*EB044*
5e	*LML002*	*EB009*	*LML032*	*EB042*	*EB015*
5f	*LML007*	*EB039*	*EB006*	*LML031*	*LML015*
5g	*EB082*	*LML036*	*LML003*	*LML045*	*EB007*
5h	*LML047*	*LML037*	*EB010*	*EB038*	*EB001*
5i	*LML032*	*EB044*	*EB006*	*EB031*	*EB009*
5j	*LML005*	*EB026*	*LML004*	*EB037*	*EB012*
5k	*EB007*	*EB058*	*EB043*	*EB053*	*EB085*
5l	*LML003*	*EB001*	*LML007*	*EB082*	*EB019*
5m	*LML038*	*LML025*	*LML037*	*EB018*	*LML015*
5n	*EB010*	*EB042*	*LML045*	*EB038*	*EB054*
5o	*EB069*	*EB015*	*LML006*	*EB075*	*LML047*
5p	*EB055*	*EB023*	*LML002*	*EB039*	*LML036*
10a	*5a*	*5b*			
10b	*5c*	*5d*			
10c	*5e*	*5f*			
10d	*5g*	*5h*			
10e	*5i*	*5j*			
10f	*5k*	*5l*			
10g	*5m*	*5n*			
10h	*5o*	*5p*			
20a	*5a*	*5b*	*5c*	*5d*	
20b	*5e*	*5f*	*5g*	*5h*	
20c	*5i*	*5j*	*5k*	*5l*	
20d	*5m*	*5n*	*5o*	*5p*	

Results

Experiment 1: Strain effect on morphology

Of the 43 strains assessed, 12 were capable of inducing rhizoid formation in *U. mutabilis* germlings. 17 strains allowed for elongation, and 11 caused a smooth shape as opposed to an irregular one, with ten others being a borderline case (classified as a 'crooked' but not 'irregular' shape). In 11 strains, colourless protrusions were no longer present. Six strains caused germlings to remain noticeably smaller in size than the axenic control, three extremely so. Meanwhile, 24 strains resulted in a germling larger than the control, of which ten a much larger germling.

The germlings cocultured with *Roseovarius* and *Maribacter* produced some unexpected effects: the *Roseovarius* germlings showed the presence of rhizoids and a smooth shape but no elongation, nor a particularly large size. They also did not show colourless protrusions. *Maribacter* on the other hand induced elongation and a large size, resulting in germlings of equal size to those cultured with both *Roseovarius* and *Maribacter*.

The scatterplot in figure 3 shows the MDI and relative size scores of all 43 strains, *Roseovarius*, *Maribacter*, and the *R.* and *M.* combination.

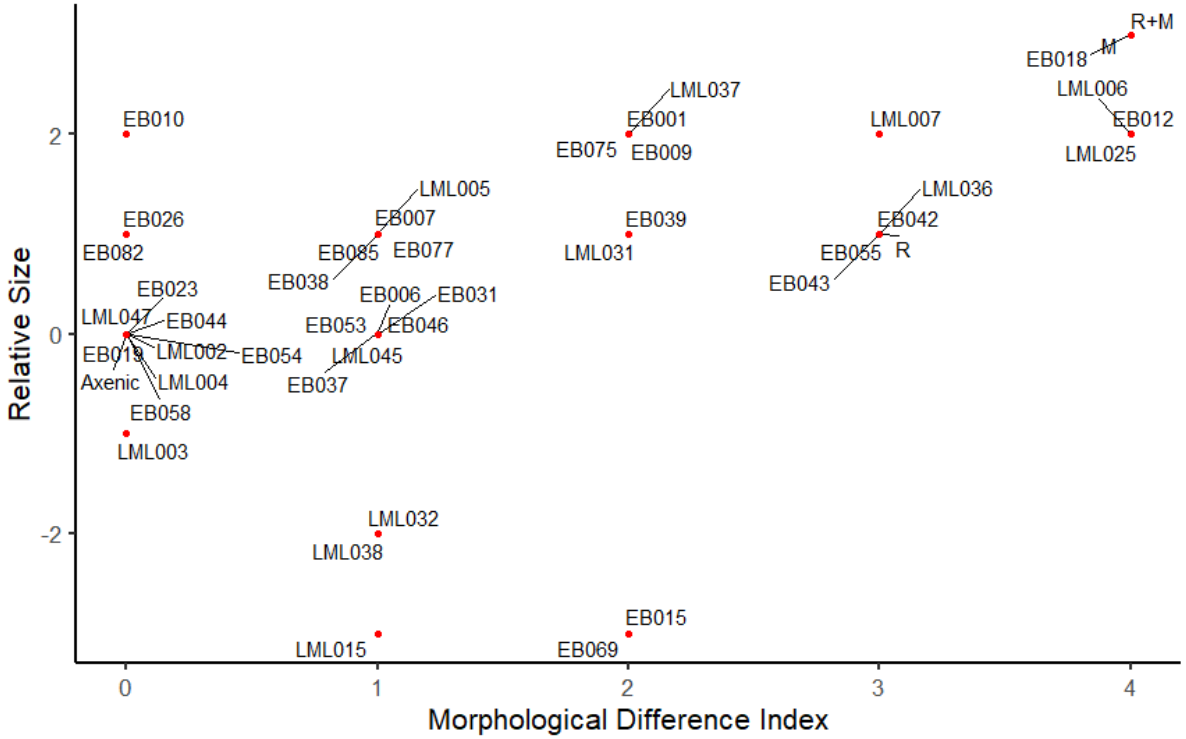

Figure 3: *MDI and relative size scatterplot of bacterial strains.*

Many strains did not induce morphology at all (the large cloud of strains tied to the point of MDI 0/Relative size 0, thus equal in both to the axenic control). The majority of strains only have a minor morphological effect. In contrast to this, there are a few strains which fully induce the normal morphology of *U. mutabilis*. These strains (EB018,12 and LML006,25) are part of the *Maribacter* genus, however, they are not the only *Maribacter* strains in the collection: not all

species of the *Maribacter* genus are equally capable of inducing typical morphology in *U. mutabilis*.

Pseudoalteromonas marina (EB015), *Dokdonia genika* (EB069), and an unknown *Winogradskyella* species (LML015) represent the smallest size category of germlings, while others also induce a smaller size than the axenic control: a *Lacinutrix* sp. (LML038) and *Maribacter* sp. (LML032), as well as *Maribacter vaceletii* (LML003).

In figure 4, six clusters are defined based on results, grouping strains based on the morphology they introduce. Cluster I and II consist of bacterial strains that induce an irregular shape, and form a gradient of germling size, with I consisting of sizes equal to the axenic control and II of larger sizes. Similarly, cluster III, IV and V define strains that cause a smooth shape, and different size increments, III being similar to the axenic control and V inducing a much larger size. Cluster VI consists of the strains that keep germlings small, as has been discussed earlier. The results visible in figure 5 preserve clustering based on size and shape, but also accurately represent other morphological traits upon comparison with the original data. Clusters I', II' and III' form a gradient of shape and size (I' control size and irregular, III' large size and smooth) and share the presence of cell wall protrusions. *Dokdonia sinensis* (EB010) is of an intermediate size between II' and III'. Clusters IV', V' and VI' lie along a similar gradient, and share the trait of rhizoid formation. Cluster VII' encapsulates the strains inducing extremely small size.

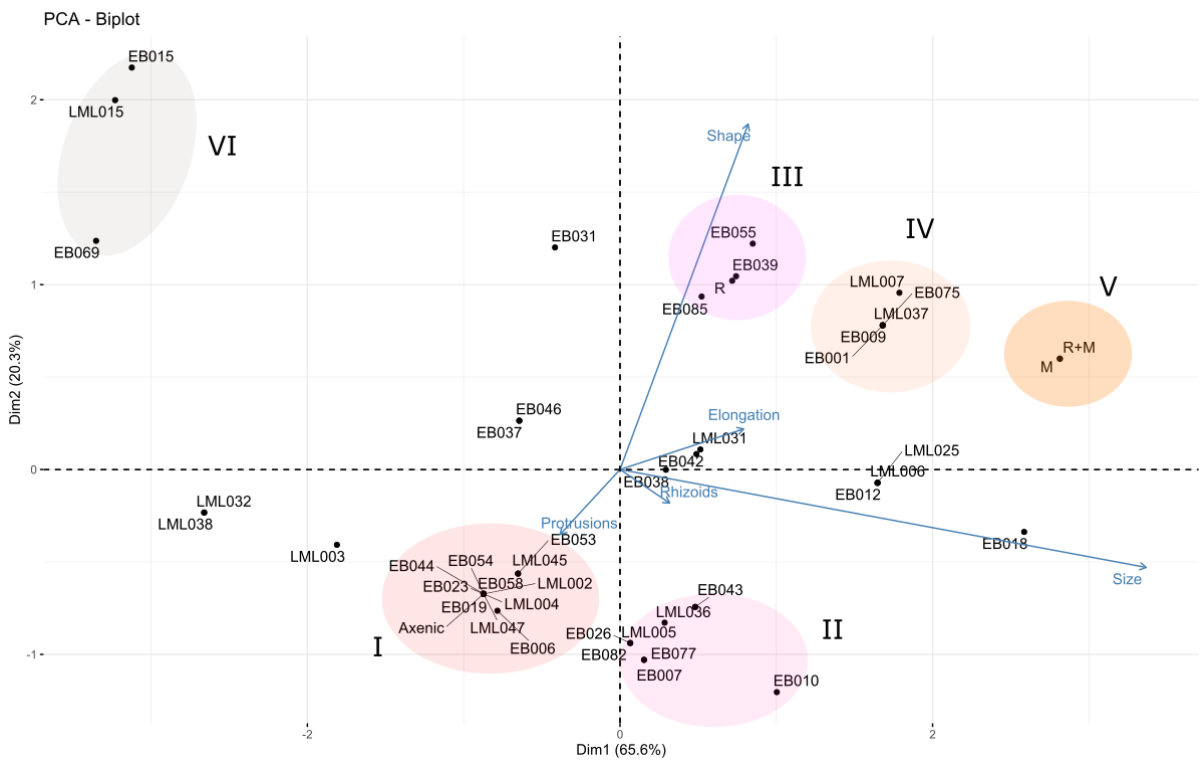

Figure 4: *PCA biplot of morphology data. Dim1 (horizontal) and Dim2 (vertical) represent the principal components that account for 65.6% and 20.3% of total data variation, respectively.*

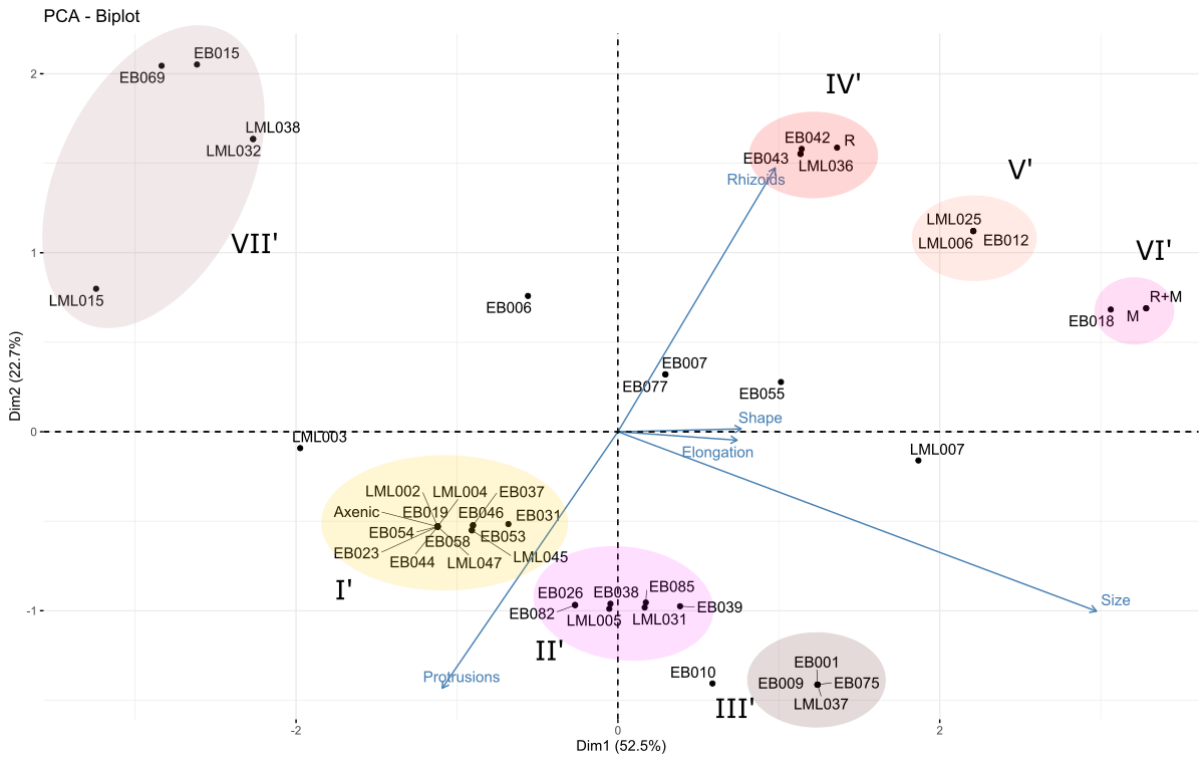

Figure 5: *PCA biplot of morphology data with additional weight attributed to the protrusion and rhizoid parameters. Dim1 (horizontal) and Dim2 (vertical) represent the principal components that account for 52.5% and 22.7% of total data variation, respectively.*

Strains found to promote both rhizoid formation and cell wall development (lack of protrusions) largely consisted of *Maribacter* species (*M. dokdonensis, M. chungangensis*, *M. litoralis* etc.). However, *Vibrio variabilis* (EB042), *Polaribacter* sp. (EB006) and *Dokdonia diaphoros* (EB007) also have these effects. A *Lacinutrix* sp. (LML038), *Maribacter* sp. (LML032) and unknown member of the Alteromonadaceae (EB077) are able to induce rhizoid formation although they lack proper cell wall development. Cell wall development and prevention of protrusions without rhizoid formation was induced by *Pseudoalteromonas marina* (EB015), *Alteromonas abrolhosensis* (EB055), *Dokdonia genika* (EB069) and *Sulfitobacter geojensis* (LML007).

Experiment 2: Strain effect on growth

Germling RGR varied between values of zero and 0.52 mm² growth per mm² per day (Figure 6). Interaction of strain and temperature conditions was highly significant according to the two-way ANOVA model ($p < 0.001$). In other words, the way that germling RGR responded to differences in temperature was dependent on the bacterial strain they were cultured with.

Pairwise comparisons of RGR between strains showed that adding any strain resulteds in a significantly higher RGR than observed in axenic germlings ($p<0.001$). Other significant results corroborated visual key differences visible in figure 6 (e.g., *Winogradskyella litoriviva* (LML031) inducing a higher germling RGR than the control Maribacter). Table 3 is a summary of significant results from additional post-hoc comparisons between germlings cocultured with the same strains, at different temperature levels.

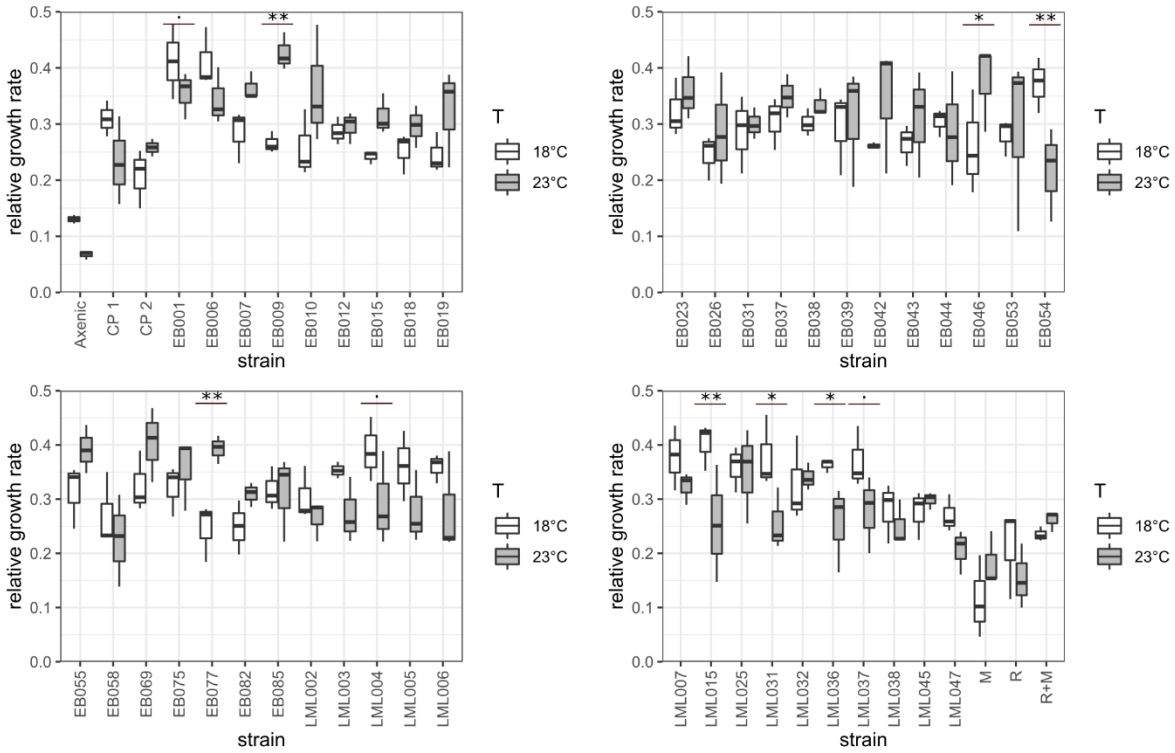

Figure 6: *Boxplots displaying the RGR of germlings inoculated with bacterial strains, under normal (18°C) and elevated (23°C) temperature. Strains that differ significantly (Tukey-adjusted pairwise contrasts with p < 0.05) in their RGR between temperatures are marked with * (p < 0.05) or ** (p < 0.01). Strains that were only barely non-significant were marked with · (p ± 0.05). R and M represent the Roseovarius and Maribacter control strains. CP1 and CP2 represent community pilots 1 and 2.*

Table 3: *Bacterial strains that induce a significantly different relative growth rate in U. mutabilis germlings under elevated temperature conditions. Their effect on RGR depending on temperature is marked with * (p < 0.05) or ** (p < 0.01).*

Pseudoalteromonas translucida (EB009)	RGR 18°C < RGR 23°C **
Vibrio atlanticus (EB046)	RGR 18°C < RGR 23°C *
Bacillus altitudinis (EB054)	RGR 18°C > RGR 23°C **
Unknown Alteromonadaceae sp. (EB077)	RGR 18°C < RGR 23°C **
Winogradskyella litoriviva (LML031)	RGR 18°C > RGR 23°C *
Maribacter spongiicola (LML036)	RGR 18°C > RGR 23°C *
Winogradskyella sp. (LML015)	RGR 18°C > RGR 23°C **

Experiment 3: Diversity effect on growth

Both community size ($p < 0.001$, ANOVA) and temperature condition ($p < 0.01$, ANOVA) had a significant effect on germling RGR (Figure 7). No clear evidence that the effect of temperature depended on community size was observed ($p = 0.086$).

Post-hoc pairwise comparisons between each pair of community sizes, and between temperature conditions for each community size separately revealed that a significant drop in RGR under elevated temperatures was only observed in axenic germlings ($p < 0.05$), and germlings cultured with communities of ten strains ($p < 0.001$). In addition, communities of 5, 10 and 20 strains induced a significantly higher RGR than an axenic treatment at 23°C ($p<0.05$), while at 18°C no such difference between strains was observed.

Taking all communities into account instead of grouping them based on size (Figure 8) also found that temperature and community have a significant impact on RGR ($p < 0.05$, ANOVA). Again however, no significant interaction between these variables was discovered ($p = 0.12$). Post-hoc testing based on Tukey's HSD showed a decrease in RGR with elevated temperature in 3 different communities ($p<0.05$), and an increase in RGR with elevated temperature in 1 community ($p<0.05$) (Table 4). Other communities displayed similar RGRs in both temperature treatments.

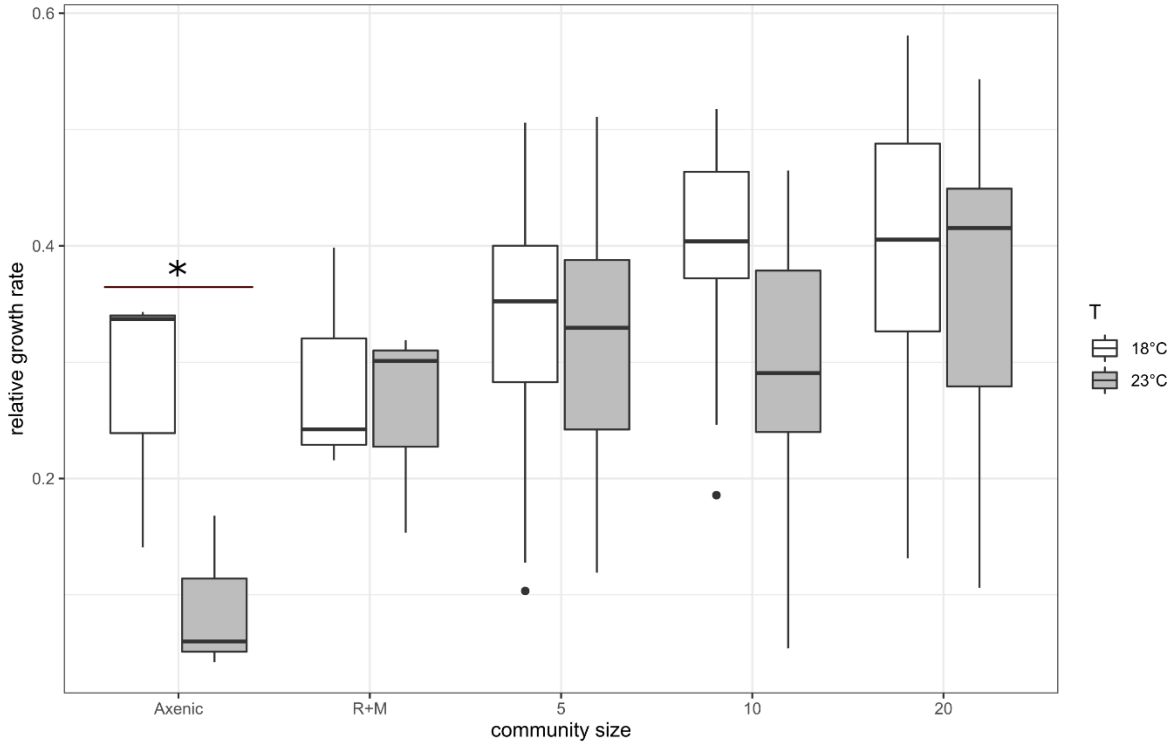

Figure 7: *Boxplots displaying the differences in relative growth rate (RGR, mm^2 growth per mm^2 per day) between germlings cultured with bacterial communities made up different numbers of strains. R and M represent the Roseovarius and Maribacter control strains. The * symbol signifies a p-value below 0.05 in post-hoc comparison between temperature conditions.*

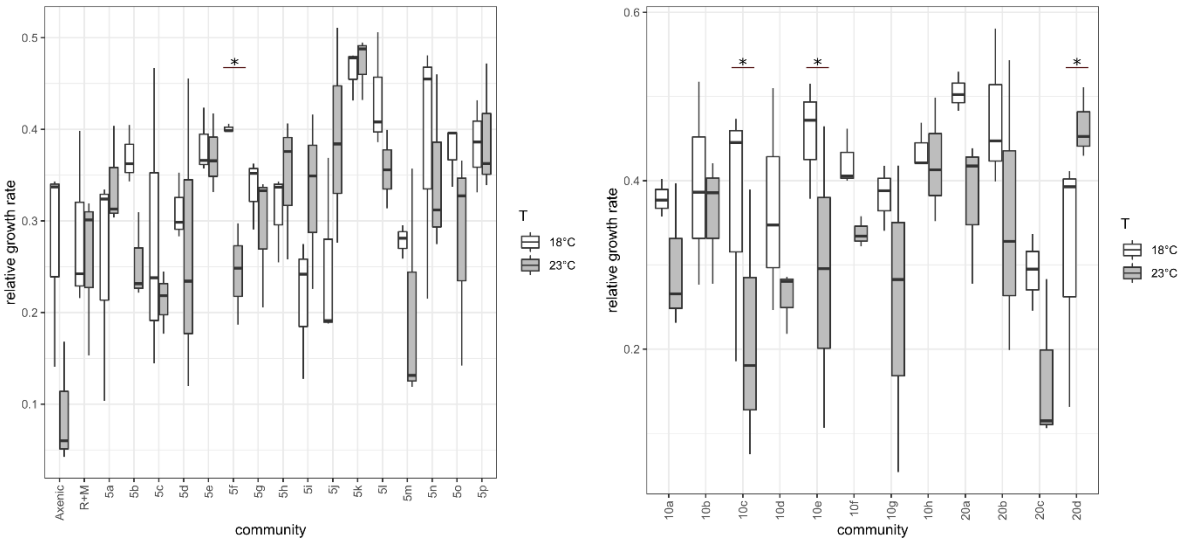

Figure 8: *Boxplots of germling RGR (mm² growth per mm² per day) for separate bacterial communities, showing the difference in relative growth rate between temperature treatments. The * symbol signifies a p-value below 0.05 in post-hoc comparison between temperature conditions.*

Table 4: *Bacterial communities that induce a significantly different relative growth rate in U. mutabilis germlings under elevated temperature conditions. The * symbol signifies a p-value between 0.05 and 0.01.*

Axenic	RGR 18°C > RGR 23°C *
5f	RGR 18°C > RGR 23°C *
10c	RGR 18°C > RGR 23°C *
10e	RGR 18°C > RGR 23°C *
20d	RGR 18°C < RGR 23°C *

Discussion

Experiment 1: Strain effect on morphology

First of all, it is worthwhile to address the unexpected effects of the *Roseovarius* and *Maribacter* strains use, as shown in the results of the morphology experiment. The developmental traits they induce in *U. mutabilis* do not wholly line up with the function that is attributed to them by Spoerner et al. (2012) and Weiss et al. (2017). The induction of rhizoids is not typical for *Roseovarius*, nor is full cell wall development (preventing protrusions from forming). *Maribacter* is characterised as inducing these traits, and while it does, its germlings also shows a large size as a result of cell division and elongation, which is what *Roseovarius* would be expected to cause (but does not). Due to the prevalence of horizontal gene transfer and transmissible genetic elements, different strains of the same bacterial species can be highly varied (Sobecky & Hazen, 2009) and the same is true for their impact on a host (Van Rossum, Ferretti, Maistrenko, & Bork, 2020). Additionally, the experiments of this thesis have shown that within the *Maribacter* genus, effects of different species also vary considerably (e.g., *M. vaceletii* does not induce any traits of proper morphology). Thus, it is not perplexing that *Maribacter* and *Roseovarius* effects deviate somewhat from their description in the surrounding literature. Furthermore, the consequence of these atypical individual effects was minimal: the strains that were used still produce a fully developed morphology when both are cultured with *U. mutabilis* together, which was the purpose of their inclusion in the growth experiments.

When examining the early visualisation of morphology results, one of the most eye-catching results is that some strains cause germling size to be much smaller than the axenic control. Effectively, they are detrimental to *U. mutabilis* growth as opposed to the majority of strains, which have a neutral or positive effect. Two of these strains are part of the *Flavobacteria*, with one being a *Dokdonia* species (*D. genika*). Many bacteria of the *Cytophaga-Flavobacterium-Bacteroidetes* group have been identified as inhibitors of algal growth, and as degraders of organic structural compounds like cellulose (Kirchman, 2002; Mayali & Azam, 2004). Though *Flavobacteria* are highly varied and frequently among growth-promoting as well as growth-reducing strains, Inaba et al. (2020) have found that *Dokdonia* is highly represented among growth-limiting bacteria in natural beds of blooming macroalgae. Yet, as seen in figure one even this observation is not universal: *Dokdonia sinensis* (EB010) induces growth more strongly than the axenic control. This confirms that even within genera and species, effects on a host can be extremely variable.

It is interesting to note that experiment two does not replicate the result of reduced size compared to axenic control. Likely, this is due to the promotion of growth by *Roseovarius* and *Maribacter*, which may overshadow any detrimental effects caused by *P. marina*, *D. genika* and other inducers of small size.

The scatterplot based on morphological difference index has been a useful tool to draw some early conclusions, like the identity of strains that induce small germling size strains. However, when assessing more nuanced effects, it must be treated with caution. Grouped strains may have the same MDI, but this only means that they induce a morphology equally different from the

axenic control. For instance, a strain inducing rhizoid growth and a strain preventing protrusion formation would be grouped together on the MDI axis.

Similarly, the first PCA analysis resulted in a biplot able to accurately explain size, but not traits like rhizoid or protrusion presence. The reason for this was that as the size variable had many levels with a larger numerical range it was overrepresented in the analysis. The *vegan* package for R includes a scaling method to standardise the impact of variables in a PCA analysis, however, percentages of variance explained by the principal components were strongly reduced when this method was applied. Furthermore, the impact of size was not well represented in the graphical output of a PCA standardised this way: effects that were extreme in reality were hardly or not at all visible in graphical displays of the model. This is the reason that a second PCA was conducted and included: changing the numerical scale of presence/absence gave rhizoid and protrusion presence more weight in the analysis without compromising the effect of size, and made the resulting graphical representations more true to reality when compared to raw data, at a minimal reduction of principal component explanatory capacity.

Though rhizoid formation and cell wall development is dominated by *Maribacter* in morphology results, clearly other species also possess this ability, some from wholly different phyla (e.g., *Vibrio variabilis* being part of the Proteobacteria rather than Bacteroidetes). In the case of *Maribacter*, establishment of *Ulva* morphology is mediated by the release of thallusin, a terpenoid morphogen described by Alsufyani et al. (2020) and Singh & Reddy (2014), the latter of which also describe a myriad of related and similar bioactive compounds. It is therefore interesting to speculate whether other Flavobacteria, or bacteria of a more distant taxonomy, may also produce thallusin or similar compounds, and to what degree this terpenoid may be conserved across bacterial evolution. Alternatively, considering the high rate of horizontal gene exchange by marine bacteria (McDaniel et al., 2010; Sobecky & Hazen, 2009) it is possible that the ability to produce thallusin and other bioactive is highly mobile across strains and broader taxa.

Experiment 2: Strain effect on growth

The second experiment highlights a few strains which differ significantly in relative growth rate depending on temperature conditions. While culturing with *Pseudoalteromonas translucida*, *Vibrio atlanticus* or Alteromonadaceae sp. (EB077) showed significantly improved growth rates at 23°C, RGR was reduced at this temperature for germlings with *Bacillus altitudinis, Winogradskyella litoriviva, Maribacter spongiicola* and *Winogradskyella* sp. (LML015).

A search for *P. translucida* and *V. atlanticus* using BacDive (Reimer et al., 2019) characterises these strains as mesophiles that grow optimally under temperatures from 25°C to 28°C. However, this does not make them unique among the isolated strains, so fails to explain their significantly higher relative growth rate under elevated temperatures. Similarly, it was difficult to pinpoint shared traits among the strains that caused germling RGR to be significantly reduced under elevated temperatures.

A clear conclusion that can be drawn from the second experiment, however, is that all bacterial strain significantly improved RGR of the germlings compared to the axenic control. The presence natural symbiont improves *U. mutabilis* growth even in culture conditions, despite not all strains

inducing a larger size in the first morphology experiment. Bacterial effects may increase as germlings age and bacteria multiply or form biofilms over time.

In addition, it is hard to ignore the clear tendency of some strains to display a visible increase or decrease in RGR under high temperature, even when they are not highlighted by the statistical analysis. Especially so since experimental replicates were limited due to the large number of strains that were included. For instance, *Maribacter* and *Roseovarius* show opposite effects on RGR under 23°C (*Maribacter* increasing germling RGR and *Roseovarius* decreasing it). When the two are combined these apparent effects seem to cancel each other out, leading to an equal RGR under 18°C and 23°C. Conducting additional experiments with a larger number of replicates to assess how *Maribacter* and *Roseovarius* specifically affect germling growth under high temperature seems advisable, and they are very well characterised with regards to morphogenesis induction. This may allow for specific morphogenic traits (and their bacterial inducers) to be linked to temperature resistance and even an improved growth under high temperatures.

Experiment 3: Diversity effect on growth

Although the first ANOVA model for the data of the third experiment did not find any significant effect of community sizes on the way germling RGR responds to temperature (no interaction between groups), this result must be taken with a grain of salt. The p-value for the interaction variable was 0.086 (close to 0.05) and additionally, the assumption of normality for the ANOVA model was deemed met only based on graphical interpretation of a histogram of residuals and a residual QQ-plot. The Shapiro-Wilk test returned a significant deviance from normal distribution in residuals ($p < 0.05$). For this reason, it must be kept in mind that the lack of a significant interaction variable may be due to a less reliable ANOVA model. Additionally, since there were more 5-strain communities than 10- and 20-strain, the number of observations per level of the community size grouping were not equal, which may have further skewed the results of the first model.

While the data of experiment two showed a significant increase in overall germling RGR when bacteria were present (as opposed to axenic germlings), the first model of experiment three describes a significant drop in axenic RGR under 23°C, but not under 18°C. This appears to show that axenic germlings are significantly more vulnerable to high temperatures that non-axenic ones. Visually, the data of experiment two also gives this impression. This results of post-hoc pairwise comparison contradicts the finding of the main model that community type did not impact the way RGR responded to temperature treatment, further questioning the accuracy of the model.

The second ANOVA model lacks some of the first's flaws: number of observations per level of the community grouping are equal. However, the same phenomenon persists. While no significant interaction is found by the main model, post-hoc testing reveals a significant difference in RGR between temperatures for some communities, but not all, indicating a community effect on temperature response. It is likely that employing a different and more complex data model is more appropriate for these results, however, this did not turn out feasible within the constraints of the study.

Community 20d presents itself as unique in its significant increase in germling RGR under an elevated temperature, while all other 20-strain communities showed decreased RGRs. It is certainly different from community 20c, with which it shares no common strains, and whose temperature response appears directly opposite to that of 20d. When compared to community 20a (which shares 9 strains with 20d) and 20b (which shares 11), an argument can be made that additional similarity to 20d improves RGR response to elevated temperature. However, 20d is made up of communities 5m, 5n, 5o and 5p, which all exhibit a neutral or negative RGR response to high temperature.

This appears contradictory, but it may imply an important interaction between strains that significantly improves *Ulva* growth under high temperature, which does not occur without a wholistic bacterial community. Bacteria are known for high rates of sociality within and between species, which persist during symbiosis and characterize the way they interact with a variety of hosts (Abisado, Benomar, Klaus, Dandekar, & Chandler, 2018). Though tyically defined by quorum sensing, social bacterial interactions are also highly predicated on metabolic exchange, which includes nutrient transfer but also mediation of metabolic plasticity through the myriad compounds that are released by communities of bacteria (Paczia et al., 2012; Ponomarova & Patil, 2015). In algal-bacterial symbiosis, the metabolism of host and microbiome are tightly linked through metabolite exchange as well (Fuentes et al., 2016; Segev et al., 2016), and macroalgae like *Ulva* (He et al. discusses *U. prolifera* specifically) repond to temperature stress primarily through metabolic strategies (Barati, Gan, Lim, Beardall, & Phang, 2019; He et al., 2018). This metabolic link between *Ulva* temperature response and bacterial sociality gives credibility to the idea that interaction between bacterial strains in a community, especially a larger one, may be as defining for algal growth under high temperature as any species- or strain-specific traits inherent in individual symbionts. This may be a valuable avenue of prospective study to elucidate bacterial effects of temperature response in *Ulva* and macroalgae in general.

The effect of community 5f and 10c on RGR temperature response are similar to one another (a significant decrease of RGR when temperature is raised), and community 10c contains the strains of community 5f, thus creating a through line of this effect being caused by the strains of community 5f. At the 20 strains level, community 5f is part of community 20a, which appears to show a drop in germling RGR under 23°C. 5f shares three strains with 20c as well, which do not occur in 20d: *Winogradskyella litoriviva* (LML031), *Polaribacter* sp. (EB006) and *Sulfitobacter geojensis* (LML007). Based on significant results of post-hoc testing and comparison of the highlighted communities, these strains appear of interest in eliciting a negative RGR response to high temperature. In the case of *W. litoriviva* this is confirmed by the findings of experiment two, where this strain also caused significantly reduced RGR under elevated temperature. *W. litoriviva* also occurs in community 10e, the remaining community with a significantly negative RGR response to elevated temperature.

Though *W. litoriviva* is not averse to high temperatures according to its characterising literature (Nedashkovskaya et al., 2015), this recurrence of its negative effect on *U. mutabilis* germlings under high temperatures does not appear coincidental and may prompt further investigation of the species as a potential algal pathogen at high temperatures. Temperature-dependent virulence is not rare and already threatens wild algae in marine environments (Mayers, Bramucci, Yakimovich, & Case, 2016). Therefore, this finding may be evidence that *W. litoriviva* was isolated as a latent pathogen on *Ulva australis*, or it could mean that while normally symbiotic, *W. litoriviva* (and other strains) turns to pathogenic activity under temperature stress.

Conclusion

In summary, there are many bacterial strains spread across phyla that can induce different aspects of *Ulva mutabilis* morphology. Even within narrow taxa their effects may vary significantly. It is likely that this variablility is related to the strong capacity of bacteria to horizontally transfer genetic material, which may include genes responsible for the production of morphogenic compounds. *Ulva* temperature response was also found to be influenced by some individual bacterial strains and also by larger communities of bacteria. *Winogradskyella litoriviva* was identified as a consistent illicitor of reduced growth rate, and could be either a pathogen or a symbiont showing virulence under elevated temperature, which is a common contributor to holobiont dysbiosis. Community results in general pointed towards within-community interactions as the defining factor for bacterial regulation of *Ulva* temperature response. This may be mediated by metabolic exhange and impact of bacterial signalling on algal metabolism, which is a promising avenue for future research.

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Appendix

APPENDIX 1: Strains isolated from wild *Ulva* specimens.

best_sample	Hit taxon name	Hit strain name	Accession	Samples with same ID	similarity	completeness	length (bp)	Final taxon	phylum	class	order	family	genus
EB001	Alteromonas addita	R10SW13(T)	CP014322	EB001	99.93%	99.20%	1461	Alteromonas addita/Alteromonas stellipolaris	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas
EB007	Dokdonia diaphoros	MSKK-32(T)	AB198089	EB007, EB047, EB050, EB060, EB066	99.54%	75.50%	1112	Dokdonia diaphoros/Dokdonia eikasta	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Dokdonia
EB009	Pseudoalteromonas translucida	KMM 520(T)	CP011034	EB009	99.73%	99.90%	1472	Pseudoalteromonas translucida	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas
EB010	Dokdonia sinensis	SH27(T)	MH681543	EB010	96.65%	99.10%	1451	Dokdonia sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Dokdonia
EB012	Maribacter dokdonensis	DSW-8(T)	LDPE01000001	EB012	99.66%	100.00%	1477	Maribacter dokdonensis	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
EB013	Shewanella electrodiphila	MAR441(T)	FR744784	EB008, EB013, EB026	99.58%	97.30%	1425	Shewanella electrodiphila	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
EB014	HM591462_s	SF-July-154	HM591462	EB005, EB014, EB016	96.56%	87.70%	1284	Glaciecola sp.	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola
EB015	Pseudoalteromonas marina	Mano4(T)	AY563031	EB015	98.86%	71.10%	1055	Pseudoalteromonas marina	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas
EB017	Glaciecola nitratireducens	FR1064(T)	CP003060	EB017, EB022	97.57%	99.10%	1452	Glaciecola sp.	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola
EB018	Maribacter_chungangensis	CAU 1044(T)	JN036550	LML040, EB018	98.35%	99.80%	1446	Maribacter_sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
EB019	Dokdonia aurantiaca	ZOW29(T)	MF614626	EB019	98.54%	100.00%	1473	Dokdonia aurantiaca	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Dokdonia
EB023	JQ218668_s	SPL24r	JQ218668	EB023	97.59%	94.00%	1375	Arenicella sp.	Proteobacteria	Gammaproteobacteria	Arenicellales	Arenicellaceae	Arenicella
EB031	Epibacterium scottomollicae	LMG 24367(T)	AM905330	EB031, EB035, EB041, EB048, EB057, EB059, EB089	100.00%	100.00%	1409	Epibacterium scottomollicae	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Epibacterium
EB033	Kordia ulvae	SC2(T)	KT589975	EB033	97.94%	70.70%	1021	Kordia sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Kordia
EB037	Ruegeria meonggei	CECT 8411(T)	FWFP01000022	EB037, EB083	99.93%	98.10%	1359	Ruegeria meonggei	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Ruegeria
EB038	Alteromonas genovensis	LMG 24078(T)	AM885866	EB038	99.08%	97.30%	1424	Alteromonas genovensis	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas
EB039	Sulfitobacter undariae	W-BA2(T)	KM275624	EB039	98.55%	99.70%	1391	Sulfitobacter undariae	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Sulfitobacter
EB042	Vibrio variabilis	R-40492(T)	GU929924	EB042	96.57%	100.00%	1492	Vibrio sp.	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
EB043	Maribacter forsetii	DSM 18668(T)	JQLH01000001	EB003, EB024, EB043	99.45%	100.00%	1486	Maribacter forsetii	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
EB044	Agarivorans aestuarii	hydD622(T)	KM203871	EB044	98.83%	99.10%	1475	Agarivorans aestuarii	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Algibacter
EB046	Vibrio atlanticus	Vb 11.11(T)	EF599163	EB046	99.79%	98.40%	1475	Vibrio atlanticus/Vibrio tasmaniensis	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
EB052	Loktanella acticola	OISW-6(T)	KY817315	EB052	98.99%	100.00%	1413	Loktanella acticola	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Loktanella
EB053	Nonlabens_ulvanivorans	PLR(T)	JPJI01000032	LML033, EB034, EB045, EB053	99.93%	100.00%	1479	Nonlabens_ulvanivorans	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Nonlabens
EB054	Bacillus altitudinis	41KF2b(T)	ASJC01000029	EB054	100.00%	100.00%	1499	Bacillus altitudinis/Bacillus xiamenensis	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus

EB055	Alteromonas abrolhosensis	PEL67E(T)	KC871606	EB055	99.86%	96.80%	1407	Alteromonas sp.	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas
EB058	Sulfitobacter donghicola	KCTC 12864(T)	JAMC01000023	EB058	97.75%	92.90%	1289	Sulfitotobacter sp.	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Sulfitobacter
EB065	Hyunsoonleella pacifica	SW033(T)	JX501247	EB065	97.18%	99.40%	1432	Hyunsoonleella sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Hyunsoonleella
EB069	Dokdonia_genika	Cos-13(T)	AB198086	LML039, EB032, EB040, EB056, EB061, EB069, EB071, EB080, EB084, EB086	99.72%	100.00%	1469	Dokdonia_genika	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Dokdonia
EB075	JXYE_s	S4079	JXYE01000009	EB064, EB067, EB074, EB075, EB078, EB079, EB090	99.06%	99.60%	1399	Yoonia sp.	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Yoonia
EB077	Alteromonadaceae sp.	THG-3.7(T)	KY694984	EB077	94.63%	99.60%	1463	not within genus limit!	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	N/A
EB082	Aquimarina amphilecti	92V(T)	JX050189	EB062, EB068, EB070, EB072, EB073, EB082, EB087	99.92%	100.00%	1483	Aquimarina amphilecti	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Aquimarina
EB085	Yoonia rosea	DSM 29591(T)	jgi.1085777	EB085	98.98%	99.20%	1372	Yoonia rosea	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Yoonia
LML002	Flagellimonas_aquimarina	ECD12(T)	KX245373	LML002, LML014, LML016, LML019, LML027, LML029, LML030, LML043, LML044, LML046, EB025, EB030	99.03%	100.00%	1469	Flagellimonas_aquimarina	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flagellimonas
LML003	Maribacter_vacelettii	DSM 25230(T)	RBIQ01000001	LML003, LML009	99.45%	100.00%	1473	Maribacter_vacelettii	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
LML004	Polaribacter_reichenbachii	KCTC 23969(T)	LSFL01000022	LML004, LML008, LML012, LML020	98.54%	100.00%	1475	Polaribacter_dokdonensis/reichenbachii	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Polaribacter
LML005	Algibacter_pectinivorans	DSM 25730(T)	jgi.1055420	LML005, LML048	99.54%	74.60%	1083	Algibacter_pectinivorans	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Algibacter
LML006	Maribacter_caenipelagi	HD-44(T)	KF748920	LML006	99.44%	74.70%	1088	Maribacter_sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
LML007	Sulfitobacter_geojensis	MM-124(T)	JASE01000005	LML001, LML007, LML034, LML041	99.93%	98.70%	1381	Sulfitobacter_geojensis	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Sulfitobacter
LML015	Winogradskyella_sp.	ABHI_s	ABHI01000004	LML015	98.75%	88.50%	1282	Winogradskyella_sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Winogradskyella
LML017	Paraglaciecola_aquimarina	GGW-M5(T)	JX508596	LML017	96.80%	96.80%	1408	Paraglaciecola_sp.	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Paraglaciecola
LML021	Octadecabacter_ascidiaceicola	CECT 8868(T)	FXYD01000004	LML010, LML018, LML021, LML022, LML023, LML028	98.64%	85.00%	1196	Octadecabacter_ascidiaceicola	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter
LML025	Maribacter_litoralis	SDRB-Phe2(T)	MG456900	LML025	99.48%	66.90%	968	Maribacter_litoralis	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
LML031	Winogradskyella_litoriviva	KMM 6491(T)	JX174421	LML031	98.90%	100.00%	1460	Winogradskyella_litoriviva	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Winogradskyella
LML032	Maribacter_sp.	REP6-13	JF769685	LML011, LML032, LML035, LML042, EB020	98.60%	98.50%	1435	Maribacter_sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
LML036	Maribacter_spongicola	W15M10(T)	JX050191	LML036	98.59%	53.90%	779	Maribacter_spongicola	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter
LML037	Octadecabacter_arcticus	238(T)	ABSK01000033	LML037	97.69%	100.00%	1411	Octadecabacter_sp.	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter
LML038	Lacinutrix_sp.	5H-3-7-4	AEYR01000002	LML038	99.17%	74.80%	1096	Lacinutrix_sp.	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Olleya
LML045	Erythrobacter_longus	DSM 6997(T)	JMIW01000006	LML045	98.70%	98.30%	1405	Erythrobacter_longus	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Erythrobacter
LML047	Erythrobacter_citreus	RE35F/1(T)	AF118020	LML047	97.70%	98.80%	1404	Erythrobacter_sp.	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Erythrobacter

APPENDIX 2: R Script for data visualisation and statistical analyses

```
####### Microbiomal composition impacts temperature response of Ulva mutabilis:
####### the macroalgalholobiont in a warming marine environment.

####### Ruben Algoet (2020-2021)

####### R Script for analyses and data visualisations


library(MASS)
library(vegan)
library(ggplot2)
library(ggrepel)
library(factoextra)
library(gridExtra)
library(carData)
library(car)
library(emmeans)

setwd("C:/Users/Ruben Algoet/Desktop/THESIS R PROJECTS") ## <- SET APPROPRIATE WORKING
DIRECTORY!

#-----------------------------------------------------------------------------------------
## EXPERIMENT 1 SCATTERPLOT (Figure 3)

ex.1.scatter <- read.table("experiment_1_scatterplot_data.csv", header = TRUE, sep = ",")
set.seed(43)
ggplot(ex.1.scatter, aes(x = Morphological.Difference.Index, y =
Relative.Size.to.Negative.Control, label = STRAIN)) +
  geom_text_repel(max.overlaps = Inf) +
  geom_point(color = 'red') +
  theme_classic(base_size = 16) +
  ylab("Relative Size") +
  xlab("Morphological Difference Index")

#-----------------------------------------------------------------------------------------
# EXPERIMENT 1 PCA & PLOTS

ex.1.data = read.table("experiment_1_morphology.csv", header=TRUE, sep = ",")
rownames(ex.1.data) <- ex.1.data[,1]
ex.1.data$X <- NULL

#Preliminary plots

ex1.pca <- rda(ex.1.data)
ex1.pca
plot(ex1.pca)
bp.ex1 <- biplot(ex1.pca, scaling = -1)

#Plot 1 (Figure 4)

options(ggrepel.max.overlaps = Inf)

set.seed(123)
ex1.pca.rpl <- prcomp(ex.1.data)
factoextra::fviz_pca_biplot(ex1.pca.rpl, repel = TRUE)

#Plot 2 (Figure 5)

ex1.data.impact <- read.table("experiment_1_morphology - high impact.csv", header=TRUE, sep =
",")
rownames(ex1.data.impact) <- ex1.data.impact[,1]
ex1.data.impact$X <- NULL

ex1.pca.impact <- prcomp(ex1.data.impact)
factoextra::fviz_pca_biplot(ex1.pca.impact, repel = TRUE)

#-----------------------------------------------------------------------------------------
## EXPERIMENT 2 BOXPLOTS (Figure 6)
ex2.data = read.table("experiment_2_data.csv", header = TRUE, sep = ",")

ex2.I <- ex2.data[c(1:72),]
```

```
ex2.II <- ex2.data[c(73:144),]
ex2.III <- ex2.data[c(145:216),]
ex2.IV <- ex2.data[c(217:294),]

ex2.I.pl <- ggplot(data = ex2.I, aes(x=strain, y=RGR, fill=T)) + geom_boxplot() + theme_bw() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1)) + ylab("relative growth
rate") +
  scale_y_continuous(expand = c(0, 0), limits = c(0, 0.5),breaks=c(0,0.1,0.2,0.3,0.4,0.5)) +
  scale_fill_manual(values=c("white", "gray"))

ex2.II.pl <- ggplot(data = ex2.II, aes(x=strain, y=RGR, fill=T)) + geom_boxplot() + theme_bw()
+
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1)) + ylab("relative growth
rate") +
  scale_y_continuous(expand = c(0, 0), limits = c(0, 0.5),breaks=c(0,0.1,0.2,0.3,0.4,0.5))+
  scale_fill_manual(values=c("white", "gray"))

ex2.III.pl <- ggplot(data = ex2.III, aes(x=strain, y=RGR, fill=T)) + geom_boxplot() +
theme_bw() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1)) + ylab("relative growth
rate") +
  scale_y_continuous(expand = c(0, 0), limits = c(0, 0.5),breaks=c(0,0.1,0.2,0.3,0.4,0.5))+
  scale_fill_manual(values=c("white", "gray"))

ex2.IV.pl <- ggplot(data = ex2.IV, aes(x=strain, y=RGR, fill=T)) + geom_boxplot()  +
theme bw() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1)) + ylab("relative growth
rate") +
  scale_y_continuous(expand = c(0, 0), limits = c(0, 0.5),breaks=c(0,0.1,0.2,0.3,0.4,0.5))+
  scale_fill_manual(values=c("white", "gray"))

grid.arrange(ex2.I.pl, ex2.II.pl, ex2.III.pl, ex2.IV.pl, nrow = 2, ncol = 2)

#-------------------------------------------------------------------------------------------
## EXPERIMENT 2 STATISTICAL ANALYSIS
##LINEAR MODEL:
ex2.aov <- aov(RGR ~ strain * T, data = ex2.data)

##TWO-WAY ANOVA ASSUMPTIONS:
##Normal distribution of residuals:
#Calculate standardised residuals:
ex2.aov.stres <- rstandard(ex2.aov)

#Histogram
ggplot(ex2.aov, aes(x = ex2.aov$residuals)) + geom_histogram(color="black", fill="white") +
  labs(title = 'Histogram of Residuals', x = 'Residuals', y = 'Frequency')

#QQ-plot
qqPlot(ex2.aov.stres)

#Shapiro test
shapiro.test(ex2.aov.stres)

#Residuals are normally distributed

#Homogeneity of variance:
leveneTest(RGR ~ strain, data = ex2.data)
leveneTest(RGR ~ T, data = ex2.data)
#Variance is homogenous between groups

## SIGNIFICANT INTERACTION VARIABLE (p < 0.0001): warrants post-hoc testing
## POST-HOC TESTS

TukeyHSD(ex2.aov, 'strain')
emmeans(ex2.aov, pairwise ~ factor(T) | strain, adjust="tukey")

#-------------------------------------------------------------------------------------------
## EXPERIMENT 3 BOXPLOTS

ex3.data = read.table("experiment_3_data.csv", header = TRUE, sep = ",")

ex3.data$com_size <- factor(ex3.data$com_size, levels = c("Axenic", "R+M", "5", "10", "20"))

ex3.I <- ex3.data[(1:72),]

ex3.II <- ex3.data[(73:180),]
```

```
ex3.II$community <- factor(ex3.II$community, levels = c("Axenic", "R+M", "5a", "5b", "5c",
"5d", "5e", "5f", "5g",
                                                        "5h", "5i", "5j", "5k", "5l", "5m",
"5n", "5o", "5p"))

#Plot 1 (Figure 7)

ggplot(data = ex3.data, aes(x=com_size, y=RGR, fill=T)) + geom_boxplot() + ylab("relative
growth rate") +
  scale_fill_manual(values=c("white", "gray")) + theme_bw() + xlab("community size")

#Plot 2 (Figure 8)

ex3.I.plot <- ggplot(data = ex3.I, aes(x=community, y=RGR, fill=T)) + geom_boxplot() +
ylab("relative growth rate") + theme_bw() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1)) +
scale_fill_manual(values=c("white", "gray"))

ex3.II.plot <- ggplot(data = ex3.II, aes(x=community, y=RGR, fill=T)) + geom_boxplot() +
ylab("relative growth rate") + theme_bw() +
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1)) +
scale_fill_manual(values=c("white", "gray"))

grid.arrange(ex3.II.plot, ex3.I.plot, ncol = 2)

#-------------------------------------------------------------------------------------------
## EXPERIMENT 3 STATISTICAL ANALYSIS

##### COMMUNITY SIZE
ex3.size.aov <- aov(RGR ~ com_size*T, data = ex3.data)

##ANOVA ASSUMPTIONS:
##Normality:
#Calculate standardised residuals:
ex3.size.aov.stres <- rstandard(ex3.size.aov)
#Histogram
ggplot(ex3.size.aov, aes(x = ex3.size.aov$residuals)) + geom_histogram(color="black",
fill="white") +
  labs(title = 'Histogram of Residuals', x = 'Residuals', y = 'Frequency')
#QQ-plot
qqPlot(ex3.size.aov.stres)
#Shapiro test
shapiro.test(ex3.size.aov.stres)

##Homogeneity of variance:
leveneTest(RGR ~ com_size, data = ex3.data)
leveneTest(RGR ~ T, data = ex3.data)
#Variance is homogenous between groups

# POST-HOC
TukeyHSD(ex3.size.aov, 'com_size:T')
emmeans(ex3.size.aov, pairwise ~ factor(T) | com_size, adjust="tukey")

##### SEPARATE COMMUNITIES
ex3.type.aov <- aov(RGR ~ community*T, data = ex3.data)
summary(ex3.type.aov)

##ANOVA ASSUMPTIONS:
##Normality:
#Calculate standardised residuals:
ex3.type.aov.stres <- rstandard(ex3.size.aov)
#Histogram
ggplot(ex3.type.aov, aes(x = ex3.type.aov$residuals)) + geom_histogram(color="black",
fill="white") +
  labs(title = 'Histogram of Residuals', x = 'Residuals', y = 'Frequency')
#QQ-plot
qqPlot(ex3.type.aov.stres)
#Shapiro test
shapiro.test(ex3.type.aov.stres)

##Homogeneity of variance:
leveneTest(RGR ~ community, data = ex3.data)
leveneTest(RGR ~ T, data = ex3.data)
#Variance is homogenous between groups

# POST-HOC
emmeans(ex3.type.aov, pairwise ~ factor(T) | community, adjust="tukey")
```