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INTERUNIVERSITY MASTER OF SCIENCE IN MARINE AND LACUSTRINE SCIENCE AND MANAGEMENT

CHASING BACTERIA IN THE GREEN ALGA *BRYOPSIS PLUMOSA*

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Abstract

Bryopsis is a siphonous green marine macroalga characterized by a complex life cycle including both sexual and asexual reproductive stages. While the diverse bacterial communities associated with *Bryopsis* have been extensively studied, little is known about the stability of these bacterial communities. We investigated the bacteria associated with *Bryopsis* spp. from different sites along the European coast. Our aim was to understand the stability of the bacterial communities over time and host reproduction. We therefore focused on tracking bacteria across selected life stages in *Bryopsis* spp. We characterized the *Bryopsis*-associated bacteria using high throughput 16S rRNA amplicon sequencing. Symbiont stability was tested by characterizing cultures which had been in the lab for an extended amount of time. Bacterial transmission during sexual reproduction was monitored by screening *Bryopsis* gametes using transmission electron microscopy (TEM). The potential for the acquisition of novel bacteria during protoplast formation, an asexual reproduction strategy, was tested using fluorescent bacteria. Proteobacteria (mostly Alphaproteobacteria and Gammaproteobacteria), Bacteroidetes, and to a lesser extent Cyanobacteria and Firmicutes dominated the communities associated with *Bryopsis*. *In situ Bryopsis* samples hosted the highest bacterial species richness while communities present in the *Bryopsis* cultures which had been maintained in the lab for at least six months were the most diverse. Significant differences in the alpha and beta diversity indices of the bacterial communities hosted by the different *Bryopsis* sample types and reproductive stages in our study illustrated limited bacterial symbiont stability over time and life cycle transitions. Spatial variations were also observed among communities associated with *in situ Bryopsis* strains. Bacterial communities associated with hosts from a single location, Marseille (France) were more similar than those found on hosts from other sites. Vertical bacterial transmission was not observed using TEM on *Bryopsis* gametes. Fluorescent bacteria were not stably acquired (horizontal transmission) during *Bryopsis* protoplast formation, suggesting that *Bryopsis* employs a selectivity mechanism against foreign bacteria during protoplast formation which possibly influences horizontal bacterial transmission. The phylogenies of *Bryopsis* did not reflect the similarity between bacterial communities associated with those hosts, implying a lack of a host-symbiont evolutionary signal which is characteristic of transient symbioses. Our study sheds light on the stability of the bacterial communities associated with *Bryopsis* sp. and how this is impacted by cultivation and the potential for (directed) horizontal and vertical transmission. Functional characterization of these bacterial communities is recommended to improve the understanding of factors determining bacterial stability in *Bryopsis*.

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1.0 Introduction

1.1 Background: Seaweed-bacterial relationships

No organism is an island. Ecosystems consist of biota living together in a variety of inter- and intraspecies relationships (Boyd & Brown, 2015) which are influenced by many factors such as variations in environmental conditions (Smale & Wernberg 2013; Goulet & Goulet, 2021). Marine macroalgae (seaweeds) are a taxonomically diverse group of organisms which provide favorable conditions for the proliferation of various marine species, including marine bacteria (Egan *et al.*, 2013). These prokaryotic partners reside either in (endobionts) or on (epibionts) their seaweed hosts (Hollants *et al.*, 2011a; Egan *et al.*, 2013; Cleary & Huang, 2020) either permanently (obligates) or transiently (facultatives) (Goecke *et al.*, 2010). Early studies on seaweed-bacterial associations, mainly based on microscopy, confirmed the presence of diverse symbiotic bacteria associated with seaweeds (Kong & yu Chan, 1979; Duan *et al.*, 1995). Subsequent advanced molecular techniques which enabled the in-depth characterization of seaweed associated communities showed that the most abundant bacterial phyla associated with seaweeds are Bacteriodetes, Firmicutes and Proteobacteria (Hollants *et al.*, 2013a, b; Singh & Reddy, 2014; Selvarajan *et al.*, 2019).

Seaweed - bacterial relationships are characterized by a broad spectrum of interactions which may be positive, negative or neutral (Relman, 2008; Theis *et al.*, 2016). The functions performed by bacteria for algae include the induction of changes in the host life cycle (Patel *et al.*, 2003, Dimitrieva *et al.*, 2006); causation of morphological changes (Marshall et al., 2006; Wichard, 2015, Ghaderiardakani *et al.*, 2020); pathogenesis (Correa *et al.*, 1993, Craigie & Correa, 1996, Egan *et al.*, 2013; Ward *et al.*, 2019); secretion of biologically active metabolites such as antimicrobial compounds which protect the host (Wiese *et al.*, 2008, Arnaud-Haond *et al.*, 2017) and nutrient provision through breakdown of complex organic matter and nitrogen fixation (Rosenberg and Paerl, 1981, Chisholm *et al.*, 1996).

The performance of these microbial-mediated functions confers physiological advantages to their hosts thus enhancing algal fitness (Stratil *et al.*, 2014). Bacteria associated with the invasive, siphonous seaweed *Caulerpa* have been suggested to be at least partly responsible for its rapid proliferation, even in oligotrophic aquatic systems (Arnaud-Haond *et al.*, 2017; Chisholm *et al.*, 1996). Understanding the functional diversity of seaweed-associated bacterial communities is essential to understand the ecological roles of these partnerships. Moreover, it enhances the exploration for novel bioactive compounds from the bacterial symbionts for diverse industrial applications (Qian *et al.*, 2009; Penesyan *et al.*, 2009).

1.1.1 Bacterial symbiont stability and transmission in seaweeds

Several factors determine the diversity, composition and structure of bacterial symbiont communities in marine macroalgae. These include seaweed species (Lachnit *et al.*, 2009; Weigel and Pfister 2019), host physiology and life cycle (Lemay *et al.*, 2018, 2021), functional thallus differentiation (Aires *et al.*, 2015), bacteria-bacteria interactions (Foster *et al.*, 2017) and the abiotic conditions (Quigley *et al.*, 2020). However, the assembly and maintenance of host-symbiont relationships in macroalgae is still considered a complex process that is poorly

understood and influenced by many more unpredictable factors (Zhou *et al.*, 2013). Hosts sharing a similar ecological niche and phylogenetically closely related species are often expected to be more similar in their symbiont community structure than their counterparts who are separated by substantial spatial and phylogenetic distances (Harvey & Pagel, 1991; Borcard *et al.*, 1992). This hypothesis has been tested by comparing the bacterial communities associated with seaweeds to the phylogenetic relation between seaweeds, inferring patterns of host-symbiont coevolution (Hollants *et al.*, 2013b). The phylogenetic signal between hosts and their respective symbiont communities is used as a proxy to assess species' evolutionary relatedness and is defined by Blomberg and Garland (2002) as *"the tendency for related species to resemble each other more in a trait than expected by chance."*

The diversity of the bacterial community in a seaweed is also determined by symbiont transmission. The mode of transmission may be either vertical (direct symbiont transfer from parent to offspring) or horizontal (symbiont uptake from the host environment or via a secondary vector) (Moran *et al.*, 2008; Aires *et al.*, 2015). Vertical transmission mechanisms that often guarantee symbiont transmission across generations are typically observed in symbionts that are extremely essential for host fitness (Moran, 1993). It may result in clear host-symbiont phylogenetic patterns as observed in certain sponge and insect symbionts (Kikuchi, 2009; Dale and Moran, 2006; Burgsdorf *et al.*, 2015). Conversely, horizontally transmitted bacterial symbionts are generally considered more facultative in nature, forming short-lived, partnerships with their respective hosts (Wahl *et al.*, 2012; Romero Picazo *et al.*, 2019). The evolution of symbiotic relationships into close co-dependencies in which the host and symbiont(s) depend on each other for ecological success and survival has resulted in the development of the holobiont concept which considers the partnership as one biological unit (Morrissey et al, 2019).

1.2 *Bryopsis* spp.

Bryopsis is a genus of the siphonous green seaweeds belonging to class Ulvophyceae. It is characterized by a feathery morphology consisting of a tube-like giant cell structure with a large, centralized vacuole surrounded by a multinucleate cytoplasmic region (Mine *et al.*, 2008). *Bryopsis* has a complex life cycle composed of multiple pathways of sexual and asexual reproduction. These pathways coincide with physiological and morphological changes during the transitions between reproductive stages (Morabito *et al.*, 2010). Protoplast formation is in *Bryopsis*, as in several other siphonous seaweeds a way of asexual reproduction (Kim *et al.*, 2001). During wounding events, the single-celled structure of these macroalgae predisposes them to a high risk of destruction due to potential rapid loss of cytoplasm (Welling *et al.*, 2009). Protoplast formation has evolved as a rescue mechanism (Menzel, 1988). The extruded cytoplasm agglutinates into spherical structures, the protoplasts, which subsequently undergo *de novo* cell wall synthesis and develop into fresh thalli (Kim *et al.*, 2001).

1.2.1 Bacterial symbiont diversity in *Bryopsis*

The intracellular environment of siphonous macroalgae, including *Bryopsis*, forms a favorable niche for diverse bacterial endobionts, whose presence has been validated using microscopy (Burr *et al.*, 1970; Dawes & Lohr, 1978; Hollants *et al.*, 2011a) and molecular analyses (Hollants *et al.*,

2011, 2012, 2013; Morrissey *et al.*, 2019). Hollants *et al.*, (2012) highlight five bacterial taxa, viz, several members of the Cytophaga-Flavobacterium-Bacteroides (CFB) group, Actinobacteria, Gammaproteobacteria, Firmicutes and Alphaproteobacteria as the main constituents of *Bryopsis'* core community. Studies into the functional diversity of the bacterial symbionts hosted by tubular green algae species such as *Caulerpa* and *Bryopsis* has revealed the performance of essential functions by specific bacterial species (Hollants *et al.*, 2011, 2012). Nitrogen fixation, for instance is a key function that has been associated with Alphaproteobacterial symbionts hosted by giant-celled macroalgae (Cocquyt *et al.*, 2010). Flavobacteria and Bacteriodes symbionts secrete morphogens and growth promoting factors which are essential for their seaweed hosts (Spoerner *et al.*, 2012), while the secretion of bioactive compounds such as the predator-repellant kahaladides produced by *Bryopsis* has been credited to its bacterial symbionts (Zan *et al.*, 2019).

1.2.2 Transmission and stability of bacterial symbionts in *Bryopsis*

In *Bryopsis*, both vertical and horizontal bacterial transmission mechanisms have been studied based on microscopy and molecular techniques (Burr and West, 1970; Kim *et al.*, 2002; Hollants *et al.*, 2011, 2013). The presence of bacterial particles in *Bryopsis* gametes, for instance has previously been described (Burr and West, 1970) although the exact mechanisms of bacterial symbiont transmission via gametes during sexual reproduction remain unknown (Arnaud-Haond *et al.*, 2017). Further, Hollants *et al.*, (2013) emphasize the role of ecological conditions and host physiology in the acquisition and maintenance of endosymbiont communities owing to observed structuring of most microbiota based on environmental gradients. This aligns with findings by Meusnier *et al* (2001) on the biogeographical distribution of seaweeds and their associated microbiota. Subsequent studies also show that changes in the microbiome structure might help the seaweed cope with altered abiotic conditions such as temperature and salinity shifts (Dittami *et al.*, 2016).

The community structure and composition of bacterial endobionts in *Bryopsis* has been shown to be the product of a tight interplay between ecological conditions, spatial variations and evolutionary forces (Hollants *et al* 2013b). Using variation partitioning analysis, Hollants and colleagues (2013b) attempted to disentangle the role of environmental factors, host physiology and evolutionary forces in structuring bacterial communities hosted within *Bryopsis*. Their study revealed that while the occurrence of Rickettsia and Mycoplasma seemed to be mainly influenced by abiotic factors, the presence of Flavobacteria and Bacteroidetes was strongly linked to the host phylogeny, illustrating the possible impact of evolutionary drivers for these specific endosymbionts. This conclusion was supported by the apparent stability of Flavobacterial and Bacteroidetes communities in *Bryopsis* samples collected at geographically separate locations in comparison to the abundance of other bacteria which varied with changes in host habitat. Spatial stability of Flavobacteria and Bacteriodes implies that they may be obligate endobionts that have evolved a high fidelity to *Bryopsis* due to the roles they play which might be essential to host survival. On the other hand, Rickettsia and Mycoplasma may be epiphytic symbionts that form facultative relationships with *Bryopsis* (Hollants, 2011a). Similar spatial stability in bacterial symbionts was observed in *Caulerpa* (Aires *et al.*, 2015), implying that this is likely to be a shared

characteristic of bacterial communities hosted by siphonous marine macroalgae. The sustenance of host-symbiont fidelity over evolutionary timescales often contributes to the gradual occurrence of coevolution and/ or co-speciation events which are often evidenced by clear host-symbiont phylogenetic congruency (Rosenblueth *et al.,* 2012).

The changes that occur during the different sexual and asexual reproduction pathways may also influence bacterial symbiont recruitment, transmission and maintenance across generations, which is still poorly understood in *Bryopsis*. Additionally, the role of protoplast formation in shaping endophytic bacterial communities in *Bryopsis* has not been studied. This process represents a unique opportunity where the intracellular constituents of *Bryopsis* come into direct contact with the external environment, including the bacterial communities therein (Kim *et al.*, 2001; Hollants et al., 2011b). Klotchkova *et al.,* (2005) concluded that *Bryopsis* employs certain mechanisms to selectively determine which particles are incorporated into the protoplast. These findings aligned with the chemical compounds such as lectins (Kim *et al.*, 2006; Yoon *et al.*, 2008) which prevent the incorporation of foreign bacterial and inorganic particles into the newly formed *Bryopsis* protoplasts.

1.3 Rationale and study objectives

Several studies have shed light on the diversity and to a lesser extent, the functions and variability of bacterial communities hosted by *Bryopsis*. However, little is known about the impact of cultivation and reproduction on the stability of the bacterial symbiont community. This study focused therefore on obtaining further insights into stability of bacterial symbionts associated with *Bryopsis* spp. from selected sites along European Coast. Here, we use the term "bacterial symbionts" to refer to the bacterial communities (both epi- and endobionts) that are associated with the *Bryopsis* spp. We aimed to (i) characterize the collected *Bryopsis* spp. and the bacterial symbionts associated with them; (ii) investigate the variations in bacterial symbiont community structure at selected life and cultivation stages of their *Bryopsis* hosts and (iii) assess the phylogenetic signal of *Bryopsis* species and its bacterial symbionts. We hypothesized that (a) the diversity bacterial communities associated with *Bryopsis* spp. would decrease with increase in the culture period (b) the composition of the bacterial symbionts would differ in the different reproductive stages of the host; (c) there would be distinct members of the bacterial community including potential endosymbiotic bacteria such as *Mycoplasma,* which would remain stable over time and across the different reproductive stages of the host and (d) the *Bryopsis*-bacterial evolutionary history would be evidenced by a phylogenetic signal showing the relatedness of the host and bacterial communities.

2.0 Materials and methods

2.1 Design of this study

The scheme below (Fig. 1) illustrates the workflow that was used to study the bacterial symbionts associated with and transmitted in *Bryopsis* spp. Experiments were conducted by the author unless stated otherwise. Briefly, samples collected at different sites were transported to the Phycology laboratory in Ghent University where *in vitro* propagation by sexual and asexual reproduction techniques was conducted. Both the *Bryopsis* hosts and bacterial communities present at different stages were characterized using molecular techniques. Microscopy was used to validate bacterial transmission. Each step is expounded in more detail in this chapter.

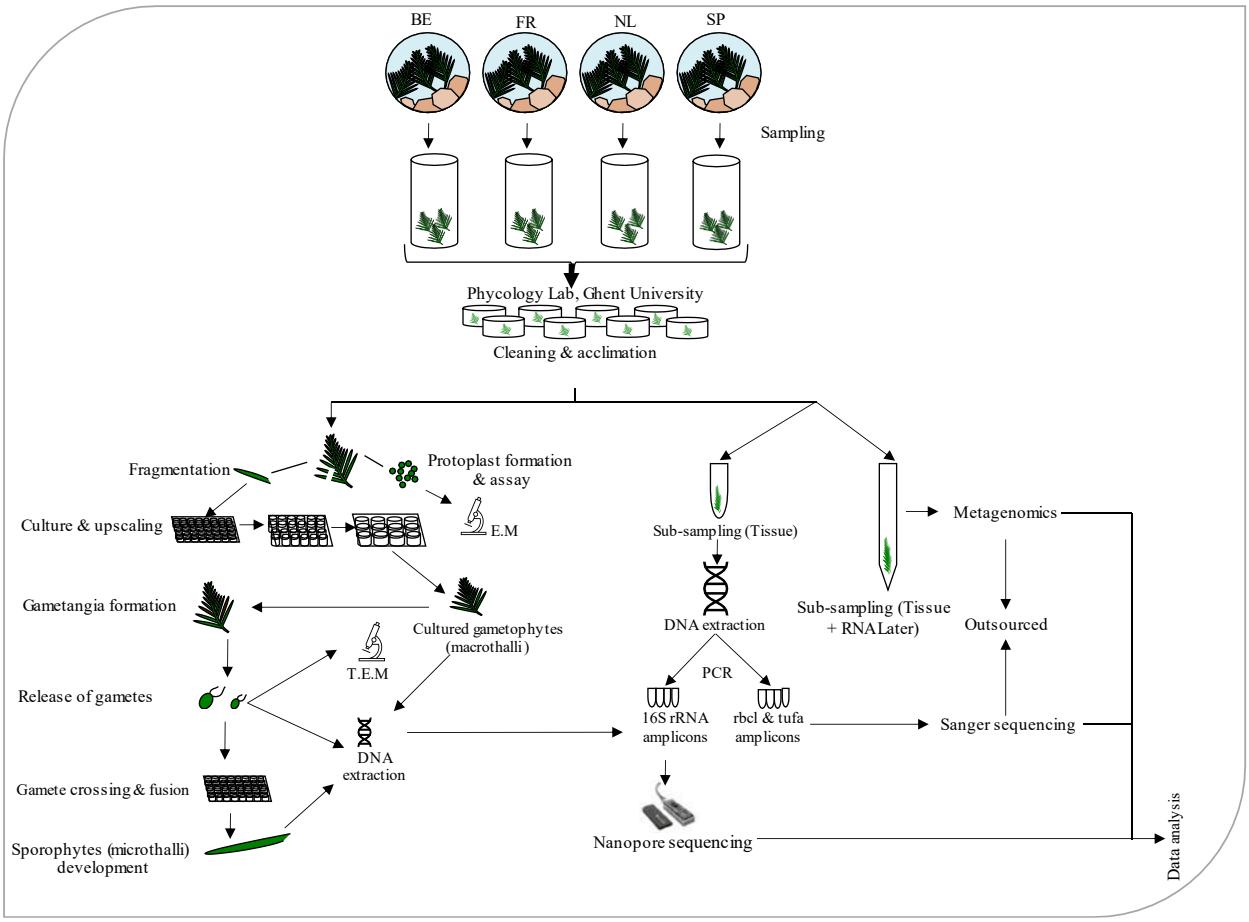


Figure 1: Summary of the study design. Top: *Bryopsis* spp. sampled at different sites and acclimated in the lab. Bottom, left: *In vitro* propagation of *Bryopsis* spp. via asexual (fragmentation and protoplast formation) and sexual (gametogenesis) reproduction. Transmission electron microscopy (TEM) used to investigate vertical bacterial transmission during gametogenesis and Epifluorescence microscopy (EM) applied to assess horizontal transmission during protoplast formation. Bottom, right: Host and symbiont characterization using molecular techniques and subsequent data analysis.

2.2 Collection and acclimation of *Bryopsis* specimens

Bryopsis samples (gametophytes) were collected from different sites located within the littoral zone of the European coast including Platja Cala Fosca in Spain, Marseille & Étang de Thauin in France, Sas van Goes & Grevelingenmeer in the Netherlands and Jachthaven Zeebrugge in Belgium (Fig. 2A). The collection of these specimen was conducted over varying periods between May and September 2021. The GPS position of each site was recorded and photographs of the

specimen taken *in situ* during sampling (Fig. 2B). Identification of the specimen before collection at the field was based on morphological traits of the characteristically featherlike thallus of *Bryopsis*. Each specimen was then placed in a separate, labelled plastic container half-filled with sea water and transported to the phycology laboratory at Ghent University. The sampling in Sas van Goes was done by the author, all other sampling were undertaken by members of the Phycology research groups. All samples were processed in the lab (as described below) by the author.

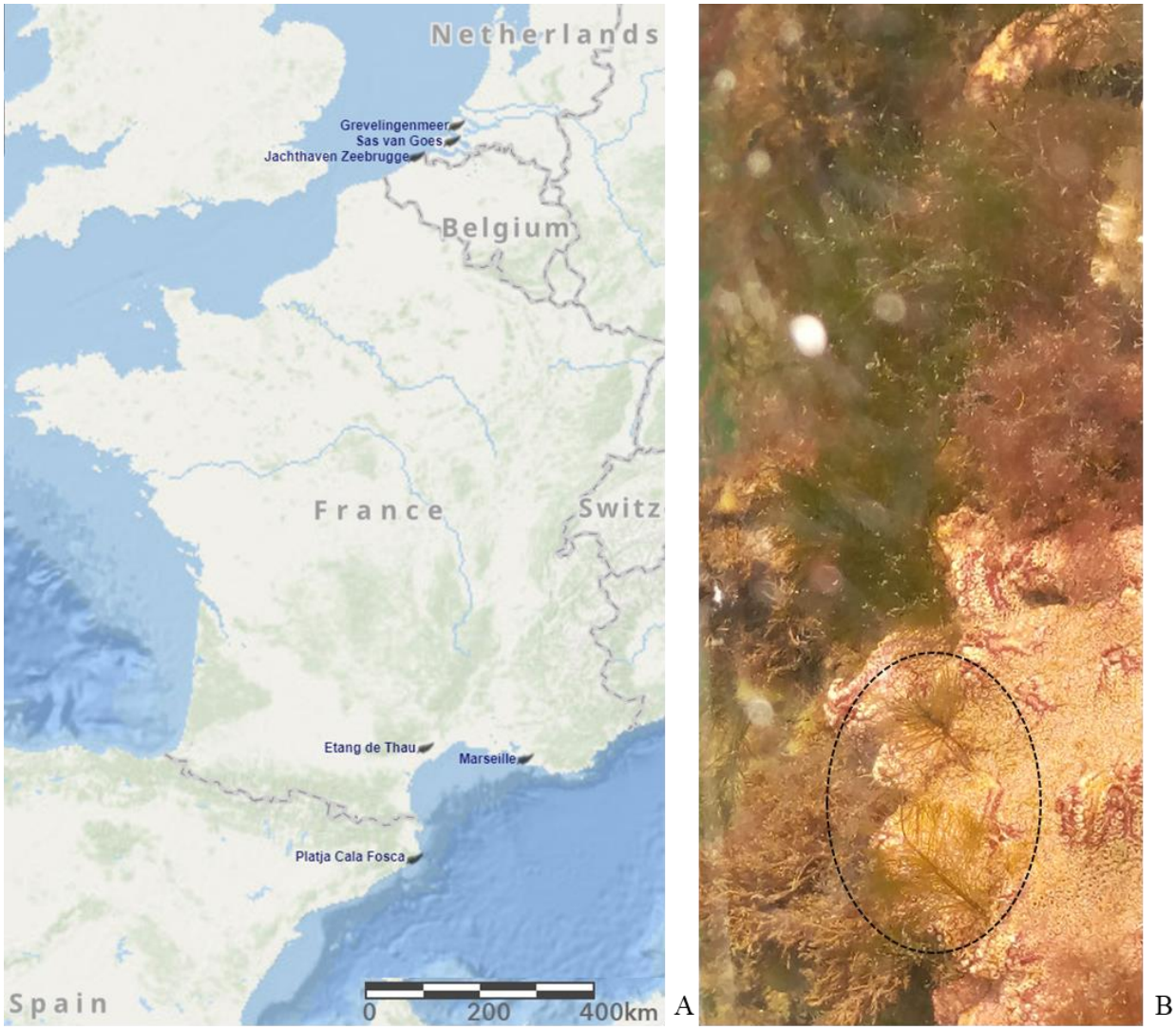


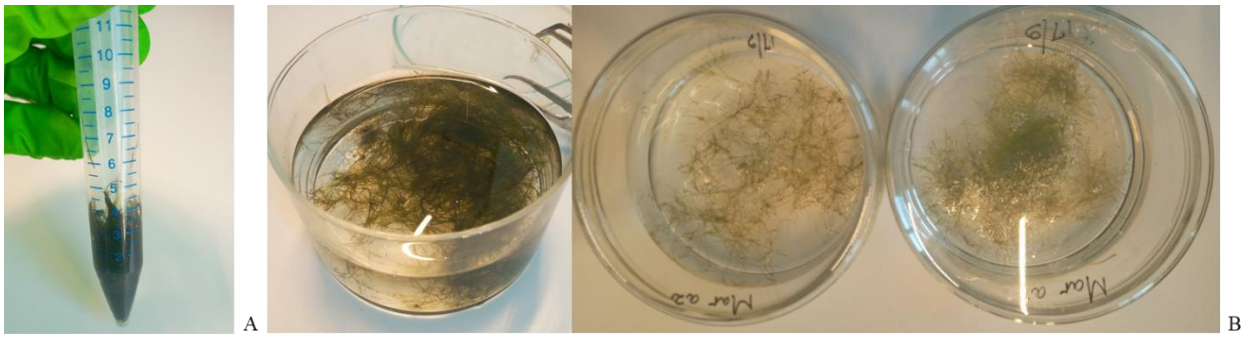
Figure 2: A. Sampling sites (ArchGIS, 2022) B. *In situ Bryopsis* sp. (encircled) before collection (Njeru J.)

At the lab, the samples were transferred from the sampling containers into sterile crystalizing dishes (Fig. 3B) using sterilized forceps. All foreign material attached to the specimens were carefully removed and the samples acclimatized in an incubator at 18°C and a 12h:12hr light-dark cycle (~12.4 µmol photons/m^2/s), for a period of one to three days. Periodic observation of the samples was conducted to monitor the acclimation of the macroalgae to laboratory conditions, identify any signs of stress and remove contaminated or necrotic sections of the algal tissues.

Successfully acclimated samples were then cultured in 1/10 MPES (Modified Provasoli Enriched Seawater) medium (West and Mcbride, 1999). All the stock cultures were maintained for at least 6 months. The medium was refreshed at intervals of seven to ten days, with removal of any sections of contaminated tissues.

2.3 Sub-sampling for preliminary molecular analysis

Each *Bryopsis* sp. sample was sub-sampled for DNA extraction. The sub-samples were preserved in RNALater solution (Thermo Fisher Scientific; Fig. 3A) for extraction of molecular material for metagenomic analyses. A second sub-sample of each strain was stored in a 2ml centrifuge tube and frozen at -80°C or -20°C for subsequent extraction of total genomic material for phylogenetic analysis of the samples and *in situ* bacterial communities (see below).



A B

Figure 3: A. *Bryopsis* sub-sample for molecular analysis B. Cultures in cyrtalizing dishes (Njeru J.)

2.4 *Bryopsis* propagation and assessment of selected life cycle stages

Collected *Bryopsis* gametophytes were used for assessing different life cycle stages of the siphonous green alga. Asexual reproduction was achieved by fragmentation and protoplasm formation, while gamete release and fusion into sporophytes constituted the sexual stages of the life cycle.

2.4.1 *In vitro Bryopsis* fragmentation subculture, maintenance and upscaling

From each acclimated sample, two replicates of a fragment of the apical tissue were aseptically transferred to separate wells of a 48 well plate containing sterile sea water enriched with 2/10 MPES medium and germanium (IV) oxide (GeO_2) which was essential for the inhibition of diatom proliferation (Shea & Chopin 2007). The samples were maintained at 18°C on a 12h: 12h light-dark cycle. Media refreshment with monitoring for growth was performed on a weekly basis. Efforts were made to eliminate the abundant contaminants including ciliates, cyanobacteria, diatoms, red algae and dinoflagellates from the cultures. This was done by washing the fragments through two wells containing the culture medium before transferring them to the final plates containing fresh media. This was done on a daily basis for a week.

Following weeks of growth in the 2/10 MPES, the fragments had grown in size and thus their nutritional requirements increased. The contaminants had also reduced significantly after several cleaning and refreshing sessions. This necessitated the increase of the nutrient concentration to 5/10 PES after 12 – 16 weeks and subsequently to 10/10 MPES after eight weeks. As the fragments increased in size, they were gradually transferred from 48 to 24, 12 and finally six well plates to allow sufficient space for growth and plume formation.

2.4.2 Induction of *Bryopsis* protoplast formation

2.4.2.1 Protoplast formation

Asexual reproduction by formation of protoplasts was investigated. Intracellular material was extruded from cultured gametophytes by cutting and squeezing the tissue or crushing *Bryopsis* tissue in a 1.5ml Eppendorf tube using a sterile pestle. The extruded cytoplasm was transferred a glass slide and observed under the microscope for the initial aggregation of chloroplasts and cellular material into spherical protoplasts. The specimen was then incubated at 16°C with periodic observation for further development of the typical spherical structure, formation of a phospholipid membrane and growth into thallus. This work was performed by Arno Felix.

2.4.2.2 Protoplast aggregation pH assay

Optimal conditions for extracellular aggregation of protoplasts were tested. The influence of extracellular pH on protoplast formation in *Bryopsis* sp. has been studied (Ye *et al.*, 2005). A trial was conducted to assess the influence of pH on the formation of protoplasts. The extruded protoplasm material obtained as described above was transferred to wells containing autoclaved sea water and artificial sea water (Tropic Marine Sea Salts) with pH adjusted (0.1M NaOH or HCl) to 6 and 8. The specimen were incubated in the climate room with daily observation to monitor aggregation into protoplasts and subsequent growth into thalli.

2.4.2.3 Fluorescence microscopy of protoplasts

The uptake of both living and non-living particles during protoplast formation was tested by introducing motile green fluorescent protein (GFP) - labelled bacteria and fluorescent beads into samples of freshly cut *Bryopsis* sp. filaments containing newly formed protoplasts. Four different GFP-labelled bacteria were used, viz *Vibrio* ME9-GFP, *Phaeobacter inhibens* DSM 17395-GFP, *Zobellia* Y24-GFP and *Escherichia coli* pBAV1K and were screened to confirm motility and adequate fluorescence (Fig. 9 - b) prior to introduction into the protoplast assays. Fluorescent microscopy and imaging were conducted using a Nikon Ni-U Epifluorescence microscope with a DS-Fi3 camera. The uptake of the fluorescent beads and bacteria by the protoplasts over minutes to several days was assessed. 500 µl each of 1/10 MPES medium, FluoSperes and bacteria were pipetted into wells, with a separate mixture prepared for each bacterial strain. A negative control was prepared that did not contain the bacteria. *Bryopsis* sp. were then added into the wells and fragmented to release the protoplasm. The assay was incubated at room temperature and observed for the first 15 minutes after fragmenting and again after 72 hours to confirm the uptake and temporal stability of the fluorophores using fluorescent microscopy.

2.4.3 Gametogenesis in *Bryopsis* sp.

2.4.3.1 Gametangia development and gamete release

During observations of the cultures, specimen that had developed plumes were observed for differentiation into gametangia, which characteristically appear as a netting structure towards the edges of the filaments. Attempts were also made to induce differentiation of the filaments into gametangia according to the procedure outlined by Burr & West (1970) and Mine *et al.*, (1996). In summary, the tip of the plumes (approx. 2 to 3cm) from each sample were transferred to the fresh wells. The plates were then incubated in the culture room with frequent observation for

development of gametangia. Upon formation of the gametangia, gamete release was halted by overnight incubation of the gametangia in darkness for a period of 10-14 hrs. The specimens were subsequently exposed to ambient light to trigger spontaneous release of the gametes. Motile gametes were then harvested by pipetting and transferred to a 1.5 ml centrifuge tube and preserved in 70% at -20°C ethanol or RNALater for subsequent molecular analysis described below. A sub-sample of the gametes was fixed for transmission electron microscopy as detailed below.

2.4.3.2 Gametogenesis induction by temperature and light variation

An experiment was set up to test the effect of temperature on the induction of gametogenesis. Apical tips of plumes from 17 *Bryopsis* strains were obtained as described above and transferred to wells containing MPES medium. A total of four replicates of each strain were obtained for subsequent incubation at different temperatures i.e., 18C° (in the culture room), 16°C, 18°C (in an incubator) and 20°C. The specimens were maintained in culture with weekly media refreshing and observation for the onset of gametogenesis and gamete release.

2.4.3.3 Inter-strain gamete hybridization and development

Gametes harvested from four strains were tested for inter-strain complementarity. 500 µl of media containing freshly released motile gametes were harvested and distributed to labelled wells in a 48 well plate. Gametes from different *Bryopsis* strains were mixed into the same well to test for successful development of the sporophytes. The inter-strain crossing was done as illustrated in Table 1 below. The gametes were incubated in the culture room with observation for fusion into zygotes and growth conducted every two weeks. Sporophytes that developed following successful gamete fusion were subsequently sampled for DNA extraction and characterization of bacterial symbiont communities using MinION (Oxford Nanopore Technology; ONT) sequencing as described below.

Table 1: Crossing of gametes from different strains. x indicates a cross between the strains indicated in the respective column and row headers. c indicates control wells containing gametes from single strains (not crossed)

	GR	**Co A**	**A2**	**Mar c**
GR	c	x	x	x
Co B	x	c	x	x
A2	x	x	c	x
Mar c	x	x	x	c

2.4.3.4 Transmission electron microscopy of *Bryopsis* sp. gametes

Transmission electron microscopy (TEM) was used to validate the presence of bacteria in the gametes. Gametangia in four selected strains containing motile gametes were isolated. After release of the gametes, the medium containing the free-swimming gametes was transferred to centrifuge tubes containing media in duplicates. The gametes were centrifuged down at 5000g for 5 minutes to sediment the motile gametes. Fixation was conducted as described by Burr and West (1970) with a few modifications. For each pair of samples per strain, 1 ml of 5% glutaraldehyde (Aurion, the Netherlands) was added to one tube and 1 ml of 4% formaldehyde (VWR, USA) to the other. The gametes were incubated on a shaker at room temperature for two hours after which 250 µl of the supernatant was removed from each tube and replaced with 250 µl of freshly prepared

0.1 M sodium cacodylate buffer adjusted to pH 7. The tubes were then incubated at room temperature for 20 min followed by a 5 min centrifuge spin at 5000g. The replacement of 250 µl of the supernatant with the buffer, mixing and centrifugation steps were repeated twice as described above with gradually increasing volumes of the supernatant replaced by the buffer (500 µl then 900 µl). The samples were then dehydrated using ethanol in increasing concentrations of 30%, 50% and 70%, each time mixing well before incubating on a shaker at room temperature for 20 minutes then centrifuging for 5 min at 5000g before transferring to the next dilution. The fixed and desalinated gametes were finally stored in 70% ethanol at -20°C (for samples fixed using formol) and subsequently delivered to the UGent TEM-Expertise center for further processing and TEM imaging.

2.5 Molecular analyses

Changes in the bacterial symbiont communities with time and reproduction were assessed by comparing the communities in different sample types. In assessing the temporal variations, we assessed the symbiont communities present in three sample types i.e., the originally sampled individuals (*In situ*), specimen cultured for (20 - 24 weeks) and samples that were maintained in culture for prolonged periods of time (> 24 weeks). For assessing the influence of reproduction on the bacterial communities, we compared four different *Bryopsis* host types i.e. (i) the original *in situ* mature gametophytes (Original gametophyte), (ii) gametophytes propagated *in vitro* by fragmentation of the original thalli (Propagated gametophyte), (iii) protoplasts developed *in vitro* by wounding selected strains (Protoplast) and (iv) sporophytes following gamete fusion (Sporophytes).

2.5.1 Extraction of total DNA from *Bryopsis* samples

Extraction was conducted on the *in situ* samples preserved on the sampling day as well as samples that had been from the cultures which had been cultivated for 6 to 12 months in the lab. The commercial QIAamp DNA Mini Kit (QIAGEN) was used for extraction of genomic DNA from the *Bryopsis* samples for subsequent phylogenetic analysis of both the *Bryopsis* strains and their respective bacterial communities. A negative control (empty 2ml Eppendorf tube) was included to identify contaminants during the DNA extraction procedure. Cell lysis was achieved by using a combination of mechanical disruption and biochemical treatments. Zirconium beads and 180 µl of lysis buffer ATL was added to each of the 2ml centrifuge tubes containing the frozen *Bryopsis* samples. Bead beating was then done at 30Hz for 5 minutes after which the samples were pelleted and 20 µl of Proteinase K was added for breakdown of portentous cellular components in the lysate. The samples were then vortex-mixed and incubated at 56°C for one hour. 200 µl of lysis buffer AL was then added to each sample, vortexed for 15 seconds and incubated at for 10 minutes at 70°C. 200 µl of absolute ethanol was then added and the mixture vortexed to mix. The supernatant of each sample was then pipetted onto a QIAamp Mini spin silica column and centrifuged at 8,000 rpm for 1 minute. The flow-through was discarded and the column washed through two steps using 500 µl buffers AW1 (8,000 rpm for 1 min.) and AW2 at (14,000 rpm for 3 min.) respectively. The columns were then placed on labelled 1.5 ml centrifuge tubes and 200 µl of elution buffer AE followed by a 1 min spin at 8,000 rpm. For acquisition of maximum nucleic

acid yield, elution was repeated, after which the columns were discarded and the genomic material stored at -20°C.

2.5.2 Marker gene amplification, assessment and sequencing

2.5.2.1 *Bryopsis* specimen

For molecular identification of the *Bryopsis* strains, two chloroplast DNA makers were used i.e., *rbcL* and *tufA* (Oliveira *et al.*, 2021). For the *rbcL* marker, two sets used i.e., 712F (5' -CATTAY TYAAATGCWACWGC- 3') with 1391R (5' -TCTTTCCAAACTTCACAAGC- 3') and 7F (5' -CCAMAAACWGAAACWAAAGC- 3') with 791R (5' -GGNAYACCNAAWTCTTTIGC- 3'). For the *tufA* marker, the F (*5*'- TGAAACAGAAMAWCGTCATTATGC- 3' and R (5' CCTTCNCGAATMGCRAAWCGC 3') primer set was used. 1 µl of DNA template from each sample was added to the PCR mix which consisted of 1.25 µl of each primer, 2.5 µl of 10X PCR buffer, 2.5 µl of 200 µM of dNTP's, 1.0 µl of 100µg/µl BSA, 14.75 µl of milli-Q water and 1.25 µl of Taq polymerase. The mixture was briefly vortexed to mix and subjected to a 40-cycle PCR run. Each cycle was characterized by a thermal profile comprising five steps i.e., initial denaturation for 3 min at 94°C, denaturation for 1 min at 94°C, annealing at 45°C for 1 min, elongation for 2 min at 72°C and final elongation for 5 min at 72°C. The quality of the PCR products was assessed using agarose gel electrophoresis as described below. Sanger sequencing of the PCR products was then outsourced.

2.5.2.2 Bacterial symbionts associated with *Bryopsis*

The 16s rRNA gene amplification and ONT sequencing on a MinION flow cell of the amplicon was performed according to the procedure described by van der Loos *et al.*, (2021) as summarized below. Primers 27F_BCtail-FW (TTTCTGTTGGTGCTGATATTGC_AGAGTTTGATCMTGGCTCAG) & 1492R_BCtail-RV (ACTTGCCTGTCGCTCTATCTTC_CGGTTACCTTGTTACGACTT) were used for amplification of the 16S rRNA genes of bacterial communities present in the total DNA samples extracted from the *Bryposis* samples. A blank PCR sample and a commercial bacterial mock community were included. The PCR mix added to 1 µl of the genomic material from each sample contained 7.5 µl Phire Tissue Direct PCR Master Mix, 0.3 µl primer mix and 6.2 µl water. The samples were vortexed to mix and subjected to a PCR run involving the thermal profile of initial denaturation at 98°C for 3 min, 30 cycles (denaturation at 98°C for 8 sec, annealing at 60°C for 8 sec and elongation 72°C for 30 sec) and final extension at 72°C for 3min after which the samples were maintained at 12°C. Guided the manufacturer's protocols, the resultant PCR products were barcoded, purified, quantified, ligated with adapters and loaded onto the MinION for sequencing (D'hondt, 2020). Molecular analysis of protoplast samples was conducted Arno Felix.

2.5.2.3 *Mycoplasma* in selected *Bryopsis* sp. strains

Five strains were selected based on amplicon sequencing data to confirm the presence of *Mycoplasma* amplicons in their bacterial communities. The selected strains had been in culture for an extended period of time (8 to 12 months) and would be assessed to confirm the persistence of *Mycoplasma* symbionts for this extended period. DNA extraction was conducted as described above. Samples where no *Mycoplasma* was detected based on the ONT 16s rRNA gene amplicon

sequencing data were used as negative controls. A blank was included as additional control. Three primer sets were selected (van Kuppeveld *et al.,* 1993; Young *et al.,* 2010) to best match to the obtained amplicon consensus sequences identified as *Mycoplasma* in our data set (Table 2). The PCR mix for each sample consisted of 2.5 µl buffer, 2.5 µl dNTP's, 1 µl BSA, 1.25 µl Taq polymerase, 14.25 µl milliQ water, 1.25 µl of each primer and 1 µl DNA template. The samples were vortexed to mix and subjected to a PCR run of 40 cycles of the thermal profile 94°C for 1 min (denaturation), 60°C for 1 min (annealing) and 72°C for 2 min (extension). The amplified sequences were analyzed by gel electrophoresis Lee *et al.,* (2012) as described below.

Table 2: Primer sets used for *Mycoplasma* detection

No.	Strand	Set	Sequence	bp length	Amplicon length
1	Sense	GPO-1	GGGAGCAAACAGGATTAGATACCCT-	25	724
	Antisense	MGSO	TGCACCATCTGTCACTCTGTTAACCTC	27	
2	Sense	GPO-3	GGGAGCAAACAGGATTAGATACCCT	25	288
	Antisense	MGSO	TGTATTACCGCGGCTGCTG	19	
3	Sense	GPO-1	ACTCCTACGGGAGGCAGCAGTA	22	277
	Antisense	Mseq-3	TGTATTACCGCGGCTGCTG	19	

2.5.2 4 Assessment of PCR product quality by gel electrophoresis

2.5.2.4.1 Preparation of agarose gel

1 g of agarose was dissolved in 60 ml of buffer 1 X TAE. The mixture was heated 2 minutes to dissolve. The molten gel was then poured into a mini-gel tray, fitted with a gel comb and allowed to solidify at room temperature for 15 minutes. The was solidified gel immersed in 1 X TAE buffer in an electrophoresis chamber.

2.5.2.4.2 Agarose gel electrophoresis run

5 µl of each the PCR-amplified products mixed with 5 µl of loading dye was pipetted into respective wells of the 1% agarose gel in the electrophoresis chamber. A 30-minute electrophoresis run was then performed. The gel was immersed in ethidium bromide for 15 min to stain the bands which were visualized using a UV transilluminator. Bands formed were compared to a standard DNA ladder to confirm successful amplification based on the expected length per amplicon. The quality of *rbcL, tufA* and *Mycoplasma* amplicons was assessed using this procedure.

2.6 Processing of sequence reads and data analysis

The chromatograms from the *rbcL* and *tufA* sequences were cleaned using Mega 11 software (version 11.0.10) and exported in FASTA format. The sequences were concatenated into the same file and used to generate a maximum likelihood phylogenetic tree on the RAxML (Randomized Axelerated Maximum Likelihood) platform. The tree was based on a Hasegawa-Kishino-Yano + Gamma (HKY+G) substitution model which was selected for both partitions using Mega X software (version 11.0.10). Data on the bacterial 16S rRNA amplicon reads was were analyzed using a modified version of the NanoCLUST pipeline described by Rodríguez-Pérez *et al.,* (2020). Briefly, the pipeline commenced by cleaning out the sequences to remove reads with lengths above or below the length of the amplicon. The remaining sequences were then converted into 5-mers to

enable effective clustering of similar sequences into operational taxonomic units (OTUs). Clusters were delineated using UMAP (version 0.4.6, McInnes *et al.,* 2018). The clustering settings were optimized based on a test conducted to cluster of a mock bacterial community.

For each cluster a consensus sequence was generated using (Canu version 2.0; Koren *et al.*, 2017). The consensus sequences were then polished using Racon (v 1.4.13; Vaser *et al.*, 2017) and Medaka (v 1.0.3; Oxford Nanopore Technologies, 2020). The consensus sequences and counts per cluster (relative abundances) were then imported into R Studio. The DADA2 package (v 1.22.0; Callahan *et al.,* 2016) was used to further clean the data through removal of bimeras. The remaining consensus sequences were aligned using ssu-align (v 0.1.1; Nawrocki, 2009) also masking ambiguous positions. A phylogenetic tree was constructed from this alignment using FastTree (v 2.1.11; Price *et al.*, 2010). Taxonomy was assigned to the consensus sequences using the SILVA database (v 123; Quast *et al.,* 2013) with vsearch. The OTUs from different samples were agglomerated based on the tree (tip_glom from the phyloseq package with h=0.03; v 3.14; McMurdie & Holmes, 2013). OTUs that could not be assigned to a bacterial taxon or that were assigned to chloroplasts were removed prior to the statistical analyses. The cleaned data was then used to compile, filter and visualize the data using the phyloseq and gglot2 packages in RStudio.

Selected alpha diversity measures (Observed, Chao1, Shannon and Simpson) were computed based on a rarefied data set (Hong *et al.,* 2022) representing the bacterial abundance present in each sample. The output was visualized using boxplots. Observed and Chao1 indices are illustrative of the species (OTU) richness. Chao1 index includes the observed and an estimate of the species that were not observed. Shannon and Simpson indices are applied to infer the OTU diversity, considering the evenness of species within a population (Thukral, 2017). The statistical significance of the differences in the Observed, Chao1 and Shannon in the different groups of samples was tested using one-way ANOVA. Data on the Simpson measure did not meet the assumptions for ANOVA and was therefore subjected to the non-parametric Kruskall test (vegan package, v 2.5.7). Post-hoc tests (TukeyHSD and Dunn) were subsequently performed to identify the statistically significant pairwise differences in the bacterial communities hosted within the sample types and life stages.

For the beta diversity analyses, non-metric multi-dimensional scaling (NMDS) and Principal coordinates analysis (PCoA) were applied to compare the bacterial communities in the *Bryopsis* sp. samples representing different sample types and life stages. Cluster analysis using a dendrogram was additionally used to visualize the differences between the symbiont communities present in the *in situ Bryopsis* strains. PERMANOVA (permutational multivariate ANOVA) analysis was then applied to test the statistical significance of the beta diversity of the bacterial communities illustrated by NMDS, PCoA and cluster analyses and plots. The host-symbiont phylogenetic congruence was visualized using a tanglegram generated based on distance matrices of the *Bryopsis* hosts and their respective bacterial communities. A mantel test (vegan package, RStudio) was performed to assess the correlation and statistical significance between the host and symbiont phylograms.

3.0 Results

3.1 Characterizing the host

3.1.1 Overview of *Bryopsis* sp. culture and propagation

Of the 22 *Bryopsis* sp. strains, collected in the field as mature gametophytes and selected for *in vitro* propagation, 20 were successfully acclimated to the laboratory conditions. These strains were propagated in the lab for subsequent culturing towards asexual (fragmentation & protoplast formation) and sexual (gametogenesis & fertilization) reproduction (Table 3; Fig. 4 - a). 17 strains grew sufficiently well to allow asexual reproduction through fragmentation of the gametophyte thalli (Fig. 4 -b). Fragments were maintained in culture and upscaled to develop into mature gametophytes over a period of 20 to 24 weeks (Table 3; Fig. 4– c). During the early stages of gametophyte development, most samples had high proportions of contaminants which gradually reduced with progressive cleaning and media refreshing.

Table 3: Summary of *Bryopsis* sp. strains propagated in vitro. + indicates that a strain was used for a specific stage while – indicates that it was excluded. * Strains that were not directly propagated by fragmentation within the scope of our study but were sourced from back-up strains in related studies for use in subsequent downstream processes. + refers to samples that were not used in the temperature variation trial.

No.	ID	Acclimated to lab conditions	Substantial vegetative growth	Protoplast formation	Used for gametogenesis induction	Gamete release observed	Upscaled fragments	Sustained in culture	Sporophytes
1	Mar a1	+	+	+	+	-	+	-	-
2	Mar a2	+	+	-	+	-	+	-	-
3	Mar a3	+	+	+	+	-	+	-	-
4	Mar a4	+	+	-	+	-	+	-	-
5	Mar b	+	+	+	+	-	+	-	-
6	* Mar c	+	-	+	+	-	-	+	+
7	Mar d	+	+	-	+	-	+	-	-
8	Mar e1	+	+	-	+	-	+	-	-
9	Mar e2	+	+	-	+	-	+	-	-
10	NJ	+	+	+	+	+	+	-	-
11	SG2	+	+	-	+	-	+	-	-
12	* GR	+	-	-	+	+	-	+	+
13	GR2	+	+	+	+	-	+	-	-
14	Jos 1	+	+	-	+	-	+	-	-
15	KB 1	+	+	-	+	-	+	-	-
16	KB2	-	-	-	-	-	-	-	-
17	Sp B1	+	+	-	+	-	+	-	-
18	Sp B2	+	+	-	+	-	+	-	-
19	* Co A	+	-	-	+	+	+	+	+
20	* Co B	+	-	-	+	-	-	-	-
21	A2	+	+	-	+	+	+	+	+
22	A3	+	+	-	+	+	+	-	-

Mature gametophytes from 20 strains that developed the typical featherlike structure were induced to develop gametangia (Fig. 4 – d) with four replicates of 17 strains being subjected to varying incubation temperatures to assess the optimum temperature for gametangia development (Table 3). However, none of the samples in the four temperature treatments developed the gametangia. Due to an error at the culture facilities (beyond our control) most cultures were lost and only a subset of five cultures (Table 3) could be used for a second attempt. These strains had been in culture for relatively longer periods of time and the second attempt resulted in gametangia development and successful gamete release. The released gametes (Fig. 4 -e) were harvested and incubated resulting in four strains yielding sporophytes (Table 3, Fig. 4 – f); including one hybrid between GR and A2 strains (GA).

Table 4: *Bryopsis* sp. strains that fertilized and developed into sporophytes. Numbers correspond to sporophyte counts with the red values indicating the inter-strain cross between A2 and GR strains. '-' denotes the strains and crosses that did not develop sporophytes.

	GR	Co A	A2	Mar c
GR	-	1	12	-
Co B	-	-	-	2
A2	15	1	-	-
Mar c	-	-	-	-

Protoplasts were developed from six strains under a related study (Table 1; Figure 1 - g). The mature gametophytes were however not successfully maintained in culture owing to an unforeseen, sudden change in culture conditions (Table 1).

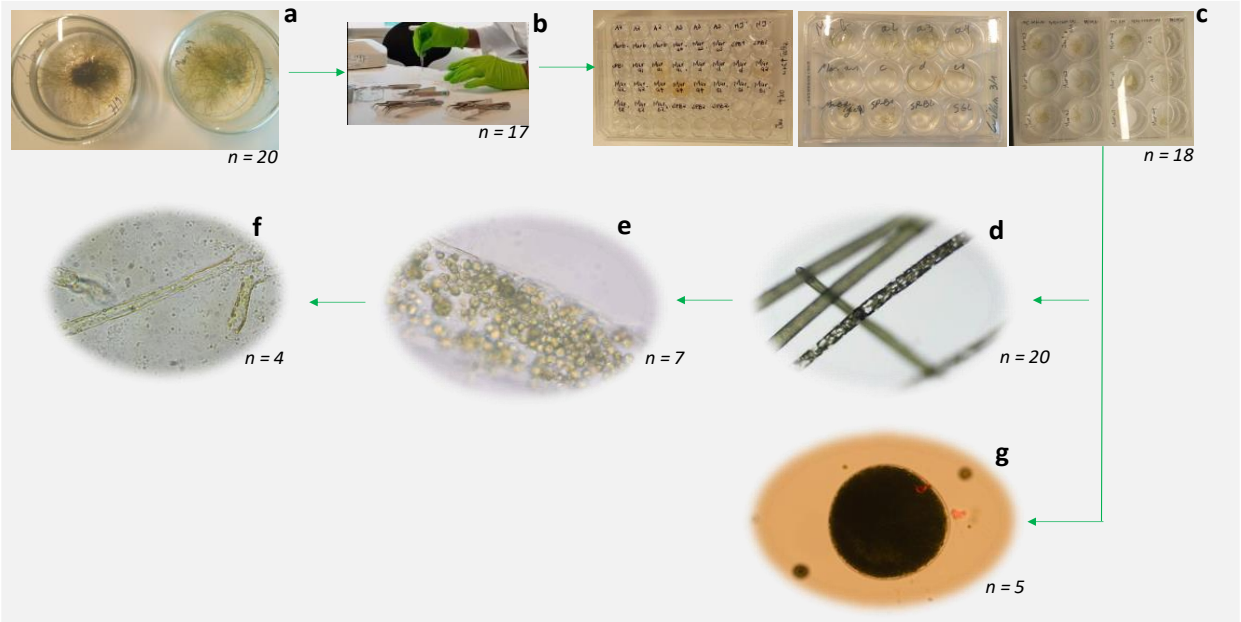


Figure 4: Summary of *Bryopsis* sp. *in vitro* propagation results: a. 21 strains acclimated to laboratory conditions b. 17 strains fragmented to induce asexual reproduction c. 18 strains maintained *in vitro* and upscaled as fragments develop into mature gametophytes d. 20 strains induced to develop gametangia (netting structure) e. Seven strains release gametes from mature gametangia f. Development of sporophytes in four strains from fertilization of gametes after crossing g. Protoplasts developed by wounding mature gametophytes of five strains.

3.1.2 *Bryopsis* sp. phylogeny

rbcL and *tufA* marker gene sequences from ten *Bryopsis* sp. strains were used to construct the *Bryopsis* spp. phylogeny (Fig. 5 - a). The phylogram showed two main clades which were not clearly distinguished based on the location of the sampling sites since the samples from different sampling sites clustered together without a clear pattern and some clades had exceptionally low bootstrap values.

To obtain a more informative representation, the sequences from our study were combined with those from Hollants et al (2011a). In the two gene-based phylogeny (Fig. 5 - b), the strains characterized in this study split in two distinct clades. The largest clade incorporated seven strains from our study identified as *Bryopsis plumosa*, with five of these being from Marseille in France. These clustered together with *B. plumosa* sampled from the USA and Atl. France in the related study. The other two strains identified as *B. hypnoides* formed a small clade with a strain from Italy. Most samples from our study that were obtained from Marseille in France thus clustered together clustered together with samples from regions with similar climatic conditions portraying a possible environmental gradient in the *Bryopsis* host distributions. A more representative sample size is however necessary to validate this pattern.

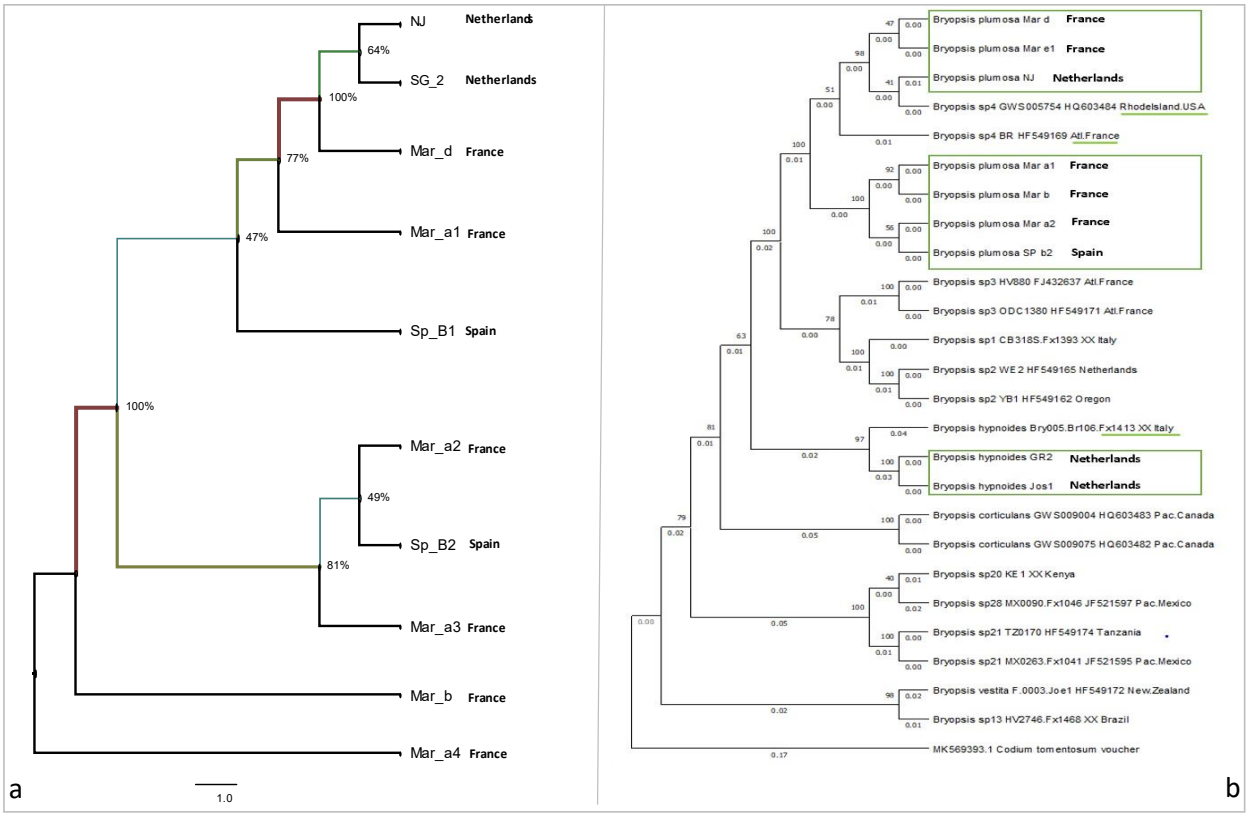


Figure 5: a. Unrooted Maximum likelihood *Bryopsis* sp. phylogeny of 10 cultures. Colours and branch thickness correspond to bootstrapping values. b. Maximum likelihood phylogenetic tree combining *Bryopsis* sp. samples from our study with selected strains from Hollants *et al.,* (2011a). The green boxes demarcate the 9 strains characterised in this study. Species names assigned to our samples are based on the best BLAST hits of the marker sequences obtained from NCBI. Both trees generated based on *rbcL* and *tufA* cpDNA marker sequences using the RAxML online tool.

3.2 Characterizing the bacterial communities

3.2.1 Amplicon reads overview

Nanopore sequencing of 16S rRNA gene from 64 samples containing *Bryopsis* spp. yielded a total of 5,901,556 raw reads. These were clustered into 2,706 consensus sequences, from which 137 chimeras were removed, resulting in 2,523 Operational Taxonomic Units (OTU's) with an average of 39 ± 19 OTUs per sample (App. 1, Fig. 2). 99.69% of the OTU's were taxonomically classified based the SILVA reference database with the taxonomic assignment score at 99% similarity to the reference sequences in the database. Using the tree, closely related taxa were merged based on a 97% similarity threshold using the tip glom function; yielding a total of 1, 287 OTUs. Sequences identified as chloroplasts (106) and those not being identified at the domain level (1) were removed, resulting in a total of 1,176 OTUs. Following this pre-processing of the raw reads, a total of 4,733,953 reads was obtained with an average of 73,968 reads per sample (min. = 494, sd. = 27,202, max. = 143,795, App. 1, Figure 2). Rarefaction curves plotted based on the rarefied phyloseq object illustrated adequate sampling depth for all samples (Appendix 1, Figure 3)

3.2.2 Bacterial community composition in *Bryopsis* sp.

The bacterial communities associated with *Bryopsis* were classified into 18 phyla, 42 classes, 73 orders, 125 families and 241 genera. The most abundant bacterial phyla were *Proteobacteria* (72%), *Bacteroidetes* (14%), *Cyanobacteria* (5%), *Tenericutes* (4%) and *Planctomycetes* (2%). Differences in the relative abundance of the bacterial phyla was observed across sample types and life stages. Based on the sample types, 61% of *Proteobacteria* and 72% of *Cyanobacteria* were hosted by cultured samples while the communities in the *in situ* samples constituted the highest proportion of *Bacteriodetes* (58%) and *Tenericutes* (80%). Cultured and prolonged culture samples both contained 41% of the *Planctomycetes* (Fig. 6, Top panel). In terms of the life stages, original gametophytes were the most abundant in *Cyanobacteria* (79%) and *Planctomycetes* (49%). Protoplasts hosted 42% of *Proteobacteria,* while the highest proportions of *Bacteriodetes* (54%) and *Tenericutes* (80%) were hosted by the original gametophytes (Fig. 6, Bottom panel).

At class level, 43% of the bacterial community consisted of *Alphaproteobacteria,* representing the most abundant class. This was followed by *Gammaproteobacteria* (24%) and *Sphingobacteria* (9%). *Cyanobacteria* and *Flavobacteria* both accounted for 5% of the bacterial abundance, while *Mycoplasma* represented 4%. Similar to the bacterial phyla, differences were observed in the relative abundance of the classes across the varied sample types and life stages. Cultured samples hosted the highest proportion of *Alphaproteobacteria* (73%), *Gammaproteobacteria* (46%), *Cyanobacteria* (72%) and *Flavobacteria* (62%); while the most *Sphingobacteria* (77%) were hosted by *in situ* samples (Figure 7, Top panel). Considering the four different life stages, protoplasts hosted the highest proportion of *Alphaproteobacteria* (53%) and *Flavobacteria* (39%); while the original gametophytes contained the highest abundance of *Gammaproteobacteria* (34%) and *Sphingobacteria* (73%). *Cyanobacteria* were most abundant in the propagated gametophyte samples at 79% (Figure 7, Bottom panel).

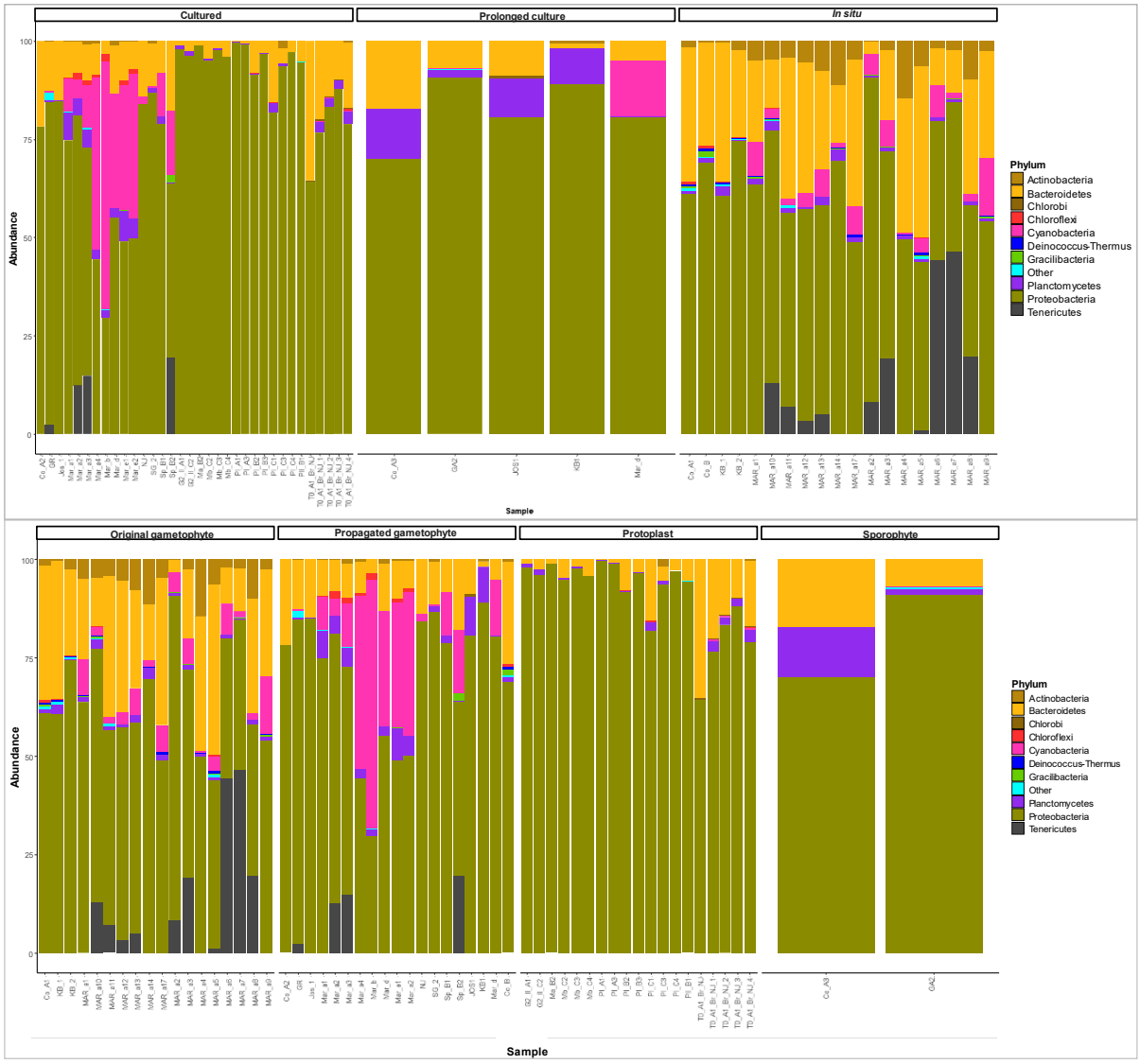


Figure 6: The relative abundance of bacterial communities in each sample. Top panel: 10 most abundant phyla grouped by sample types; Bottom panel: 10 most abundant phyla, grouped based on life stages. The remaining phyla are lumped together in the Others category.

Rhodobacterales, Alteromodales and Sphingobacteriales were the most abundant bacterial orders representing 28%, 11% and 9% of the entire community respectively (App. 1, Fig. 4). At family level, *Acanthopleuribacteraceae* and *Sphingobacteriaceae* accounted for 12% of the total community each; while *Rhodobacteraceae* and *Halieaceae* each represented 9% of the total bacterial abundance (App. 1, Fig. 5). Finally, *Granulosicoccus* was the most abundant genus at 10% of the total community; followed by *Hoeflea* and *Mycoplasma* at 8% and 6% respectively (App. 1, Fig. 6).

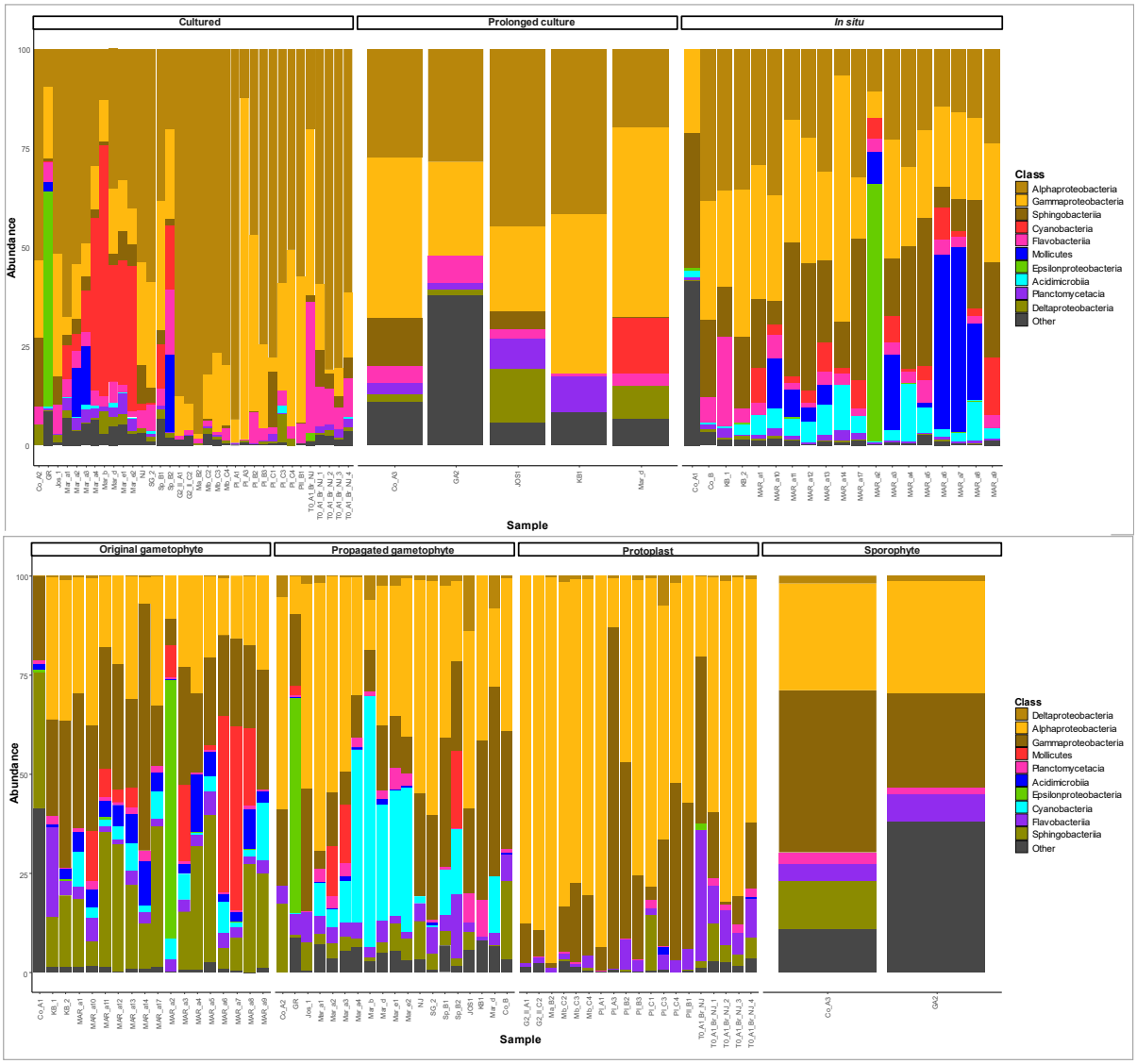


Figure 7: The relative abundance of bacterial communities in each sample. Top panel: 10 most abundant classes grouped by sample types; Bottom panel: 10 most abundant classes, grouped based on life stages. The remaining phyla are lumped together in the "Other' category.

At genus level, variations were observed in the composition of the bacterial symbionts both in the sample types and the life stages with Proteobacteria representing the most dominant genera in both categories (App. 1, Table 3 & 4). *In situ* hosts were dominated by Proteobacterial genera represented by four of the five most abundant genera. The five most abundant genera hosted by the *in situ Bryopsis* hosts were *Granulosicoccus* (Chromatiales), *Mycoplasma* (Mycoplasmatales), Parvularcula (Parvularculales), *Thiothrix* (Thiotrichales) and *Arcobacter* (Campylobacterales). In the cultured samples the dominance of the Proteobacteria persisted with different representative genera from this phylum dominating the samples. The five most abundant bacterial genera hosted by the cultured samples were *Hoeflea* (Rhizobiales), *Dinoroseobacter* (Rhodobacterales), *Leptolyngbya* (SubsectionIII), *Aliiglaciecola* (Alteromonadales) and *Granulosicoccus* (Chromatiales). In the prolonged culture samples, Proteobacteria still accounted for the most

abundant genera which were *Methylotenera* (Methylophilales), *Glaciecola* (Alteromonadales), *Sulfitobacter* (Rhodobacterales), *Spongiibacter* (Cellvibrionales) and *Neptuniibacter* (Oceanospirillales) (Table 5, Top).

Table 5: Five most abundant genera based on the *Bryopsis* sample types (Top) and life stages (Bottom). % represent relative abundance of the respective genera within the specific groups

	Sample types					
	In situ	**%**	**Cultured**	**%**	**Prolonged culture**	**%**
1	Granulosicoccus	23.2%	Hoeflea	13.8%	Methylotenera	16.6%
2	Mycoplasma	13.5%	Dinoroseobacter	8.2%	Glaciecola	14.9%
3	Parvularcula	11.1%	Leptolyngbya	6.3%	Sulfitobacter	6.6%
4	Thiothrix	9.0%	Aliiglaciecola	6.2%	Spongiibacter	6.3%
5	Arcobacter	5.3%	Granulosicoccus	4.2%	Neptuniibacter	6.3%

	Life stages							
	Original gametophyte	**%**	**Propagated gametophyte**	**%**	**Protoplast**	**%**	**Sporophyte**	**%**
1	Granulosicoccus	23.2%	Leptolyngbya	13.6%	Hoeflea	20.4%	Methylotenera	24.4%
2	Mycoplasma	13.5%	Roseobacter	6.2%	Dinoroseobacter	14.0%	Glaciecola	16.0%
3	Parvularcula	11.1%	Arcobacter	5.5%	Aliiglaciecola	10.6%	Spongiibacter	10.7%
4	Thiothrix	9.0%	Glaciecola	4.7%	Granulosicoccus	5.6%	Sulfitobacter	9.3%
5	Arcobacter	5.3%	Celeribacter	4.6%	Phaeobacter	5.2%	Roseobacter	7.8%

Based on the *Bryopsis* life stages, the dominance of Proteobacteria was also observed among the most abundant bacterial genera in the original and propagated gametophytes. The most abundant genera present in the original gametophytes were *Granulosicoccus* (Chromatiales,), Mycoplasma (Mycoplasmatales), *Parvularcula* (Parvularculales), *Thiothrix* (Thiotrichales) and *Arcobacter* (Campylobacterales). In the propagated gametophytes, the most abundant genus *Leptolyngbya* (SubsectionIII) while the remaining four of the five most abundant genera were represented by the Proteobacterial, *Roseobacter* (Rhodobacterales), and *Arcobacter* (Campylobacterales), *Glaciecola* (Alteromonadales) and *Celeribacter* (Rhodobacterales) (Table 5, Bottom).

3.2.3 Bacterial diversity in *Bryopsis* sp.

Differences in alpha diversity measures were observed for the bacterial communities in *Byopsis* sp. samples grouped by sample type and life stage (Fig. 8). The richness of the bacterial communities in all samples ranged from 9 ± 0.15 to 96.42 ± 11.33 OTUs. Sporophytes recorded the highest richness (Chao1 = 51.3 ± 8.57) and diversity (Shannon = 2.95 ± 0.26). Based on the sample types, the *in situ* samples ranked highest in OTU abundance (Chao1 = 50.1 ± 8.25) while the samples subjected to prolonged culture were highest in terms of their species diversity (Shannon = 2.74 ± 0.23). One-way ANOVA tests conducted to statistically assess differences in the Observed, Chao1 and Shannon indices and a non-parametric test for the Simpson measure revealed that the differences were statistically significant ($p < 0.05$; App. 1; Table 1).

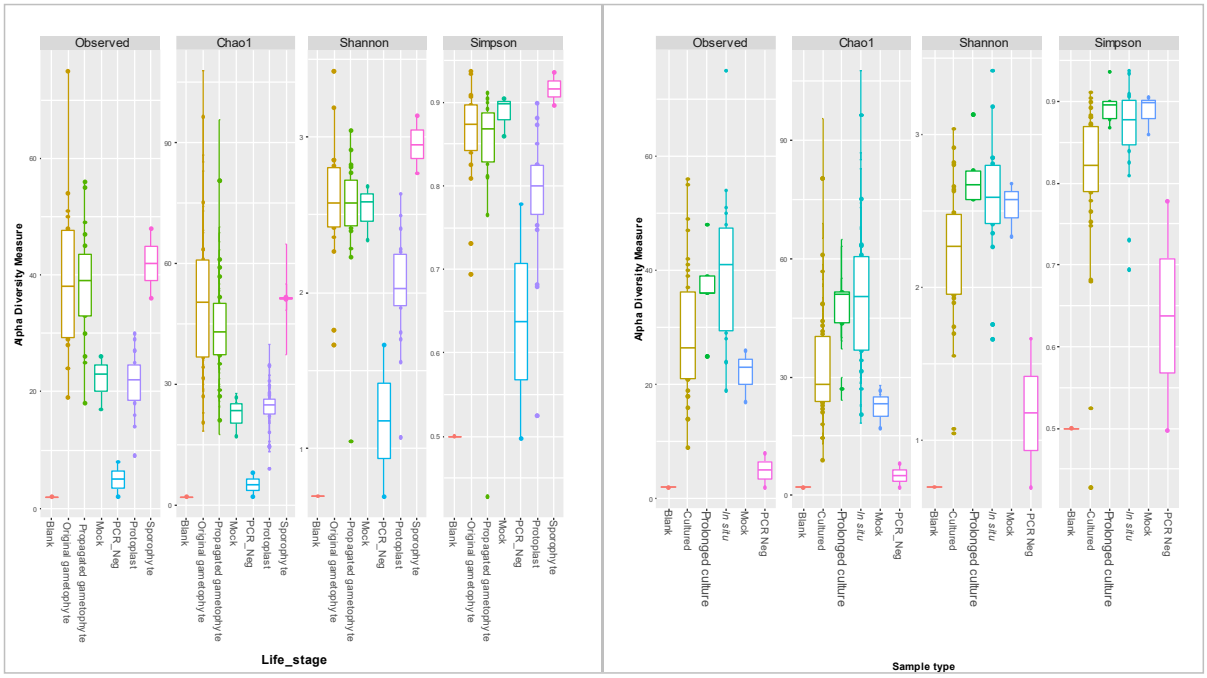


Figure 8: Alpha diversity measures (y-axis) of bacterial communities. Left - Samples grouped by life stage; Right - Samples grouped by Sample type. Colours correspond to x-axis labels [Life stage (Blank, Original gametophyte, Propagated gametophyte. Mock, PCR Negative, Protoplast, Sporophyte); Sample type (Blank, Cultured, Prolonged culture, *In situ*, Mock, PCR Negative).

Post hoc tests showed that in the sample type category, species richness and diversity were significantly different between the *in situ* and cultured groups ($p < 0.05$). The comparative diversity cultured vs prolonged culture samples were also statistically significant ($p < 0.05$) (App. 1, Table 1). Based on the grouping of samples according to life stages highly significant differences ($p < 0.001$) of both richness and diversity were noted for the comparisons between protoplasts and the other three stages (App. 1, Table1).

NMDS plots (Figure 9, Top panel) generated based on a Bray Curtis matrix illustrated the dissimilarity between the bacterial communities in the different samples. Clustering of samples into distinct groups was observed. (Figure 9). *In situ* samples clustered together while the cultured and prolonged culture samples seemed to form one large cluster. Based on the life stages, the separation of the clusters was more distinctive, with three main clusters being observed. Sporophytes, which were represented by only two samples, did not differ from the gametophytes. Similar clustering patterns were observed for in PCoA plots generate based on weighted UniFrac distances (Figure 9, Bottom panel). The proportion of variation in the bacterial communities in the *Bryopsis* samples explained by the first and second ordination axes was 34.6% & 14.9% for the sample type category and 35% & 12% for the life stage category respectively.

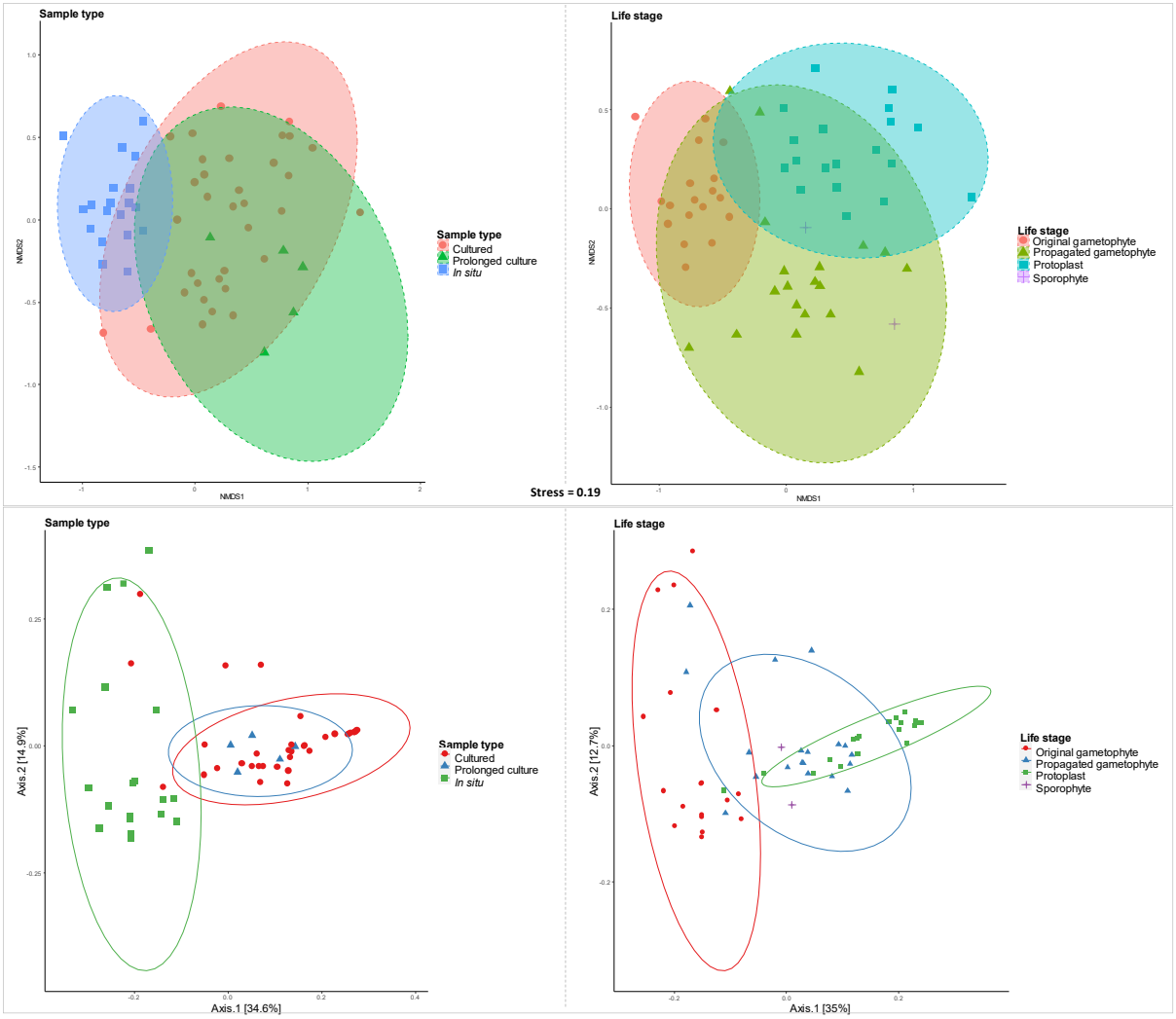


Figure 9: Top panel: Non-metric multidimensional scaling (NMDS) plots. The points represent samples with different shapes and colours used based on the different groups of sample types (left) and life stages (right). Ellipses demarcate clusters based on the closeness of the samples, which shows the similarity of the microbial communities associated with these samples. Bottom panel: Principal coordinates analysis (PCoA) plots. Plot based on weighted Unique Fraction (UniFrac) metric of the bacterial communities in the *Bryopsis* samples grouped according to sample type (left) and life stages (right).

A PERMANOVA (permutational multivariate ANOVA) analysis (Table 2) showed that the differences illustrated in the clustering patterns based on the beta diversity of the bacterial communities were highly statistically significant ($p < 0.001$).

Table 6: Summarized results of the PERMANOVA analysis performed based on a Bray Curtis distance matrix of the bacterial communities in the *Bryopsis* sp. samples grouped by sample type and life stage.

Factor	R-squared	*P*-value	Sig. code
Sample type	0.09103	0.001	***
Life stage	0.12641	0.001	***

Post-hoc tests were tests revealed that within the sample type grouping, differences in the bacterial communities in the cultured vs *in situ* and prolonged culture vs *in situ* pairs were statistically significant ($p < 0.05$) while the bacterial communities within the cultured samples and those exposed to prolonged culture period were similar ($p > 0.05$, Appendix 1, Table 2). Within the life

stage category, statistically significant differences were found between the original and propagated gametophytes as well as the protoplasts and both gametophyte types ($p < 0.05$). Communities in the sporophyte and the three other life stages were however all comparatively similar ($p > 0.05$, App. 1, Table 2).

Bacterial communities hosted within the *in situ* samples revealed some degree of spatial differentiation. Most of the samples were collected in Marseille, France and these appeared to cluster together while samples from other sites, although comparatively fewer also clustered together (Fig. 10). A PERMANOVA analysis testing the significance of the spatial differences showed that the variations were highly significant ($p = 0.001$).

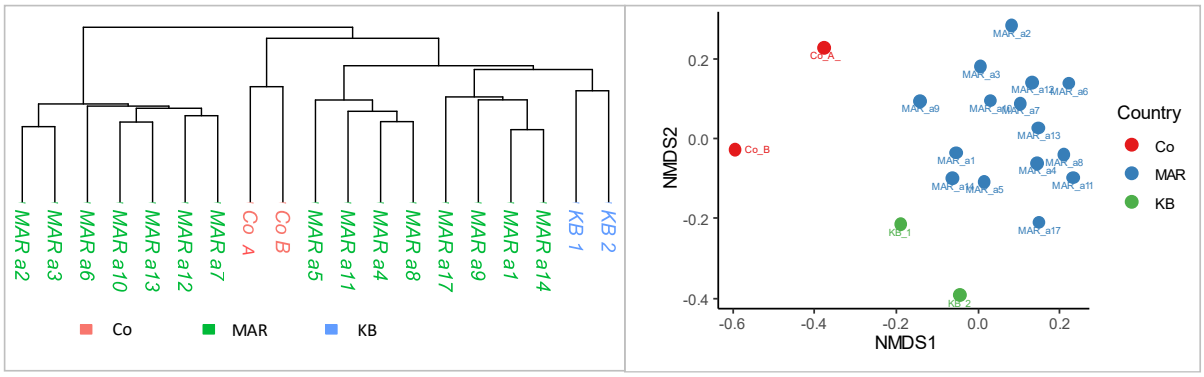


Figure 10: Spatial variability of bacterial communities hosted in the *in situ Bryopsis* strains based on Unique Fraction (UniFrac) metric of the bacterial communities. (Left) Ward.D2 linkage dendrogram. Tip label colours represent the three strains sampled from different locations. (Right) NMDS plot. Colours correspond to host strains from different locations.

3.3 Phylogenetic relationships between *Bryopsis* sp. hosts and their bacterial communities

A mantel test comparing the *Bryopsis* sp. hosts phylogeny to the similarity in their respective bacterial communities revealed the absence of a phylogenetic signal as also illustrated by the tanglegram (Fig. 11). The mantel test confirmed that the *Bryopsis*-bacterial relationship was not statistically significant at a confidence level of 0.05 (Mantel statistic $r = 0.3146$, p - value = 0.1003, permutations = 9999). The r value showed a relatively weak correlation between the hosts and their respective symbiont communities which supported output illustrated by the tanglegram (Figure 11). This value implies that while the variations in the bacterial community structure are related to differences in the *Bryopsis* sp. hosts, the host-symbiont symbiont phylogenetic link is relatively weak and statistically insignificant ($p > 0.05$).

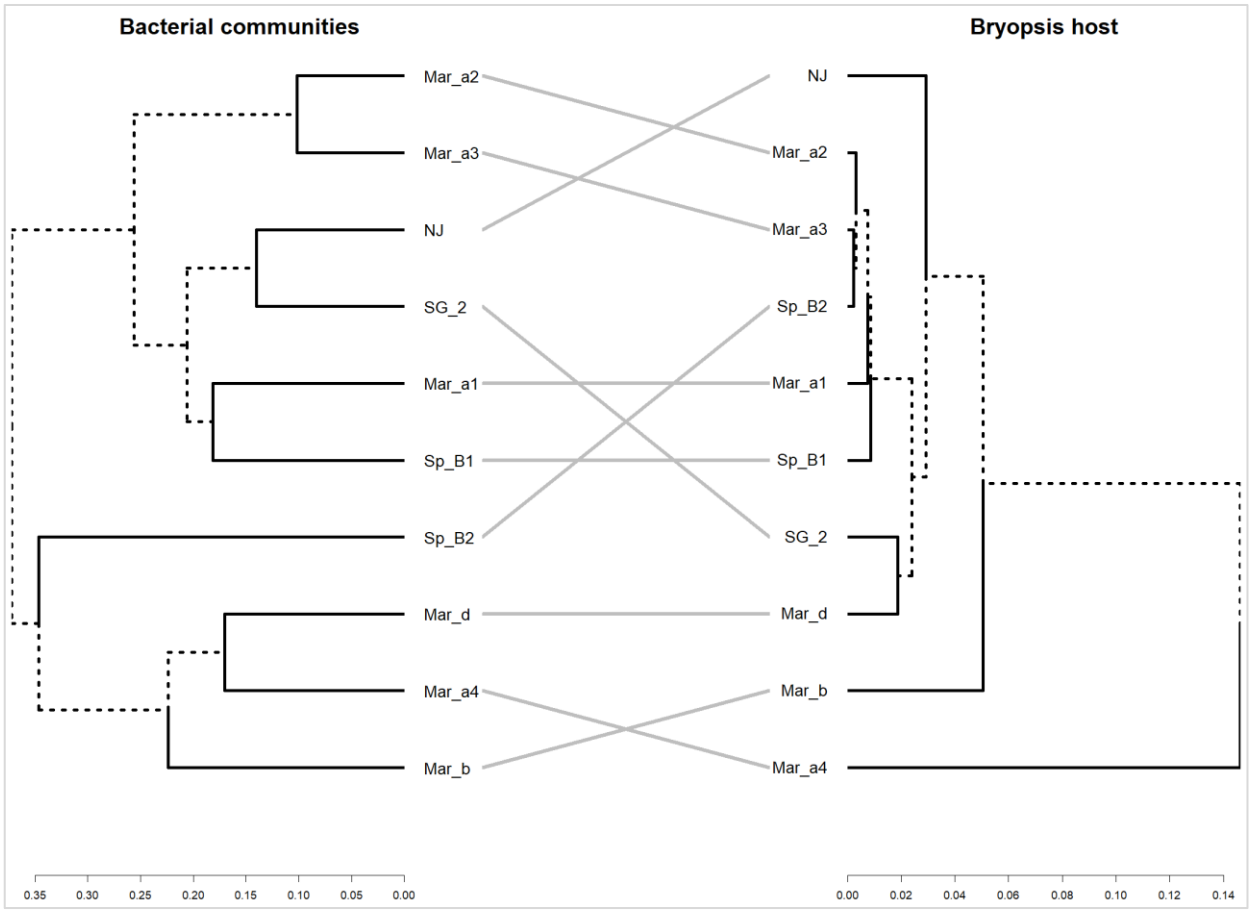


Figure 11: Tanglegram of *Bryopsis* sp. hosts phylogeny (right) and the respective bacterial communities dendrogram (left). Host phylogram generated using RAxML based on a partitioned model comprised of *rbcL* and *tufA* sequences for 10 cultured samples. Bacterial dendogram created based on a weighted UniFrac distance matrix of the communities hosted by the the different hosts. Grey lines illustrate the absence of strong host-symbiont phylogenetic signals.

3.4 Bacterial stability and transmission in *Bryopsis* sp.

3.4.1 *Mycoplasma* in specific *Bryopsis* sp. hosts

A comparison of the amplicon sequences of the selected samples revealed the presence of the *Mycoplasma* whose abundance seemed to be maintained across different sampling types as illustrated in samples Mar_a2 and Mar_a3 in Fig. 12- a, below. However, the results obtained from both runs using the three different primer sets used (Table 2) did not yield conclusive results. The first primer set yielded ambiguous multiple bands across all the samples. Although clearer bands were observed with the second and third primer sets, the samples that were expected to contain the *Mycoplasmas* (Targeted samples) did not show clear bands, while some negative controls had bands (Fig. 12 -b).

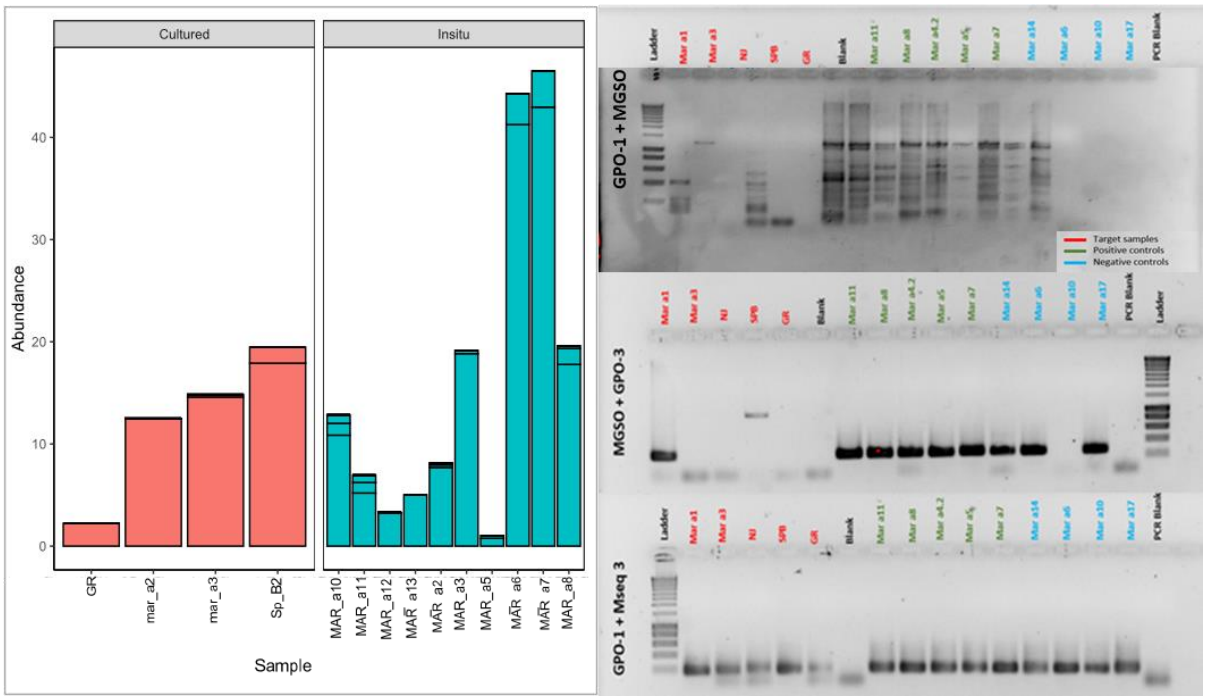


Figure 12: a. (Left) Relative abundance of *Mycoplasma* in *Bryopsis* samples grouped by sample type. b. (Left) Agarose gel electrophoresis image for PCR products generated using two primer sets to assess the presence of Mollicutes in selected *Bryopsis* sp. samples

3.4.2 Vertical bacterial transmission during gametogenesis in *Bryopsis* sp.

Transmission electron microscopy was used to check for the presence of bacterial in gametes of one *Bryopsis* sp. strain (Co A). This would be used a proxy for vertical transmission of the symbionts during asexual reproduction. The electron micrographs (Figure 13), however did not show the presence of any bacterial particles in the gametes.

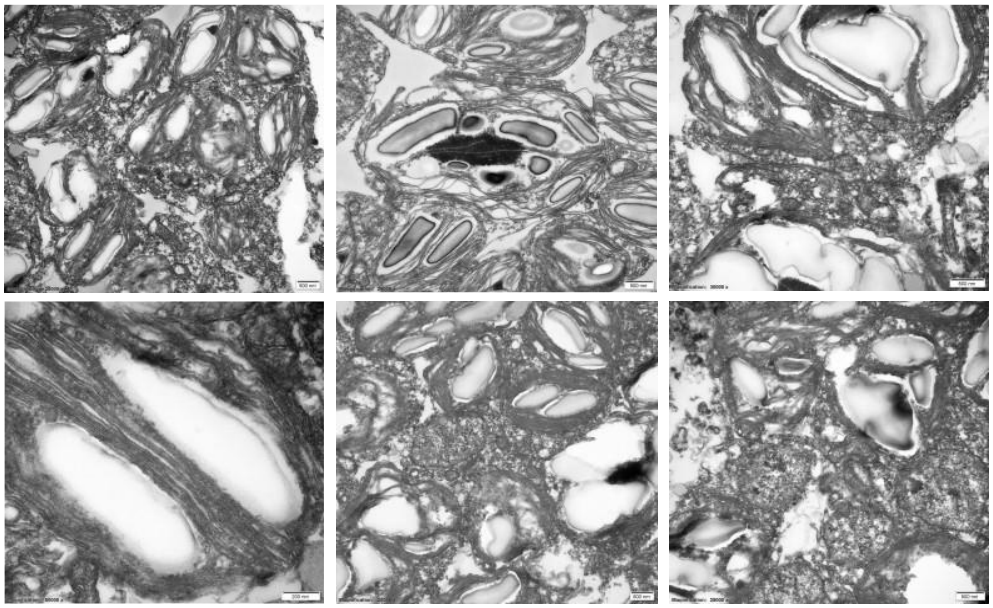


Figure 13: Electron micrograph showing the ultrastructure of *Bryopsis* sp. (strain Co A) containing gametes. Bacterial particles absent in the gametes.

3.4.3 Horizontal bacterial transmission during protoplast formation in *Bryopsis* sp.

Prior to introduction into assays containing the newly formed protoplasts (Fig. 14– a), the four different GFP-labelled bacteria were motile and with adequate fluorescence (Figure 14 - b). Both fluorophores were observed in and on the surface of the protoplast with intact primary envelope immediately upon the onset of protoplast formation as well as in protoplasts with compromised primary membrane membranes (Figure 14 - c & d).

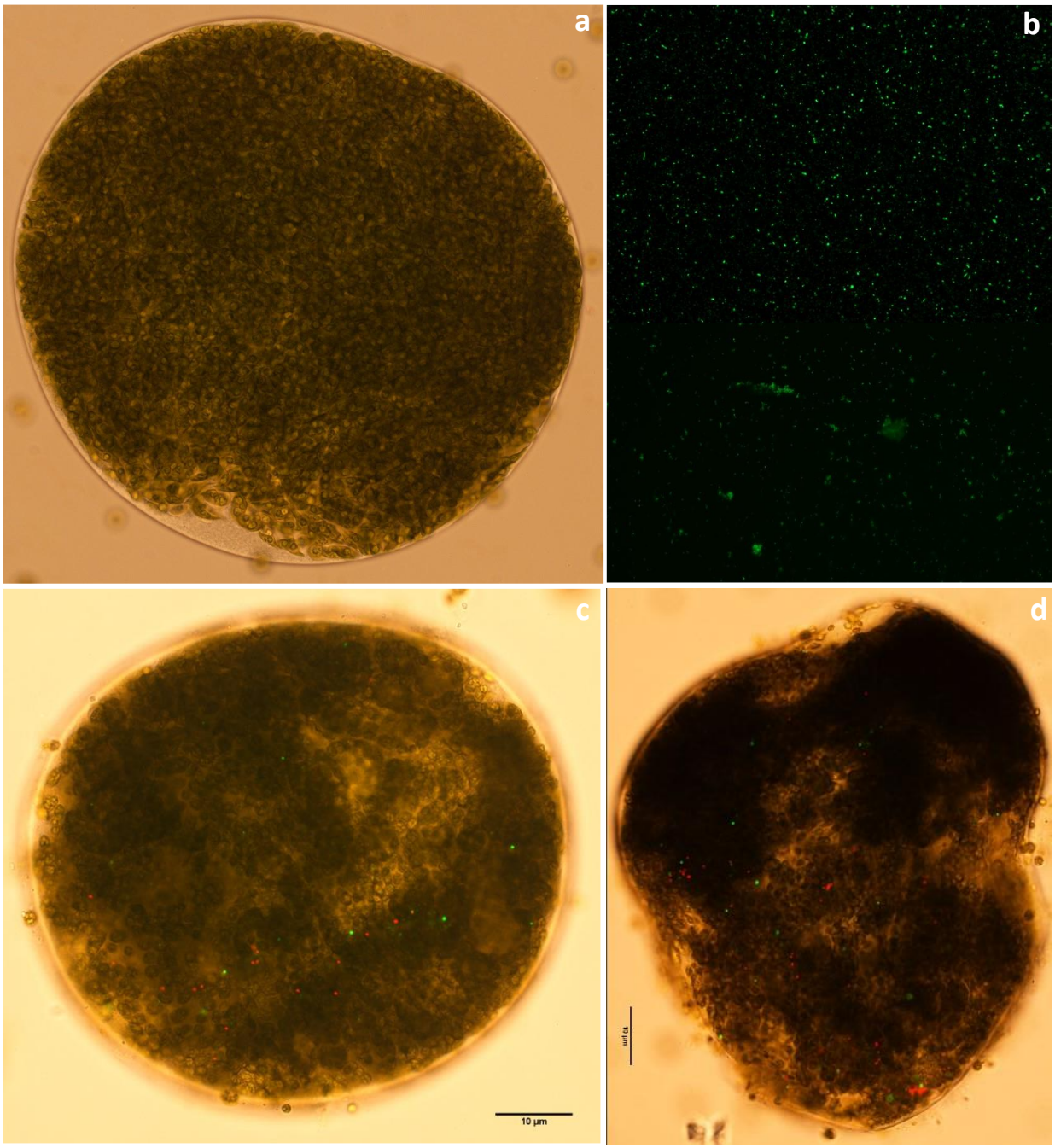


Figure 14: a. a. Newly formed *Bryopsis* sp. protoplast with intact primary envelope. b. Motile GFP-labelled bacteria showing high fluorescence and dense concentration prior to introduction into *Bryopsis* sp. protoplast assays. c. Incorporation of both GFP-labelled bacteria (green) and fluorospheres (red) into a newly formed protoplast with an intact primary envelope. d. Protoplast with a compromised primary envelope containing both bacteria and fluorospheres.

However, after a 3-day incubation period, the fluorophores seemed to be completely lacking within the protoplasts (now with permanent membranes) or restricted only to the surface or peripheral margins of the protoplasts (Fig. 15). Fluorescent bacteria were also observed in the negative control, to which no bacteria had been added, implying that there was cross contamination during preparation of the assay.

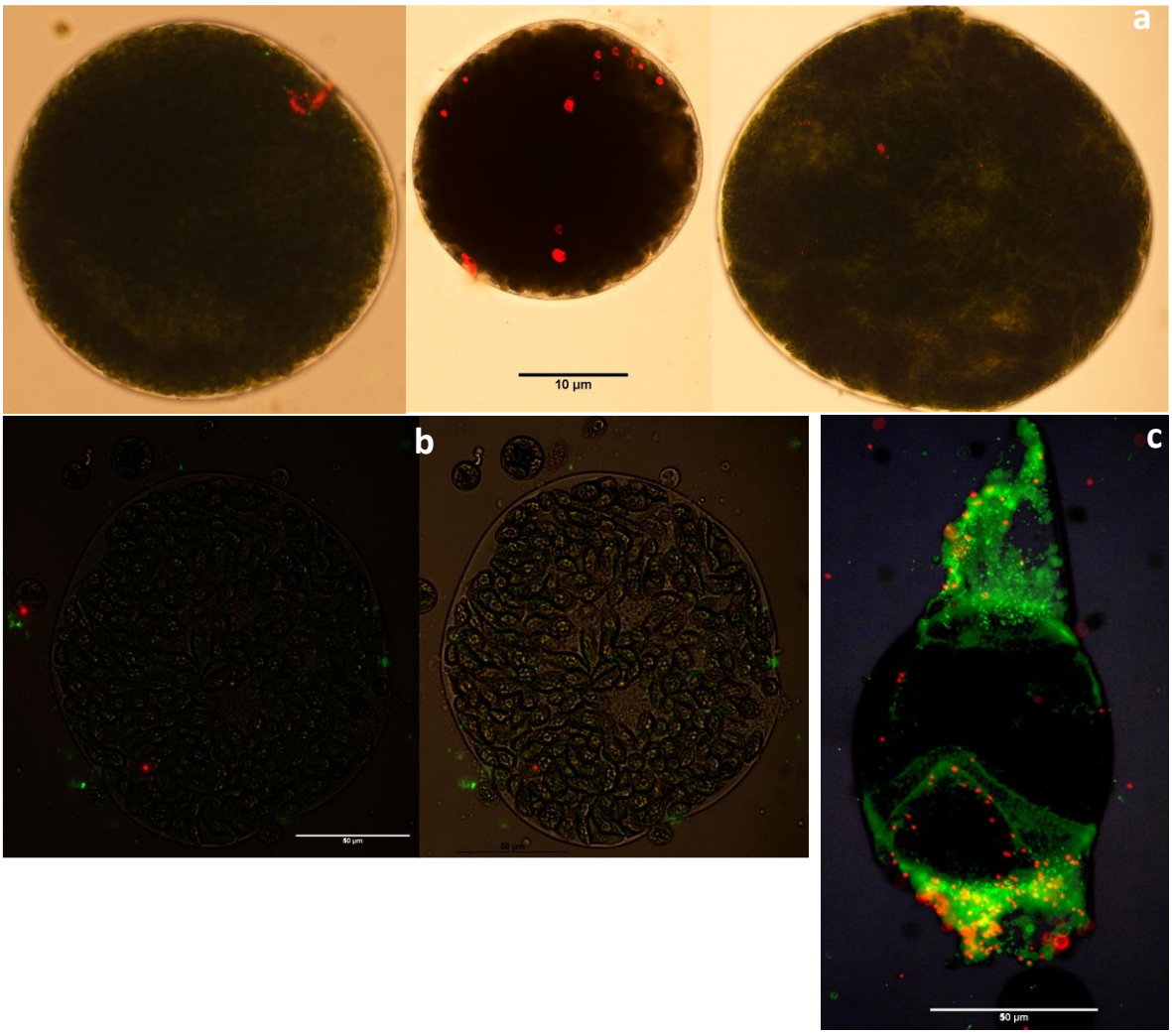


Figure 15: Protoplasts three days after the assay: a. Three protoplasts with a few scattered fluorophores localized to the periphery and surface. b. Two protoplasts with neither GFP labelled bacteria, nor fluorospheres within the internal structure. c. Protoplast formed within a *Bryopsis* filament with a mixture of fluorophores localized to the surface.

4.0 Discussion

4.1 Overall bacterial composition in *Bryopsis* spp.

In this study, Bacterial symbionts associated with *Bryopsis* were taxonomically diverse, with a dominance of Proteobacteria in all samples. The most abundant genera across samples including *Granulosicoccus, Arcobacter, Hoeflea* and *Glaciecola* were all Proteobacteria. Other studies have found these bacteria dominating seaweed-associated bacterial communities (Hollants *et al.*, 2011a, Marzinelli *et al.*, 2018, Florez *et al.*, 2017, Juhmani *et al.*, 2020). Many of these bacteria, including the pathogenic *Arcobacter*, are however not exclusively associated with seaweeds (Mizutani *et al.*, 2019). Different beneficial functions of Proteobacteria toward seaweeds have been discovered , such as remineralization of complex macroalgal polysaccharides such as fucoidan (de Oliveira *et al.*, 2012; Singh *et al.*, 2014). Singh and colleagues (2014) however refer to Proteobacteria as weed microbial species owing to their ability to proliferate in association with diverse species inhabiting varied marine habitats.

Bacteriodetes, the second most bacterial phylum identified in our study has been widely studied as a main constituent of the *Bryopsis* endophytic community, particularly with reference to Flavobactariaceae (Hollants et. al., 2011; Zan *et al.*, 2019). Bacteriodetes also perform essential functions for their macroalgal hosts including induction of morphological changes (Matsuo *et al.*, 2003), carbon turnover (Pinhassi *et al.*, 2006) and secretion of antimicrobial compounds (Srinivasan *et al.*, 2021). *Candidatus Endobryopsis kahalalidefaciens* is one unique *Flavobacterium* endobiont hosted by *Bryopsis* spp. which secretes kahaladides - toxic compounds that protect the host from predation (Becerro *et al.*, 2001). This symbiont is thought to be horizontally transmitted to the *Bryopsis* grazer *Elysia rufescens* (Hawaiian sea slug) which tolerates the bacterial toxin and continues to benefit from the chemical defence conferred by the bacterium (Rao *et al.*, 2008; Zan *et al.*, 2019). Cyanobacteria were also present in all *Bryopsis* spp. samples. In *in vitro* samples their presence may be linked to their documented ability to outcompete other bacterial species in eutrophicated aquatic systems (Rastogi *et al.*, 2015) which may be comparable to the relatively nutrient-rich *in vitro* culture conditions. Conversely, *Cyanobacteria* in *in situ* samples may be linked to their nitrogen fixation activity which is essential for seaweeds (Rosenberg & Paerl,1981) and other marine organisms (Lesser *et al.*, 2007).

4.1.1 Taxonomic vs functional diversity in *Bryopsis*-associated bacteria

Similar to our findings, seaweed bacterial symbioses including in *Bryopsis* hosts have been characterized as highly diverse consortia especially from a taxonomic point of view (Hollants *et al.*, 2011a; Morrissey *et al.*, 2019). At higher taxonomic levels, we observed the occurrence of bacterial symbionts belonging to similar taxa, particularly the highly abundant Proteobacteria. However, at lower taxonomic levels, *Bryopsis* hosts were observed to host taxonomically different bacterial communities and these differences were even observed at the level of individuals subjected to the same conditions. Hollants *et al.*, (2011a) made similar observations and attributed it to the roles played of different bacterial symbionts associated with *Bryopsis*. The characterization of seaweed-associated bacterial communities based on functional diversity of has gradually gained prominence over taxonomic characterization. Burke and co-investigators (2011)

postulate that symbiont assembly in seaweeds is possibly driven by host-specific requirements which drive the selection of bacteria that would best promote host fitness based on the functions that they perform. This may contribute to the observed variability in bacterial composition in individuals since different hosts belonging to the same population may select different combinations of bacterial taxa which perform the similar functions (Fan *et al.,* 2012). In our study, we observed different combinations of Proteobacterial genera in the different *Bryopsis* individuals. Consequently, while the taxonomic diversity of bacterial communities at genus or species level might be high, the associated functional variability might be quite limited owing to redundancy in roles performed by different bacterial taxa as previously observed in macroalgal hosts (Burke *et al.,* 2011; Roth-Schulze *et al.,* 2018). This redundancy is however considered essential to the maintenance of bacterial community stability at a functional level in the event of ecological perturbations which might impact certain sensitive members of the symbiotic consortium (Shade & Handelsman *et al.*, 2012; Burke *et al.,* 2011; Allison and Martiny 2008).

4.2 Variations in bacterial community structure associated with *Bryopsis* spp.

4.2.1 Spatial variations

In the bacterial communities associated with the *in situ Bryopsis* samples, we observed that strains collected from similar sites hosted similar bacterial communities. This supports the assertion that the environmental conditions of the host's habitat might strongly influence the bacterial community structure (Campbell *et al.*, 2015) due to their effects on host physiology (van der Gucht *et al.*, 2007). Studies also show that changes in abiotic conditions such as temperature and salinity may induce the development of adaptations in macroalgae and modifications in the microbiome structure has been highlighted as one of these coping strategies (Dittami *et al.*, 2016). Hollants and colleagues (2013b) observed a correlation between temperature variations and the structuring of Flavobacterial symbionts in *Bryopsis.* This finding was associated with either a bacterial preference to the specific temperature ranges or the shifts in host physiology which impact symbiont composition (Hollants *et al.*, 2012). Temperature is a key environmental factor which determines the spatial distribution patterns of seaweeds (Breeman, 1988). As such, it has a major influence on macroalgal physiology and evolutionary patterns, which in turn impacts the composition of the associated bacterial symbiont communities. Our study design incorporated a temperature variation experiment to gauge the optimum temperature for gametogenesis in *Bryopsis* and subsequently assess the bacterial communities present in gametes produced at different temperatures. This experiment was however unsuccessful, thus, we recommend further research into the influence of abiotic factors on the *Bryopsis* life cycle and bacterial symbionts. The unique constitution of the bacterial community composition even in *Bryopsis* individuals inhabiting the same ecological niche however, infers the influence of additional strain-specific mechanisms that each host possibly employs in structuring and maintaining a unique host-symbiont complex even within the same host community (Hollants *et al.*, 2011a).

4.2.2 Temporal variations

Temporal variations in bacterial species richness and diversity were observed. Richness was highest in *in situ* samples while prolonged culture samples contained the highest bacterial species

evenness. *In situ* & cultured *Bryopsis* samples were significantly different in the species richness while the diversity of cultured samples was significantly different compared to both *in situ* and prolonged cultured samples ($p < 0.05$). The richness and diversity of bacterial communities was expected to decrease with increase in cultivation time because owing to changes in the ecological conditions imposed by laboratory culture and possible dominance of opportunistic bacterial strains over time (Ghoul & Mitri, 2016). This explains our observation of the highest richness in *in situ Bryopsis* samples. The high diversity in our prolonged culture samples in comparison to the other sample types may be a result of the emergence and proliferation of bacterial species adopted for *in vitro* conditions and the stabilization of the bacterial community over the prolonged culture time. However, *in vitro* laboratory culture conditions can never mirror the complex dynamics characteristic of the marine environment. Ecological changes induced by laboratory conditions might have had an impact on host physiology thus affecting the associated bacterial communities. Hollants and colleagues (2013) shared a similar opinion on the influence of laboratory conditions on endophytic bacterial communities in *Bryopsis* and advised that this is an important factor to take into consideration when interpreting findings obtained from host-symbiont studies based on *in vitro* seaweed propagation.

Seaweed culture often requires the incorporation of enrichment media containing nutrients necessary to maintain the vitality of the specimen (Andersen, 2006). In natural ecosystems, the high organic matter content associated with the surfaces of seaweeds has been associated with the abundance of bacterial communities in macroalgal beds (Armstrong *et al.*, 2001). The additional nutrients incorporated to propagate macroalgae is comparable to this conclusion and may constitute one of the main factors that might be responsible for the rapid proliferation of the opportunistic Cyanobacteria which often flourish in eutrophic environments. This theory possibly justifies the steep increase in the proportion of Cyanobacterial abundance in the prolonged cultured samples in comparison to the cultured and *in situ* strains. The failure of some *Bryopsis* strains in our study to acclimate to laboratory conditions while others seemingly flourished under similar *in vitro* environments might be associated with individual or strain-specific differences in microbiome communities which possibly confer an adaptive advantage to some strains over others. The direct influence of host physiology on microbiome structure dictates that environmental changes that affect the host will invariably have an impact on symbiont community structure and functionality as reported by Marzinelli *et al.,* (2018).

Differences between *in situ* and cultured communities may further be explained by the absence of other algal species that would ideally associate with the *Bryopsis* hosts in the natural environment. These species may have an influence on the bacterial communities associated with *Bryopsis* and vice versa through competition within the microbiome and horizontal uptake mechanisms such as host switching. Other species present in natural ecosystems may also act as vectors to transfer the bacterial communities between hosts as observed during the predation of *Bryopsis* by sea slugs (Händeler *et al.*, 2010) or shed their own symbionts into the shared environment followed by subsequent uptake by the adjacent *Bryopsis* hosts. The bacterial communities associated with the sampled *Bryopsis* strains in our study have been identified as symbiotic partners in other marine

macroalgae species. Sea lettuce (*Ulva sp*.) and the red seaweed *Delisea pulchra,* for instance, host Rhizobales on their surfaces (Burke *et al.*, 2011; Longford *et al.*, 2007) while Rhodopsedomonads have been characterized as part of the endophytic community of *Caulerpa taxifolia* (Chisholm, 1996).

The observation of *Mycoplasma* in relatively constant abundance in selected samples in our study may be related to their characterization as obligate pathogenic symbionts of marine macroalgae **(**Ward *et al.*, 2019). *Mycoplasma* have been specifically identified as members of the endobiotic bacterial community associated with *Bryopsis* spp. (Hollants *et al.*, 2011a). The validation of *Mycoplasma* stability in selected samples in our study was however unsuccessful due to possible cross contamination which resulted in inconclusive PCR results.

4.2.3 Reproduction-mediated variations

Bacteria associated with selected life stages in our study were composed of different communities. Sporophytes hosted the community with the highest richness and diversity, with the bacteria in the protoplasts being significantly different in richness and diversity relative to the other three life stages ($p < 0.05$). Our findings on the variability of the bacterial community structure hosted by *Bryopsis* with reproduction are in line with other studies that associate these differences to varying mechanisms of symbiont recruitment at the different life stages (Xue *et al.*, 2021). The life cycle of *Bryopsis* consists of several pathways including both sexual and asexual reproduction stages (Morabito *et al.*, 2010). Thallus differentiation in seaweeds involves physiological changes in the hosts and result in the development of new structures within or external to the host (Morrissey *et al.*, 2019). While the impact of the thallus differentiation during reproduction on *Bryopsis* physiology remains unknown, it might have an impact on the bacterial community structure as illustrated by the significant differences in the consortia observed within *Bryopsis* samples representing different life stages in our study. This conclusion might be supported in part by findings on the siphonous seaweed *Caulerpa* showing that bacterial communities differ in different parts of host thallus (Morrissey *et al.*, 2019; Ranjan *et al.*, 2015).

Protoplast formation in *Bryopsis* is an asexual reproduction stage which includes a transient stage where the extruded cytoplasm is exposed to direct contact with the external environment (Burr & Evert, 1972, Kim *et al.*, 2009). This provides ample leeway for environmental contaminants, including bacteria to be integrated into the developing protoplasts (Hollants *et al.*, 2011). The protoplast assay conducted in our study provided some evidence that implies selectivity during protoplast formation in *Bryopsis*. Although newly formed protoplasts appeared to take up both living bacterial and non-living particles, the extended assay period resulted in the exclusion of these particles from the internal environment of the protoplasts, restricting the particles to the surface of the protoplasts. A combination of mechanisms involving the physiology, biochemical processes and morphology of *Bryopsis* have been associated with the observed selectivity of the protoplasts against these foreign particles (Goecke *et al.*, 2010). These mechanisms include the presence of a selectivity barrier that keeps away the alien particles (Klotchkova *et al.*, 2005). Moreover, Bryohealin, a lectin secreted to mediate the agglutination of cytoplasmic contents from

siphonous macroalgae has been shown to possess antibacterial properties which are potent against alien bacteria (Klotchkova & Kim, 2006; Yoon *et al.*, 2008).

The concept of microbial gardening based on chemically-mediated symbiont recruitment mechanisms as reported in *Ulva* might also explain how the *Bryopsis* host or its bacterial symbionts keeps away unwanted visitors during this vulnerable life stage while selecting only those partners that are essential for the partnership (Kessler *et al.*, 2018; Saha and Weinberger 2019). The cooperative partnership that exists between macroalgae and their bacterial symbionts implies that while the bacteria have a strong influence of host physiology, the host may in turn also contribute to the structuring of the bacterial communities. Kessler and colleagues (2018) provide evidence to support this hypothesis by suggesting that the secretion of dimethylsulfoniopropionate (DMSP) by the algae serves as a chemical signal that enables bacterial symbionts to trace photosynthates produced by the host thereby sustaining the symbiotic relationship.

4.3 Bacterial symbiont stability in *Bryopsis* and its evolutionary implications

Our study revealed the dominance of *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Mycoplasma* across different life stages and sample types of *Bryopsis*. This implies the relatively high reliance on, or importance of these bacterial to the host, necessitating the evolution of mechanisms to guarantee transmission to and recruitment by the host. However, an assessment of the host-symbiont phylogenetic relationship in our study showed a weak correlation between the evolutionary history of *Bryopsis* and its bacterial symbionts. This implies that the bacterial community is possibly dominated by generalists which form temporary relationships with the *Bryopsis* host.

Molecular analyses targeting the characterization of endobiotic bacterial symbionts of *Bryopsis* revealed the presence and spatio-temporal stability of related types of bacteria including Flavobacteriaceae, Phyllobacteriaceae, *Rickettsia*, Rhizobiaceae, *Mycoplasma*, Bacteroidetes and *Labrenzia* (Hollants *et al.*, 2011, 2013). The apparent host-symbiont fidelity observed in these studies inferred close, obligate relationships that might have been a product of vertical transmission. Closer scrutiny of the phylogenetic signals of the entire bacterial community revealed that Labrenzia, Phyllobacteriaceae and Rhizobiaceae communities were closely related to epiphytic, free-living strains implying that their occurrence as endobionts in *Bryopsis* may be transient through horizontal transmission during protoplast formation (Kuykendall, 2005; Hollants *et al.*, 2011). Similarly, although certain bacteria found in our study such as *Arcobacter*, *Flavobacteria* and *Mycoplasma* are documented as endobiotic in *Bryopsis* spp., (Hollants *et al.*, 2011a), the absence of a clear *Bryopsis*-bacterial phylogenetic signal in our study implies that they may be transient symbionts. Hollants and colleagues (2013a) attribute the similarity of bacterial communities of related hosts to the evolution of related mechanisms of symbiont uptake and maintenance by these hosts. However, this uptake selectivity may be influenced by habitat-driven, host-specific physiological factors and the *in situ* composition of bacterial communities for each individual strain which might influence the host-symbiont evolutionary relationship.

4.4 Limitations of our study

This study relied on the *in vitro* propagation of unialgal *Bryopsis* specimen to study changes in bacterial symbiont communities with time and across different life stages. However, obtaining unialgal cultures was quite daunting owing to the abundance of other organisms deemed as contaminants in the cultures. The complete eradication of these contaminants within the allocated time was not achievable. The challenge of obtaining unialgal seaweed cultures is not novel as it has been experienced by pioneer seaweed researchers and coincidentally contributed towards the comprehension of the strong ties that bind bacteria and their bacterial symbionts both *in vitro* and *in situ* (ZoBell, 1946; Andersen, 2006). The loss of several cultures in the course of the experiment also had a significant influence on our sample size and could not be remedied owing to the long period of time that would be required to obtain unialgal cultures with minimal contaminants.

5.0 Conclusion and recommendations

Our result show that the structuring of the bacterial communities hosted by *Bryopsis* sp. is influenced by spatio-temporal variations and reproduction processes. Diverse bacteria are associated with *Bryopsis* spp. from the European coast, with these communities being dominated Proteobacteria. Species richness reduced with increase in cultivation time while the communities hosted in the different sample types consisted of significantly different compositions. The *Bryopsis*-bacteria relationships in our study were however not stable across time and life cycle changes implying they may be predominantly facultative, transient relationships. This was confirmed by the absence of a clear host-symbiont phylogenetic signal between the bacterial and *Bryopsis* sp. phylograms in our study. Horizontal bacterial transmission during protoplast formation was observed to be controlled process that limits the uptake of unwanted bacteria from the environment thereby promoting the long-tern stability of only those strains that are essential to the host. Vertical bacterial symbiont transmission via gametes was not observed.

While our study focused on applying high throughput sequencing technology to characterize entire communities represented in the *Bryopsis* strains, further studies distinguishing endobionts from ectobionts present in the different life stages and sample types will provide a clearer understanding of symbiont fidelity across varying conditions in host physiology and environmental factors. Improvements on *in vitro Bryopsis* propagation are necessary to limit the impact of laboratory conditions on host physiology and obtain a more representative sample size. These improvements may include inclusion of the same species sampled from different locations, attempts to fast-track *in vitro Bryopsis* propagation to reduce time spent *in vitro* while significantly reducing/ eliminating the concentrations of contaminants.

Characterization of the functional diversity of the bacterial symbionts our study was mainly based on literature. Inclusion of metagenomic analyses in subsequent studies will improve the characterization of the specific roles played by the diverse bacteria associated with *Bryopsis* and probably provide insights on the dynamics of the functional profile across time and reproductive changes. This recommendation aligns with recent studies that base the characterization of microbiomes on characterization of functional genes and transcriptomes (Burke *et al.*, 2011; Fan *et al.*, 2012) which provides a more realistic illustration of the status of the bacterial community at a specific point of interest. In depth analysis of bacterial genomes may also provide insights into co-evolved host-symbiont dependencies which are characterized by the apparent loss in gene function (genome reduction) owing to the development of the partnerships.

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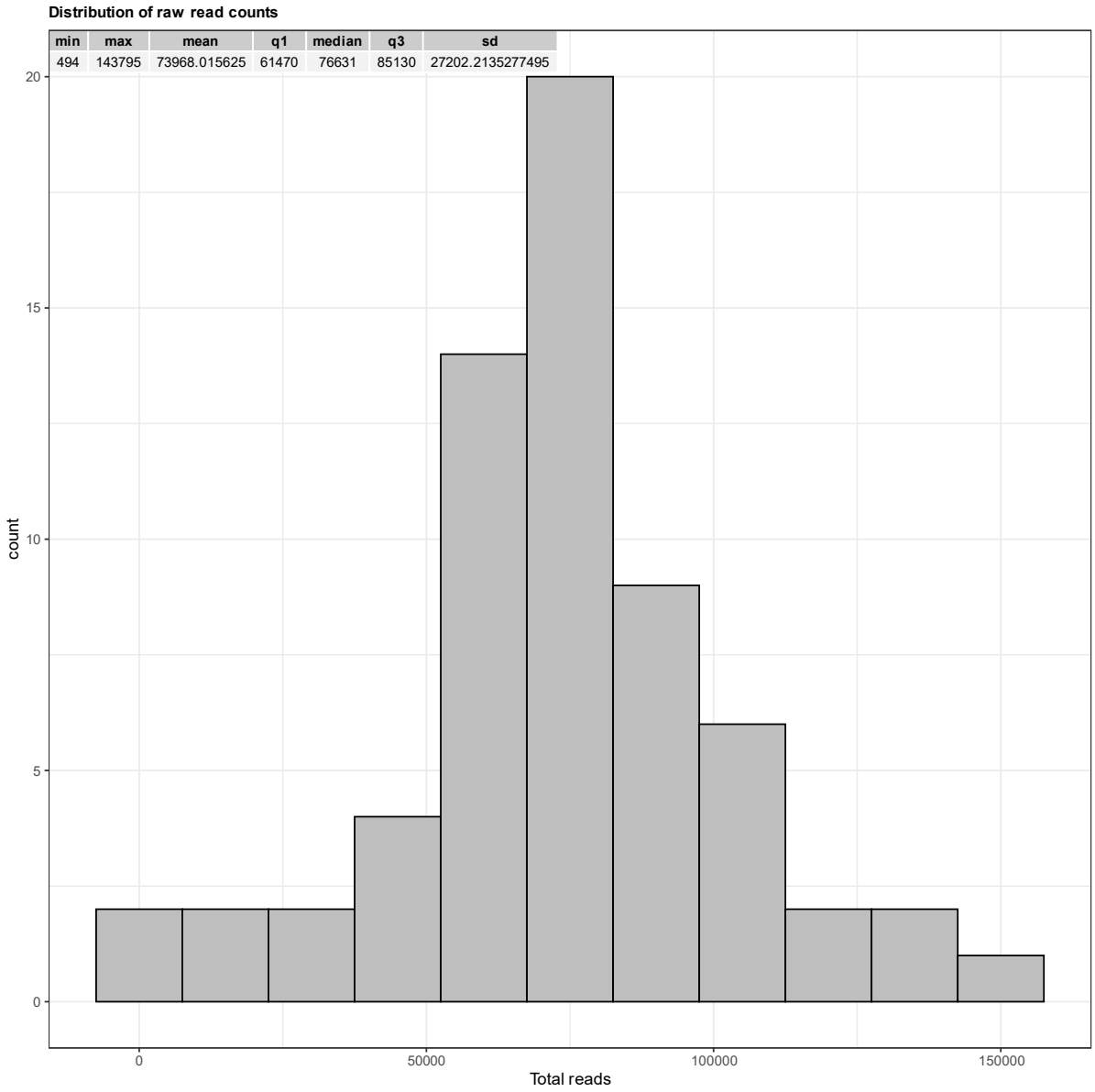
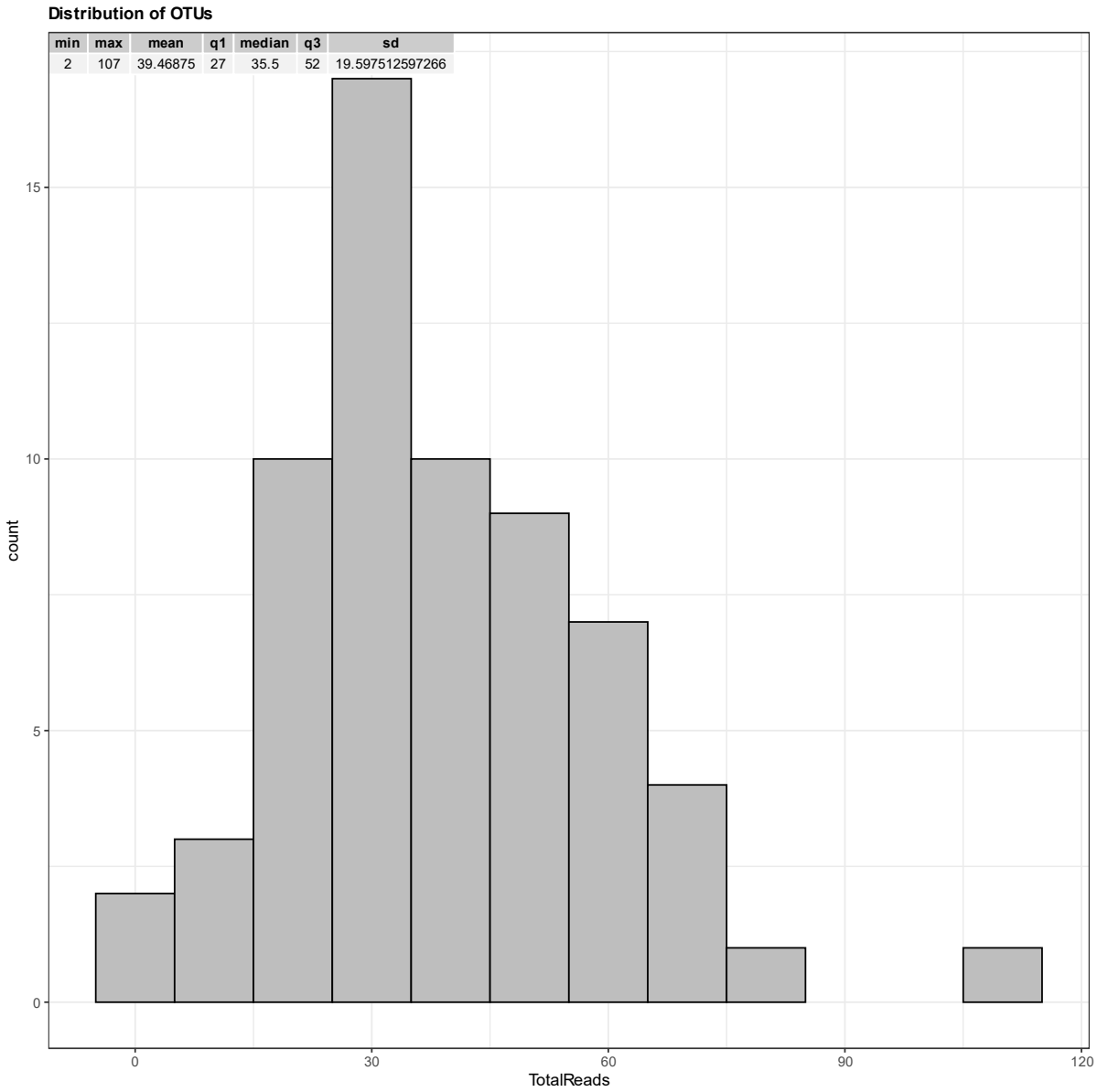
8.0 Appendices

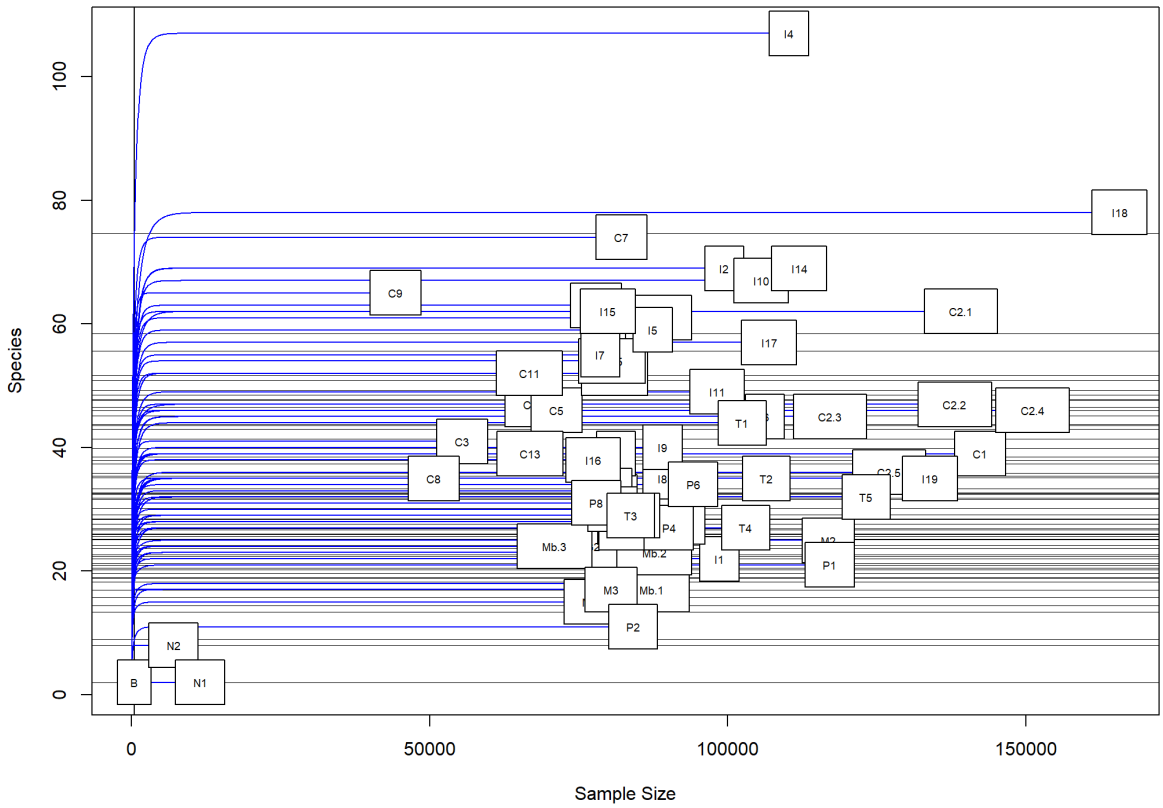
Appendix 1: Additional illustrations



Appendix 1, Figure 1: Distribution of amplicon sequencing raw read counts across the *Bryopsis* sp. samples



Appendix 1, Figure 2: Distribution of OTU counts across the *Bryopsis* sp. samples



Appendix 1, Figure 3: Rarefaction curves: Each curve represents a single sample. Number of species (OTUs) are plotted against the number of sequences (sequencing depth). The plateauing of the curves with increasing sequencing depth indicates the saturation in the number of species which implies that the sequencing depth was sufficient to cover the species diversity in all the samples represented.

Appendix 1, Table 1: Alpha diversity measures of the different sample types and life stages. Highlighted values indicate the highest measure in the respective selected category.

Sample type	**Observed**	**Chao1**	**se.chao1**	**Simpson**	**Shannon**
Blank	2.00	2.00	0.00	0.50	0.69
Cultured	29.00	32.66	3.59	0.80	2.22
Prolonged culture	**36.80**	44.97	7.23	**0.90**	**2.75**
In situ	39.37	**50.16**	8.26	0.86	2.58
Mock	22.00	22.31	0.70	0.89	2.53
PCR_Neg	5.00	5.00	0.00	0.64	1.18
Life stage	**Observed**	**Chao1**	**se.chao1**	**Simpson**	**Shannon**
Blank	2.00	2.00	0.00	0.50	0.69
Original gametophyte	39.06	49.67	8.23	0.86	2.57
Propagated gametophyte	38.16	44.40	5.10	0.84	2.51
Mock	22.00	22.31	0.70	0.89	2.53
PCR Negative	5.00	5.00	0.00	0.64	1.18
Protoplast	21.37	23.58	2.79	0.79	2.02
Sporophyte	**42.00**	**51.30**	**8.57**	**0.92**	**2.95**

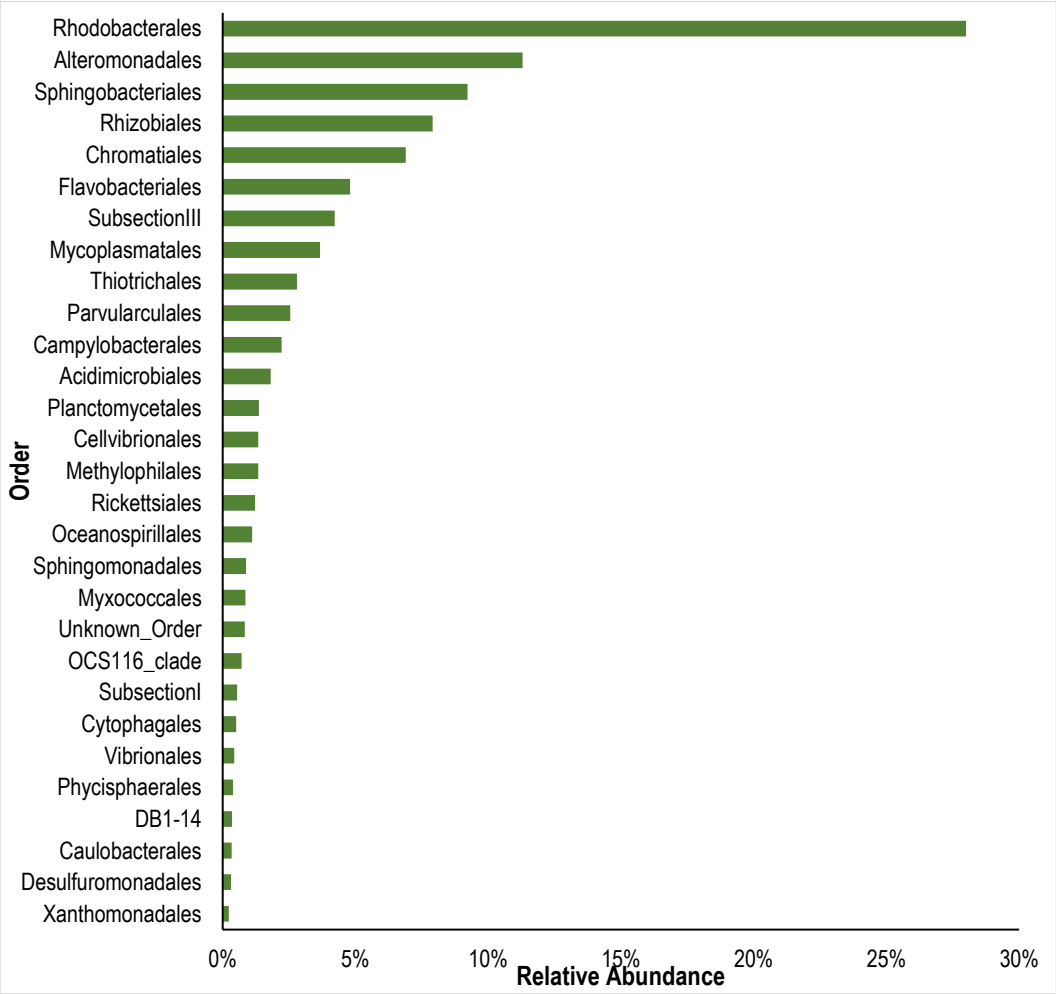
Appendix 1, Table 2: Post-hoc tests on statistical significance of differences in alpha diversity measures of *Bryopsis* sp. samples. Entries marked in green are statistically significant.

Sample type	Measure	p value	Code	Post Hoc Test	
				Observed	***p value***
ANOVA	**Observed**	0.00962	**	Prolonged culture-Cultured	0.244837041
				In situ-Cultured	0.010529409
				In situ- Prolonged culture	0.983295225
				Chao1	***p value***
	Chao1	0.000777	***	Prolonged culture -Cultured	0.134266425
				In situ-Cultured	0.000814636
				In situ-Prolonged culture	0.934327231
				Shannon	***p value***
	Shannon	0.00389	**	Prolonged culture -Cultured	0.037044313
				In situ-Cultured	0.014156341
				In situ- Prolonged culture	0.730999007
				Simpson	***p value***
Kruskall	**Simpson**	0.002091		Cultured - Prolonged culture	0.009922925
				Cultured – *In situ*	0.017409799
				Prolonged culture – *In situ*	0.309770434

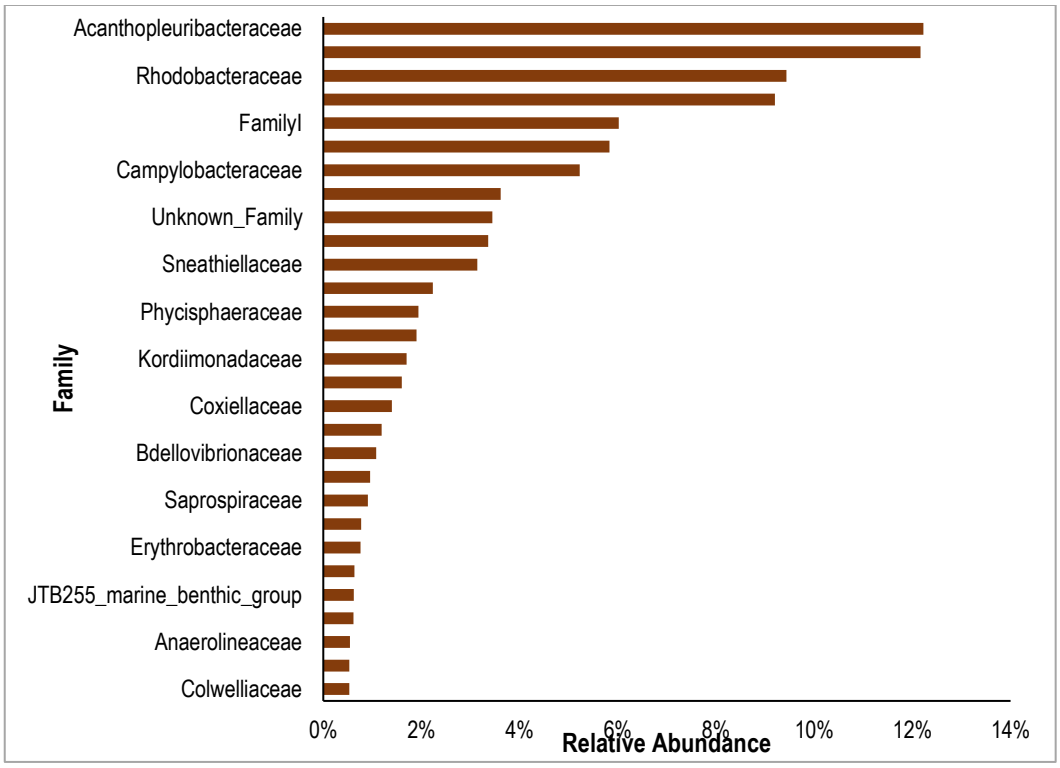
Life stage	Measure	p value	Code	Post Hoc Test	
				Observed	***p value***
				Propagated gametophyte-Original gametophyte	0.999999967
				Protoplast- Original gametophyte	2.51237E-06
ANOVA	**Observed**	1.19e-07	***	Sporophyte- Original gametophyte	0.953246769
				Protoplast- Original gametophyte	1.91118E-06
				Sporophyte- Propagated gametophyte	0.952402271
				Sporophyte-Protoplast	0.017171045
				Chao1	***p value***
				Propagated gametophyte - Original gametophyte	0.845477213
	Chao1	3.32e-08	***	Protoplast- Original gametophyte	1.40E-07
				Sporophyte-Gametophyte	0.978309995
				Protoplast- Propagated gametophyte	2.15E-06
				Sporophyte- Propagated gametophyte	0.871131917
				Sporophyte-Protoplast	0.009283739
				Shannon	***p value***
				Propagated gametophyte - Original gametophyte	0.965336263
	Shannon	9.78e-05	***	Protoplast- Original gametophyte	0.000580787
				Sporophyte- Original gametophyte	0.580879934
				Protoplast- Propagated gametophyte	0.002203753
				Sporophyte- Propagated gametophyte	0.451067437
				Sporophyte-Protoplast	0.014278216
				Simpson	***p value***
				Gametophyte - Propagated gametophyte	0.651125425
Kruskall				Original gametophyte - Protoplast	0.006533872
	Simpson	0.00102		Propagated gametophyte - Protoplast	0.012988961
				Original gametophyte - Sporophyte	0.252018016
				Propagated gametophyte - Sporophyte	0.217710094
				Protoplast - Sporophyte	0.013780433

Appendix 1, Table 3: Post-hoc statistical tests on significance of differences in beta diversity measures (PERMANOVA) of *Bryopsis* sp. samples (paiwise.adonis, vegan package). Entries marked in green are statistically significant.

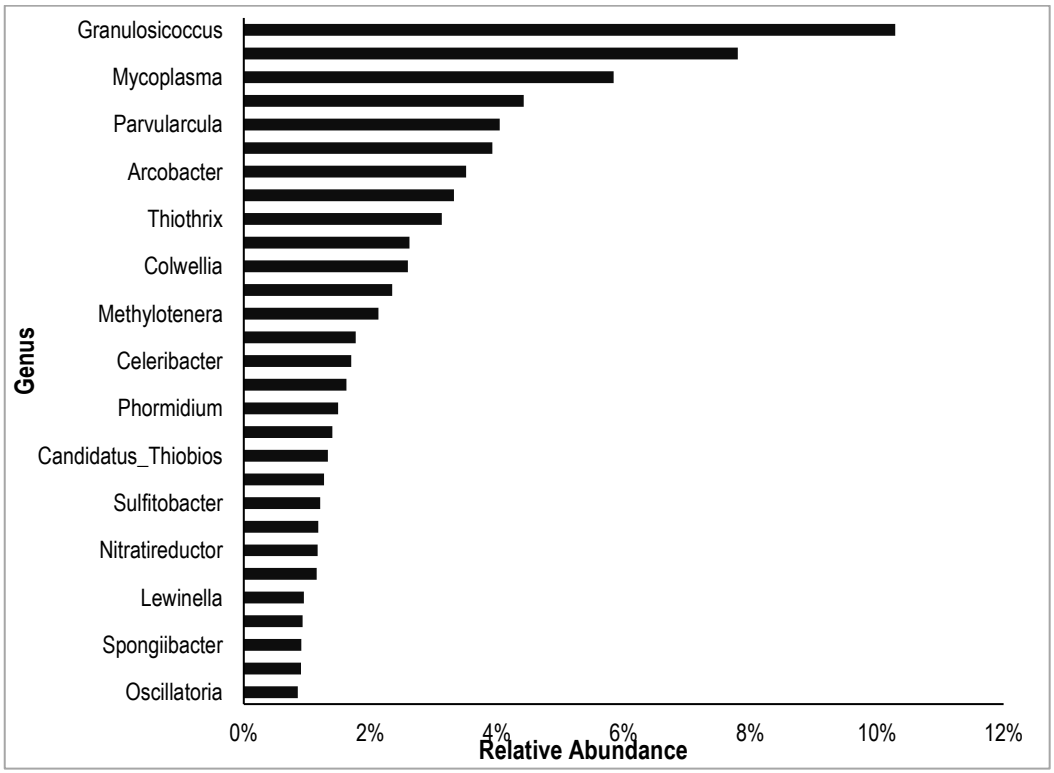
	Sample type					
	Pairs	***Df***	***R2***	***p-value***	***p-adjusted***	***sig***
1	Cultured vs Prolonged culture	1	0.03441	0.079	0.237	
2	Cultured vs *In situ*	1	0.07398	0.001	0.003	*
3	Prolonged culture vs *In situ*	1	0.09282	0.001	0.003	*
	Life stage					
	Pairs	***Df***	***R2***	***p-value***	***p-adjusted***	***sig***
1	Propagated gametophyte vs Sporophyte	1	0.0540	0.297	1	
2	Propagated gametophyte vs Protoplast	1	0.0632	0.001	0.006	*
3	Propagated gametophyte vs Original gametophyte	1	0.0860	0.001	0.006	*
4	Sporophyte vs Protoplast	1	0.0665	0.108	0.648	
5	Sporophyte vs Original gametophyte	1	0.0831	0.01	0.06	
6	Protoplast vs Gametophyte	1	0.1090	0.001	0.006	*



Appendix 1, Figure 4: Relative abundance of the top 30 bacterial orders associated with *Bryopsis* sp.



Appendix 1, Figure 5: Relative abundance of the top 30 bacterial families associated with in *Bryopsis* sp.



Appendix 1, Figure 6: Relative abundance of the top 30 bacterial genera associated with in *Bryopsis* sp.

Appendix 1, Table 4: Top 50 bacterial genera present in the different sample types. Cells highlighted in red indicate genera that are present in the three sample types, white cells represent genera unique to the specific sample types

	In situ	%	Cultured	%	Prolonged culture	%
1	Granulosicoccus	23.2%	Hoeflea	13.8%	Methylotenera	16.6%
2	Mycoplasma	13.5%	Dinoroseobacter	8.2%	Glaciecola	14.9%
3	Parvularcula	11.1%	Leptolyngbya	6.3%	Sulfitobacter	6.6%
4	Thiothrix	9.0%	Aliiglaciecola	6.2%	Spongiibacter	6.3%
5	Arcobacter	5.3%	Granulosicoccus	4.2%	Neptuniibacter	6.3%
6	Rhodobium	5.0%	Colwellia	3.7%	Sedimentitalea	5.8%
7	Candidatus_Thiobios	3.8%	Roseobacter	3.4%	Leptolyngbya	4.6%
8	Phormidium	3.2%	Celeribacter	3.2%	Roseobacter	4.6%
9	Rubidimonas	2.2%	Arcobacter	3.1%	Hoeflea	3.8%
10	Portibacter	2.2%	Phaeobacter	3.1%	Methylophaga	3.7%
11	Leucothrix	1.8%	Paraglaciecola	2.6%	Planctomyces	3.5%
12	Colwellia	1.7%	Mycoplasma	2.2%	SM1A02	3.5%
13	Ulvibacter	1.4%	Nitratireductor	2.1%	Ascidiaceihabitans	2.6%
14	Amylibacter	1.4%	Lacinutrix	1.7%	Kordia	2.3%
15	Blastopirellula	1.2%	Oscillatoria	1.6%	Blastopirellula	2.2%
16	Sulfitobacter	1.2%	Roseovarius	1.6%	Fabibacter	1.2%
17	Ruegeria	0.8%	Sphingorhabdus	1.6%	Polycyclovorans	1.0%
18	Erythrobacter	0.8%	Lewinella	1.4%	Maribacter	0.8%
19	Luteibacter	0.6%	Glaciecola	1.4%	Alcanivorax	0.7%
20	Lewinella	0.6%	Alteromonas	1.3%	Porticoccus	0.7%
21	Pleurocapsa	0.6%	Amylibacter	1.3%	Pseudospirillum	0.7%
22	Algicola	0.6%	Vibrio	1.2%	Aestuariibacter	0.5%
23	Dokdonia	0.6%	Ahrensia	1.2%	Leisingera	0.5%
24	Spirulina	0.5%	Marinobacter	1.2%	Planktotalea	0.5%
25	Winogradskyella	0.5%	Neptuniibacter	0.9%	Dinoroseobacter	0.4%
26	Pseudoalteromonas	0.5%	Aquimarina	0.9%	Pseudophaeobacter	0.4%
27	BD1-7_clade	0.5%	Ruegeria	0.9%	Oleiphilus	0.4%
28	Kordiimonas	0.5%	Aestuariibacter	0.9%	Haliea	0.4%
29	Subsaxibacter	0.4%	Pseudophaeobacter	0.8%	Erythrobacter	0.3%
30	Aquimarina	0.4%	Rubidimonas	0.8%	Kordiimonas	0.3%
31	Truepera	0.3%	Kordia	0.8%	Cohaesibacter	0.3%
32	Sufflavibacter	0.3%	Phormidium	0.7%	Jannaschia	0.3%
33	Roseobacter_clade_NAC11-7_lineage	0.3%	Defluviimonas	0.7%	Labrenzia	0.3%
34	Acaryochloris	0.3%	OM60[NOR5]_clade	0.7%	Pirellula	0.3%
35	Glaciecola	0.2%	Pseudomonas	0.6%	Escherichia-Shigella	0.3%
36	Schleiferia	0.2%	Owenweeksia	0.6%	Rhodopirellula	0.2%
37	Kordia	0.2%	Maribacter	0.5%	Propionibacterium	0.2%
38	Maribacter	0.2%	Antarctobacter	0.5%	Owenweeksia	0.2%
39	Thiohalophilus	0.2%	Rhodopirellula	0.5%	OM27_clade	0.2%
40	Pelagibacterium	0.2%	Loktanella	0.5%	Sphingorhabdus	0.2%
41	Aquibacter	0.2%	Blastopirellula	0.4%	Gemella	0.2%
42	OM27_clade	0.2%	Winogradskyella	0.4%	Thalassotalea	0.1%
43	Croceitalea	0.1%	Primorskyibacter	0.4%	Colwellia	0.1%
44	Cocleimonas	0.1%	Planctomyces	0.4%	Balneola	0.1%
45	Thiohalorhabdus	0.1%	Algimonas	0.4%	Pir4_lineage	0.1%
46	Planctomyces	0.1%	Parvularcula	0.4%	Oceanospirillum	0.1%
47	Marinicella	0.1%	Marinifilum	0.4%	Balneatrix	0.1%
48	Nitratireductor	0.1%	Pir4_lineage	0.3%	Maritalea	0.1%
49	Peredibacter	0.1%	SM1A02	0.3%	Marinomonas	0.1%
50	Reichenbachiella	0.1%	Mesorhizobium	0.3%	Defluviicoccus	0.1%

Appendix 1, Table 5: Top 50 bacterial genera present in the different life . Cells highlighted in red indicate genera that are present in the three sample types, white cells represent genera unique to the specific sample types

	Original gametophyte	%	Propagated gametophyte	%	Protoplast	%	Sporophyte	%
1	Granulosicoccus	23.2%	Leptolyngbya	13.6%	Hoeflea	20.4%	Methylotenera	24.4%
2	Mycoplasma	13.5%	Roseobacter	6.2%	Dinoroseobacter	14.0%	Glaciecola	16.0%
3	Parvularcula	11.1%	Arcobacter	5.5%	Aliiglaciecola	10.6%	Spongiibacter	10.7%
4	Thiothrix	9.0%	Glaciecola	4.7%	Granulosicoccus	5.6%	Sulfitobacter	9.3%
5	Arcobacter	5.3%	Celeribacter	4.6%	Phaeobacter	5.2%	Roseobacter	7.8%
6	Rhodobium	5.0%	Hoeflea	4.4%	Colwellia	4.4%	Methylophaga	6.2%
7	Candidatus_Thiobios	3.8%	Mycoplasma	4.0%	Paraglaciecola	4.3%	SM1A02	5.9%
8	Phormidium	3.2%	Neptuniibacter	3.0%	Nitratireductor	3.6%	Kordia	3.9%
9	Rubidimonas	2.2%	Oscillatoria	2.9%	Lacinutrix	3.0%	Hoeflea	2.1%
10	Portibacter	2.2%	Roseovarius	2.9%	Amylibacter	2.2%	Polycyclovorans	1.6%
11	Leucothrix	1.8%	Granulosicoccus	2.6%	Sphingorhabdus	2.1%	Blastopirellula	1.5%
12	Colwellia	1.7%	Rhodobium	2.6%	Marinobacter	1.5%	Alcanivorax	1.2%
13	Ulvibacter	1.4%	Sedimentitalea	2.4%	Aestuariibacter	1.5%	Porticoccus	1.2%
14	Amylibacter	1.4%	Alteromonas	2.4%	Kordia	1.3%	Planctomyces	0.8%
15	Blastopirellula	1.2%	Vibrio	2.2%	Rubidimonas	1.2%	Dinoroseobacter	0.7%
16	Sulfitobacter	1.2%	Colwellia	2.2%	Celeribacter	1.2%	Neptuniibacter	0.6%
17	Ruegeria	0.8%	Ahrensia	2.1%	Pseudophaeobacter	1.1%	Oleiphilus	0.6%
18	Erythrobacter	0.8%	Thiothrix	1.9%	Defluviimonas	1.1%	Haliea	0.6%
19	Luteibacter	0.6%	Planctomyces	1.9%	OM60[NOR5]_clade	1.1%	Maribacter	0.6%
20	Lewinella	0.6%	Ruegeria	1.6%	Neptuniibacter	1.1%	Erythrobacter	0.6%
21	Pleurocapsa	0.6%	Lewinella	1.5%	Pseudomonas	1.1%	Cohaesibacter	0.6%
22	Algicola	0.6%	Aquimarina	1.4%	Lewinella	1.0%	Jannaschia	0.6%
23	Dokdonia	0.6%	Phormidium	1.3%	Antarctobacter	0.8%	Rhodopirellula	0.4%
24	Spirulina	0.5%	Methylotenera	1.3%	Loktanella	0.8%	OM27_clade	0.3%
25	Winogradskyella	0.5%	Blastopirellula	1.2%	Maribacter	0.7%	Gemella	0.3%
26	Pseudoalteromonas	0.5%	Ascidiaceihabitans	1.1%	Primorskyibacter	0.6%	Pirellula	0.2%
27	BD1-7_clade	0.5%	Fabibacter	0.8%	Mesorhizobium	0.5%	Oceanospirillum	0.2%
28	Kordiimonas	0.5%	Owenweeksia	0.8%	Rhodopirellula	0.5%	Maritalea	0.1%
29	Subsaxibacter	0.4%	Kordiimonas	0.7%	Parvularcula	0.4%	Marinomonas	0.1%
30	Aquimarina	0.4%	Marinifilum	0.6%	Winogradskyella	0.4%	Aliiglaciecola	0.1%
31	Truepera	0.3%	Sphingorhabdus	0.6%	Algimonas	0.4%	Neptunomonas	0.1%
32	Sufflavibacter	0.3%	Pir4_lineage	0.6%	Owenweeksia	0.4%	Pontibacter	0.1%
33	Roseobacter_clade_NAC11-7_lineage	0.3%	Rubidimonas	0.6%	Spongiibacter	0.3%	Marinoscillum	0.1%
34	Acaryochloris	0.3%	Thalassococcus	0.5%	Reichenbachiella	0.3%	Fabibacter	0.1%
35	Glaciecola	0.2%	Subsaxibacter	0.5%	Sandaracinus	0.3%	Marinicella	0.1%
36	Schleiferia	0.2%	Methylophaga	0.5%	Aquimarina	0.3%	Parvularcula	0.1%
37	Kordia	0.2%	Marinobacter	0.5%	Nonlabens	0.3%	C1-B045	0.1%
38	Maribacter	0.2%	Sulfitobacter	0.4%	Magnetospira	0.2%	Aureispira	0.0%
39	Thiohalophilus	0.2%	Pseudospirillum	0.4%	Pseudoalteromonas	0.2%	Maricaulis	0.0%
40	Pelagibacterium	0.2%	Limnothrix	0.4%	Cyclobacterium	0.2%	Crocinitomix	0.0%
41	Aquibacter	0.2%	Anderseniella	0.4%	Marinicella	0.2%	Burkholderia	0.0%
42	OM27_clade	0.2%	SM1A02	0.4%	Arcobacter	0.2%	Thalassobaculum	0.0%
43	Croceitalea	0.1%	Pseudophaeobacter	0.4%	SM1A02	0.2%		
44	Cocleimonas	0.1%	Aquibacter	0.3%	Blastopirellula	0.2%		
45	Thiohalorhabdus	0.1%	Maribacter	0.3%	Luteivirga	0.1%		
46	Planctomyces	0.1%	Rhodopirellula	0.3%	Rhodovulum	0.1%		
47	Marinicella	0.1%	Pseudoalteromonas	0.3%	Kordiimonas	0.1%		
48	Nitratireductor	0.1%	Flexithrix	0.3%	Sedimentitalea	0.1%		
49	Peredibacter	0.1%	OM27_clade	0.2%	Leucothrix	0.1%		
50	Reichenbachiella	0.1%	Winogradskyella	0.2%	Pseudofulvibacter	0.1%		