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https://doi.org/10.25903/enjb%2Dgf32

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Molecular insights into a novel coral model, *Heliofungia actiniformis*

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and the

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James Cook University

This thesis is presented for the degree for Doctor of Philosophy of James Cook University

Dedication

I dedicate this thesis to my parents and sister; since the day I decided to be a marine biologist you have supported my dreams unconditionally.

Chloë, age 6: “I want to be a sea doctor!”
Chloë, age 29: “Nailed it.”

Acknowledgements

Firstly, to my advisory team:
David Miller, thank you for giving me one of the most amazing opportunities of my life. If someone had told me four years ago that I was about to get a Facebook message asking if I wanted to do a PhD with you, I would think they were mad (clearly dreams do come true). I have learnt so much from you and count myself extremely fortunate for the freedom I got in the lab and the field to explore my unique ideas.

Aurelie Moya, they say 'don't meet your heroes' but I'm so glad I did. I was a massive fangirl of your work before I started and getting to work with you these past years has been incredible. You have been a fantastic role model and taught me so much, I could not have got over the finish line without you.

Ira Cooke, your knowledge bombs, kind patience and advice have been invaluable during this process. You always take the time to explain a difficult subject or concept to me which I will be forever grateful for.

Roger Huerlimann, my amazing unofficial advisor. You taught me magic in the lab (I cannot believe the science I now know how to do thanks to you) and I also gained a great friend whilst learning.

Natalia Andrade, my beautiful mentor, you stepped in when I needed you most and gave me the encouragement and support, I had lost in myself.

To my Orpheus family: Marta Espinheira, Bryn Jones, Anders Zimny and Jimmy. My fieldwork would not have been possible without you, and you made every trip feel like Orpheus was my second home.

Thank you to my lab mates for all your help on spawning and dissection trips, you are an amazing team and the memories of our time on Orpheus will last a lifetime. Also, thank you to Katie Motson for coming out with me extremely last minute so I could get extra research done.

Thank you to Steve Robbins for our 'microbiome nerd outs' and help with the Archaea/Bacteria primers at the start of my thesis journey. Thank you again to Roger and all the wonderful people in MEEL who taught me how to do library prep and sequencing. I could not have got through the 1000 samples without you: Alyssa Budd, Sandra Infante Villamil and Carolyn Smith-Kleune.

Thank you to David Bourne, Grace Cole, Casey Whalen and Katarina Damjanovic for all the discussions on FISH and how to make it work. Thank you Lit Chien Cheah for making me the most

beautiful histology slides. A special thank you to Shane Askew also, without you teaching me how to use the confocal microscope, I would likely still be sitting there.

Stats, bioinformatics and using R are not easy tasks when you start a PhD, but I was extremely lucky to have some great teachers (Ira & Roger) and peers that I could speak to (instead of throwing my computer out the window); thank you Jia Zhang, Ramona Brunner and everyone in the CodeR club for making a difficult process easier.

Thank you to Liz Tynan for all your help and wisdom with scientific writing when I started my PhD.

To all the wonderful people in my life:
PhDs are tough and each comes with their own unique trials and tribulations. I was lucky enough to have an amazing group of people around me to help me through the difficult times. Thank you to Katy, Ricardo and Manish for your wisdom over the years. Thank you to my incredible friends in my PhD cohort, without you all, my PhD would have been pretty lonely, in particular, my dog walking trio; Britt and Suze (plus Beau and Cara), housemate, Sage and my forest ladies; Motson, Sambrook and Tessa, our focused planting group has got me through this last year and a half. To the fantastic friends outside of JCU; Dan, my hiking buddy, Em, soul sister, Brooke and all you lovelies that have kept me sane over the years. To my friends back home, thank you for supporting my dream even though it took me far away from you all and we will hopefully be reunited soon Ellie, Elly, Bex and James.

Finally, thank you to my incredible family. Thank you to Ruth and Greg for accepting me into your family and giving me a home in Australia when mine was so far away. My family, you have all supported me since I was little and have been big believers in my dreams. Thank you to my cousin Imy for always looking out for me. My grandparents, Michael and Audrey, you always knew I'd make it (even when I doubted myself) and encouraged my sense of adventure and curiosity from birth, I miss you both. To my brilliant Uncle John, my Godfather, thank you for all the proofreading, supporting my nerdiness and all the geeky chats swapping ideas. To my beautiful boys, Josh and our doggo Obi-Wan, you have been my rocks these past 3 years, thank you for all your love and support and kicking me over the finish line. Finally, my mum, Alison, dad, Paul and sister, Hannah, you have believed in me since I decided 'I wanted to make the sea better', you have been my constant cheerleaders and I am so grateful for all your support in achieving my dream, we did it!

Statement of contributions of others

Funding:
The JCU Postgraduate Research Scholarship.

Covid-19 Student Support Scholarship.

College of Public Health, Medical and Veterinary Sciences – SSA funds

ARC Centre of Excellence for Coral Reef Studies

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Title page image:
Dr Aurelie Moya, ARC Centre of Excellence for Coral Reef Studies, James Cook University

Laboratory work:
Lit Chien Cheah, JCU Histology, James Cook University

Abstract

Coral reefs are highly diverse ecosystems that presently face a range of threats, the most obvious of which is coral bleaching, the loss of symbionts caused by rising sea surface temperature. While coral bleaching has been extensively researched, the underlying mechanisms remain unclear. Because the photosynthetic Symbiodiniaceae reside in the endodermal tissue layer (also known as the gastrodermis), one idea is that data on transcriptional differences between that tissue in the bleached and normal states might provide novel insights into bleaching. Whilst tissue-specific transcriptomics is not feasible in colonial corals, in this thesis I took the novel approach of using the fungiid coral *Heliofungia actiniformis* to specifically address biological questions at the level of individual tissues. The large size of this solitary coral allowed physical separation of individual coral tissues for the first time, opening new avenues of research in coral biology. In the present thesis, these characteristics were taken advantage of to undertake the tissue-specific microbiome and transcriptome in both the normal and bleached states.

Chapter #2 provides microbiome data for the mucus, ectoderm, acrosphere, endoderm and gut of healthy *Heliofungia* individuals based on partial 16S rRNA sequence data. Fluorescent *in situ* hybridization with general bacterial and *Endozoicomonas*-specific probes was used to visualise bacteria in coral tissues.

The second data chapter (chapter #3) compares the tissue-specific microbiomes of bleached *Heliofungia* collected during the April 2017 bleaching event, and of unbleached corals a year later. The results show significant differences in the diversity of bleached and unbleached individuals, particularly in the ectoderm, endoderm and gut.

The final data chapter (Chapter #4) compared the transcriptomes of the host ectoderm and endoderm of bleached and unbleached *Heliofungia* specimens. The largest differences in gene expression occurred in the endoderm, and application of this approach allowed inference of the physiological states of the tissues in the bleached and unbleached states.

Overall, this thesis provides a number of novel perspectives on interaction of corals with microorganisms and highlights the potential informativeness of studying tissue regions separately.

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Thesis declaration

I, Chloë Georgina Boote, declare that the thesis presented here fulfils the requirements for the degree of Doctor of Philosophy at James Cook University.

The research presented is my own and written by myself. Any literature or sources I have used have been referenced in the thesis.

This thesis has not been submitted to any other institution as part of a degree or professional qualification.

This work in no way infringes or violates another person's copyright, trademark, or patent.

Signed:

Chloë Georgina Boote

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Chapter 1: *Heliofungia actiniformis* as a novel model coral

1.1 Model organisms in coral reef research

1.1.1 The importance of coral model organisms

Coral reefs cover approximately 0.1% of the planet, but support a minimum of 25% of known marine species (Fisher et al., 2015). For humankind, reefs supply fundamental ecological services directly and indirectly, from coastal protection to novel products that can be used in drug therapies (Beck et al., 2018; de Groot et al., 2012; Hoegh-Guldberg et al., 2017; Moberg & Folke, 1999). However, coral reefs are significantly impacted by anthropogenic climate change, leading to the large-scale degradation of reef ecosystems (Hoegh-Guldberg et al., 2018; Hughes, Barnes, et al., 2017). As of 2019, it was estimated that 19% of reefs have been degraded beyond recovery (Pratchett & Hoogenboom, 2019).

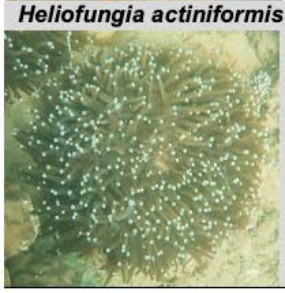
To improve the chances of coral reefs surviving, one of the areas that needs to be studied further is the mechanistic response of corals to climate change, in particular, the bleaching response. When corals are exposed to extended heat stress, they may undergo bleaching by either engulfing or expelling their Symbiodiniaceae, however, it is disputed on which party initiates this. There are several theories on the triggers behind symbiont expulsion or engulfment; photosystem II damage (Warner et al., 1996), inhibition of photosynthetically fixed carbon dioxide (Jones et al., 1998), the Symbiodiniaceae over-producing reactive oxygen species (ROS) causing toxicity in the host cells (Smith et al., 2005), nitric oxide synthesis and caspase activity triggering apoptosis (Hawkins et al., 2013), host and coral species-specific tolerance levels (Berkelmans & van Oppen, 2006; Loya et al., 2001l), nutrient ratios in the host (Pogoreutz et al., 2017), amongst others. In addition to the numerous causative theories, even some of the most established (those with respect to photosystem damage and ROS) have come under scrutiny (Nielsen et al., 2018) and it has been hard to pinpoint the bleaching mechanism with research essentially generating more questions than answers. Current studies are based on whole polyp sampling with findings portraying an average result of the response across both symbiotic and aposymbiotic tissues making results unclear. It is probable that the coral tissues - the aposymbiotic ectoderm and symbiotic endoderm (also known as the gastrodermis) - display a different molecular response with respect to bleaching pressures and need to be measured separately to isolate the bleaching mechanism. What is needed is a coral model that can split the molecular responses to bleaching between ecto- and endoderm as one factor significantly limiting previous bleaching studies is the lack of fine-

scale molecular analysis. Developing such a coral model will facilitate greater understanding of the biological mechanisms that lead to coral dysbiosis and current states of ecosystem degradation, and may also expand our understanding of germ layers and cnidarian evolution (Cleves et al., 2019).

Model organisms (henceforth referred to as models) have been used to understand biological principles and create research cohesion. Model organisms often have traits that make them particularly suited to answering certain biological questions. This, combined with the focussed investment in molecular resources that can be justified for model organisms, often facilitates rapid progress in a field. Selecting a model is dependent on some key features: their phylogenetic position, species significance and ease of care, culture and accessibility to experimental approaches (Hedges, 2002; Mendel & Mangelsdorf, 1965; Müller & Grossniklaus, 2010). For example, *Mus musculus* is an extremely useful model because of the molecular similarities it shares with humans, its high fecundity, ease of care and short generation time (Ericsson et al., 2013).

There seems to be a bias against selecting new models when a well-studied, safe model already exists. It is difficult to study a new organism when so many resources already exist for developed models however, this selection bias has created inertia in research (Bolker, 1995). Therefore, to continue advancing our biological understanding, science must move away from the 'models of convenience and convention' and towards atypical species (Bolker, 2012; Jenner & Wills, 2007; Milinkovitch & Tzika, 2007). Fortunately, with the advances made in molecular techniques, establishing new models has become more achievable (Newmark & Sanchez Alvarado, 2002). A novel model being developed is in this respect, the solitary coral *Heliofungia actiniformis* (henceforth referred to as *Heliofungia*) has the potential to facilitate research in several areas of coral biology. Below, I discuss; i) species currently regarded as model corals, ii) the limitations that apply to these species as "models" and, iii) how *Heliofungia* compares with the more commonly studied corals.

Table 1.1: The current coral models and their advantages and disadvantages. Image references: *Acropora millepora* and *Heliofungia actiniformis*; Boote C. G. *Stylophora pistillata*; Tambutté E., (https://www.eurekalert.org/multimedia/640942o). *Montastraea faveolata*; Wikipedia, (https://en.wikipedia.org/wiki/Orbicella_faveolata). *Aiptasia*; Brown T. & Rodriguez-Lanetty M., (Brown & Rodriguez-Lanetty, 2015).

Species	Advantages	Disadvantages
Acropora millepora 	Large amount of research already carried out on the species. Prolific on the reef. Gonochoric. Predictable spawning time. Genome and transcriptome available. Genome under 400Mbp. Calcification.	It is not part of the understudied clade (Robusta). Bleaches easily. Regarding tissue-specific analysis: - it is a colony. - small, interconnected coral polyps. - difficult to access regions.
Stylophora pistillata 	Research already undertaken particularly on climate change and symbiosis. Predictable spawning date. Part of the understudied Robusta clade. Genome and transcriptome available. Calcification. Genome is ~400Mbp.	It is a brooder (releases larvae). Regarding tissue-specific analysis: - it is a colony. - small, interconnected coral polyps. - difficult to access regions.
Montastraea faveolata 	Coral model of convenience for the Caribbean. Predictable spawning time. Gonochoric. Part of the understudied Robusta clade. Genome and transcriptome available. Calcification. Commonly, does not perish after bleaching.	Limited geographically. Large genome size (~700Mbp). Regarding tissue-specific analysis: - it is a colony. - small, interconnected coral polyps. - difficult to access regions.
Aiptasia 	Can live symbiotically and aposymbiotically. Research already undertaken particularly on symbiosis and epigenetics. Genome under 400Mbp. Gonochoric. Individual organism.	It does not calcify. Larvae difficult to settle in the lab, hard to close their life cycle. Small organism. Some individuals are hermaphroditic.
Heliofungia actiniformis 	Individual organism. Large polyp (adults over 20cm diameter). Long permanently extended tentacles. Gonochoric. Predictable spawning time. Calcification. Commonly, does not perish after bleaching.	Understudied species. Genome and transcriptome not yet public/complete.

1.1.2 Non-scleractinian models and their limitations

Nematostella vectensis and *Hydra* spp. were selected as cnidarian models due to their ease of culture and evolutionary positions (Fritzenwanker et al., 2004; Kusserow et al., 2005). In the freshwater *Hydra,* the molecular mechanisms behind body patterning, regeneration and morphogenetic gradients were investigated (Bode, 2003; Steele, 2012; Technau & Bode, 1999). More recently, *Hydra* has also been used to understand co-evolution of host species with the microbiome, as shown by the use of the animals gastrointestinal microbes to control and regulate gut peristalsis (Murillo-Rincon et al., 2017). Studies on *Hydra* date back to the 1740s when Trembley started using the organism to understand the processes behind regeneration (Lenhoff & Lenhoff, 1991). Since then, the animal has become an established study model for a number of areas in biology (Augustin et al., 2017; Gierer et al., 1972; Quinn et al., 2012). In regards to *Hydra* as a model, Hydrozoa were originally assumed to be the basal class within the Cnidaria (Hyman, 1940). However, examination of characteristics of the mitochondrial genome essentially disproves this theory - amongst cnidarians, only in Anthozoa does the mt genome resemble those of higher metazoans in being circular (Bridge et al., 1995; Bridge et al., 1992), indicating that the Anthozoa (which includes anemones, like *Nematostella*, and corals) are the ancestral class (Medina et al., 2001; Miller et al., 2005; Moya et al., 2016; Technau et al., 2005). This updated view of cnidarian phylogeny led to selection of *Nematostella* as a representative of the earliest diverging class in order to investigate molecular mechanisms behind axial patterning and nervous system specification that are shared between cnidarians and higher metazoans (Darling et al., 2005; Miller et al., 2005; Rentzsch & Technau, 2016). *Nematostella* has also been used to increase understanding of cnidarian host - microbiome interactions and in particular how these may influence the phenotype of an animal (Bonacolta et al., 2020; Fraune et al., 2016).

Another anemone gaining prominence as a model organism in cnidarian biology is *Aiptasia* which hosts a similar range of Symbiodiniaceae as do corals, but (unlike reef-building corals) has the ability to live both symbiotically and aposymbiotically (i.e., with or without these symbionts). Its ease of culture and short generation time make *Aiptasia* simple to work with and these advantages have already been exploited to investigate, for example, symbiotic interactions, and responses to stress (Ahmed et al., 2019; Weizman & Levy, 2019). Another advantage is the available whole genome assembly for *Aiptasia* (Baumgarten et al., 2015). The ability of *Aiptasia* to live aposymbiotically enables comparative analyses of molecular responses in both symbiotic and non-symbiotic states without compromising the health of the animal (Belda-Baillie et al., 2002; Lehnert et al., 2012; Sunagawa et al., 2009; Weis et al., 2008). For example, how gene regulation under heat stress is impacted with and without

symbionts (Hubner & Spector, 2010; Weizman & Levy, 2019). and, how heat stress alters the microbiome away from its normal healthy state (Ahmed et al., 2019). *Aiptasia* larvae have been used to study Symbiodiniaceae acquisition, providing insights into candidate genes for roles in the establishment of symbiosis (Wolfowicz et al., 2016). Whilst *Aiptasia* has certainly provided insights into several aspects of coral biology (Weis, 2019) it does come with certain limitations as a coral model.

Unfortunately, sea anemones and other Medusozoans cannot be used to study some aspects of coral biology - for example, how calcification is impacted by environmental changes and the role of symbiosis in calcification. Research has shown that the Symbiodiniaceae are linked to increasing the pH of the calcifying fluid the host uses for calcification (Inoue et al., 2018) and these mechanisms can only be studied in Scleractinia. Due to increasing pressures from ocean acidification and local threats (pollutants, sedimentation etc.), calcification and growth is one of the key challenges hermatypic corals face in predicted climate change scenarios (De'ath et al., 2009; Lebrec et al., 2019; Mollica et al., 2018). Therefore, using a species that does not secrete a calcium carbonate skeleton, subsequently building reefs, as a 'coral' model makes it impractical for ocean acidification studies. Additionally, when considering developmental studies, it can be problematic extrapolating results from anemones to corals as, although they have developmental processes similar to corals (Schlesinger et al., 2010) they are not the same as anemones and do not need to start secreting a calcium carbonate skeleton once metamorphosed and settled.

As model organisms, sea anemones continue to advance our understanding of several areas of coral biology. However, they cannot be informative with respect to several important traits of corals, including calcification and broadcast spawning. The limitations that apply to the established representative justify exploring the potential of other coral species, such as *Heliofungia*, to provide new perspectives on traits that are coral specific and more general.

1.2 The current coral models

1.2.1 Complexa coral species

Acropora millepora (henceforth referred to as *Acropora*) is a member of the Complexa coral clade that is widespread and common in the shallow waters of the Indo-Pacific and was the first coral to be extensively studied at the molecular level (Miller & Ball, 2000). *Acropora* was the first anthozoan to be extensively exploited to expand our understanding of the evolution of animal complexity (Bosch et al., 2009; Chen et al., 2002; Miller & Ball, 2000)

highlighting ‘vertebrate-specific’ gene repertoires relating to morphological complexity (Miller et al., 2005). Furthermore, the predictable mass spawning events of *Acropora* enable collection of large amounts of essentially synchronous developmental stages, facilitating developmental studies (Babcock et al., 1986; Miller & Ball, 2000). As the species is a broadcast spawner, the release of gametes allows individual cell stages to be observed (Fritzenwanker et al., 2007; Marlow & Martindale, 2007). *Acropora* early development has many similarities (spherical embryogenesis, prawn-chip phase, gastrulation process) with that of other cnidarians and more complex animals, opening up questions on the ancestry of gene repertoires that coordinate development (Ball et al., 2002; Miller & Ball, 2000). Furthermore, *in situ* hybridization (ISH) in *Acropora* has provided further evidence for conservation of function for some organiser and gastrulation genes (Ball et al., 2002; Hayward et al., 2015) as well as genes involved in specifying body axes (Hayward et al., 2001), and patterning the nervous system (Pax genes (Ball et al., 2002; Miller et al., 2000)).

Advances in DNA sequencing technology have also enabled substantial progress in coral biology, in particular, the whole genome assembly of *Acropora digitifera* in 2011 (Shinzato et al., 2011). The assembly of the *A. digitifera* genome and subsequent comparative analyses by a relatively small team demonstrated the feasibility of *de novo* genomics in non-model species, creating a knock-on effect for coral research (Baumgarten et al., 2015; Lin et al., 2015; Shinzato et al., 2011; Voolstra et al., 2017). Consequently, making the genome available proved foundational in facilitating transcriptomic and metagenomic studies on corals and their symbiotic communities in general (Miller et al., 2011; Shinzato et al., 2014; Thompson et al., 2015; Weston et al., 2012; Wood-Charlson et al., 2015). Using phylogenomics, these insights also facilitated a better understanding of the Complexa and Robusta classification which are the two scleractinian superfamilies (Kitahara et al., 2014; Lin et al., 2014). One recent discovery highlighted that, uniquely amongst animals (and unlike complex corals and anemones), robust corals are capable of *de novo* histidine biosynthesis, these results reflect the first major biological difference between the two clades (Ying et al., 2018). It is important to note that, in comparison to complex corals, the robust corals are an understudied group. For example, whereas (as of the beginning of 2021) 13 whole genome assemblies of complex coral species were publicly available whereas only four were available for robust corals on NCBI (Table 1.2).

Table 1.2: The publicly available Hexacorallia genomes available from NCBI split into their respective coral clades (https://www.ncbi.nlm.nih.gov/genome).

Complexa:	NCBI id:	Robusta:	NCBI id:
Acropora digitifera	10529	*Orbicella (Montastraea) faveolata*	13173
Acropora florida	23154	*Pocillopora damicornis*	22550
Acropora hyacinthus	15620	*Pocillopora verrucosa*	94504
Acropora millepora	2652	*Stylophora pistillata*	12040
Acropora muricata	23207		
Acropora nasuta	23156		
Acropora tenuis	7192		
Acropora yongei	23189		
Astreopora myriophthalma	33299		
Montipora cactus	7570		
Montipora capitata	82295		
Montipora efflorescens	80926		
Porites rus	69593		

1.2.2 Robusta coral models

When considering which corals to study to further our understanding of cnidarian development it became apparent that the data available for the Robusta clade were inadequate. In developmental studies so far, assessment of Robusta corals has been preliminary even though results showed the process to differ significantly from the complex. What is known of early development of robust corals comes from two main studies using ISH (Okubo et al., 2016; Okubo et al., 2013), expression tags and microscopy which show their early developmental morphology but not the molecular mechanisms behind the changes. One significant difference is that robust species lack a prawn chip stage, instead favouring a pseudoblastopore stage (for extended explanation see Box. 1) (Marlow & Martindale, 2007; Okubo et al., 2016). Also, although planula larvae in robust corals are not as elongated as in the complexa, they have demonstrated the ability to revert between the spherical and elongated-planula stage like in some Acroporids. The reasons, and functional significance, behind the additional stages and changes in robust coral development are not understood.

Box. 1: Developmental morphology of robust corals

Rather than a prawn chip phase, the unipolar blastula goes through two invaginations at the pseudoblastopore and coeloblastula stages before undergoing gastrulation (Marlow & Martindale, 2007; Okubo et al., 2016; Okubo et al., 2013). Although the pseudoblastopore resembles the 'fat donut' stage of complex species (Miller & Ball, 2000) it is vastly different in structure; a single cell layer is present which surrounds a hollow blastocoel filled with mucopolysaccharides – a coeloblastula (Fukui, 1991; Okubo et al., 2013). Invagination is then induced by the movement of lipids and cell particles into the blastocoel and gastrulation follows shortly after this (Okubo et al., 2016; Okubo et al., 2013). Even with the additional stages in robust development, robust species seem to develop more rapidly than complex species, reaching gastrulation at ≤ 23h post-fertilisation (species dependent; *Pocillopora meandrina* 20-23h, *Fungia scutaria* 16-21h and, *Heliofungia actiniformis* 14-20h) whereas *Acropora millepora* begins gastrulation at 12h and, under normal temperature conditions, is completed by 36h (Ball et al., 2002; Hayward et al., 2015). Following gastrulation, the robust gastrula develops cilia which allow the embryos to swim freely (Marlow & Martindale, 2007; Okubo et al., 2013).

Currently, the most extensively studied robust coral is *Stylophora pistillata.* Before whole genome sequencing, an EST library was produced from *Stylophora* to assist in filling the knowledge gap in the robust dataset. The library had a sizable coverage of functional transcripts, highlighting signalling pathways like *BMP* and the *Wnt* pathways (Karako-Lampert et al., 2014). Other gene repertoires identified in *Stylophora* include functions relating to; carbonic anhydrases (Bertucci et al., 2011), skeletal organic matrix proteins (Drake et al., 2013), bicarbonate transporters (Zoccola et al., 2015) and, extracellular signal-regulated kinase (ERK) (Courtial et al., 2017). These pathways have been studied in *Stylophora* as the coral has been used extensively as a climate change response model, particularly in relation to calcification, and how the process may be impeded by environmental change.

The assembly of the genome of *Stylophora* in 2017 (GenBank ID: 415215/281535) facilitated more thorough analysis and an orthologue-focused study was undertaken in comparison to the complex *A. digitifera* genome (Voolstra et al., 2017). Similar to previous *Stylophora* studies, genes relating to symbiosis, innate immunity and stress were analysed in the context of responses to climate change predictions. Also, it was demonstrated that orthologues of several gene families involved in innate immunity were expanded in Scleractinia in comparison *Nematostella* (Aranda et al., 2016; Shinzato et al., 2011; Voolstra et al., 2017). Utilising a broader range of coral genomes and corals as molecular models will give a better idea of how individual species will respond to climate change as well as the coral reefs they support (Voolstra et al., 2015) and currently there is a large gap with respect to robust corals.

Unfortunately, *Stylophora* is of limited use for comparative developmental studies as it is a brooding species. Brooders release fully developed planula larvae, therefore sampling or observing developmental stages would be extremely challenging. Nor is it possible to track parental lineage from brooding species as they may be hermaphroditic or have stored sperm from unknown males in the wild (Brazeau et al., 1998). As *Montastraea faveolata* has an available reference genome and is a broadcast spawning species it could be a potential robust model however, it is exclusively a Caribbean species. Although *Montastraea* has been studied extensively, it is necessary to establish if these findings apply more generally, particularly with respect to non-Caribbean species.

Stylophora has also been used in studies relating to coral feeding and digestion where chymotrypsinogen, an enzyme solely involved in digestion, was identified (Raz-Bahat et al., 2017). However, more is needed to understand the role microbial symbionts may play in coral digestion. Corals are known to be both autotrophic and heterotrophic feeders, but research has focused on autotrophic feeding as this method is the most impacted during bleaching events. When corals bleach, they lose their Symbiodiniaceae and therefore a considerable amount of energy and resources they supply to the host through autotrophy (Goreau et al., 1971). However, a large factor in coral resilience and recovery is the extent to which a particular species can undertake heterotrophic feeding (Grottoli et al., 2006). Heterotrophic compensation has been suggested as a possible explanation as to why some species are more resilient to climate disturbances. Corals are not solely dependent on their Symbiodiniaceae and can acquire energy through heterotrophy, possibly assisted by other microbial symbionts (Cardini et al., 2015; Hughes & Grottoli, 2013; Meunier et al., 2019). Even though the roles of microbes in coral nutrition have only recently been considered due to analogies with the gut microbiomes of mammals (Bourne et al., 2016; Ley et al., 2008), these may be very significant. For example, in higher metazoans a ‘healthy gut microbiome’ is known to have a positive impact on overall wellbeing, although whether similar relationships can be seen in corals is still debatable (Mackay, 2020). Previous studies have demonstrated potential similarities between the coral gastric microbiome and those of the guts of more advanced metazoans (Agostini et al., 2009). However, the small polyps of corals make studying the gastric cavity extremely challenging. Previous studies have attempted sampling from the gastric cavity using complex techniques and probes (Agostini et al., 2012) but with very limited success due to size and morphology.

Size and physiology are considerable limitations on the application of many techniques to all corals that have been extensively studied to date. Unlike Nematostella, Hydra and Aiptasia,

the majority of corals are colonial animals made up of many tiny interconnected polyps. Multicellular organisms have individual tissue compartments, structures and cell types, and corals are no different (Peters, 2015). Molecular processes are likely to be different in each of these various compartments, however, corals studied so far are not amenable to physical separation of tissues. Although *Acropora* has been extensively studied and has well represented the processes behind coral development and complexa evolution, the fact that it is colonial limits investigation of many aspects of coral biology. A potential solution to this could be the use of advancing techniques, for example single-cell sequencing, subcellular imaging and fluorescent ISH (Cooke et al., 2019). These advanced techniques have made it possible to target single cells and regions in a study species. Combining enhanced microscopic resolution and methods could open the door for compartmental studies of corals, but these remain technically challenging in their application (Wada et al., 2016). These limitations would be largely overcome in the case of solitary corals with large individual polyps, such as members of family Fungiidae (mushroom corals).

1.2.3 Heliofungia as a potentially informative organism in coral biology

Heliofungia actiniformis is a large solitary coral (Figure 1.1) that has been recorded to grow up to 33 cm in diameter (Bos & Hoeksema, 2015). The species belongs to the understudied Robusta clade of scleractinians (Kitahara et al., 2014; Okubo et al., 2016). *Heliofungia* is often mistaken for a sea anemone due to its long, permanently extended tentacles that are used for both predation and defence (Bos & Hoeksema, 2015). Its unique appearance has made it popular in the live aquarium trade, especially as it is easy to collect due to it being unattached to the reef and living in the first 15m (Knittweis et al., 2009). *Heliofungia* has exceptional regenerative capabilities, being shown to heal even when split in two (Mondal & Raghunathan, 2017). Additionally, *Heliofungia* has been recorded in turbid waters where it is predominately heterotrophic and, does not depend on its Symbiodiniaceae as heavily as other corals and, it is known to consume salps and small fish (Hoeksema & Waheed, 2012). The fact that this species is so sizable, hardy, has extended tentacles and is unattached makes it easy to collect and work on in a laboratory setting. These characteristics alone make it potentially useful as a means of overcoming many of the limitations that apply to more highly studied corals.

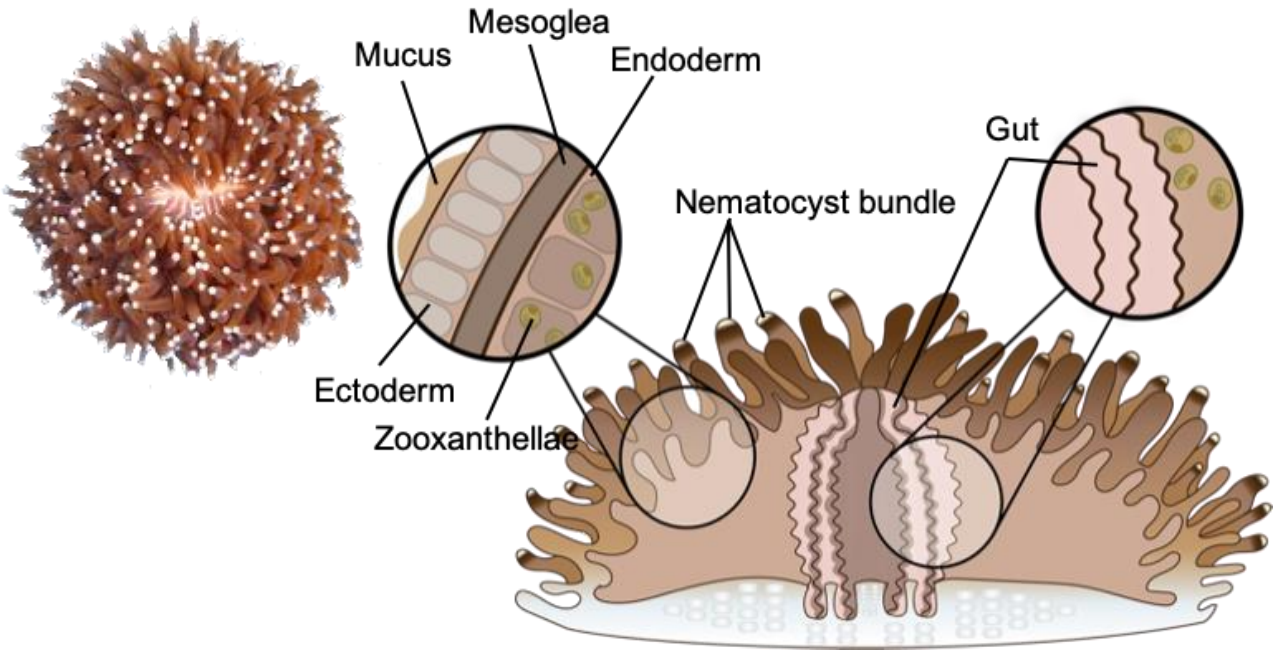


Figure 1.1: an above view of a *Heliofungia actiniformis* individual (photo credit: Moya) and a schematic side view showing the different, accessible regions of *Heliofungia*

1.3 Application of the *Heliofungia* model

1.3.1 Heliofungia as a potential model organism

Heliofungia has been recorded as both brooder (Abe, 1937) and gonochoric spawner (Baird, Guest, et al., 2009). On the Great Barrier Reef, it is a gonochoric spawner with a predictable reproduction window (Willis et al., 1985), which allows controlled crosses to be made, unlike brooding species. Transcriptomic analyses of early development have been undertaken for several members of the Complexa with detailed studies on particular important developmental genes (Hayward et al., 2001; Hayward et al., 2015; Hayward et al., 2004; Hayward et al., 2002; Miller et al., 2000; Shinzato et al., 2008; Yasuoka et al., 2016). However, our understanding of the molecular mechanisms of development in robust corals are limited to two studies by Okubo (2013 & 2016) which focuses on imagery and targeted gene expression of three important developmental genes (*brachyury, chordin* and *forkhead*). *Heliofungia*, as a species that is easy to collect and a spawner, could be used to fill these knowledge gaps.

For research consistency, it would be appropriate to use the same coral for both developmental and adult studies which *Heliofungia* could achieve. As well as studying both the host and microbial changes observed during each developmental stage. Most studies

currently on the coral microbiome sample at set time periods, not when cleavage or cellular changes occur. The point of gastrulation, when the tissue layers are formed, may be of particular interest for microbial acquisition as microbes from the seawater may be brought into close proximity with cells and trapped as the embryo folds in on itself. Recent research has shown that the microbiome undergoes changes during the development of corals, *Hydra* and sponges (Damjanovic, 2019; K. Damjanovic et al., 2019; Franzenburg et al., 2013; Webster & Thomas, 2016), but these papers generally include small numbers of time points and have limited resolution. Microbial acquisition has both vertical and horizontal contributions in cnidarians (Apprill et al., 2012; Damjanovic et al., 2019; Lema et al., 2014; Zhou et al., 2017), and the mechanisms behind acquisition remain inconclusive (Webster & Thomas, 2016). The advantages of *Heliofungia* for developmental studies include that it would allow us to better understand how microbiomes develop over time and how microbes are acquired.

1.3.2 Size and physiology

Corals are diploblasts; they have two tissue layers, an outer ectoderm and inner endoderm separated by a mesoglea. The Symbiodiniaceae are exclusively housed in the coral endoderm, so this tissue is of particular interest in the context of coral bleaching. To date, it has not been possible to investigate tissue-specific gene expression in corals in relation to coral bleaching. However, the size of *Heliofungia* polyps permits physical separation of the ectoderm and endoderm by applying a method originally developed in sea anemones (Richier et al., 2006), thus permitting tissue-specific analyses of the bleaching response and the microbiome.

1.3.3 The use of Heliofungia in microbial studies

Our understanding of the composition of the coral microbiome has significantly expanded over the past decade and has highlighted the potential contributions of microbes to many aspects of coral biology (Bosch & McFall-Ngai, 2011; Bourne et al., 2016). However, only limited spatial information is available about the location of bacteria associated with corals. Tissue fractionation may improve our understanding of the roles of specific bacteria in coral biology (Hernandez-Agreda et al., 2016; Rees et al., 2018) with the different regions containing unique microbiomes. In higher metazoans and plants, specific microbiomes are associated with particular organs and tissues and these relate to their function, processes and health (Thaiss et al., 2016; Turner et al., 2013). Furthermore, analysing each tissue separately may show microbiota that are normally lost in the data of whole polyp extraction, for example,

compartment-specific study of *Nematostella* demonstrated the presence of individual microniche microbiomes with *Spirochaeta* being prevalent in the capitulum, a finding that would normally be lost in whole body analysis (Bonacolta et al., 2021; Bonacolta et al., 2020).

In addition to the various tissue layers, *Heliofungia* may help us understand more about coral heterotrophy. Size and morphology have previously limited access to the gastric microbiome of corals (Agostini et al., 2012), but these limitations do not apply in the case of \ *Heliofungia*. Considering that heterotrophic feeding may provide 15-35% of daily metabolic needs in healthy corals and up to 100% in bleached corals (Grottoli et al., 2006), it is reasonable to assume that specific microbes facilitate digestion (Bourne et al., 2016; Grottoli et al., 2006). Furthermore, that gastric microbes may provide the coral metaorganism with additional nutrients and vitamins during times of stress for example, B_{12} which was found to be present in the gastric cavity of *Galaxea fascicularis* (Agostini et al., 2009; Agostini et al., 2012; Houlbrèque et al., 2004). However, the mechanisms by which the microbiome participates in coral digestion are still relatively unknown (Bourne et al., 2016; Herndl & Velimirov, 1986). Applying *Heliofungia* as a model to this problem may allow a better understanding of the role of the microbiome in coral heterotrophic processes, digestion and energy storage.

1.3.4 How this model and techniques can be applied to coral bleaching

It is not possible to understand the impacts of climate change on the ecology of a species without understanding the basic biology of that species, and understanding tissue-specific molecular characteristics, including the microbiome, and gene expression profiles during and following stress are likely to be fundamental in understanding responses at the individual and community levels (Teplitski & Ritchie, 2009).

In addition to immunity-focused research, further understanding is needed on how this relates to coral bleaching particularly, with respect to the molecular mechanisms that may assist with symbiont removal. During bleaching, over-production of reactive oxygen species (ROS) is one of the leading factors causing dysbiosis between the coral and their Symbiodiniaceae (Bosch & Miller, 2016; Brown et al., 1995; Smith et al., 2005; Tchernov et al., 2004). Following bleaching, whether the coral recovers and re-establishes its symbiosis after bleaching is dependent on the mechanisms protecting it (Grottoli et al., 2006; Thomas & Palumbi, 2017). However, whether dysbiosis is host or symbiont mediated, is still undetermined. Research has shown bleaching to result in differential gene expression associated with survival mechanisms such as DNA repair, cytosis, immune response and anti-apoptosis (DeSalvo et al., 2010; Moya et al., 2016; Seneca & Palumbi, 2015). In corals, apoptosis has been associated to cell death

during bleaching stress (Pernice et al., 2011), for example, a caspase-mediated apoptotic cascade initiated by ROS, and therefore, research in to the mechanisms that inhibit this response are critical for coral survival (Moya et al., 2016; Tchernov et al., 2011). However, the link between apoptosis and bleaching is still tentative and the tissue-specific mechanisms of these response pathways remains unclear (Quistad et al., 2014; Seneca & Palumbi, 2015). Linking the apoptotic repertoire with its tissue gene expression may explain if it has a role in bleaching, particularly in regard to the endoderm. Overall, much can be learned from the molecular response of the endoderm pre- and post-bleaching, which can now be achieved by physically separating the tissue regions in *Heliofungia*. Furthermore, assessing the endoderm and gut microbiome during bleaching may yield a better understanding of how microbial shifts may exacerbate the bleaching response (Rosenberg & Falkovitz, 2004; Rosenberg et al., 2009), as these regions are most likely to show the most prominent changes. With this model, these findings may give coral a much brighter and healthier future.

1.4 Conclusion

Heliofungia can be applied to numerous areas of research; from understanding the changes that cause it to go from a gamete to settling larvae to tissue-specific responses in adult corals. The large size and physiology of *Heliofungia actiniformis* polyps make it an excellent candidate to investigate tissue level mechanisms that have not been possible to study previously. Furthermore, it has physiological characteristics that can be utilised for example, its large singular mouth can be easily accessed for coral digestion research and its nematocyst bundles for biotechnology. The species is physiologically hardy, making it useful for repetitive sampling and climate change experimentation. As well as it already having an annotated transcriptome and whole genome near completion which can facilitate in depth molecular analysis. *Heliofungia* could be used to fill the knowledge gap present in the Robusta clade whilst adding novel findings with respect to localised expression data. Overall, *Heliofungia* is a promising species as a novel coral model and, in this thesis, will be used to explore; i) if there is a tissue-specific microbiome, ii) how are the tissue microbiomes affected by bleaching and, iii) if there is a tissue-specific host response with respect to bleaching.

Chapter 2: Characterisation of the tissue-specific microbiomes of *Heliofungia*

2.1 Introduction

Microbial communities play important roles in the health and disease status of most organisms (Coyte et al., 2015; Lee & Mazmanian, 2010). They are a principal part of the coral holobiont - the term used to describe the coral animal, its photosymbionts, viruses, fungi, bacteria, archaea and other symbionts (Bourne et al., 2009; Rohwer et al., 2002). Previous coral studies have linked the microbial communities to numerous important biological roles, including immunity, stress resilience, disease, feeding and digestion (Bourne et al., 2016; van Oppen & Blackall, 2019). Due to the increasing ease of high-throughput sequencing on atypical study species, there has been a significant rise in data and analyses surrounding the coral holobiont (Cooke et al., 2019). However, despite this increase in research, there are still various knowledge gaps that surround the coral microbiome.

One of the significant problems with studying coral microbiomes is the lack of localised tissue level data. Tissue-specific analysis would facilitate a greater understanding of the functions of the microbes, particularly as metagenomic approaches are still in their infancy due to the lack of coral-specific microbial genomes (Cooke et al., 2019; Huggett & Apprill, 2019). While tissue-specific microbiomes have not previously been investigated in corals, core microbiomes and micro-niche habitats have been (Ainsworth et al., 2015; Hernandez-Agreda et al., 2016; Pollock et al., 2018). Ainsworth et al. (2015) used 16S rRNA sequencing and FISH to show that the microbiome of the holobiont could be split into three groupings: the whole coral community, the endosymbiotic community and the symbiotic community. Ainsworth and colleagues showed that these communities had set core members, even across great spatial distances (Ainsworth et al., 2015). Furthermore, the meta-analysis by Hernandez-Agreda (2017) implied that the coral is likely to have a different microbiome between the external mucus, soft tissue and hard skeleton, which would be impacted by different environmental variables. Evidence for tissue-specific microbiomes can also be found in other non-coral cnidarians. For example, *Hydra has* been used to show the presence of several microbial niches coexisting together within the organism (Deines & Bosch, 2016; Deines et al., 2020). One could theorise that these micro-niches could easily be separated between tissues. In addition to this, the analysis of separate body compartments of *Nematostella* has shown

regionally specific microbiomes with the capitulum (the body structure just beneath the tentacles) consisting of 66% Spirochaetes. These results would normally have been lost in the sequencing data as the physa (its basal borrowing organ) and mesenteries contain less than 1.3% Spirochaetes (Bonacolta et al., 2020). These findings highlight the usefulness of studying microbiomes at a tissue level. Using 16S metabarcoding, regional microbiomes have also been shown in other structurally simplistic marine animals, including the giant clam (*Tridacna maxima*) and Pacific oyster (*Crassostrea gigas*), which was demonstrated by separating the gills, guts and mantle tissues (Lokmer et al., 2016; Rossbach et al., 2019). While a simplistic animal like coral may not contain regional and tissue-specific microbiomes of similar complexity to higher metazoans (Arumugam et al., 2011), the preliminary evidence in other cnidarians and early coral studies show that a basic level of tissue-specific differentiation is possible, especially when considering the coral anatomy. Corals are diploblasts; they have two germ layers - the ectoderm and endoderm (also known as the gastrodermis). These are separated by a mesoglea and each polyp has a mouth connected to a gastrovascular cavity (Miller & Ball, 2000). To date, it has been unfeasible to examine the tissue-specificity of coral microbiomes due to their small size and colony lifestyle. However, the development of *Heliofungia actiniformis* (from now on referred to as *Heliofungia*) as a new coral model has eliminated these prerequisites.

Unlike most colonial coral species, Fungiidae, which *Heliofungia* belongs to, are large, individual species made up of a singular polyp. *Heliofungia* in particular has sizable tentacles that are always extended and from which we can dissect the two tissue layers in order to observe ecto- and endodermal microbiomes, in that particular case. It is likely that ectoderm and endoderm contain different microbiomes due to functional roles. For example, only the endoderm contains Symbiodiniaceae, the photosymbiotic constituent of the holobiont. This may likely impact the microbial community as cultured Symbiodiniaceae have demonstrated their own unique microbiome (Lawson et al., 2018; Lawson et al., 2020). Identification of the core microbiome of several different Symbiodiniaceae clades using 16S rRNA metabarcoding identified three core genera associated with all clades (present in 100% of samples) and several other abundant associated genera (for example Alteromonas) were identified (Lawson et al., 2018). In addition to this, symbiotic and aposymbiotic (symbiont free) *Aiptasia* have been used to demonstrate that their association with Symbiodiniaceae, or lack thereof, significantly altered their microbiome (Röthig et al., 2016). The findings of Röthig et al. (2016) also indicated that the microbiome may play an important role in the successful establishment of symbiosis between cnidaria and algae, which has been hypothesised previously (Webster, 2014). Overall, analysing the tissue-specific microbiome should clarify the extent to which the ecto- and endoderm function as distinct microbial niches.

Not only does the large size of *Heliofungia* allow access to the tissue layers, but it also means easier access to the gastrovascular cavity, and gut, as the species has one singular mouth/anus. Previous studies have highlighted the likelihood of a digestive microbiome similar to what is seen in higher metazoans. However, research into this area has been cumbersome due to the physiology of the study species (Ghanbari et al., 2015; Shreiner et al., 2015). Digestion and metabolism are an interesting topic to study in corals as they are mixotrophic organisms; they can acquire nutrients from both feeding directly on microorganisms in the water column and photosynthetically fixed carbon from their Symbiodiniaceae (Grottoli et al., 2006; Porter, 1976). This mixotrophic capability may influence digestion, as each species has a varying level of dependency on auto- or heterotrophy; therefore, individual corals may require different gut microbiomes. *Heliofungia* provides a unique opportunity to study the digestive microbiome as it not only has a large mouth but is also known for being more heterotrophic and has even been reported to eat salps and small fish (Hoeksema & Waheed, 2012; Sisson, 1973). Earlier studies have already suggested the presence of a gut microbiome in corals. Fluid was sampled from the gastric cavity of *Galaxea fascicularis,* and radio assays were used to determine the concentrations of B_{12} (Agostini et al., 2008; Agostini et al., 2012). Agostini et al. hypothesised that B_{12} came from the gastric microbiome as bacteria have shown to be a key part of B_{12} biosynthesis (Agostini et al., 2012; Albert et al., 1980; Degnan et al., 2014). In addition to this, histology and isolation-cloning processes have been used in *Stylophora pistillata* to identify a coral chymotrypsinogen gene, an inactive precursor of the digestive enzyme chymotrypsin (Raz-Bahat et al., 2017), which has been shown to also have microbial origins. Previously, chymotrypsinogen has been isolated from the archeon Halobacteria and activated by the bacteria Streptomyces (Awad Jr & Wilcox, 1963; Stan-Lotter et al., 1999) which have both been identified in corals previously (Fu et al., 2013; Wegley et al., 2004). Additionally, a study exposing the cold-water corals *Lophelia pertusa* and *Madrepora oculata* to various diets (carnivorous, herbivorous and a mix) showed that this altered the microbiome (Galand et al., 2020). Overall, being able to study the coral gut microbiome with ease will expand our understanding of the microbes involved in digestion, and may explain the observed variance in community composition across species.

Endozoicomonas is an extremely abundant bacterial genus found in symbiotic partnership with many marine organisms: cnidarians, echinoderms, fish, molluscs, sea squirts and porifera (Gao et al., 2020; Howells et al., 2021; Høj et al., 2018; Neave et al., 2017; Schreiber et al., 2016). The genus is highly diverse, which makes it challenging to study when there are numerous potential species established in coral reefs (Shiu et al., 2018). *Endozoicomonas* is shown to dominate the coral microbiome; however, its role is still inconclusive as it has been

linked to numerous biological processes, for example; nutrient acquisition, transport and recycling and host health and disease (Bayer et al., 2013; Bayer et al., 2013; Hernandez-Agreda et al., 2016; Neave et al., 2016). Recent genome studies of *Endozoicomonas* species are rapidly improving our understanding of their functional role in the coral holobiont (Ding et al., 2016; Neave et al., 2014; Tandon et al., 2018). A species isolated from the stony coral *Montipora aequituberculata*, now classified as *Endozoicomonas montiporae*, exhibited gene repertoires present for gene transfer with the host, for entering the host symbiotically through endocytosis, and for stress mitigation for the host (Ding et al., 2016). Furthermore, a species isolated and cultured from an *Acropora* sp., *Endozoicomonas acroporae* (Tandon et al., 2018), has shown a likely role in the coral sulphur cycle due to its ability to use dimethylsulphoniopropionate as a carbon source and metabolise into dimethylsulphide (Tandon et al., 2020). These findings may explain the prominence of *Endozoicomonas* in marine organisms (Tandon et al., 2018; Tandon et al., 2020). As there are so many unclassified species of *Endozoicomonas*, another way to help understand its functional role could be through phylogenetics and tissue localisation; observing whether different species have a preference to certain coral tissues and microniches may help elucidate function. For example, the analysis of several Ascidian species showed the presence of a pharynx-specific *Endozoicomonas* species which feeds off the mucin found in this region and metabolises it into by-products that the ascidian host can use (Schreiber et al., 2016). In addition to this, the giant clam study mentioned previously (Rossbach et al., 2019) showed that the abundance and composition of Endozoicomonadaceae OTUs differed between regions, with the gill showing the greatest variation. From these results, it may be probable that corals have a variation in regional *Endozoicomonas* abundance. Using the large individual species *Heliofungia* could be the key to elucidating this knowledge gap.

This study has three main aims. First, to investigate whether specific microniches exist in *Heliofungia* that display different microbial communities. Second, if it is possible to study the gut microbiome in this larger species and observe the genera present. Third, whether *Endozoicomonas* exhibits spatial preference or is dominant throughout all regions of the host. From this research, a better understanding of the coral microbiome will be achieved. Moreover, for the first time, tissue-specific microbial data will be produced in corals.

2.2 Methodology

2.2.1 Collection and maintenance of Heliofungia actiniformis specimens

Sampling was conducted in November 2016, August 2017, November 2017, April 2018 and August 2018. At each time point, five individuals of the scleractinian coral *Heliofungia actiniformis* (>15 cm diameter) were collected by scuba diving from the reef adjacent to Orpheus Island on the Central Great Barrier Reef (18.60°S, 146.49°E). The corals were transported in separate Nally bins filled with seawater to the aquarium facilities at Orpheus Island Research Station. The specimens were maintained in outdoor flow-through aquaria filled with 0.2-µm-filtered seawater and under natural light irradiance for a minimum of 24 hours prior to sampling. Coral collection and experimentation were carried out under GBRMPA Permit No. G11/34573.1 and G17/39908.1.

2.2.2 Tissue collection

Five tentacles were dissected per individual, at each of the five time points. Cut tentacles were placed in a sterile petri dish and rinsed with 0.2 µM filtered seawater to remove outside contaminants and dabbed dry with sterile wipes. The tentacle was cut vertically along the axis with fine scissors. Following the incision, the tentacle could be opened up flat and the endoderm (n = 5 per individual) scraped from the ectoderm (n = 5 per individual) with the smooth, curved edge of the tweezers. Tools were exposed to UV for one hour and autoclaved prior to dissections. Between individual dissections, the tools were soaked and washed in 100% ethanol and then rinsed with filtered seawater. External mucus (n = 5 per individual) was collected by running sterilised cotton swabs over the outside of the whole coral. Four buccal swabs were taken of the gastrovascular cavity in the morning (10:30) and four in the afternoon (16:30) of each of the five individuals to sample the gut microbiome, gut samples were taken at all time points except November spawning. Five environmental samples were taken from the seawater in the raceway, seawater at sample site and filtered seawater used for rinsing and were collected by filtering one-litre of water through a Sterivex-GP 0.2 µM barrel filter using a syringe (new syringe per sample replicate) (Bourne & Munn, 2005). In addition to this, the acrosphere was only sampled three out of five time points as the nematocyst bundle had not been discovered before the commencement of this thesis. During dissections, it was observed that the region was expelled from the tip of the tentacle when agitated and could be pulled off with fine sterilised tweezers and sampled separately. A survey of the coral literature revealed two papers that noted this acrosphere (Bos & Hoeksema, 2015; Hoeksema, 2014).

All samples were immediately snap-frozen in liquid nitrogen and transported to James Cook University for analysis.

2.2.3 Extraction of DNA

To reduce contamination, all equipment and consumables were wiped with ethanol, autoclaved, and exposed to a minimum of one hour of UV radiation prior to DNA extraction. Furthermore, DNA was extracted in a laminar flow cabinet using the 'Isolation of Genomic DNA from Tissues' protocol from the QIAamp Micro Kit (Qiagen). The DNA yield was assessed using spectrophotometry (NanoDrop 1000, ThermoFisher Scientific). The extracted DNA was aliquoted and diluted to a concentration of 100 ng/µl using sterilised UltraPure DNase/RNase-Free Distilled Water (ThermoFisher Scientific). The diluted samples were stored at -20^{o}C until library preparation and the remaining sample stock at -80^{o}C.

2.2.4 Library preparation and sequencing

The 803/1392 primer pair was used for polymerase chain reaction (PCR) amplification (forward: 5'- TTAGAKACCCBNGTAGTC-3 (made from a mix of four 803Fs: 2a:b:c:d); reverse: 5- ACGGGCGGTGWGTRC-3) (Table S2.1) which covers both bacterial and archaeal phylotypes (Robbins et al., 2021; Robbins et al., 2019). The Illumina protocol for '16S Metagenomic Sequencing Library Preparation' (Illumina, 2013) was followed. However, instead of 'KAPA HiFi HotStart ReadyMix', 'Platinum SuperFi DNA Polymerase' (ThermoFisher Scientific) was used. The SuperFi reagent quantities were changed for the first 'Amplicon PCR' in the Illumina protocol as follow 0.5 µl of each primer (10 µM), 12.5 µl of Platinum SuperFi, 9 µl of ddH_2O and 2.5 µl of template in a total reaction volume of 25 µl. The PCR products were cleaned using Sera-Mag Speedbeads (ThermoFisher Scientific) on the Zephyr G3 NGS Workstation (PerkinElmer) (Infante-Villamil et al., 2019; Villamil et al., 2018) and gel electrophoresis was used to check amplicon quality after cleaning. The amplicons were indexed using the Illumina indexing adapters (Nextera XT Indices Kits A, B and C, Illumina), resulting in a final library size of 736 bp. The indexed libraries were cleaned again using Sera-Mag Speedbeads. The final library fragment size was confirmed post-clean using the EnSpire Alpha Plate Reader (PerkinElmer) and QuantiFluor dsDNA system kit (Promega). The libraries were normalised to 0.5 nmol/L and pooled by combining 5 µl of each individually indexed library. The DNA concentration of the pooled libraries were quantified using the Qubit (dsDNA HS Assay Kit, Life Technologies). Library size and concentration were again checked using the Tapestation System (2200, Agilent). The final library concentration was 4nM and

was sequenced following the 'MiSeq System User Guide' protocol (Illumina, 2014), using the MiSeq benchtop sequencer with a MiSeq Reagent Kit V3, 600 cycles (Illumina) across four runs; each run contained all regions from certain time points (run 1 = November 2016, run 2 = April and August 2017, run 3 = April 2018 and, run 4 = November 2017 and August 2018) as well as samples from different projects to increase heterogeneity. These methodologies were selected due to their previous success and viability (Infante-Villamil et al., 2019; Villamil et al., 2018).

2.2.5 Fluorescent in situ hybridization

Samples were collected for Fluorescent *In Situ* Hybridization (FISH) to visually validate the presence of the microbes in the ecto- and endoderm recorded in the sequencing data. Tentacles were collected and fixed in 4% paraformaldehyde before being sent for histology work at JCU by Lit Chien Cheah where the samples were set in paraffin wax and cut to 6µM thickness. Half the slides were stained with hematoxylin and eosin for light microscopy and the other half with no staining for FISH. Non-stained slides were placed in 100% xylene for two periods of three minutes and then placed in an ethanol dehydration series for five minutes each (50%, 80% and 100%). Following air-drying, barriers were drawn around the slides with a PAP pen. Two ml of Hybridization buffer (360µl 5M NaCl, 40µl 1 M Tris-HCl (pH 8.0), 600µl 100% formamide and 1ml of autoclaved milli-Q water) was made per slide and gentley pipetted onto the slide, 2µl of the probe was added to this then slides were incubated in the dark in moist chambers for two hours at 46°C. The probes used were: a negative, non-bacterial (NONEUB), general bacterial (EUB338iii) (Ainsworth et al., 2006; Wada et al., 2016) and an Endozoicomonas-specific one (ENDOZ) (Bayer et al., 2013; Damjanovic et al., 2019), for breakdown of probes used and dyes see Table S2. After incubation, slides were kept in the dark and placed in falcon tubes containing preheated wash buffer (1020µl 5 M NaCl, 1ml 1 M Tris-HCl (pH 8.0), 50µl 10% SDS, up to 50ml with autoclaved milli-Q water) at 48°C for 12 minutes. Soak in ice-cold autoclaved milli-Q water for 10 seconds. Air dry with pressurised air and seal with a coverslip. Once the slide had been set, confocal laser-scanning microscopy was performed to acquire images of the bacteria *in situ* (Ainsworth et al., 2006; K. Damjanovic et al., 2019; Wada et al., 2016).

2.2.6 Statistical analysis

The large PCR product size (736bp) meant that minimal overlap between 300bp paired-end reads was expected from this experiment therefore, only the forward reads were

used for analysis. Quality control and mapping were achieved using QIIME 2 (v 2019.7, (Bolyen et al., 2018)). Demultiplexing and denoising were achieved using the dada2 plugin in QIIME2 (Callahan et al., 2016). The data tables produced from this were downloaded and the package microDecon (v 1.0.2, (McKnight et al., 2019)) was used to clean any contamination from the data. The decontaminated tables were then put back into QIIME2 and the feature-table function was used to merge the runs into one data table. The QIIME2 plugins; mafft (Katoh et al., 2002) and fasttree (Price et al., 2010) were used for sequence alignment and phylogeny, samples with read depths lower than 2500 were removed. Initial analyses were undertaken using the QIIME2 diversity features; 'alpha-rarefaction' and 'core-metrics-phylogenetic' which created alpha and beta diversity results (weighted and unweighted Unifrac, Jaccard distances, Bray-Curtis dissimilarity (Faith, 1992; Lozupone & Knight, 2005; Lozupone et al., 2007)). It also performs dimension reduction using Principal Coordinate Analysis (PCoA). Taxonomy was assigned to amplicon sequence variants (ASVs) *de novo* using the QIIME2 feature-classifier (Bokulich et al., 2018) and Silva taxonomy database, the classifier was trained using the QIIME2 tutorial (https://docs.qiime2.org/2019.10/tutorials/feature-classifier/).

Analysis and visualisation of the ASV output tables were carried out using R. The package qiime2r (v 0.99.6,(Bisanz, 2018)) was used to bring the QIIME2 artifacts into R and apply the metadata and shannon vector to the data. Tidyverse (v 1.3.1, (Wickham et al., 2019)), qiime2r (v 0.99.6,(Bisanz, 2018)), radiant.data (v 1.3.12, (Nijs, 2020)), ggplot2 (v 3.3.5, (Wickham, 2016)) and ggsci (v 2.9, (Xiao, 2018)) were used with the shannon data and jaccard PCoA results from QIIME2 to plot α-diversity box plots and PCoAs with normal data ellipses (student-t distribution multivariate confidence level = 90) (Ahlmann-Eltze & Patil, 2021) to test the significance of differences between regions in community composition. Statistics were run on the α- and β-diversity using the packages; agricolae (v 1.3-5, (de Mendiburu & de Mendiburu, 2019)), permute (v 0.9-5, (Simpson et al., 2019)) and vegan (v 2.5-7, (Oksanen et al., 2007)). Phyloseq (v 1.36.0, (McMurdie & Holmes, 2013)), microbiome (v 1.14.0, (Lahti & Shetty, 2018; Shetty & Lahti, 2019)), hilldiv (v 1.5.1, (Alberdi & Gilbert, 2019)), RColorBrewer (v 1.1-2, (Neuwirth & Neuwirth, 2011)), ggpubr (v 0.4.0, (Kassambara & Kassambara, 2020)), ggplot2 (v 3.3.5, (Wickham, 2016)) and dplyr (v 1.0.7, (Wickham et al., 2021)) were used to calculate and plot relative abundance of samples. For the analysis of the gut, ggplot2 (v 3.3.5, (Wickham, 2016)) was used to create bubble plots and METAGENassist ((Arndt et al., 2012), http://www.metagenassist.ca/METAGENassist/faces/Docs/Tutorial.jsp) was used to create oxygen requirements pie chart and metabolic function bar graphs and log fold differences between morning and afternoon sampling points.

2.2.7 Phylogeny

Qiime2r (v 0.99.6,(Bisanz, 2018)) and phyloseq (v 1.36.0, (McMurdie & Holmes, 2013)) were used to create a phyloseq object and tidyverse (v 1.3.1, (Wickham et al., 2019)) was used to filter for the family Endozoicomonadaceae. Ggtree (v 3.13, (Yu, 2020; Yu et al., 2017)), ape (v 5.5, (Paradis et al., 2004; Paradis & Schliep, 2019)), ggstance (v 0.3.5, (Henry et al., 2020)) and ggridges (v 0.5.3, (Wilke, 2018)) were used to create a phylogenetic tree with read numbers per region alongside each ASV branch (ASVs with less than 1500 reads were pruned). The most abundant *Endozoicomonas* ASVs (n = 14) had their sequences imported into Geneious Prime (v 2021.1.1, https://www.geneious.com); as well as six *Endozoicomonas* strains isolated from *Acropora* (NCBI ascension number: NZ_SAUT01000292.1, NZ_SAUT01000296.1, NZ_SAUT01000298.1, NZ_SAUT01000299.1, NZ_SAUT01000297.1 and NR_169415.1 (W.-M. Chen et al., 2019; Tandon et al., 2020)), two isolated from soft corals (*Eunicea fusca*, JX488684.2 and *Plexaura* sp., JX488685.1 (Pike et al., 2013)), three strains from *Montipora aequituberculata* (KJ372462.1, KJ372475.1 and KJ372457.1 (Rua et al., 2014; Yang et al., 2010)), four strains of *Parendozoicomonas* isolated from the sponge genus *Haliclona* (NZ_FWPT01000026.1, NZ_FWPT01000034.1, NZ_FWPT01000035.1 and NZ_FWPT01000038.1 (Bartz et al., 2018)) and three strains from *Hahella* spp. (*Hahella cheujensis*, NR_114540.1 (Lee et al., 2001) and *Hahella ganghwensis*, NZ_AQXX01000001.1 and NZ_AQXX01000004.1). *Hahella ganghwensis* was selected as the outgroup as suggested by the literature (Shiu et al., 2018; Tandon et al., 2020). Multiple trees were run in Geneious, following the user manual (https://assets.geneious.com/documentation/geneious/GeneiousPrimeManual.pdf) the final tree settings were selected as; alignment type - global alignment with free end gaps, cost matrix - 70% similarity (IUB) (5.0/4.5), genetic distance model - Tamura-Nai and tree build method - neighbour joining.

2.3 Results

2.3.1 Alpha diversity of the tissue regions

The overall diversity, measured by Shannon Index, across all sampling time points except the bleaching time point (see Chapter 3) showed significant differences between the individual tissues (Figure 2.1). The mucus and the gut showed similar diversity to each other, which was significantly higher than the acrosphere, endoderm, and ectoderm (Figure 2.1). The ectoderm showed the lowest Shannon diversity. A linear model was fitted to the Shannon diversity including all the independent factors; region, time point and individual as fixed effects.

Using a Tukey's honestly significant difference (HSD) test with a threshold of p <0.05 on the factor 'region' showed there were three groups with significantly different α-diversity (group a; the ectoderm), (group b; endoderm and acrosphere) and group c (mucus and gut).

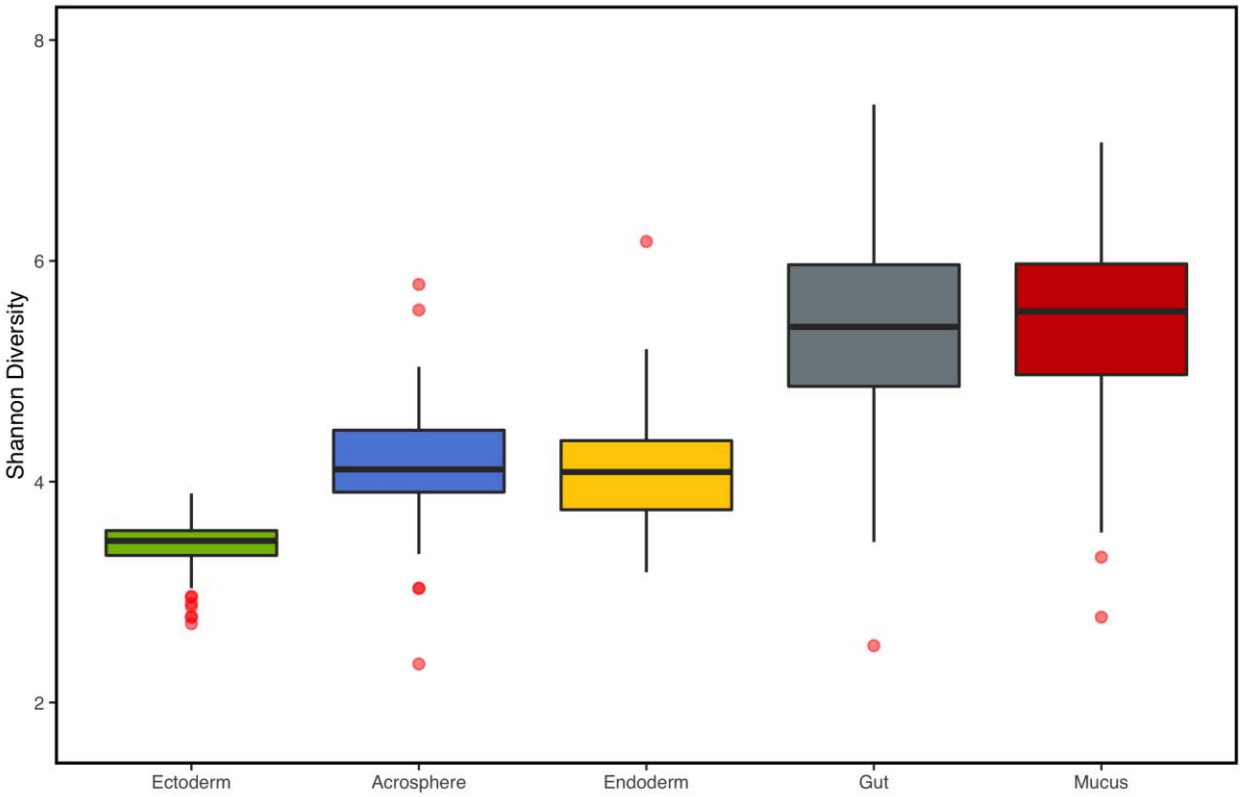


Figure 2.1. Shannon diversity was calculated from the ASVs present in the five tissue regions from *Heliofungia actiniformis*. Each boxplot is represented by 125 points comprising five technical replicates taken from each tissue from five individual corals at each of five time points.

2.3.2 Beta diversity of the tissue regions

Principal coordinates analysis PCoA based on Jaccard distances showed that samples clustered according to tissue region across all timepoints. The appearance of visibly distinct clusters on the PCoA suggest distinct microbial communities in the different tissues. However, one exception was the gut and mucus which showed some overlap between clusters in all three of the time points at which gut samples were taken (Figure 2.2). The acrosphere was more similar to the endoderm than the ectoderm in the community, which is consistent with the idea that the acrosphere would be more similar to the ectoderm as the outer tissue is present in the region. An adonis test on the Jaccard distance matrix data showed that, in order of significance, the factors region, time point and coral all had p values > 0.01.

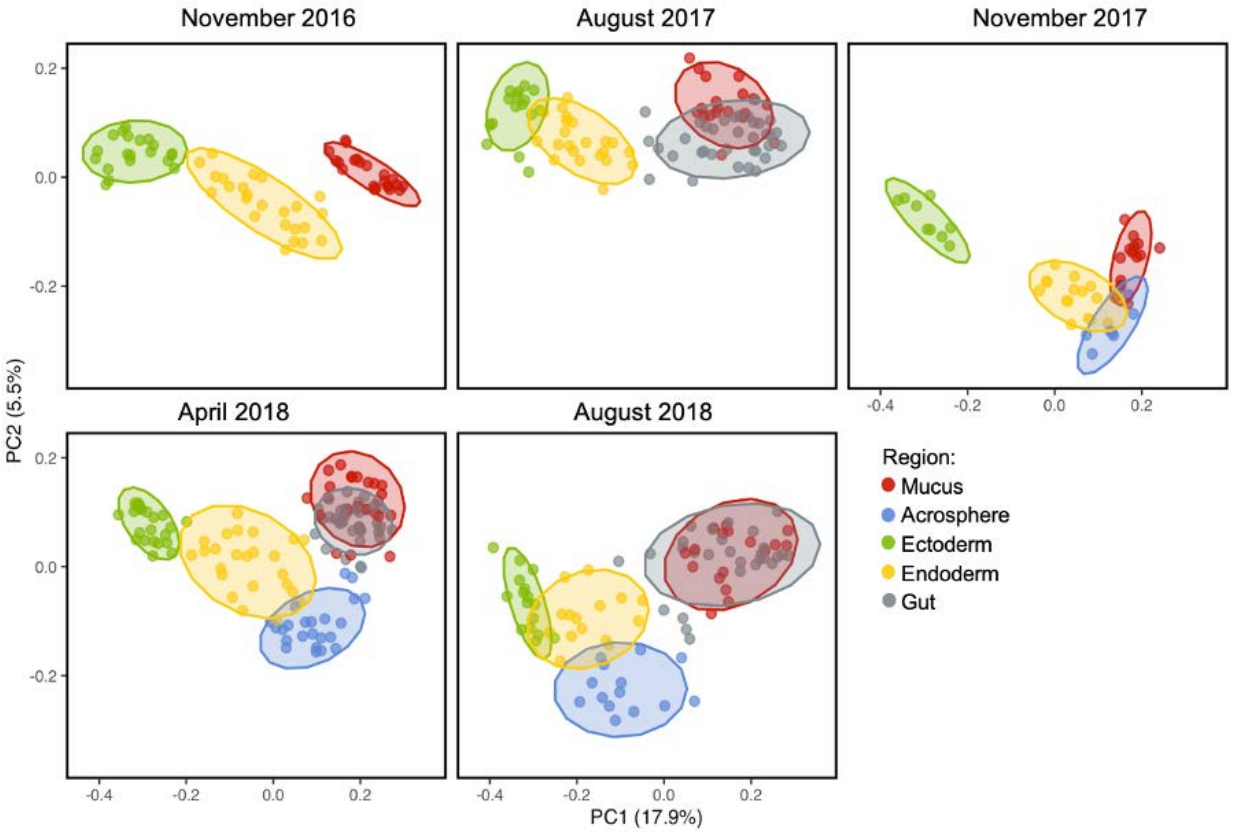


Figure 2.2. PCoAs of the tissue regions microbial β-diversity across five time points (November 2016 - August 2018), based on ASVs. Ellipses include samples that share a 90% confidence level with one another, and colour dictates the region represented. The facets contain 25 points per tissue which represent the five technical replicates taken from the five corals.

To ascertain whether the community in each region remained similar across the timepoints, PCoAs were also calculated on the basis of Jaccard distances between samples from a single time point but across all regions (Figure 2.3). The acrosphere and mucus showed the most variation in the community across the timepoints, as well as the endoderm showing some variation in November 2017 and August 2018. Whereas the ectoderm and gut seemed more stable in comparison to the other regions over time.

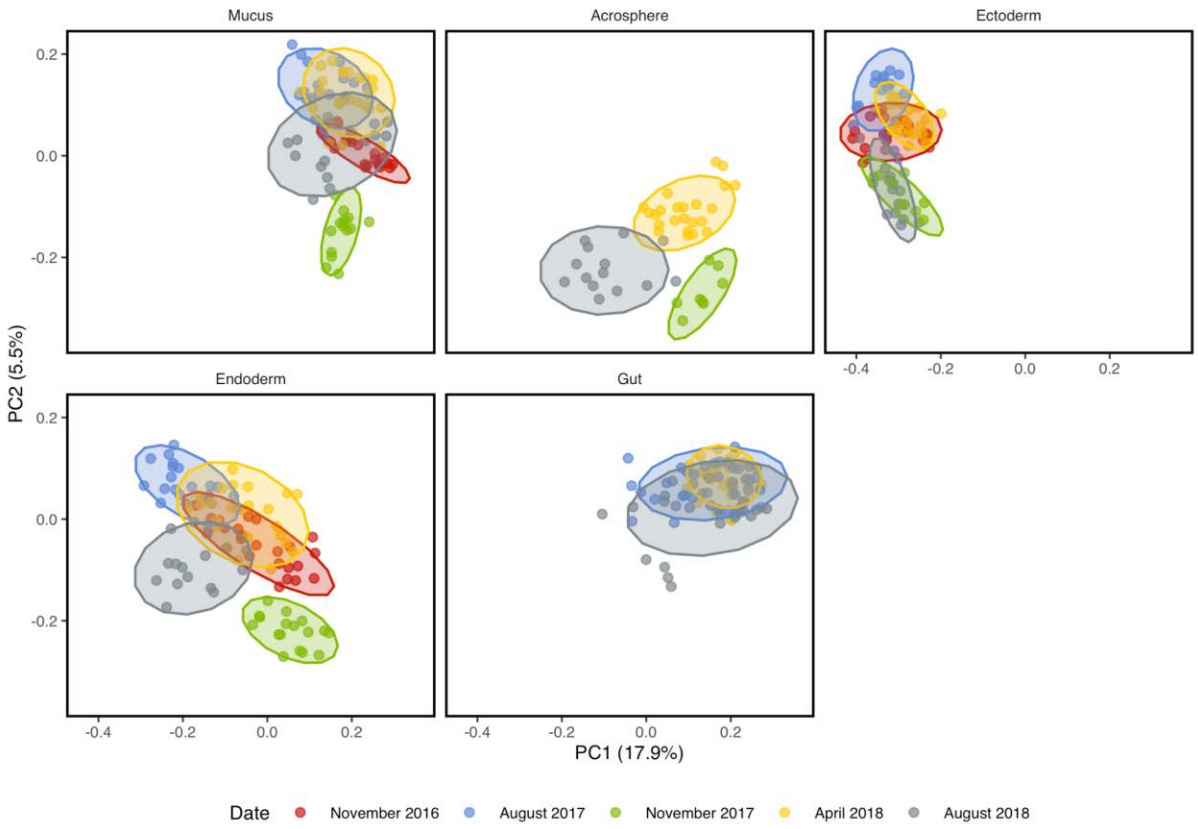


Figure 2.3. PCoAs of each tissue microbial β-diversity based on the ASVs present, separated by the sampling timepoints (November 2016 - August 2018). The more separated the clusters are, the larger the difference in community composition within the region over time, colour denotes the time point and is explained in the bottom legend. Ellipses include samples that share a 90% confidence level with one another, and colour dictates the region represented. Each facet plot shows 25 points per region comprising the five technical replicates taken per the five individuals from each of the time points.

2.3.3 Validation of bacterial presence through microscopy

FISH was used to confirm the localization of bacteria in the tissue regions, as determined by the sequencing data. Presence of bacterial aggregates in the tissue implies that it is possible for the coral to harbour separate microbial communities within its regions. Probes were designed to target both general bacteria (EUB338iii) and non-bacterial structures that would cause autofluorescence in *Heliofungia* (NONEUB).

First, histology was used to find the regions and identify points of interest in the tentacles (Figure 2.4a). Then the NONEUB image (Figure 2.4b) was used to demonstrate whether staining was successful and which cells in *Heliofungia* were naturally autofluorescent, which included the Symbiodiniaceae (zooxanthellae) and nematocysts. The large size of *Heliofungia* tentacles allows for visualisation of clearly defined tissue regions and better resolution than previously achieved in corals. Figure 2.4a (H & E staining) shows the ectoderm (Ec) as the

outer layer, filled with small dark purple cells. The structure of the outside of the ectoderm looks similar to microvilli in the way it folds in on itself. The ectoderm and endoderm are clearly demarcated by the presence of the mesoglea (Mg) which appears to have tissue tendrils seeping into it from either tissue. The endoderm (En) is the inner layer that contains numerous zooxanthellae cells (Zx, pink spherical cells with a purple disc within). Those zooxanthellae cells are clustering towards the ectoderm. In Figure 2.4b (FISH with NONEUB probe), the same physiological structuring is seen; however, the fluffy, pink border surrounding the microvilli and granular cells in the ectoderm (Ec) are no longer obvious. The mesoglea (Mg) that still clearly separates the two regions and the endoderm (En), is packed with autofluorescent zooxanthellae (Zx).

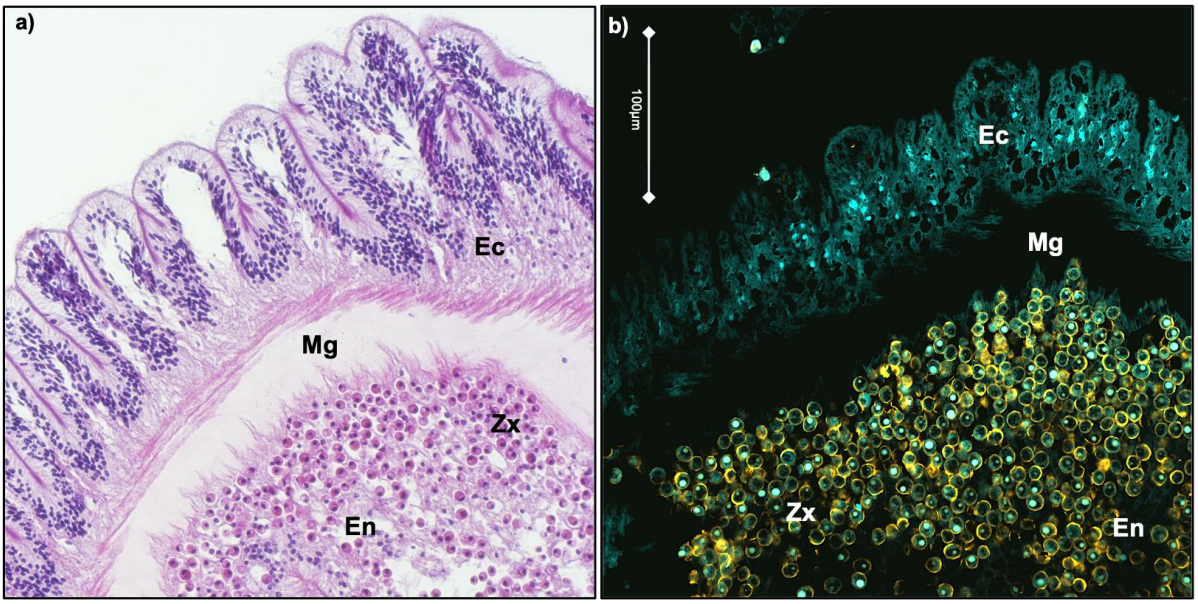


Figure 2.4: a) H & E histological slide at 20x magnification clearly showing the ectoderm (Ec), mesoglea (Mg), endoderm (En) and zooxanthellae (Zx) and, b) is an enlarged (40x magnification) area of the same tentacle from a) showing the same tissue layers. Anything fluorescing yellow represents cells that are autofluorescent in corals - here being the photopigments present in the zooxanthellae.

During tissue separation, it was observed that the nematocysts seemed to be concentrated to the white tip of the tentacle and a new method was developed to extract this region for sequencing. Only two studies (Bos & Hoeksema, 2015; Hoeksema, 2014) had previously mentioned this in *Heliofungia*. Histology was used to validate this hypothesis and indeed demonstrated that the nematocysts are all aggregated at the tip of the tentacle. Figure 2.5 (a-c) shows the tip of a tentacle where numerous nematocysts were present in the ectoderm (Ec) and a mucus-like protrusion also full of stinging cells. During dissection, agitating the white tip of the tentacle with sterilised tweezers causes a gelatinous substance to be excreted from the acrosphere. When removed from the seawater, this substance resembles snowflake structures that could then be pulled from the tentacle to be sampled. In 2.5a) the acrosphere

(Ac) had just expelled upon fixation showing a mucus-like patch attached to the tip of the tentacle. Figure 2.5b shows a magnification of this, highlighting the high number of nematocysts in that region. 2.5c) is at 100x magnification and shows a number of the stinging cells (Ne) clustered close to the outside edge of the ectoderm, discernible by their characteristic coiled appearance and pink colouration. There are many granular cells (Gr) at the ectoderm edge, these are a dark pink/purple colour and are sometimes mistaken for small bacterial aggregates, but on closer inspection under the microscope, one can see multiple tightly packed spherical cells with clear borders packed together, whereas bacterial aggregates look like a cluster of fluffy cotton wool rather than defined little circles under light microscopy. The NONEUB images are used to show how the naturally fluorescing nematocysts, yellow lines at the outer edge of ectoderm, dissipate in abundance from the tip (2.5d-f) down the trunk of the tentacle (2.5f). In other corals, the nematocysts are spread throughout coral tissue leading to blurring and excessive autofluorescence when trying to find bacterial aggregates. Whereas in *Heliofungia*, the nematocysts are packed at the edge of the tip tissue and excluded from the bulk of the tentacle, which leads to clearer images of the aggregates and other structures within coral tissue. It was also consistently observed that *Heliofungia* has a much lower abundance of zooxanthellae in the tip and the majority of the endophotosymbionts are packed in the endoderm of the tentacle trunk (2.5e-f). The symbionts cluster towards the mesoglea/ectoderm boundary and away from the open channel in the middle of the tentacle, the coelentric cavity that is filled with seawater.

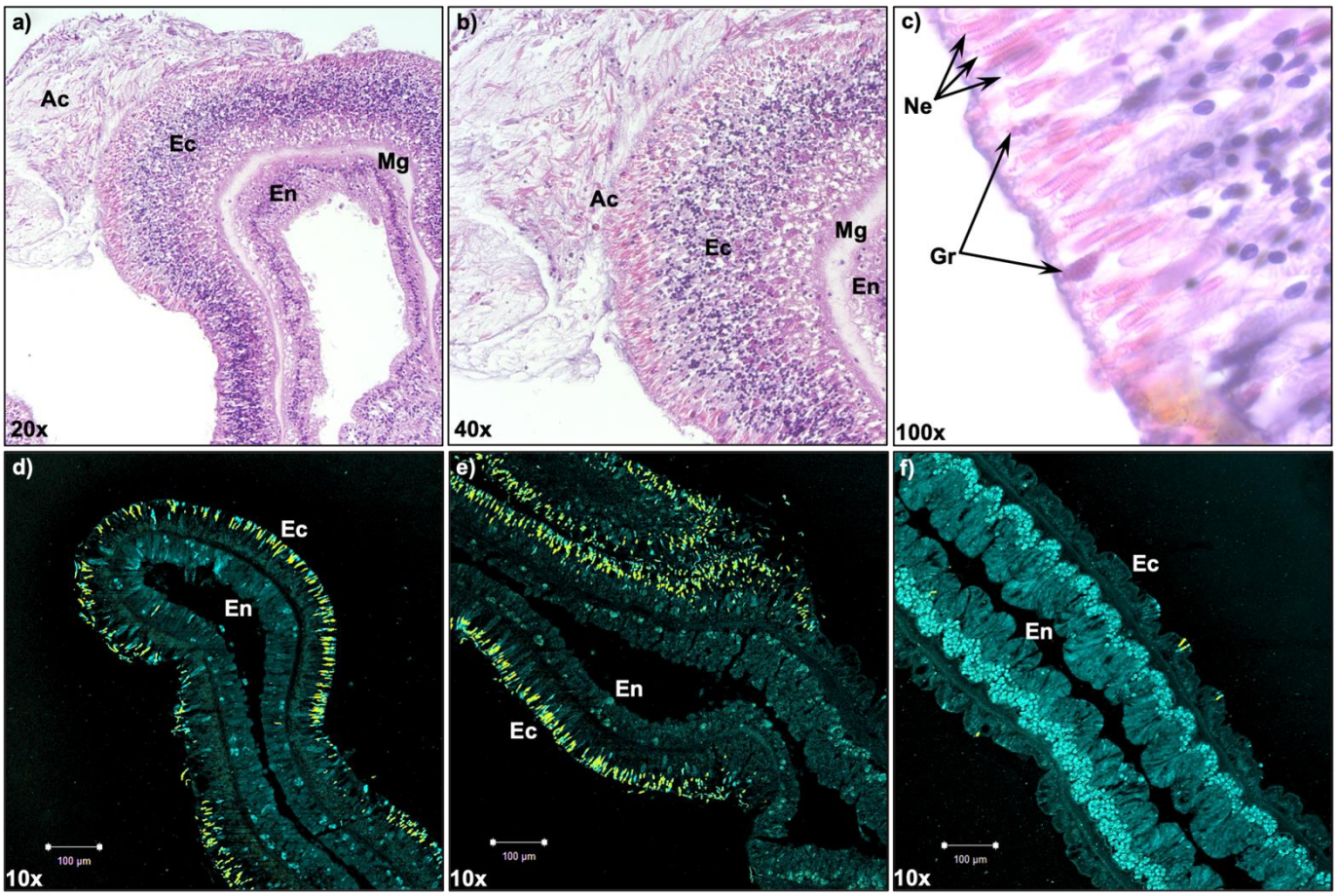


Figure 2.5: Top row of images (5a-c) show histology slides from the tip of *Heliofungia* tentacles and bottom (5d-f), is the NONEUB FISH images showing position of nematocysts in the tentacle. 5a) is at 20x magnification and shows the tip with the tissue regions - endoderm (En) and ectoderm (Ec), separated by the mesoglea (Mg) and the acrosphere (Ac), snot-like protrusion coming from the outer edge of the ectoderm which is packed full of nematocysts (pink spiral structures). 5b) is a closer magnification (40x) of the ectoderm (Ec) and acrosphere (Ac) showing all the nematocysts clustered tightly against the outer edge and within the acrosphere. 5c) is a 100x magnification image showing the characteristic coiled structure of coral stinging cells, against the ectoderm edge and a couple of granular cells (Gr) aggregates in deep pink/purple, on closer inspection on can tell these are granular cells and not bacteria as they do not have the classic 'fluffy' appearance of bacteria and can see spherical cell definition. 5d - e) show NONEUB images of the *Heliofungia* tip with the internal endoderm (En) and outer ectoderm (Ec) clearly demarcated by the mesoglea. Yellow dashes represent the autofluorescent nematocysts and these vanish as one moves down the tentacle. 5f) shows what the tentacle trunk looks like generally, with the occasional fluorescing granular cell cluster, the outer ectoderm (Ec) with the endoderm (En) within with zooxanthellae (bright blue spherical cells) packed against the mesoglea/ectoderm edge of the tissue.

The third panel of images (Figure 2.6) shows the presence of bacteria in the ectoderm and endoderm using the general bacterial probe (EUB338iii). Figures 2.6a and 2.6b show a bacterial aggregate (Ag) in the ectoderm (Ec) at 63x and 100x magnification. The dense, 'fluffy' aggregate is similar to what has previously been seen in the literature (Ainsworth et al., 2006; Ainsworth et al., 2015; Damjanovic, 2019; Wada et al., 2019) and the aggregates did not

fluoresce under the non-bacterial (NONEUB) probe. Figure 2.6c and 2.6d are also at 63x and 100x magnification respectively and also show two bacterial aggregates in the ectoderm as well as what appear to be tightly coiled or spiral-shaped bacteria (Sp). Figures 2.6e and 2.6f show the endoderm (En) at 63x and 100x magnification with the coelentric cavity (Co) present between both sides of the tentacle. Symbiodiniaceae (Zx) are shown as slightly fluorescing orbs in the endoderm and there are more spiral bacteria present throughout the endoderm which are clearer to see in the 100x image (Figure 2.6f).

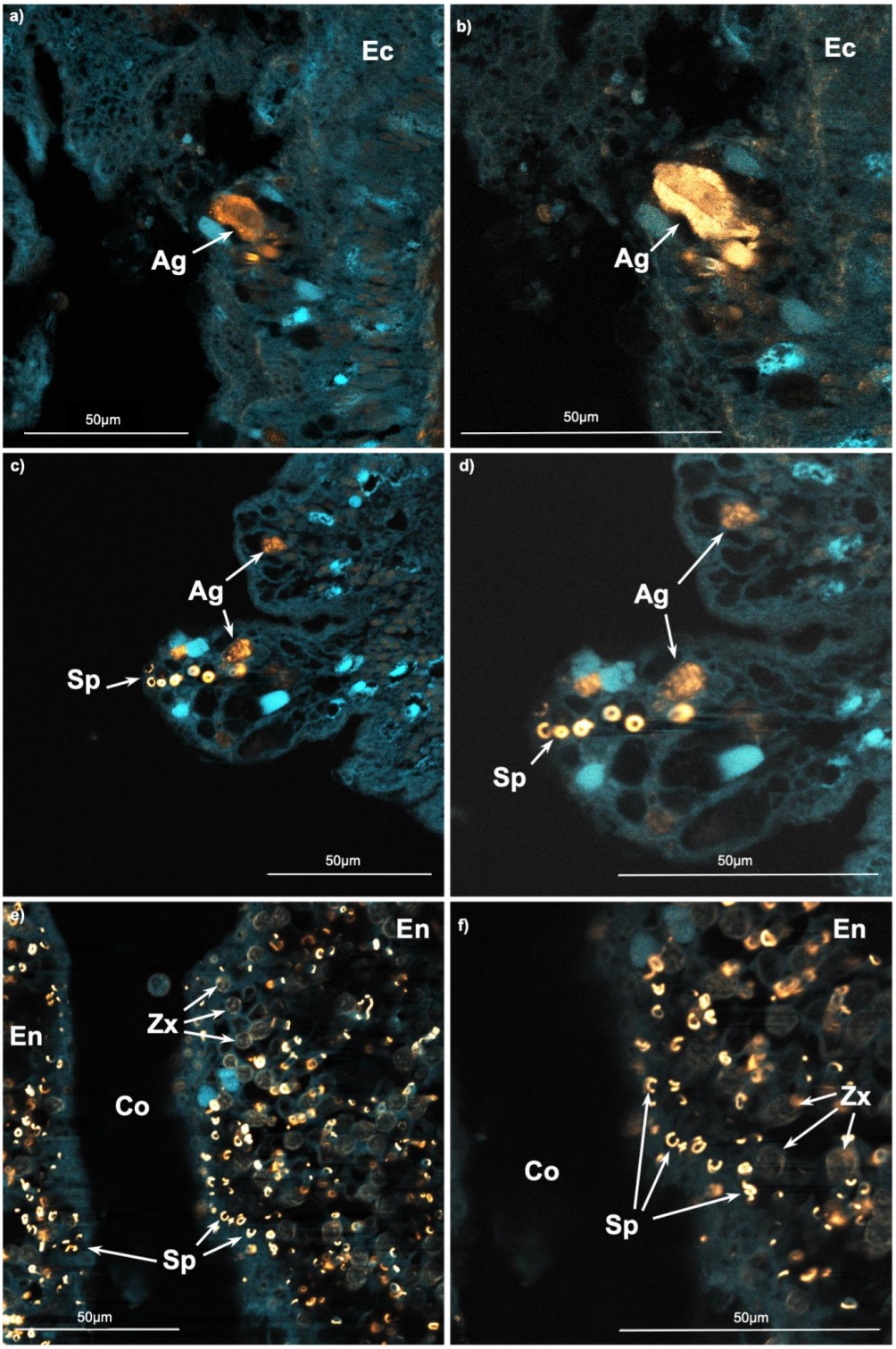


Figure 2.6: Confocal images of *Heliofungia* using an EUB338iii probe that highlights any bacterial cells within the coral. All a) images are at 63x magnification and b) close-ups of the a) regions at 100x. 1a) and b) show a region of the ectoderm that had a large bacterial aggregate present at the edge of the tissue (orange), there are other dense aggregates present next to the bacterial one in light blue which may be granular cells or another microbe. 2a and b) show the presence of orange bacterial aggregate in the ectoderm and some unknown densely coiled, potentially bacterial, cells. 3a) and b) show the fluorescing structures present in the endoderm, spherical cells are zooxanthellae, spiralling/coiled cells look bacterial in nature. Labelling - ectoderm (Ec), endoderm (En), mesoglea (Mg), coelentric cavity (Co), Symbiodiniaceae (Zx), bacterial aggregates (Ag) and spiral, coiled bacteria (Sp).

2.3.4 The top prokaryotic contenders - relative abundance

An analysis of the top 15 prokaryotic families based on relative abundance showed marked tissue specific differences (Figure 2.7). Endozoicomonadaceae was abundant in all regions but the family was most dominant in the ectoderm (≥90% relative abundance) as there was only a small percentage of other families in the outer tissue. All regions contained a proportion of 'other' families, lowest being in the ectoderm and highest in the mucus and acrosphere, as observed in the α- and β-diversity. Alteromonadaceae, Cyclobacteriaceae and Nitrinocolaceae were present in the endoderm, mucus and acrosphere. The mucus and endoderm both contained Rhodobacteraceae. The endoderm also comprises Spirochaetaceae which could be the tightly coiled, spiral bacteria observed in the FISH results above. The presence of spirochetes in sponge tissues was previously observed by microscopy (Neulinger et al., 2010). Families with larger proportions in the gut and mucus included Clade I, Flavobacteriaceae and Vibrionaceae. Families that were more dominant in singular regions were Sphingomonadaceae and Saccharospirillaceae in the acrosphere, and SS1-B-06-26 in the mucus.

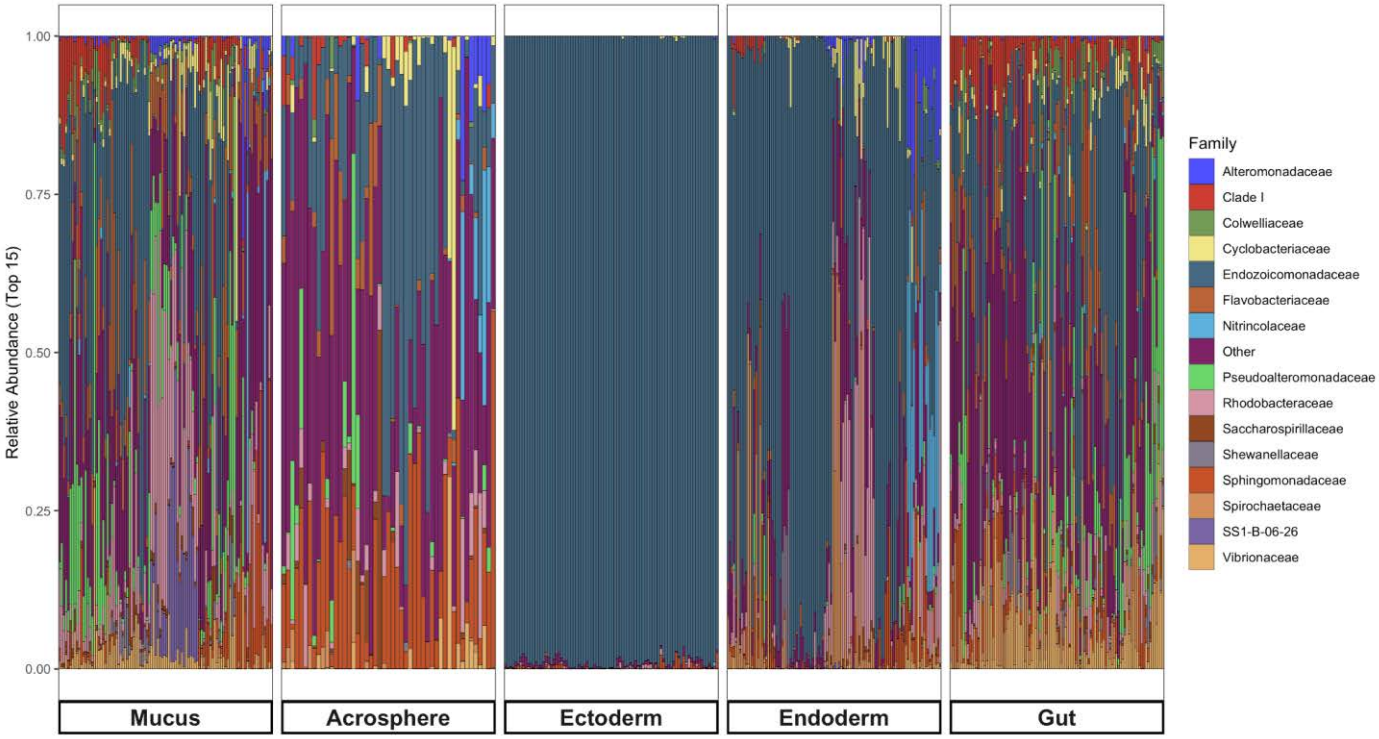


Figure 2.7. Relative abundance bar plots of the ASVs in each region of *Heliofungia* and the top 15 prokaryotic families that they belong to, from left to right; the ectoderm, endoderm, mucus, gut and acrosphere. Legend shows each family and the colour represented by them in the stacked barplot (five technical replicates were taken per tissue region per individual, n = 25).

2.3.5 The gut microbiome of Heliofungia

The large mouth of *Heliofungia* had a buccal swab taken morning and afternoon to assess the microbes present in the gastrovascular cavity and determine whether time impacted the community. Time did not show a significant effect on microbial composition, as there was considerable overlap between the morning and afternoon samples and the PC values were low for both axes (Figure 2.8). Although, there were some outliers that did show a significant change between a.m. and p.m. which included samples from individuals number 2 and 4 from August 2017 and individual number 4 from August 2018.

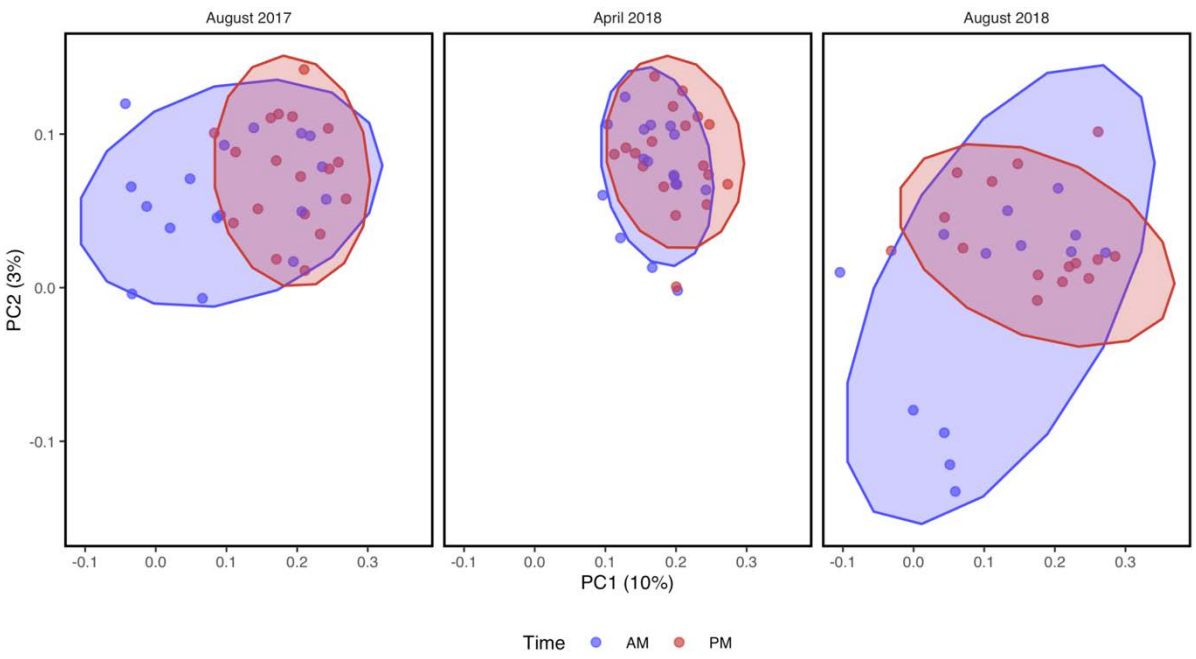


Figure 2.8: PCoA of the gut microbiome composition from three time points - August 2017, April 2017 and August 2018, where colour represents either a.m. (blue) or p.m. (red) sampling points. Ellipses denote the samples that share a 90% confidence level with one another with regards to community composition (four technical replicates were taken per morning and afternoon, per individual (five per time point)).

The mean sum abundance of each microbial family was calculated and the top 15 were plotted across the sampling points (August 2017, April 2018 and August 2018) and split by time period - a.m. (10:30) and p.m. (16:30) (Figure 2.9). The most abundant family was Vibrionaceae, but even though this family was highly abundant in August 2017, it was less abundant in the 2018 time points, which may explain some of the differences in PCoA clustering. Overall, time did not seem to have a significant effect (ANOVA, p = > 0.05) on all family abundance, although there was a large difference in the abundance of Lentisphaeraceae and Colwelliaceae in August 2017, morning to afternoon. As well as between morning and afternoon samplings of Pseudoalteromonas in April 2018. In April 2017 the most abundant family was Fusobacteriaceae and in August 2018, unknown families and Pseudoalteromonas.

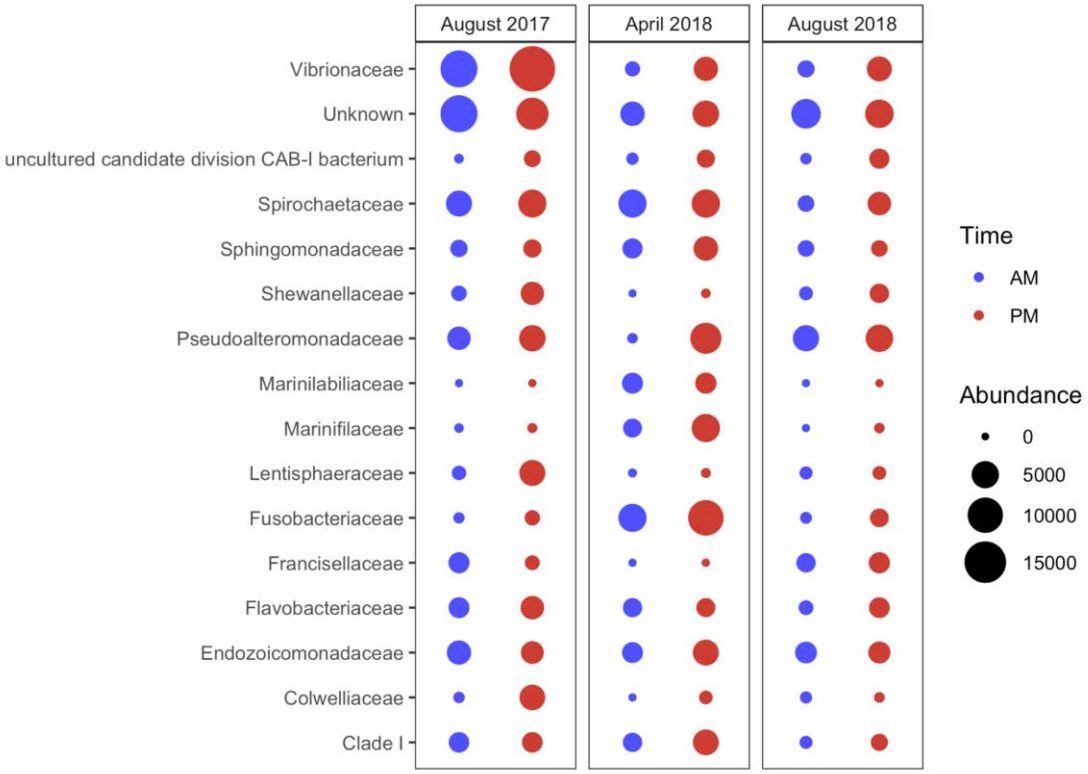


Figure 2.9: The top 15 bacterial families present in the gut of *Heliofungia actiniformis* across the three sampling points and split into time - blue, AM (10:30) and red, PM (16:30). The size of the points represents the abundance of each family; abundance was acquired from the eight technical replicates taken from the five individuals at each time point.

Metagenassist was used to infer the function of the microbiome in the gut; however, overall, most functions were unknown as the bacteria in question are lacking publicly available metagenome data. For example, when the energy sources were assessed the bacteria in the gut of *Heliofungia* showed; 1.1 % chemoorganotroph, 2.1 % autotroph, 10.6 % heterotroph and 85.1 % unknown. Although there was not a significant difference between morning and afternoon sampling in the abundance of bacterial families (ANOVA, p = > 0.05), there was a significant 2.6 log-fold increase from the norm in Chemoorganotroph at the a.m. sampling in comparison to p.m., no other energy source showed a significant change. Oxygen requirement was also assessed as this may give an inclination into the roles undertaken by the gut microbiome, 7.6% of constituents were anaerobic, 41.5 were aerobic and 50.5% were unknown (Figure 2.10).

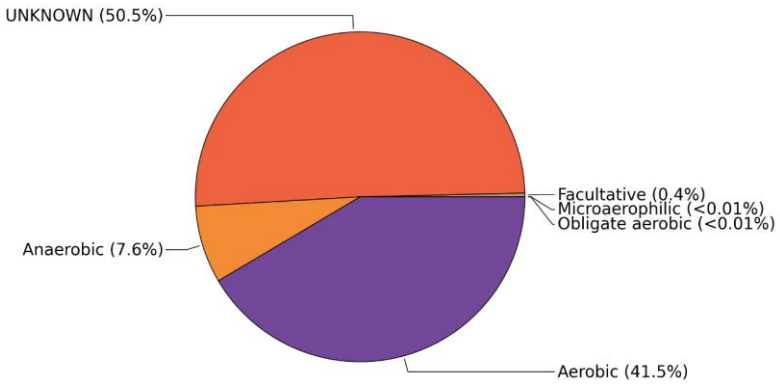
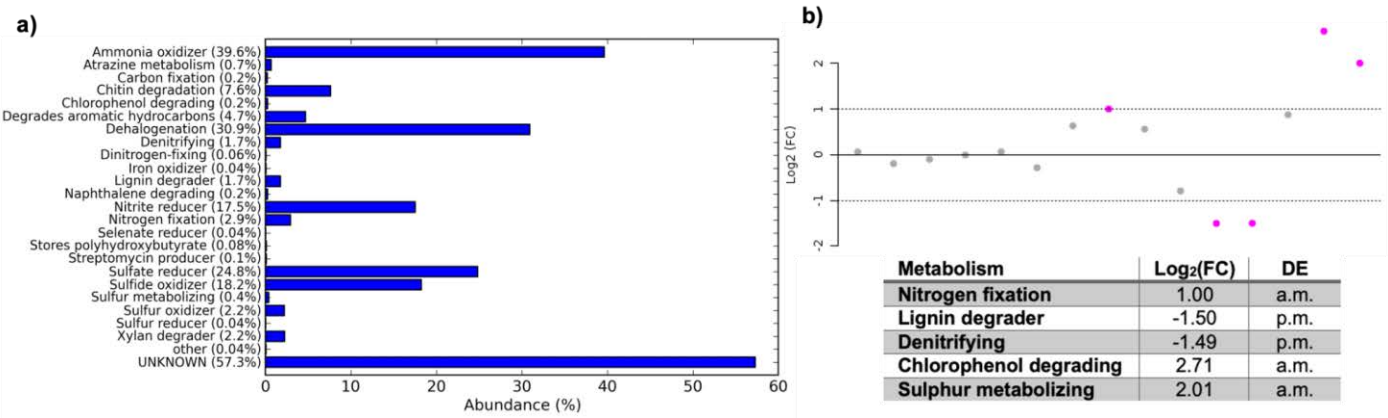


Figure 2.10: Pie chart showing the proportional representation of the oxygen requirements of the ASVs present in the gut of *Heliofungia actiniformis*, coloured segments denote the type of representation (14,000 ASVs).

By assigning metabolic pathways to the ASVs (14,000 total), the results showed half of them to be unknown; however, there were still several categories that showed a substantial percentage (>15%) (Figure 2.11a). Ammonia oxidation was seen to be a large part of the gut microbiomes metabolism (39.6% of ASV, ~5600 ASVs), followed by dehalogenation (30.9%), sulphate reduction (24.8%), sulphide oxidation (18.2%) and nitrate reduction (17.5%) as the largest proportions (Figure 2.11a). Additionally, a fold-change comparison of metabolism types between the morning and afternoon showed genes related to five types of metabolism being significantly more abundant in samples from the morning or afternoon. These metabolic processes were in the morning: nitrogen fixation, chlorophenol degradation and sulphur metabolism and, in the afternoon: lignan degradation and denitrification (Figure 2.11b).



Metabolism	Log_2(FC)	DE
Nitrogen fixation	1.00	a.m.
Lignin degrader	-1.50	p.m.
Denitrifying	-1.49	p.m.
Chlorophenol degrading	2.71	a.m.
Sulphur metabolizing	2.01	a.m.

Figure 2.11: Percentage of types of metabolism occurring in the gut microbiome of *Heliofungia actiniformis*, 10a) y represents metabolism type and x is abundance. 10b) shows the log fold-change of metabolism types between a.m. (10:30) and p.m. (16:30) sampling, pink points portray the terms that had a significant differential expression (DE) and the table beneath the plot explains the type of metabolism significantly DE at which time point and by how much (Log_2(FC)).

2.3.6 Archaeal families present in Heliofungia tissues

The regions with the highest relative abundance of Archaea were the mucus and gut, whilst the region with the lowest relative abundance was the ectoderm (Figure 2.12). The most abundant archaeal family came under the grouping of 'unknown'. Following this, Haloferacaceae was the next most prominent family in the mucus and ectoderm (maximum ~3,750 counts in the mucus). Although the acrosphere and endoderm also contained Haloferaceae, their top second abundant family was Nanoarchaeota archaeon (SCGC AAA011-G17) (which was also present in the mucus, ~2400 ASVs). All regions except the acrosphere contained a small abundance of Halomicrobiaceae (less than 1,000 ASVs) and all regions except the ectoderm contained Nitrosopumilaceae (less than 4,000 ASVs, highest in gut). Finally, uncultured bacterium and uncultured crenarchaeote were present in a low abundance (~500 ASVs) in the mucus, gut and acrosphere.

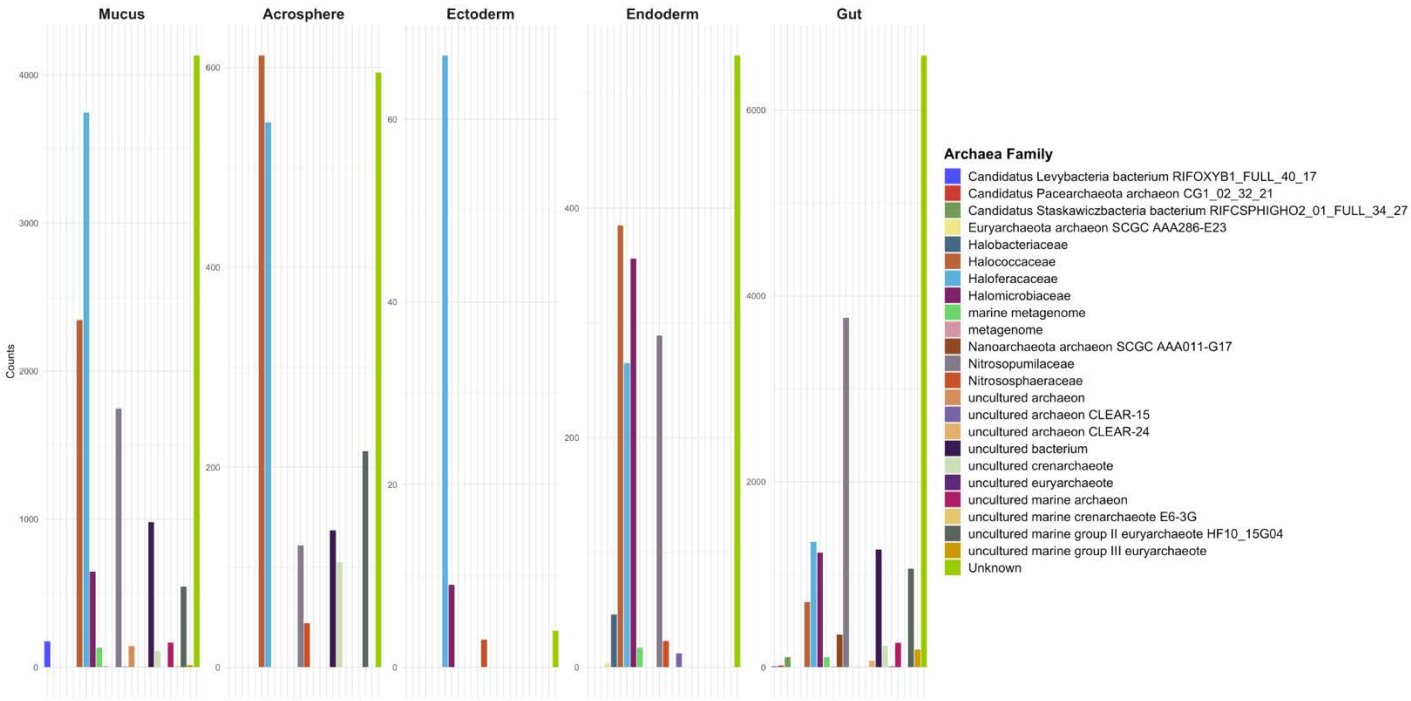


Figure 2.12: The number of ASVs that identify as members of archaeal families present in the tissue regions, based on normalised counts, note the x-axis changes between regions.

2.3.7 Dominant Endozoicomonas genus

A common outgroup for *Endozoicomonas* phylogenetics is the genus *Kistimonas* (Bartz et al., 2018; Shiu & Tang, 2019). However, no outgroup was set for the preliminary tree as during taxonomic assignment a known *Oceanospirillales bacterium LUC14_002_19_P2* belonging to the genera *Kistimonas* was identified in *Heliofungia* therefore, *Kistimonas* could not be used as an outgroup. The preliminary phylogenetic tree was needed to determine which genera or species the most abundant ASVs (>1500) were related to (Figure 2.13). The abundance plot (Figure 2.13b)) showed that the most abundant *Endozoicomonas* ASVs fell under branches of unknown or uncultured species and only one low abundance ASV was *Oceanospirillales bacterium LUC14_002_19_P2.* In addition to this, amongst the ASVs there seemed to be many that, although classed as individual ASVs, were likely the same species that contained single-nucleotide polymorphisms (SNPs) of one another, shown by the identical branches next to one another. Overall, there were 14 ASVs with an abundance greater than 1500 present in *Heliofungia* and the two most dominant strains (≥15k) belonged to uncultured and unknown species in the endoderm and ectoderm respectively. Looking into region abundance, the ASVs were shown to be present in all regions, although the acrosphere only showed a tiny proportion in the uncultured dominant strain. Overall, the greatest abundance

of *Endozoicomonas* reads were in the endoderm (~17k) followed by the ectoderm (~15k), mucus (10k), gut (~5k) and acrosphere (~3k).

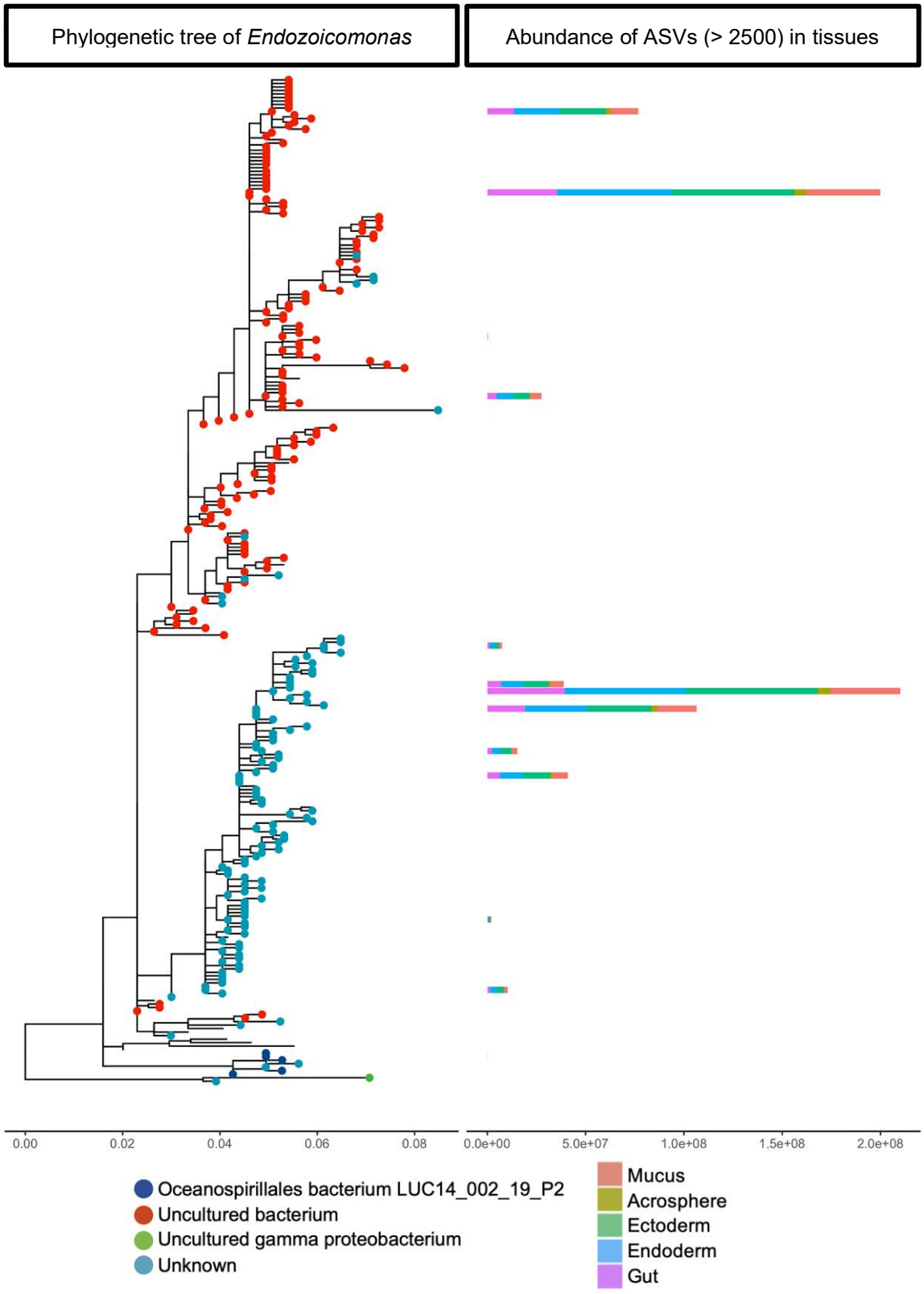


Figure 2.13: The phylogenetic tree was rooted with the known species *Oceanospirillales bacterium LUC14_002_19_P2* as the outgroup and coloured tip points were used to identify the species group it belonged to. The abundance of each individual ASV was plotted to the left in line with its corresponding ASV tip point and

abundance was deprecated by the region the sample came from. For clarity, ASVs with less than 2500 reads were removed from the left plot and the scale is shown on the x-axis (n = 373 ASVs).

The 14 most abundant *Endozoicomonas* ASVs were compared to isolated *Endozoicomonas* species from other corals and a sponge which included *Montipora*, *Acropora*, *Pleuxaura* and *Haliclona* (Bartz et al., 2018; Tandon et al., 2018). Although, after initial assessment the coral *Endozoicomonas* species were too closely related to the *Heliofungia* ASVs to act as an outgroup so members from the family Hehallaceae, *Hahella ganghwensis*, were selected due to previously being used in the coral literature (Shiu et al., 2018; Tandon et al., 2020). The new phylogenetic tree (Figure 2.14) showed the dominant *Endozoicomonas* ASVs from *Heliofungia* to be most closely related to the soft coral and *Montipora* isolates although, the unknown ASVs from *Heliofungia* fell under their own node beneath these. The *Oceanospirillales bacterium* ASV was on the same terminal branch that included a *Hahella chejuensis* species. These results imply that, although the *Heliofungia Endozoicomonas* ASVs are closely related to a few of the already isolated coral strains, there may be a *Heliofungia*-specific *Endozoicomonas* and further study is needed to elucidate this.

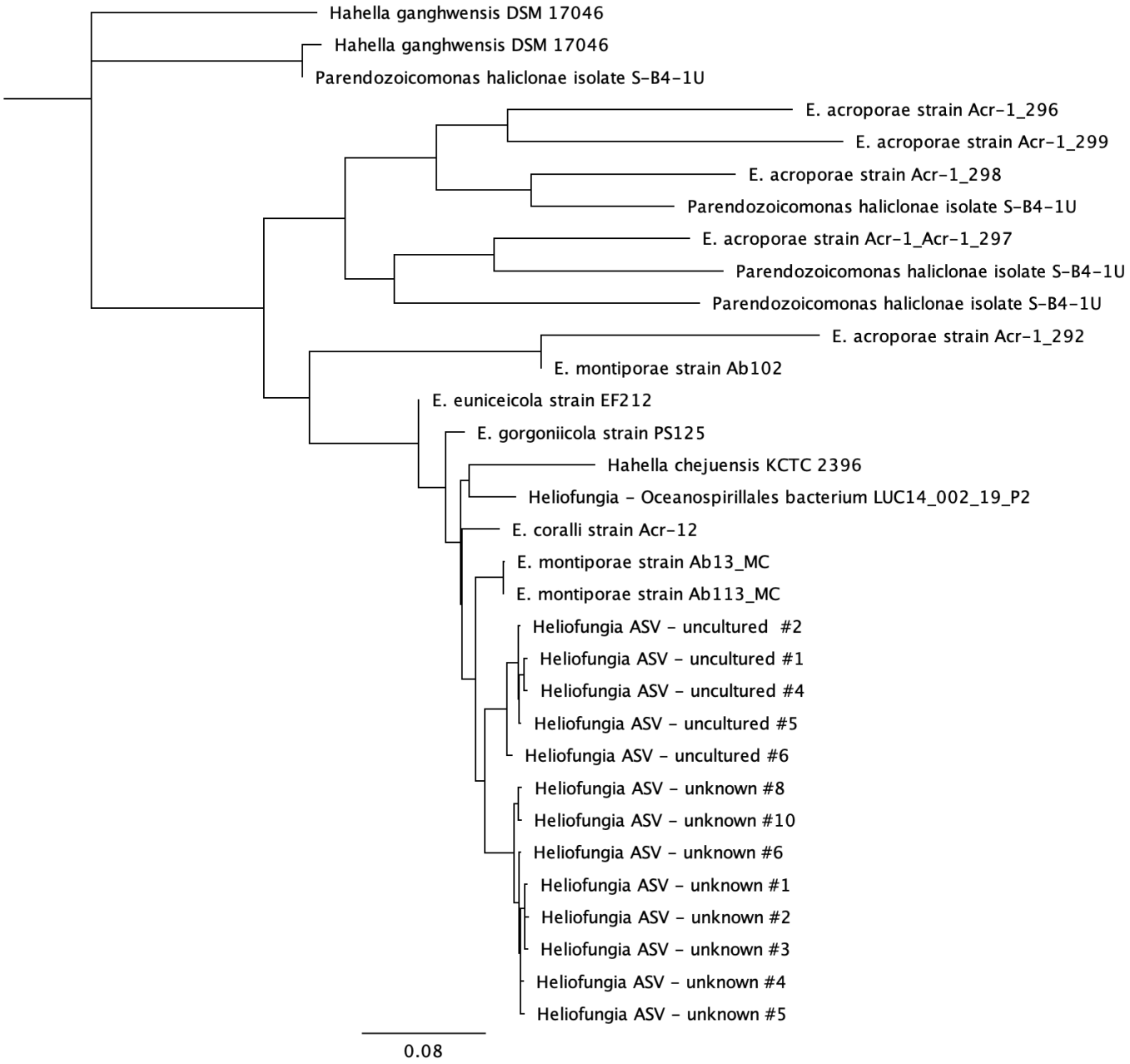


Figure 2.14: A phylogenetic tree containing the 14 most abundant *Endozoicomonas* ASVs from *Heliofungia actiniformis* ("Heliofungia ASV - unknown ##") and *Endozoicomonas* species isolated from corals (*Montipora, Acropora, Pleuxaura*) and a sponge (*Haliclona*) with *Hahella ganghwensis* as the outgroup.

2.3.8 Validation of presence of Endozoicomonas through FISH

Endozoicomonas-specific aggregates were viewed in the ectoderm, as well non-*Endozoicomonas* ones which had light up under the general EUB338iii probe but not the *Endozoicomonas*-specific probe (Figure S2.2), implying that some aggregates are *Endozoicomonas* specific and others different taxa. An interesting finding was the presence of a dense 'fluffy' aggregation of *Endozoicomonas* throughout the internal edge (with respect to the coelentric cavity, the external edge would be the side closest to the ectoderm) of the endoderm (Figure 2.15). Bacteria are predominantly seen in corals as smaller aggregates therefore the presence of *Endozoicomonas* seemed questionable, however, numerous images across multiple tentacles using light and confocal microscopy with a number of probes and staining showed that these large aggregates present as bacteria. The presence of bacteria in microscopy is regularly referred to as looking like 'fluffy' structures (Hirai et al., 2010; Ratnayake et al., 2012) and in the panel beneath one can see in the 100x images that these cloud-like structures fit this description and, in the confocal images, individual dots or bacterial cells can be observed. The assumed *Endozoicomonas* aggregates only fluoresce when exposed to a general bacterial probe (EUB338iii) and the *Endozoicomonas*-specific probe (ENDOZ).

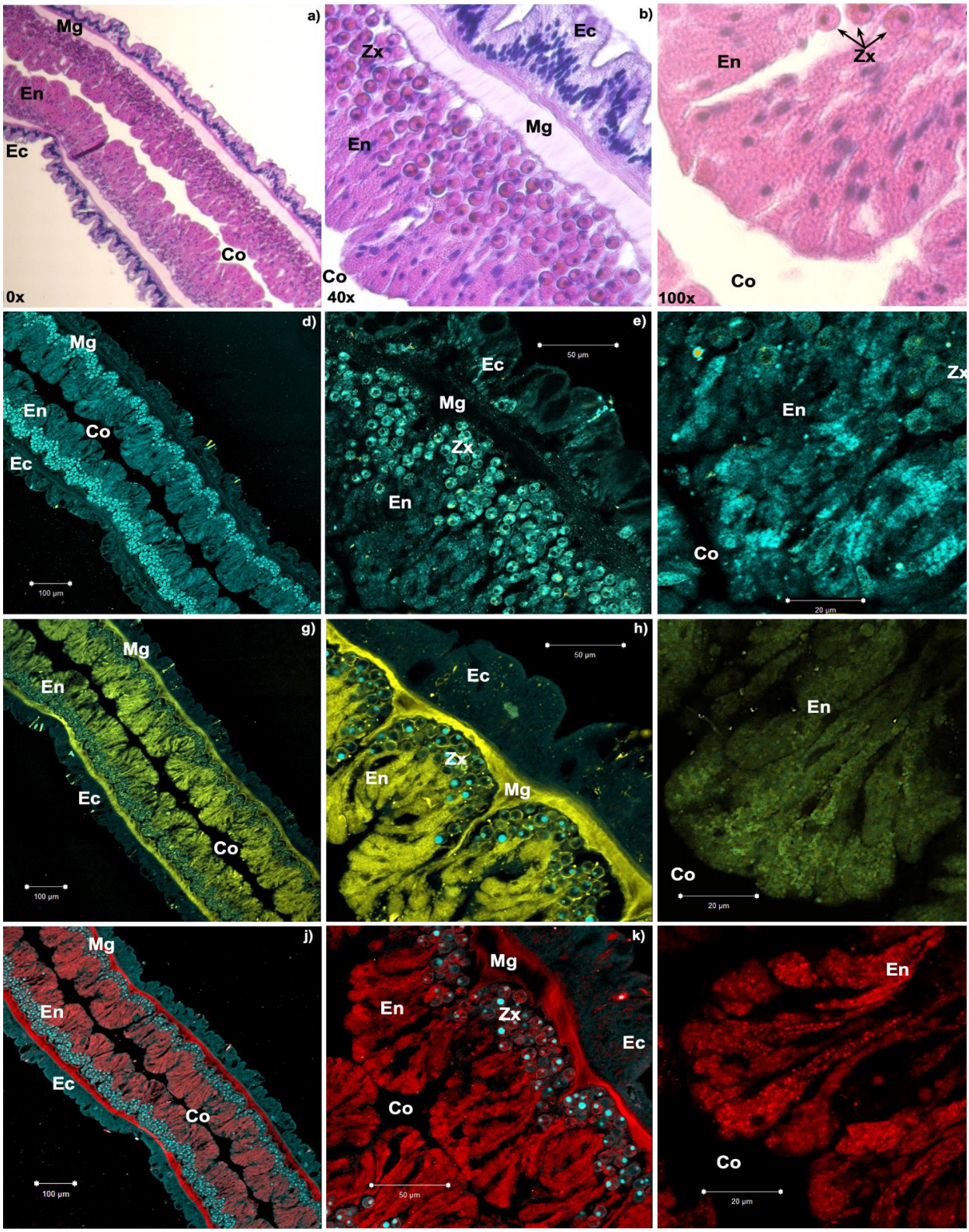


Figure 2.15: a panel of microscopy images of the same *Heliofungia* tentacle processed under several different techniques; the first row shows histology of *Heliofungia* tentacles cut longitudinally and H & E stained. From left to right the images zoom in on the inner side of the endoderm (En) that faces the coelentric cavity (Co). The first image shows the full tentacle at 10x magnification where the outer ectoderm (Ec), darker purple, mesoglea (Mg), endoderm (En) and coelentric cavity (Co) are all visible. The second image is a magnification (40x) of the endoderm where all layers are still visible with the Symbiodiniaceae (Zx) clearly defined and the final image shows the area

of interest, a 100x snapshot of the internal endoderm (En). The second row follows the same pattern as above at magnifications 10x, 63x and 100x with confocal microscopy and a NONEUB probe, objects that fluoresce are non-bacterial with a couple of cnidocytes in the first image and the Symbiodiniaceae (Zx) being brighter in the 63x image. The third and fourth rows contain images taken through confocal microscopy with a general bacterial probe (EUB338iii) and Endozoicomonas-specific probe (ENDOZ) respectively. Non-specific binding is visible in the mesoglea (Mg) and at both 100x images a bacterial presence is observable due to probe binding and 'fluffy' appearance of the bacterial clouds present between the endoderm Symbiodiniaceae and the coelentric cavity.

2.4 Discussion

2.4.1 Heliofungia tissues harbour distinct microbiomes

Cnidarians are often viewed as morphologically simple organisms that are unlikely to provide distinct niches for microorganisms; however, the results of this study imply otherwise. The α- and β-diversity plots above (Figures 2.2 and 2.3) show that with the exception of the gut and mucus, each of the regions studied harboured clearly distinct microbial populations. The tissue-specific microbiomes of *Heliofungia* were relatively stable across five sampling points from 2016 to 2018, as has previously been observed in *Hydra* and *Nematostella* (Bonacolta et al., 2020; Deines et al., 2020).

2.4.2 FISH microscopy highlights bacteria present in the tissues

The application of FISH technology to *Heliofungia* provided greater resolution than has been possible in previous studies of corals. At relatively low magnification, the bacterial aggregates seen in the *Heliofungia* ectoderm were similar to those observed in previous coral studies (Ainsworth et al., 2006; Damjanovic et al., 2020; Damjanovic et al., 2019; Wada et al., 2016). However, at 100x magnification, these aggregates to have a 'fluffy' appearance similar to that of bacteria in a growth medium, the xylem of plants and the ectoderm of a hydrozoan (Di Camillo et al., 2012; Hirai et al., 2010; Ratnayake et al., 2012). Whereas it is generally assumed that bacteria in coral tissues reside predominantly within aggregates (Wada et al., 2019) in the work presented here, spiral-like bacteria seen in the ectoderm and endoderm appeared not to be constrained to aggregates. However, Wegley et al (2004) reported observing free-floating bacteria and archaea with spirillum-like bacteria in the tissue of *Porites astreoides* (Wegley et al., 2004). Other coiled and spiral bacteria, in particular Spirochaetaceae, have been observed through FISH in sponges (Neulinger et al., 2010). Considering the high abundance of Spirochaetaceae in the endodermal microbiome (Figure

2.4) and previously in coral, *Nematostella* and Holothurian microbiomes (Bayer et al., 2013; Bonacolta et al., 2021; Bonacolta et al., 2020; Lawler et al., 2016; Weigel, 2020), the coiled, spiral structures presented here may belong to this family. However, further testing with specific probes would need to be undertaken to clarify this.

2.4.3 The mucus microbiome

The mucus is an open system, exposed to the environment; therefore, species diversity is likely to be higher due to environmental disturbances, creating niche openings for new species and leading to higher variation in ASV composition (Figure 2.7). Several studies on coral mucus have shown that the coral mucus is highly diverse and community composition can be affected by many factors including season, pH, oxygen levels and pollution (Hussien et al., 2019; Koren & Rosenberg, 2006; Meron et al., 2013; Zhang et al., 2015). Other factors affecting community composition are consequences of host-specific selection pressures, such as specific bacterial binding receptors on the coral surface, biocides and signalling molecules that may affect mucus composition (Brown & Bythell, 2005; Kvennefors et al., 2008). Quorum-sensing signals responsible for bacterial communication have been shown to impact bacterial associations within the coral mucus in several coral species (Li et al., 2017). In general, coral mucus contains predominantly α- and γ-proteobacteria (Ceh et al., 2011; Hussien et al., 2019; Li et al., 2017; M. Sweet et al., 2011), and the results of the present study indicate that is also true of *Heliofungia*. There were 15 families that were observed to be the overall most abundant across all regions; however, some families were dominant in certain regions but not others. In the *Heliofungia* mucus a few families were more abundant in this region than the others, these included: the α-proteobacteria; Clade I, Rhodobacteraceae and γ-proteobacteria; Vibrionaceae, SS1-B-06-26 and, the bacteroidetes; Flavobacteriaceae. The Clade I family belongs to the order SAR11 clade which has members commonly found in the oxygen-rich photic zone or oceanic oxygen minimum zones (OMZ) (Ganesh et al., 2014; Morris et al., 2002). Members of the SAR11 clade have genes required for life in OMZs, including low oxygen nitrate reductases (Tsementzi et al., 2016). The Clade I in *Heliofungia* belongs to the OMZ and oligotrophic SAR11 order (Thrash et al., 2014; Tsementzi et al., 2016). As corals thrive in oligotrophic waters (D'elia & Wiebe, 1990), the mucus of *Heliofungia* would be a good niche for Clade I to utilise and may feed off the nitrate available to it in the region. In *Platygyra acuta,* Rhodobacteraceae differ in abundance between the mucus, soft tissue and skeleton, implying ecologically distinct compartmental microbiomes (Luo et al., 2021) which validates what was observed in *Heliofungia*. Luo et al. demonstrated that Rhodobacteraceae isolated from the skeleton and mucus of *P. acuta* were distinct species; the mucus-specific isolate

encoded more genes related to osmolyte breakdown. These authors suggested that conditions within the coral compartments might impose distinct selection pressures on coral microbiomes (Luo et al., 2021), which is clearly supported by the results presented here for *Heliofungia*. Little information is available on the bacterial family SS1-B-06-26, which belongs to the Oceanospirillales and has been linked to denitrification and the biodegradation and metabolism of oil and microplastics (Ribicic et al., 2018; Xie et al., 2021). SS1-B-06-26 may inhabit the mucus niche to access pollutants from motor oil in the seawater as the waters surrounding OIRS are a popular area for boat traffic; however, water quality testing alongside isolating the mucus SS1-B-06-26 family and validating it contains these hydrocarbon metabolism genes would be needed to test this hypothesis. Unlike SS1-B-06-26, Flavobacteriaceae and Vibrionaceae have frequently been reported as components of coral microbiomes, particularly those of coral mucus (Ceh et al., 2011; Hussien et al., 2019; Li et al., 2017; M. Sweet et al., 2011). Both families have been suggested to be opportunistic pathogens (M. Sweet et al., 2011). It is thought that mucus effectively acts as a selection barrier, preventing most bacteria entering the host ectoderm (Ceh et al., 2011), which may account for the low relative abundance of Flavobacteriaceae and Vibrionaceae observed in the ectoderm and endoderm and that all corals sampled appeared to be in good health.

2.4.4 The ectodermal microbiome

Little is known about the coral ectoderm as previously coral soft tissue sampling has comprised of both ecto- and endoderm (Ainsworth et al., 2015; Hernandez-Agreda et al., 2016; Pollock et al., 2018; M. Sweet et al., 2011). Whereas the microbiome of the *Heliofungia* mucus layer, showed high community diversity and variation, the ectoderm was essentially monopolised by Endozoicomonadaceae (~95%) to the exclusion of other microbes (Sousa, 1979). This Endozoicomonadaceae monoculture could be occurring for a number of reasons as suggested in the coral literature (predominantly nutrient cycling) (Bayer et al., 2013; Bayer et al., 2013; Neave et al., 2017; Sheu et al., 2017; Tandon et al., 2020; Yang et al., 2010). The family is metabolically diverse, and can utilise a large number of metabolites for growth (Neave et al., 2017). Therefore, there are varying genotypes of the bacteria that have diversified dependent on their coral hosts by-products (Neave et al., 2016; Neave et al., 2017); determining whether Endozoicomonadaceae present different genotypes in the regions could aid in understanding if tissue-specificity impacts speciation also. The genome of *Endozoicomonas montiporae* gives the impression it is a facultative endosymbiont that can interact with its coral host (Ding et al., 2016). Whether there is a potential benefit of Endozoicomonadaceae still remains unclear. There are no cnidarian specific studies that

discuss the bacteria in the ectoderm; if the family were to benefit the host it may be to assist in ectoderm growth and pathogen resistance, as these roles have been identified as important in the ectoderm of *Hydra* and *Aiptasia* (Augustin & Bosch, 2010; Bosch, 2013; Fraune et al., 2015; Passamaneck & Martindale, 2012; Singer, 1971; Tivey et al., 2020). In the body regions of the giant clam, the largest abundance of Endozoicomonadaceae was found in the gills which is a tissue region exposed to the environment similar to the ectoderm (Rossbach et al., 2019). Functional analysis showed Endozoicomonadaceae to be involved in nitrogen cycling and the authors also suggested that the high density of bacteria may act as a protective mechanism against pathogen entry (Rossbach et al., 2019) therefore, indirectly assisting in pathogen resistance as hypothesised. The pharynx of several ascidians studied have shown to be dominated by Endozoicomonadaceae and these authors also suggest a facultative symbiosis between host and bacteria, they state that Endozoicomonadaceae prefer to inhabit areas with access to mucus as this is where they derive the majority of their food sources from (Schreiber et al., 2016), this fits with what is observed in the gills of the giant clam and the ectoderm of *Heliofungia*.

2.4.5 The endodermal microbiome

The endoderm on the other hand had higher bacterial diversity than the ectoderm. The β-diversity demonstrated the endoderm community to be closest to the ectoderm and acrosphere than the mucus and gut (Figure 2.2). With respect to the endoderm-specific microbiome, there are two clear contributing factors that may impact it; immunity and Symbiodiniaceae. In *Hydra*, the endodermal tissue has some characteristics in common with intestinal tissue in higher metazoans (Augustin & Bosch, 2010), including contributing to digestion, the ability to phagocytose bacteria, house symbiotic α-proteobacteria and aid in innate immunity through antimicrobial activity (Augustin & Bosch, 2010; Bosch, 2013; Bosch et al., 2009; Cheng et al., 1981). Endodermal tissue seems to be key in immunity of the Hydra host, and the endodermal microbiome is thought to be important in this context (Bosch, 2013; Thaiss et al., 2016). Rhodobacteraceae, one of the bacterial families seen to be prominent in the endoderm, has been previously suggested to be involved in gut health and immunity and was shown in sea cucumbers to have a probiotic response by activating the NF-κB signalling pathway (Weigel, 2020; Yang et al., 2015; Zhou et al., 2007). Endozoicomonadaceae may be present to assist in nutrient cycling and tissue health (Neave et al., 2017; Shiu & Tang, 2019; Tandon et al., 2020). Although microscopy has shown the family to be present in the endodermal region of *S. pistillata* in close-proximity with the Symbiodiniaceae (Bayer et al., 2013) it seems unlikely that Endozoicomonadaceae is associated with Symbiodiniaceae as it

is also highly abundant in the ectoderm which contains no photosymbionts and this observation was also noted in the giant clam (Rossbach et al., 2019). Families that show roles that may assist the Symbiodiniaceae are Cyclobacteriaceae and Nitrinocolaceae. Cyclobacteriaceae have been linked to carbon metabolism (Rosenberg et al., 2014) and have been reported in corals (Chan et al., 2019; Durante et al., 2019) with links made between Symbiodiniaceae, bacteria and carbon cycling (Matthews et al., 2020; Silveira et al., 2017). Nitrinocolaceae has been reported in *Aiptasia* and *Pocillopora* (J. Li et al., 2021; Randle et al., 2020), as well as samples from a hypoxic reef (Johnson et al., 2021). The family is suggested to be important in nitrogen cycling with mixotrophic denitrification capabilities, it also seems to be able to live in low oxygen, high ammonia environments and thermoclines (Mori et al., 2019). Nitrogen cycling has been connected with Symbiodiniaceae and bacteria (Matthews et al., 2020; Rädecker et al., 2015; Silveira et al., 2017) therefore, Nitrincolaceae may choose to live in the endoderm due to the presence of the Symbiodiniaceae and the osmolytes they give off. The association between these two families and Symbiodiniaceae are still hypothetical however, some families have been associated with five Symbiodiniaceae clades; the ones observed in the *Heliofungia* endoderm include Alteromonadaceae, Rhodobacteraceae, Flavobacteriaceae and Sphingomonadaceae (Lawson et al., 2018). *Aiptasia* has also been used as a model to compare the microbiome between aposymbiotic and symbiotic individuals, highlighting the importance of bacteria in symbiosis (Röthig et al., 2016). The results showed Alteromonadaceae to be more abundant in the symbiosome (Röthig et al., 2016) corroborating Lawson et al (2018) study, and the larger proportion of Alteromonadaceae in the *Heliofungia* endoderm observed in the present study.

2.4.6 The acrosphere microbiome

For the first time, the microbiome of the acrosphere was isolated. The diversity results presented here (Figures 2.1 and 2.2) indicate that the microbiome of the acrosphere region and its nematocyst bundle are distinct from those of other tissue compartments. The α-diversity presented a similar number of ASVs as the endoderm. The acrosphere and gut contained the largest proportion of 'other' bacterial families, taxa that did not make up the most abundant 15. Considering the specialised function of the acrosphere, we could hypothesize that the 'other' families may have a role in animal defence and may have toxic or antimicrobial properties; as unique peptides with potential antimicrobial functions have been described previously in the acrosphere of *Heliofungia* (Schmidt et al., 2020). Saccharospirillaceae was one of the dominant families in the acrosphere of *Heliofungia*, previously identified in the

mesenteries of *Nematostella vectensis* (Bonacolta et al., 2020). Mesenteries are mobile filaments that have some similar physiological roles as the *Heliofungia* tips; capturing prey. Furthermore, Saccharospirillaceae have been implicated in toxin production in *Alexandrium catenella*, a toxic dinoflagellate that is prominent in harmful algal blooms (Tang et al., 2018). Another family that is known to have toxin-producing genera, Sphingomonadaceae (He et al., 2017; Marizcurrena et al., 2019; Saeb, 2016), was also prevalent in the acrosphere.

2.4.7 The gut microbiome

The α- and β-diversity of the gut was shown to be fairly similar to the mucus although more stable, as the mucus showed greater variation in β-diversity across time points. This similarity may be due to the fact that some coral species are known to eat their mucus to recycle the nutrients available within (Brown & Bythell, 2005). Gut samples were taken in the morning and afternoon which did not show a significant difference in composition between the time points (Figure 2.8). Previously, three coral species from the Robusta clade (the same as *Heliofungia*) were sampled at multiple timepoints across two days and the results showed that the gut microbiomes altered in composition during the diel cycle, although the authors state that the changes were stochastic and a diel pattern was not present (Caughman et al., 2021). These results are contradictory to what was observed here, potentially *Heliofungia* having a much larger gut than other species sampled allows for greater community stability. However, similar bacterial families were observed between Caughman et al (2021) and *Heliofungia* (Figure 2.9).

Endozoicomonaceae, Flavobacteriaceae, Spirochaetacaea and Sphingomonadaceae were consistent throughout the time points sampled. Spirochaetaceae have previously been identified in the gut microbiome of Surgeonfish (Parata et al., 2020), Holothurians (Weigel, 2020), sea urchins (Ketchum et al., 2021) and the pharynx of *Nematostella* (Bonacolta et al., 2021; Bonacolta et al., 2020). Spirochaetes are assumed to be important in nitrogen and carbon fixation (Lilburn et al., 2001) and they have been found in a number of corals; Gorgonians, *Corallium rubrum* (van de Water et al., 2016; van de Water et al., 2018), deep-sea species (Lawler et al., 2016), cold water corals (Kellogg et al., 2009), *S.pistillata* (Bayer et al., 2013) and octocorals (Holm & Heidelberg, 2016; Wessels et al., 2017). Therefore, it was not surprising to see Spirochaetaceae so abundant in the gut of *Heliofungia.* When the abundance of the top 15 bacterial families in the gut were compared between morning and afternoon (Figure 2.9), a few showed a large change in abundance, which may explain the changes observed in the PCoA (Figure 2.8). These included; Lentisphaeraceae,

Colweliaceae (in August 2017), Pseudoalteromonadaceae, Marinifilaceae, Fusobacteriaceae (in April 2018) in the afternoon and Francisellaceae (in August 2017) in the morning. Potentially these families are linked to the metabolism types seen to be significantly expressed in the morning or afternoon (Figure 2.11); nitrogen fixation, chlorophenol degradation and sulphur metabolism in the morning and lignin degradation and denitrification in the afternoon. Francisellaceae have been shown to metabolise sulphur (Colquhoun et al., 2014; Parker et al., 1951) which fits with the increased abundance and sulphur metabolism in the morning. With respect to the metabolic processes shown to be higher in the afternoon; Pseudoalteromonadaceae, Marinifilaceae, Colwelliaceae and have been linked to denitrification (An et al., 2021; Bowman & McMeekin, 2015; Ivanova et al., 2004; Zhou et al., 2021). Pseudoalteromonas are also known to be able to degrade lignin (Díaz-García et al., 2020; Zhu et al., 2018). These characteristics may explain why the overall community composition in the gut may not alter during the day but the abundance of certain taxa which are known for certain metabolic processes does.

Some families showed a larger abundance at the different time points (Figure 2.9), for example, there was a larger abundance of Vibrionaceae, Lentispharaceae and Colwelliaceae in August 2017 in comparison to the other dates. The presence of Vibrionaceae in August is likely a response to the bleaching event that occurred four months earlier as heat stress is shown to increase the families abundance (Kushmaro et al., 2001; Meenatchi et al., 2020). One species of Colwelliaceae, *Thalassomonas loyana,* is assumed to be a coral pathogen (Thompson et al., 2006) and may present at a higher abundance in August 2017 for the same reasons as *Vibrio*. Colwelliaceae are also known for the decomposition of organic material (Bowman, 2014). Therefore, they may be present for the dissolved organic matter that *Heliofungia* picks from the water column with its tentacles (Bos & Hoeksema, 2015) and at a higher abundance in August 2017 as *Heliofungia* still requires a larger proportion of energy from heterotrophy as symbiosis may still have been recovering at this point (Grottoli et al., 2006). Lentisphaeraceae are prevalent in marine systems but at low abundances and are chemoheterotrophic degraders which produce extracellular polysaccharides (Cho, 2014). A greater abundance of polysaccharides would be useful if, as hypothesised, *Heliofungia* is still stressed following bleaching and may have larger heterotrophic demands (Grottoli et al., 2006) that the microbes can assist with, either directly or indirectly. There is little in the coral literature with respect to the Lentisphaeraceae but, the phylum Lentispharae have been found to be abundant in recovering *Mussismilia hispida* following a disease outbreak (de Castro et al., 2010). Bacterial families seen to be more abundant in the gut in April 2018 included; Fusobacteriacaea, Marinifilaceae and Marinilabiliaceae. Marinifilaceae has been found in the

gut microbiome of sea urchins (Ketchum et al., 2021) and Marinifilaceae and Marinilabiliaceae in crab guts, suggesting that these families are part of their digestive microbiome (An et al., 2021). Fusobacteriaceae have also been observed in earthworm gut microbiomes and can digest amino acids (Zeibich et al., 2019), to further this study it would be useful to analyse the amino acids and organic matter present in the gut of *Heliofungia*. The family Fusobacteriaceae have previously been observed as part of *Porites australiensis, P. lobata* and *P. lutea* microbiome when exposed to competition stressors (Brown et al., 2019). Fusobacteriaceae are abundant in Surgeonfish and their faeces (Ezzat et al., 2019; Ezzat et al., 2021; Miyake et al., 2015) which may explain how this family entered the coral gut from particulate matter in the water column. No families stood out to be dominant in August 2018 during analysis.

2.4.8 Heliofungia contains several archaeal families

Previously it has been challenging to identify archaea in coral microbiomes but due to work by Robbins et al (2019) this was feasible in *Heliofungia* (Robbins et al., 2021; Robbins et al., 2019). In *Heliofungia* there were 11 known archaeal families and 13 unknown or uncultured families (Figure 2.12). The gut and the coral mucus contained the highest abundance of archaea, these could be caught in the mucus from the environment and then brought to the gut to either digest or aid with digestion. Unfortunately, very little is known about the roles of archaea in the coral holobiont. In the mucus the most abundant family (<4,000 counts) was Haloferaceae. There is little applicable information about Haloferaceae, it is an archaeal family that is most commonly found in high salinity environments (S. Chen et al., 2020; Gupta et al., 2015; Mani et al., 2020), its abundance drops from nearly 4,000 counts in the mucus to 1,500 in the gut, this reduction implies it does not have an associated role with the coral gut and is likely being digested from the mucus. Nitrosopumiliaceae on the other hand, were prevalent in the gut (~4000), the mucus (~1750) and the endoderm (~300). The increase in Nitrosopumiliaceae in the gut suggests that the archaea may be entering through the mouth (in the mucus or from the environment) and continue to grow in the region rather than being digested. Furthermore, the family may be being passed on from the gut to the endoderm. Nitrosopumiliaceae have been observed in numerous corals, octocorals and sponges (O'Brien et al., 2021). They are known for their ability to oxidise ammonia (Könneke et al., 2005) and have been shown to be a key part of this pathway in two sponges (Moreno-Pino et al., 2020; Robbins et al., 2021). Considering its location in the mucus, gut and endoderm, it may play a part in nitrification through ammonia oxidation and these regions all have access to ammonia from the seawater (endoderm through the coelentric cavity which is filled with seawater). In addition to this, in the giant clam urea is supplied to the nitrogen-limited

Symbiodiniaceae as the symbionts encode urease, a protein that converts urea to ammonia (Ip et al., 2020) which is another ammonia source for nitrifying bacteria, hypothetically, a similar process may occur in *Heliofungia*. Other archaeal families observed abundantly in *Heliofungia* included Halococccaceae and Halomicrobiaceae, but little is known on whether they have any association with the coral regions. Overall, the results show that the mucus, gut and endoderm, but not the ectoderm, harbour an abundant population of archaea, hypothetically this may be due to the microniche of the ectoderm being dominated by *Endozoicomonas* and the resources are not available for the archaea. Further studies should be undertaken to understand the large need these regions have for archaeal families.

2.4.9 Endozoicomonas ASVs in Heliofungia are predominantly unknown taxa

The phylogenetic tree of the *Endozoicomonas* ASVs (Figure 2.13) showed that only five out of 373 ASVs belonged to a known genus, *Oceanospirillales bacterium LUC14*, the rest were unknown. *Oceanospirillales bacterium LUC14* was seen to be on its own branch apart from the abundant unknown ASVs, suggesting that there may be an *Endozoicomonas* ASV that is *Heliofungia*-specific as shown by other corals that harbour their own specific *Endozoicomonas* ASVs (W.-M. Chen et al., 2019; Sheu et al., 2017; Tandon et al., 2018; Yang et al., 2010). Most of the *Endozoicomonas* counts were seen in the endoderm, followed closely by the ectoderm, and with lower abundance in the mucus and gut, with very little occurring in the acrosphere. *Endozoicomonas* strains have been isolated from many marine animals, ones specific to corals and sponges include species from; *Acropora* sp. (strain Acr-14 (Sheu et al., 2017; Tandon et al., 2018) and Acr-12 (W.-M. Chen et al., 2019)), *Montipora aequituberculata* (Yang et al., 2010), octocorals - *Eunicea fusca* and *Plexaura* sp. (Pike et al., 2013) and the sponge genus *Haliclona* (Bartz et al., 2018). As there were 14 ASVs that had over 1500 reads in the present study, these were selected to be compared to the other coral and sponge isolated *Endozoicomonas* sp. with *Hahella ganghwensis* as an outgroup (Figure 14), as suggested by the literature (Shiu et al., 2018; Tandon et al., 2020). The results showed that all of the *Endozoicomonas* ASVs found in *Heliofungia* clustered together on their own branch, the most similar known species being *E. montiporae* isolated from *M. aequituberculata*. Comparative genomics studies on *Endozoicomonas acroporae* identified the gene repertoires necessary for DMSP metabolism (Tandon et al., 2020), and these represent unique adaptations to life in *Acropora* hosts. It would be extremely useful to undergo such analysis in *Heliofungia* as it may identify whether the *Heliofungia* strains have unique adaptations to their host. Previously, genotyping and single-cell sequencing has been applied to study the *Endozoicomonas* communities of *S. pistillata* and *P. verrucosa* (Neave et al., 2017). Results

showed that *P. verrucosa* associates with the same *Endozoicomonas* independent of geographic location whereas *S. pistillata* contained *Endozoicomonas* with spatially distinct genotypes. This was suggested to be due to the mode of spawning; *P. verrucosa* is a broadcast spawning and *S. pistillata* is a brooder, as brooding may have a strong influence on the microbiome (Neave et al., 2017). *Heliofungia* is a broadcast spawner, acquiring its symbionts from the environment, therefore, *Heliofungia* from a different geographic location may contain different *Endozoicomonas* strains. To test this hypothesis, *Heliofungia* samples should be taken from reefs further afield and their microbiomes compared to those of other host species within the same reefs. No *Heliofungia*-specific strain of *Endozoicomonas* was shown to be region-specific, implying that the bacteria are not adapted to distinct metabolomic environments, which is consistent with the idea that they are metabolically diverse (Neave et al., 2017).

2.4.10 Large aggregates of Endozoicomonas are present in Heliofungia tissues

The *Endozoicomonas*-specific probes developed by Bayer et al (2013) and previously employed on other coral species (Damjanovic et al., 2019; Damjanovic et al., 2019) detected these bacteria in the *Heliofungia* tissues. Staining of large, 'fluffy' aggregates, seen in the *Heliofungia* endoderm (Figure 2.15), has not previously been observed in corals; however, no corals the size of *Heliofungia* have been studied using FISH before. All previous FISH work on corals has been on colonial species (Ainsworth et al., 2006; Wada et al., 2019; Wada et al., 2016) with close-packed small, interconnected polyps and lacking a visible coelentric cavity that is within the endoderm. In the images (Figure 2.15) the *Endozoicomonas* appear to be present between the coelantric cavity and the Symbiodiniaceae harboured at the ectoderm edge of the endoderm. One hypothesis could be that *Endozoicomonas* may have a role in transporting products and nutrient transfer between the two; taking nutrients from the seawater in the coelentric to the Symbiodiniaceae and waste products from them to be removed in the coelentric fluid, which corroborates with theories stated on bacteria-Symbiodiniaceae metabolic exchange (Matthews et al., 2020; Neave et al., 2017) and nutrient transfer between plants and bacteria (Croft et al., 2005; Remigi et al., 2016). Studies into the genomes of *Endozoicomonas* have already shown they contain genes that code for transport, including dicarboxylic acid transporters which are common in plant-bacteria symbiosis (Neave et al., 2017, Remigi et al., 2016), and have been hypothesised to be used in interacting with the Symbiodiniaceae (Neave et al., 2017; Neave et al., 2017). This theory should be tested using fluorescent dyes to label nutrients known to go in and out of the coral (Nielsen et al., 2018) or, develop a way to use FISH on live cells (Batani et al., 2019).

2.5 Concluding remarks

Overall, tissue-specific analysis of *Heliofungia actiniformis* has shown many new outlooks and avenues of questioning with regards to coral biology. The demonstration of tissue-specific microbiomes have ramifications on how corals respond to environmental stressors. The microbiomes of the endoderm and gut are highly diverse and variable regions that should be studied at greater length. Particularly regarding how bacteria are potentially involved with Symbiodiniaceae communication and coral digestion. The discovery that the microbiome of the ectoderm is dominated by *Endozoicomonas* and that the strain found in *Heliofungia* has not been identified in corals previously, is intriguing and warrants further investigation. The introduction of tissue layer specificity into the coral-symbiont-bacteria interaction is critical to advancing our understanding.

Chapter 3: The effect of bleaching stress on the tissue-specific microbiome

3.1 Introduction

The holobiont concept recognises that corals are metaorganisms, made up of the coral host, photosymbionts and a diverse community of microbes (Bosch & McFall-Ngai, 2011; Bourne et al., 2009; Rohwer et al., 2002). The microbial component is known as the microbiome and has been shown to be integral to coral health and metabolic functioning (Bourne et al., 2016; O'Brien et al., 2019). Inoculation experiments have shown that the microbiome is pivotal in protecting corals against disease and improving stress resilience (Glasl et al., 2016; Rosales et al., 2019). So much so that several theories have been proposed with respect to the use of microbes in boosting the immunity and heat resilience of coral which have been dubbed 'coral probiotics' (Peixoto et al., 2017; Peixoto et al., 2021; Rosado et al., 2019). For example, *Pocillopora damicornis* have been inoculated with a beneficial microbial consortium (BMC) believed to be helpful for coral health and then exposed to a heat stress and/or *Vibrio* pathogens; the BMC fragments showed little to no response to the stressors (Rosado et al., 2019). Although this area of research is still in its infancy and questions remain over the legitimacy of the process, there is still much to be gained by studying the benefits of microbes on coral health and how the microbiome alters during stress.

While the microbiome and photosymbionts are often thought of as separate components of the holobiont there is evidence to suggest that the microbiome may play a role in modulating coral bleaching in response to stress, and in subsequent recovery (Bourne et al., 2008; Rosenberg et al., 2009). When corals bleach, they lose their photosymbionts and consequently a significant amount of resources supplied to them (Grottoli et al., 2006), this (dependent on species) may lead to coral death (Baumann et al., 2014). Coral bleaching is a large threat to reef ecosystems, reducing biodiversity and destroying the habitat (Brown, 1997; Hughes, Barnes, et al., 2017; Suggett & Smith, 2020). The microbiome, as part of the holobiont, has been hypothesised to be important in either mitigating or exacerbating the stress response (Morrow et al., 2018). Rosenberg and Falkovitz (2004) showed *Vibrio shiloi* to be the causative agent of annual bleaching in *Oculina patagonica* , and attributed the pathology to the production by the bacteria of toxins that inhibit photosynthesis of Symbiodiniaceae (Rosenberg & Falkovitz, 2004). Furthermore, in Thurber et al (2009) when

colonies of *Porites compressa* were exposed to multiple types of stress (heat, nutrient, carbon and pH) the community composition of their microbiome changed, and this was also associated with an increase in microbial genes linked to virulence, chemotaxis, secondary metabolism, catabolism of energy sources and stress resistance, indicating that stress impacts the coral microbiome (Thurber et al., 2009). One way in which we can understand which microbes are important to mitigate the coral bleaching response is to observe which microbes are lost during bleaching. The mushroom coral *Fungia repanda* was exposed to a heat treatment to monitor the changes observed in the three main constituents of the coral holobiont - photosymbionts, bacteria and viruses (Nguyen-Kim et al., 2015). Significant differences in microbial community composition were identified between healthy and bleached corals, and this included an increase in pathogenic viruses under heat stress (Nguyen-Kim et al., 2015). Furthermore, the comparison of the microbiomes of several coral species (*A. muricata, A. gemmifera, P. lutea* and *Coelastrea aspera*) through 16S rRNA sequencing demonstrated that, during a natural bleaching event, the more tolerant species assessed (*P. lutea* and *C. aspera*) had greater species richness and community evenness than the less tolerant species (Gardner et al., 2019). These results implied that a bacterial diverse microbiome may assist in bleaching events (Gardner et al., 2019).

Studies on *Hydra* and *Nematostella* have shown that they contain regional microbiomes for different parts of their bodies (Bonacolta et al., 2020; Deines et al., 2020). Furthermore, studies into corals have hypothesised that the coral regions may contain set core microbiota that differ between the soft tissue, mucus and skeleton (Ainsworth et al., 2015; Hernandez-Agreda et al., 2016). These theories were shown to be true in Chapter 2 using *Heliofungia* and different regional microbiomes were identified in the mucus, ectoderm, endoderm, acrosphere and gut. To date, tissue-specificity and the microbiome have not been studied in relation to bleaching. Therefore, sequencing the tissue-specific microbiome exposed to heat stress will improve our understanding of the different roles that microbes play in the distinct functions of tissues. In a bleaching context, the changes in microbiome composition that occur in different tissues are likely to reflect tissue specific alterations in metabolic, immune and environmental factors.

Here this study proposes to define whether a natural bleaching event leads to a significant change in the tissue-specific microbiome of *Heliofungia*. As part of this, it aims to define the unique and core microbes present in the different regions during bleaching to theorise whether they may have beneficial or negative roles in the microbiome and determine what other factors may influence the microbiome. Overall, this research hopes to give the first insights into the bleached tissue-specific microbiome and lay the groundwork for further studies in this area.

3.2 Methods

3.2.1 Sample collection

Samples collected for this chapter were taken from the same individuals used to study the coral bleaching response (Chapter 4) and used the same collection and sampling methods as in Chapter 2 (“Collection and maintenance of *Heliofungia actiniformis* specimens” and “Tissue collection”). Full details of sampling locations are provided in Chapter 4, briefly; Individuals were collected from around Orpheus Island Research Station (OIRS) during the bleaching event of April 2017 and a year after on the same dates in 2018 (Figure 3.1). The specimens were placed in raceways at the station in filtered (0.2µM) seawater and left to acclimatise for a minimum of 24 hours following which, tissue dissection was performed as described in Chapter 2 and samples were snap frozen in liquid nitrogen and stored at -80°C. Five technical replicates were taken from each region (except the gut) per individual (n = 5) from both time points. Four technical replicates were taken from the morning and afternoon of the gut per each individual (n = 5), as well as five environmental samples taken from: i) the seawater surrounding the individuals when collected, ii) the raceway, iii) the autoclaved filtered seawater used for tentacle rinsing pre-collection and iv) the sterile tissue wipes used to dab excess water from the tentacles. These additional samples were used to check for contamination from the surrounding environments and sampling method. Specimen collection and sampling were undertaken under GBRMPA Permit No. G11/34573.1 and G17/39908.1.

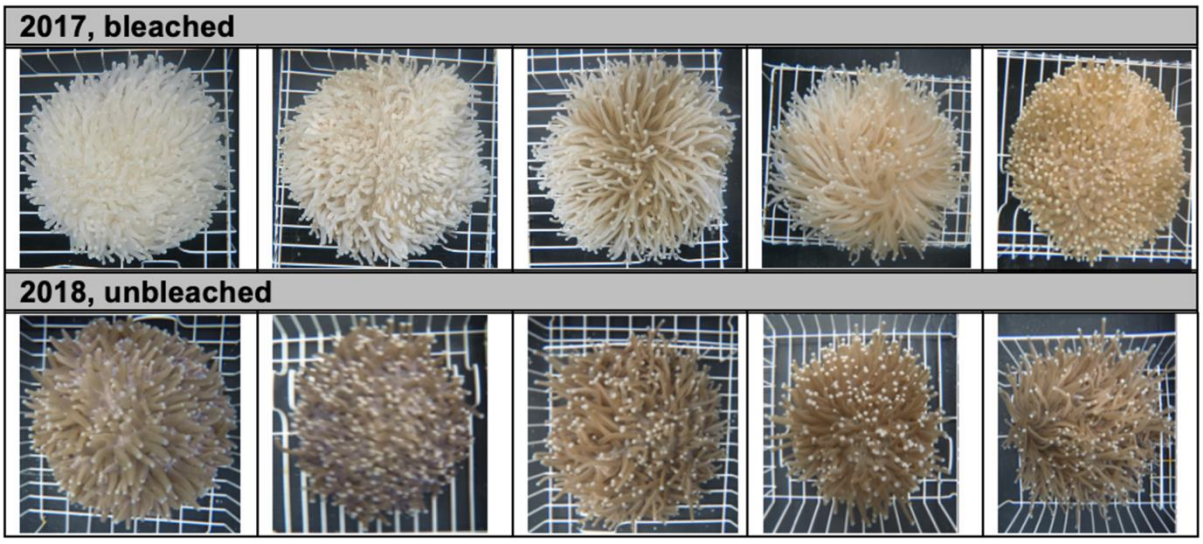


Figure 3.1: Corals sampled during the 2017 bleaching event (top row) on OIRS and a year after in 2018 when the island's population of *Heliofungia* had recovered (bottom row).

3.2.2 DNA extraction and data analysis

The same extraction method was followed as in Chapter 2; the QIAamp Micro Kit was used to extract DNA from samples collected and primers were used to identify both archaea and bacteria in the coral microbiome (see Chapter 2 methodology “Extraction of DNA” and “Library preparation and sequencing”). Following sequencing, the same workflow with QIIME2 (v 2019.7, (Bolyen et al., 2018)) and R (v 4.1.1 (Team, 2013)) were used as in Chapter 2 (for a breakdown of plug-ins, packages and plots used see Chapter 2 methodology “Statistical analysis”). Shannon diversity and jaccard PCoA results were used to calculate α- and β-diversity, acquired through QIIME2 (v 2019.7, (Bolyen et al., 2018)) features ‘alpha-rarefaction’ and ‘core-metrics-phylogenetic’ (Faith, 1992; Lozupone & Knight, 2005; Lozupone et al., 2007). A multi-way ANOVA followed by pairwise comparisons were used to determine if the α-diversity of each region was significantly different between bleached and unbleached treatments. In addition to this, Calypso (v 8.84 (Zakrzewski et al., 2017)) was used to produce non-metric dimensional scaling (NMDS) plots to determine whether genotypic grouping influenced the microbiome. The NMDS function of calypso uses the metaMDS() function from the R package vegan (Oksanen et al., 2007) and Bray-Curtis dissimilarity is used to calculate pairwise sample distances.

3.2.3 Network and core analysis

Additional to the box plot and PCoA methods used for analysis (details see Chapter 2 methodology “Statistical analysis”). The network analysis and core/unique analysis methods from Calypso (v 8.84 (Zakrzewski et al., 2017)) were also used.

Network analysis in Calypso (v 8.84 (Zakrzewski et al., 2017)) identifies co-occurring and mutually exclusive microbes based on an ensemble method using Bray-Curtis, Pearson's correlation and Spearman’s rho similarity measures. Pairwise correlations are undertaken to plot the nodes in a 2-D PCoA. The nodes represent the different genera which are coloured based on which treatment group they are present, bleached (red) and unbleached (blue), and edges represent correlation. Size of the point represents how abundant the genera are and nodes that correlate are plotted close to one another, whereas anti-correlating nodes are plotted further apart.

Core/unique analysis is a technique used to visualise the core and unique bacteria present in each region and how these change between bleached and unbleached samples. A genus is determined as ‘core’ when they are highly abundant in both treatments across replicates.

Whereas to be deemed as ‘unique’ the genus must be significantly abundant in only one of the treatment groups (bleached or unbleached). Abundance in the treatments group is calculated as significant through multiple parametric and non-parametric statistical tests (ANOVA, Bayesian test, Mann-Whitney U test, Nested ANOVA, paired t-test, t-test and Wilcoxon-rank test) (Calypso (v 8.84 (Zakrzewski et al., 2017))).

3.3 Results

3.3.1 Alpha diversity

Samples taken from each of the tissue regions were sequenced and analysed to determine the difference in community composition between April 2017 (during an in-field bleaching event) and a year after in April 2018, when the corals had recovered in the field and no longer showed signs of bleaching stress. The Shannon diversity of the different tissue regions of *Heliofungia* individuals were compared between a bleaching event and a year after the event. The Shannon diversity had a linear model fitted to it that included the independent factors; region, date and individual as fixed effects. A Tukey’s honestly significant difference (HSD) test, threshold $p < 0.05$, on the factor ‘date’ showed that the Shannon diversity of each region, except the mucus, was significantly different between the bleached and unbleached dates (Figure 3.2).

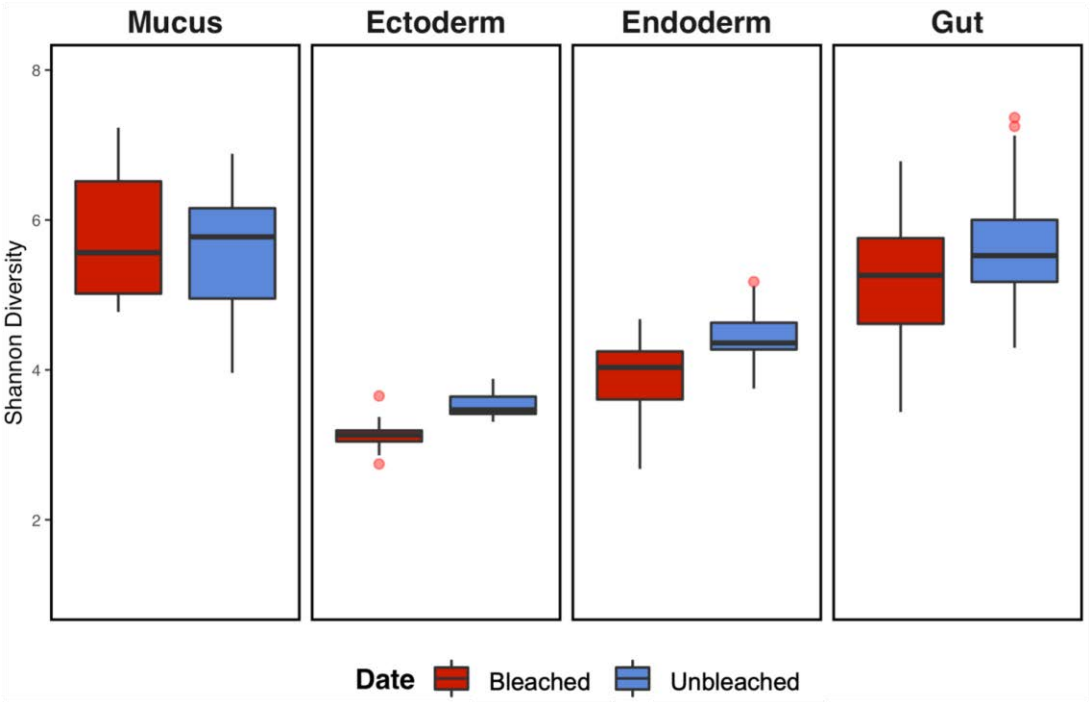


Figure 3.2: Shannon diversity box plots showing the α-diversity of each region sampled between April 2017 (bleached - red) and April 2018 (unbleached - blue). Each boxplot represents 25 points comprising five technical replicates taken from the tissues of the five individual corals at the two time points.

3.3.2 Beta diversity

The β-diversity (Jaccard PCoA) differed between the two years, similar to the α-diversity, the largest changes in community composition were in the ecto- and endoderm (Figure 3.3). The gut showed that some of the clusters overlapped in β-diversity, portrayed by the cross-over of the bleached and unbleached hulls, implying a similarity in certain samples community composition between treatments. Overall, it was apparent that the different taxa are driving the differences seen in the β-diversity.

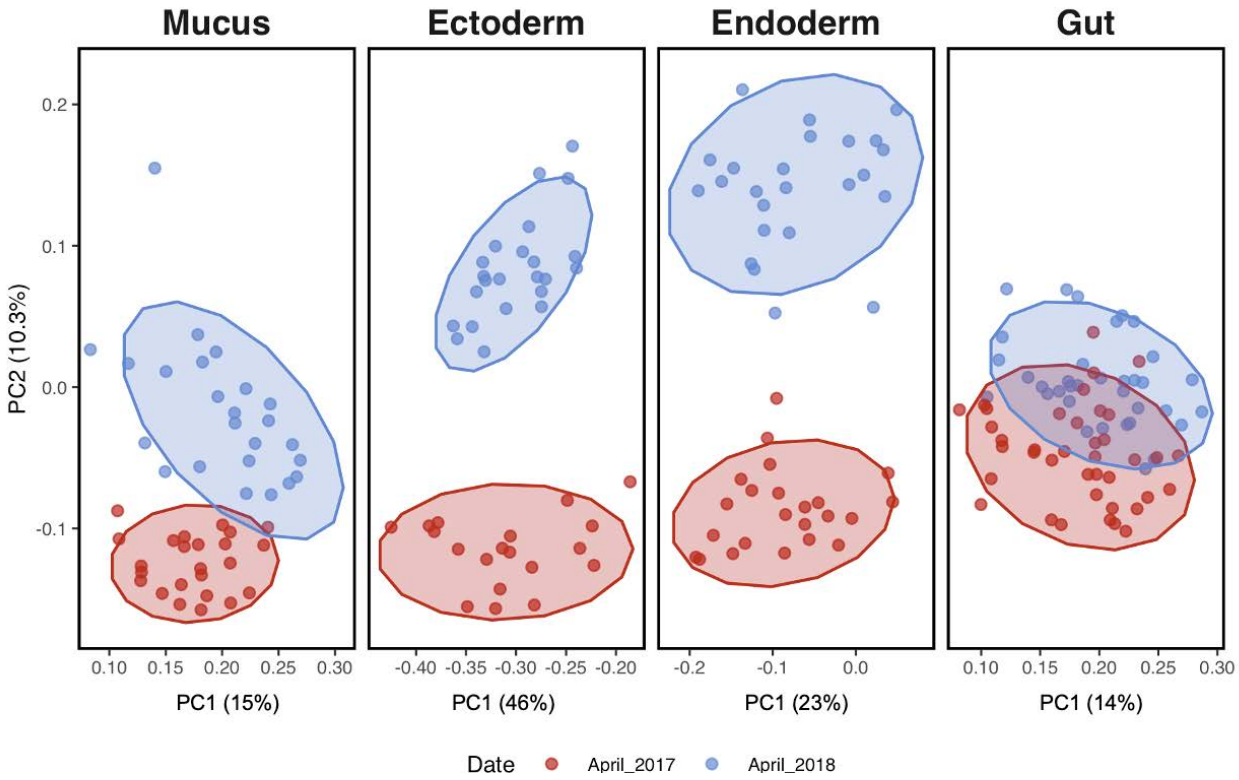


Figure 3.3: PCoAs of the β-diversity of each region sampled in *Heliofungia* during bleaching (red) and a year after a bleaching event (blue). Each point represents one of the five technical replicates taken from the five *Heliofungia* individuals per region per year. Hulls represent normal data ellipses based on multivariate confidence level from a student-t distribution, confidence level was set to 90.

3.3.3 Network analysis

Network analysis was used to determine co-occurring and exclusive genera between the bleached and unbleached individuals (Figure 3.4). Blue and red points portray genera that are abundant to either unbleached samples or bleached samples, respectively. Whereas grey points represent the genera that are abundant in both treatments and the grey lines connect taxa with highly correlated abundance across samples. The ectoderm and endoderm showed genera strongly separated by bleaching and bleaching as seen by the bright blue/red points separated in space with less connecting grey lines between. Whereas the mucus and gut microbiome showed that there were genera present associated with the 2017 and 2018 specifically (blue/red bright dots on clustering on either side of the plot) but, there was a greater number of co-occurring and less exclusive genera in the regions (as seen by the darker coloured and grey points). Furthermore, the greater number of points in the mucus and gut (in comparison to the dermal layers) showed a much larger number of connections between them, portrayed by the grey lines attached to each point. Furthermore, the size of each point represents the abundance of each genus, showing that each region had several genera in

bleached and unbleached *Heliofungia* which were high in abundance compared to the smaller points.

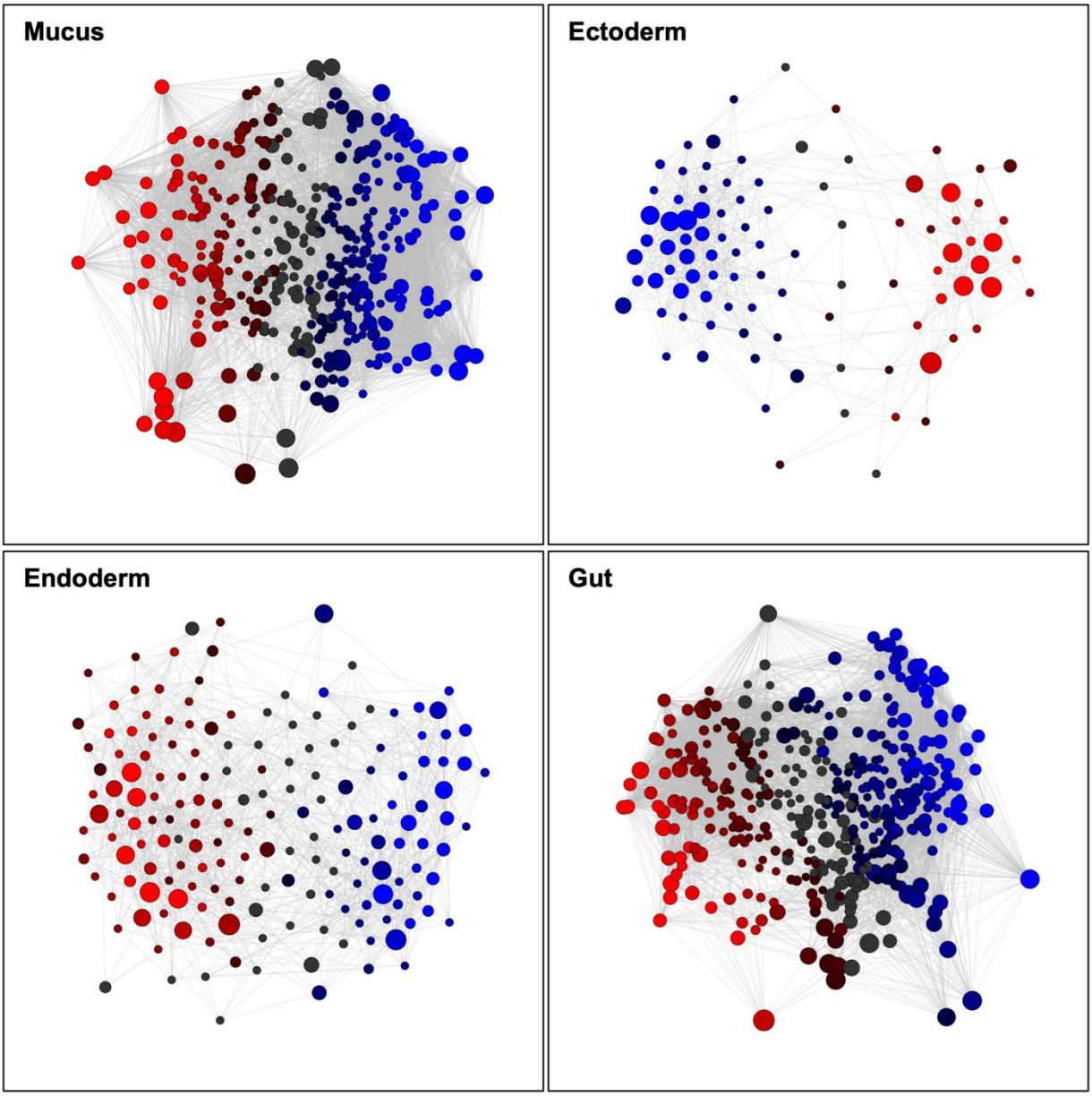


Figure 3.4: network analysis of the genera presents in the region-specific microbiomes of *Heliofungia* (mucus, ectoderm, endoderm and gut). Blue dots represent genera seen exclusively when the corals are unbleached and red is bleached, grey represents co-occurring and the darker coloured dots are genera that are less exclusive between time points. Grey lines demonstrate connections between the genera.

3.3.4 Genotype may influence microbiome

Initial analysis of the β-diversity of the microbiome showed that 'biological replicate' was a factor influencing the community composition therefore, genotype results taken from Chapter 4 (see "Key factors affecting gene expression", page 91) were used in conjunction

with the diversity results. A linear model was applied to the Shannon diversity, accounting for the independent factors; genotype, region, and individual as fixed effects. The Tukey's HSD test following this (threshold value of p <0.05), focussed on 'genotype', resulted in three groups; group 1 included genotypic group e, group 2 included genotypic groups a, b and d and, group 3 included genotypic group c, implying genotype has some influence on diversity but other factors likely influence it also. To visualise this, an NMDS (Bray-Curtis) per region was plotted. Clustering was most clear in the ectoderm, where groups c and e can be seen to have different β-diversity than a, b and d (Figure 3.5). Clustering was also present in the endoderm and mucus but with some overlap. Group d in the gut overlapped with all other groups, this implies that genotype may influence microbiome composition in the closed regions, the tissues, but the open and semi-open systems, gut and mucus, are likely influenced more by other factors like the environment.

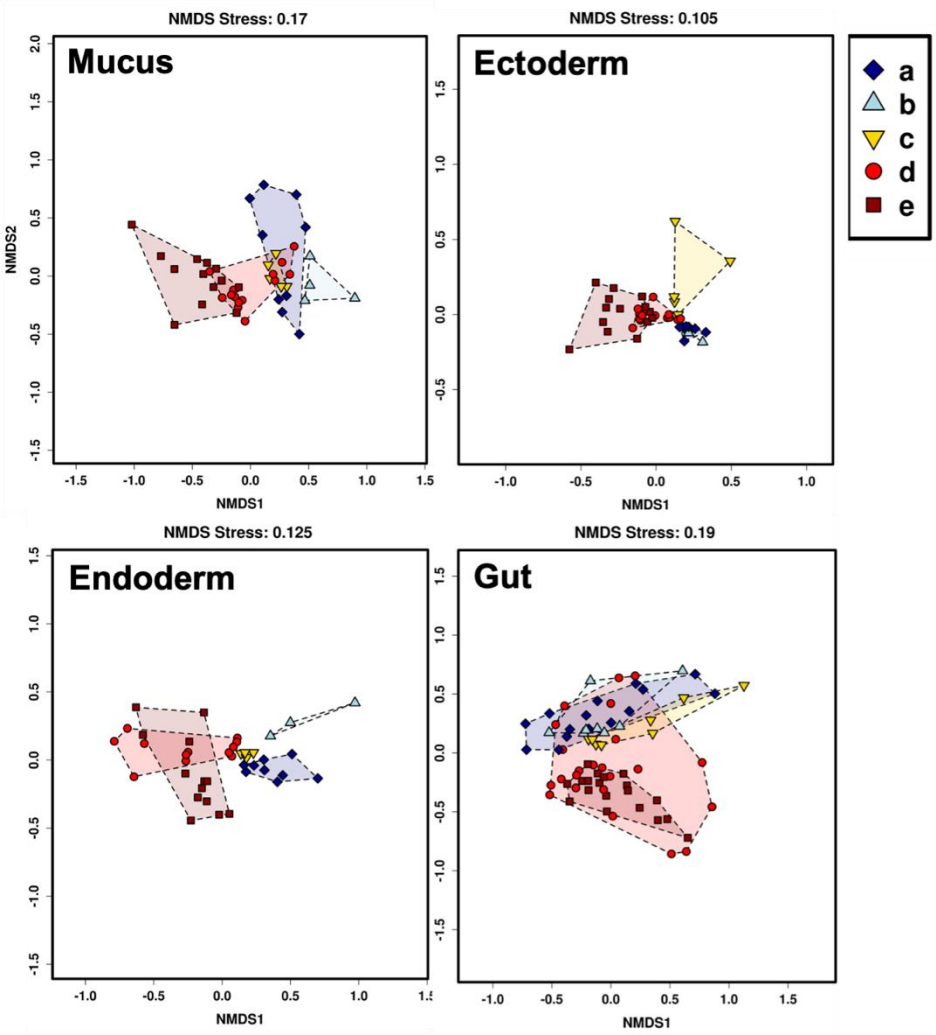


Figure 3.5: An NMDS plot using a Bray-Curtis distance matrix to plot the difference in β-diversity between samples in a 2D space, samples are clustered by genotypic grouping (data taken from Chapter 4) and faceted by region of *Heliofungia*. The points in each facet are the five technical replicates taken from each region from the 10 individual corals sampled, five were unbleached and five bleached.

3.3.5 Tissue-specific core and unique bacterial taxa

3.3.5.1 Core genera

Core genera means bacteria that are significantly abundant in both unbleached and bleached corals. The core genera presented in Table 2 are the top five abundant genera in each region per year (for a full breakdown see Table S1), the ectoderm and endoderm had less than five core genera whereas, the mucus and gut had more than five core genera, so these altered in top abundance when bleached and unbleached. In the present study, *Endozoicomonas* is the only genus found to be core in all tissue regions. In the mucus, endoderm and gut, *Endozoicomonas* shared core-genera status with other taxa, including *Candidatus Actinomarina, Pseudoalteromonas, Vibrio* and *NS5 marine group* (mucus - 2017),

Pseudoalteromonas, Synechococcus, Thalassotalea and *Vibrio* (mucus - 2018), *Synechococcus* and *Ca. Actinomarina* (endoderm), *Sphingomonas, Pseudoalteromonas, Spirochaeta* and *Vibrio* (gut - 2018) and, *Synechococcus, Sphingomonas, Spirochaeta* and *Vibrio* (gut - 2018). *Endozoicomonas* was the only core genus observed in the ectoderm which fits with the low diversity results for the tissue above.

Table 3.2: the top five most abundant bacterial genera seen to be 'core' in the tissue-specific microbiome of *Heliofungia*, during a bleaching event in 2017 and a year after in 2018. For all genera deemed 'core' in each region see Table S1.

Region	2017	2018
Mucus	*Endozoicomonas*	*Endozoicomonas*
	Candidatus Actinomarina	*Pseudoalteromonas*
	Pseudoalteromonas	*Synechococcus*
	Vibrio	*Thalassotalea*
	NS5 marine group	*Vibrio*
Ectoderm	*Endozoicomonas*	*Endozoicomonas*
Endoderm	*Endozoicomonas*	*Endozoicomonas*
	Synechococcus	*Synechococcus*
	Candidatus Actinomarina	*Candidatus Actinomarina*
Gut	*Endozoicomonas*	*Endozoicomonas*
	Sphingomonas	*Synechococcus*
	Pseudoalteromonas	*Sphingomonas*
	Spirochaeta	*Spirochaeta*
	Vibrio	*Vibrio*

3.3.5.2 Unique genera

Unique genera are bacteria observed to be significantly abundant in only one of the treatment groups, either bleached or unbleached individuals. Only the top five abundant unique genera are displayed in Figure 3.6., for a full list of unique genera see Table S3.2. The ectoderm had the lowest number of unique genera (similar to what was seen in the core results) with only three and five unique genera in bleached and unbleached corals respectively (Figure 3.6). In both the ecto- and endoderm, *Vibrio, Bacillus* and *Pseudoalteromonas* were seen to be 'unique' during bleaching. The endoderm also contained the archaea *Halococcus* and *Streptococcus* in bleached specimens. In healthy corals, both ectoderm and endoderm contained *Sphingomonas, Enhydrobacter* and *Spirochaeta* as unique genera. The ectoderm also contained *Synechococcus* and *Kistimonas* and the endoderm contained *Acinetobacter* and *Methylobacterium.* Both the mucus and the gut showed a higher level of variation amongst the top five most abundant unique genera, which is in accordance with the α- and β-diversity results above. In bleached *Heliofungia* corals, the mucus unique genera were; *NS10 marine group, Aestuariibacter, Parvulacula, Caedibacter* and *Rubritalea*. In unbleached corals, one year following the stress event, the mucus harboured *Psychrosphaera, Enhydrobacter, Litoribrevibacter, Thiomicrorhabdus* and *Arcobacter* as the five most abundant genera. In the gut; *Brevibacillus, Caedibacter, Algicola, Micrococcus* and *NS10 marine group* were unique during bleaching and, when unbleached; *Cetobacterium, Shewanella, OM27 clade, Salinisphaera* and *NS2b marine group*.

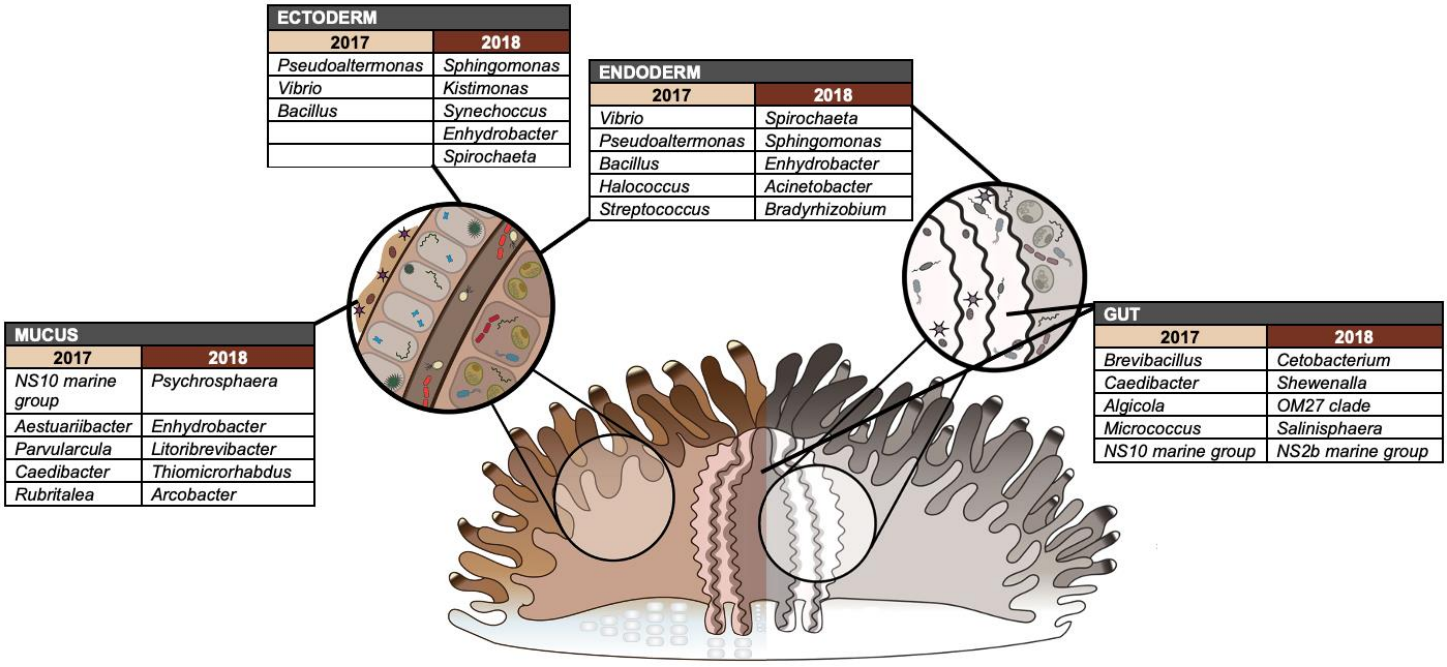


Figure 3.6: Each table represents the top five most abundant unique bacterial genera present in that region of *Heliofungia*, less than five genera means that there were not five unique genera present. Tables are divided into the bleached 2017 samples and the unbleached 2018 genera.

3.4 Discussion

The Orpheus Island population of *Heliofungia actiniformis* experienced a natural in-field bleaching event in April 2017. When the individuals were bleached, tissue regions from five individuals were sampled for microbiome analysis based on 16s rRNA sequencing. These regions included the mucus, ectoderm (outer dermal layer), endoderm (inner dermal layer) and the gut. A year after bleaching (April 2018) by which time *Heliofungia* appeared to have fully recovered, sampling was repeated at the same location. The microbiomes of each tissue region were compared to observe the significant differences between communities when bleached and a year after a bleaching event.

3.4.1 Alpha and beta diversity

Overall, the results pertaining to the α and β-diversity of the tissue-specific microbiome of *Heliofungia* demonstrate that bleaching stress significantly alters the community composition (Figures 3.2 and 3.3), the largest changes occurring in the ectoderm and endoderm; in the latter case, possibly as a consequence of the loss of the Symbiodiniaceae. The literature is divided as to whether dysbiosis leads to microbiome changes. For example, Sharp et al., (2017) stated that season has a much larger impact on microbiome composition

than loss of symbionts in symbiotic and aposymbiotic corals. Furthermore, Pogoreutz et al., (2018) showed that the core genus *Endozoicomonas* remained stable throughout experimental manipulation in *Porites verrucosa* when subjected to nutrient-induced bleaching, leading the authors to suggest that the microbiome of *P. verrucosa* is rigid and may not be able to acclimatise to environmental changes quickly. Whereas other studies have shown a significant compositional change in the coral microbiome during bleaching (Gardner et al., 2019; Koren & Rosenberg, 2006) which fits with the results seen here. One limitation of this study is that the tissue-specific microbiome under bleaching pressure have not been studied previously and it is unknown if interannual variation or local stressors are impacting the microbiome, further testing in a laboratory setting could be used to answer this. Finally, Chapter 2 of this thesis showed that there were slight variations in the tissue-specific microbiome across the time points that may likely be seasonal. However, there was still some overlap between the time points whereas the difference between bleached and unbleached regions was much more significant in these results, particularly in the ecto- and endoderm.

In the present study, the region showing least variation in community composition between bleached and unbleached individuals was the mucus. This is similar to what has been observed in *O. patagonica* (Koren & Rosenberg, 2006). This result may be due to the mucus being an open system with greater access to the surrounding environment than the other regions. The sloughing and regeneration of mucus (Ritchie, 2006) may also lead to more rapid changes in community composition. When comparing microbiomes between regions, the mucus and gut, although distinct and not overlapping, were closest to the cluster of seawater samples (Figure S3.1), implying that these regions may share some similarity with the environmental microbiome. A study on the coral *Porites lobata* presented similar findings, showing little change in the mucus community composition between bleached and healthy colonies in the Arabian sea (16S rRNA sequencing). The authors did see some changes in the unique members of the microbiome of *P. lobata* and hypothesised this was due to the need for changes in sulphur and nitrogen cycling during bleaching (Hadaidi et al., 2017), perhaps this is why a change in the unique mucus genera is observed.

The gut microbiome is likely to be influenced by the mucus microbiome because the region is a semi-open system and corals are known to eat their mucus (Brown & Bythell, 2005; Engelen et al., 2018). Genera may vary in abundance depending on the roles needed at each particular point, as well as some being more prevalent when bleached or unbleached due to more favourable growth conditions. This could explain the many connections in the gut network analysis (Figure 3.4) as opposing genera from different time points filled similar roles. There are few studies on the coral gut microbiome due to the difficulty in accessing the region

however, a recent study on azooxanthellate corals showed that the gut microbiome of *Rhizopsammia goesi* was more similar to its mucus microbiome, likely due to a substantial part of its diet being mucus (Engelen et al., 2018).

3.4.2 Other factors influencing the microbiome of Heliofungia

Studies have suggested that intraspecific variation influences the microbiome of sponges and corals (Glasl et al., 2019; van Oppen et al., 2018; Weigel & Erwin, 2016). Sampling of the base, middle and tip of branches of *Acropora cervicornis* across multiple colonies demonstrated that overall microbiome composition showed little intra-individual variation, but varied significantly between individuals (Miller et al., 2020). Furthermore, Goldsmith et al (2018) showed that sampling location influenced the microbiome in two cold-water coral species from the *Primnoa* genus and suggested that this was likely due to environmental differences and genotype. This hypothesis was further confirmed by Gong et al (2020) looking at factors most affecting heterogeneity of the coral microbiome of *Porites lutea*. The results corroborate the importance of coral genotype on the microbiome, particularly during a stress event as the genotype clustering was stronger in bleached corals (Figure 3.5). However, further testing with a larger sample size would be needed to confirm this clearly. Throughout the project samples were collected from the same dive site and dissections taken from the same tentacle area in order to minimise the effects of physical and sampling location on response. Therefore, the clustering effect observed in the individuals may also be due to Symbiodiniaceae present, previous heat exposure and even age but greater testing would be needed to confirm these.

3.4.3 Core and unique differences in regional bacterial taxa during and after a bleaching event

Before considering the core (taxa with similar high abundance in both treatments) and unique (taxa with high abundance only in bleached or unbleached corals, low to zero abundance in opposing treatment) genera in each region between bleached and unbleached individuals it is important to note that these regions were assessed individually. Rather than previous studies which have evaluated the core/unique microbes of the whole coral sample, the results above analyse what is rare/common in each region, giving a new retrospective into the coral microbiome. Therefore, this may be why some of the results highlight unique genera that have previously been categorised as core coral microbes in whole polyp studies. For

example, Pseudoalteromonas, which was shown to be unique in the bleached ecto- and endoderm but core in the mucus and gut. Similar to Bonacolta et al (2021), where the separate microniches of *Nematostella* had unique relationships (in their case, the dominance of Spirochaeta in the capitulum) that may normally be missed when sampling a whole organisms microbiome (Bonacolta et al., 2021). For a synopsis of the potential functions of the bacteria see Table S3.3.

3.4.3.1 The mucus

3.4.3.1.1 Core genera observed in the mucus

Exposure of *A. muricata* to heat stress caused major shifts in the mucus microbiome, including dominant bacteria, with noticeable increases in the abundance of a few genera including *Sphingobacteria, Flavobacteria* and *Vibrio* spp. (Lee et al., 2015); similar trends were also observed in *Heliofungia* (Figure S3.2). *Endozoicomonas, Pseudoalteromonas* and *Vibrio* were shown to be core genera (Table 3.2), similar to results in the literature (Bayer et al., 2013; Osman et al., 2020; Pogoreutz et al., 2018) and Chapter 2 results. *Vibrio* spp. are commonly found in the coral microbiome (Osman et al., 2020; Sweet & Bulling, 2017) although it was assumed *Vibrio* would not be so abundant when the corals were unbleached as the genus is more often linked to bleaching and disease in corals (Bourne et al., 2008; Kemp et al., 2018; Rubio-Portillo et al., 2020). For example, in healthy *Acropora palmata* mucus the relative abundance of *Vibrio* was less than 0.1 and around 0.25 in diseased individuals (Kemp et al., 2018), whereas the abundance in mucus of *Heliofungia* was around 0.3 both years. Potentially *Heliofungia* is not as affected by pathogenic *Vibrio* as the mucus contains other bacteria thought to be beneficial, for example, Glasl et al (2016) showed that removing *Porites astreoides* mucus bacteria (*Endozoicomonaceae* and *Oxalobacteraceae* dominant) with antibiotics led to necrosis and bleaching. The other core microbes in the mucus during bleaching were *NS5b marine group* and *Ca. Actinomarina*. *NS5b marine group* are a *Flavobacteria* that are known to degrade high molecular weight organic matter (Bennke et al., 2016) which may be useful during periods of stress and dysbiosis as an energy source. *NS5b marine group* and *Ca. Actinomarina* have been previously observed in other coral microbiomes including *Eunicella labiate* and *P. porites* (Apprill et al., 2016; Biagi et al., 2020; Bourne et al., 2008; Keller-Costa et al., 2017).

When the individuals were unbleached the five most abundant core genera in the mucus included *Thalassotalea* and *Synechococcus*, instead of *NS5 marine group* and *Ca. Actinomarina*. *Thalassotalea* spp (Table 3.2). have also previously been isolated from a range

of corals that includes *Euphyllia glabrescens* (D.-S. Sheu et al., 2018; Sheu et al., 2016), *Montipora aequituberculata* (Chen et al., 2016) and *M. capitata* (Summers et al., 2018). *Thalassotalea* are known for their ability to degrade complex natural compounds and their involvement in nutrient cycling (Kim et al., 2020). This may explain the presence of the genus in the coral mucus as they may degrade detritus caught on the coral and supply useful by-products to other microbes (Kim et al., 2020; Miller et al., 2020). *Synechococcus* was also prominent in the recovered coral mucus which was not surprising as healthy *Fungia* corals have shown to trap *Synechococcus* with their mucus (Naumann et al., 2009). In addition to this, some studies have suggested that *Synechococcus* in corals and other reef invertebrates also possess antimicrobial activity, demonstrated by the presence of microcystins and the mcyB gene (Barboza et al., 2017) but whether *Synechococcus* is associated with *Heliofungia* is unknown.

3.4.3.1.2 Unique genera observed in the mucus

In the bleached state the unique genera were identified as: the *NS10 marine group, Aestuariibacter*, *Parvularcula, Caedibacter* and *Rubritalea* (Figure 3.6)*. NS10* belongs to the *Cryomorphaceae* which are a *Flavobacteriale* and have previously been observed in sponges exposed to the 2100 climate scenario (Luter et al., 2020), implying they prefer warmer temperatures and may explain their abundance in bleached *Heliofungia. Similar to these findings, Rubritalea has only been observed in bleached Acropora tenuis (Littman et al., 2010). Aestuariibacter* has been observed in *Platygyra carnosus* colonies bearing tumours (Chiu et al., 2012), implying it prefers to inhabit stressed hosts. *Parvularcula* is more likely to be associated with the coral as a species has been isolated from a soft coral (Yu et al., 2013). It is known to be present in the mucus and tissue of *Orbicella faveolata* (Apprill et al., 2016) and as part of a coral-focussed probiotic consortium used in remediation of oil spills near reefs (Silva et al., 2021).

The five most abundant genera unique to the mucus of unbleached *Heliofungia* were; *Psychrosphaera, Enhydrobacter, Litoribrevibacter, Thiomicrorhabdus* and *Acrobacter* (Figure 3.6)*. Psychrosphaera* is closely related to the family *Pseudoalteromonas* (Pheng et al., 2017) and has been observed in high abundance in *Acropora digitifera* mucus (Gajigan et al., 2017) and in other healthy corals (Moree et al., 2014; Nissimov et al., 2009; Osman et al., 2020; Rosado et al., 2019). *Psychrosphaera* isolated from hard and soft corals have also shown to contain antimicrobial activity (Martínez-Luis et al., 2011; Nissimov et al., 2009; Shnit-Orland et al., 2012). Potentially, *Psychrosphaera* shares a similar niche role to other *Pseudoalteromonas* species explaining its abundance in the mucus as it can take advantage

of the microbes present from the seawater, possibly having an indirect knock-on effect on *Heliofungia* as a pathogen control. Conversely, Arcobacter has been linked to coral disease and hypoxic reefs (Frias-Lopez et al., 2002; Garren et al., 2009; Sweet & Bythell, 2012; Johnson et al., 2021) *Thiomicrorhabdus* has not been referenced in the coral literature previously and the only study that names it directly describes it as a sulphur oxidiser (Glavaš et al., 2018). Litoribrevibacter has been observed in coral previously (Pootakham et al., 2017) and isolated from E. glabrescens (S.-Y. Sheu et al., 2018) but little is known on its definitive function. It would be useful to analyse these bacteria further as unique genera seen in *Heliofungia* mucus to understand if they have roles in the region or if it is purely environmental.

3.4.3.2 The ectoderm

3.4.3.2.1 Core genera observed in the ectoderm

Endozoicomonas was the only core genus seen in the ectoderm (Table 3.2). This corroborates results presented in Chapter 2; *Endozoicomonas* accounted for roughly ≥90% of the ectodermal microbiome under normal conditions. Furthermore, this result aligns with previous research showing *Endozoicomonas* to be abundant and a member of the core coral microbiome (Bayer et al., 2013; Neave et al., 2017; Pogoreutz et al., 2018). Some studies link *Endozoicomonas* abundance to the presence of Symbiodiniaceae (Glasl et al., 2016; Lee et al., 2015; Ziegler et al., 2017). However, a recent dark-induced bleaching experiment showed that the relative abundance of *Endozoicomonas* is not linked to Symbiodiniaceae density in *E. glabrescens* (Shiu et al., 2020) and is more likely linked to the negative environmental conditions that induce dysbiosis. Consistent with there being no direct link between the two organisms, in *Heliofungia* Symbiodiniaceae and *Endozoicomonas* dominate two different tissue layers - the endoderm and ectoderm, respectively.

3.4.3.2.2 Unique genera observed in the ectoderm

When *Heliofungia* was bleached there were only three genera unique to the ectoderm - *Pseudoalteromonas*, *Vibrio* and *Bacillus* (Figure 3.6). *Vibrio* is likely pathogenic and its high abundance is due to the stressed state of the coral during bleaching creating a weaker immune response (Ben-Haim et al., 2003; Zhou et al., 2019). Opposingly, the antibacterial capabilities of *Pseudoalteromonas and Bacillus* (Martínez-Luis et al., 2011; Nissimov et al., 2009; Shnit-Orland et al., 2012; Chaudhari et al., 2020; Elshaghabee et al., 2017; Hashem et al., 2019) may explain their prevalence in the ectoderm during bleaching. As Bacillus isolated from various Brazilian corals, showed them to contain positive antimicrobial activity (Pereira et al.,

2017). Similar to the role of the ectoderm and glycocalyx in *Hydra*, which has been shown to protect the host from pathogen infection (Augustin & Bosch, 2010; Bosch, 2013), the coral ectoderm may house bacterial taxa that directly or indirectly reduce pathogens entering the host further. *Bacillus* also contain a multitude of various enzymes that could be useful in coral metabolism, for example, a *Bacillus* species isolated from a soft coral (*Siuaria* sp.) was shown to contain amylase capable of facilitating starch hydrolysis, producing many different malto-sugars and glucose (Puspasari et al., 2011). The *Bacillus* spp. observed in the *Heliofungia* ectoderm may be filling beneficial roles of a tissue region experiencing bleaching stress either as a defence against pathogenic opportunists or, degrading by-products for energy. Therefore, *Bacillus* should be considered in the future when determining beneficial microbes to expose unhealthy corals to and whether it has a direct role in corals should be researched further.

The unique genera in the ectoderm when unbleached (Figure 3.6) may also represent other bacteria that could be useful in a beneficial microbe mixture. *Kistimonas* belongs to the family *Endozoicomonadaceae* (Bartz et al., 2018) and has been found to be present in the microbiome of *Porites lutea* under control conditions but not in heat-stressed specimens (Pootakham et al., 2019). The genus contains genes encoded for nitrogen assimilation (Lim et al., 2019), which was a common function observed in the ectoderm as *Synechococcus* and *Spirochaeta* have been linked to nitrogen fixation in cold and warm-water corals (Cleary et al., 2021; Spiller & Shanmugam, 1987; Lawler, 2016; van de Water et al., 2016; Weiler et al., 2018). *Spirochaeta* and *Enhydrobacter* are also known for their roles in the breakdown of complex carbohydrates (W.-M. Chen et al., 2019; Cleary et al., 2021; Hespell & Canale-Parola, 1970; Premalatha et al., 2015). *Enhydrobacter* can break down carbohydrate cellulose and dietary fibre (X. Chen et al., 2019; Premalatha et al., 2015) and has been observed in other corals (Leite et al., 2018; Shore-Maggio et al., 2015; Zanotti et al., 2021).

Some of the same bacteria were observed in the ectoderm and the mucus, this may be due to the ectoderm having unicellular glands across it that secrete the mucus, as shown in *Fungia scutaria* (Lampert et al., 2006). Unlike the other genera, the role of *Sphingomonas* in the ectoderm of *Heliofungia* may be more complex. In the literature *Sphingomonas* is known for its ability to degrade contaminants like hydrocarbons and polyethylenes, inhibiting pathogenic fungi and producing sugars (Chaudhary & Kim, 2016; Kawai & Enokibara, 1996; Wachowska et al., 2013; White et al., 1996). However, it has been referred to as a coral pathogen (Rosenberg & Ben-Haim, 2002) as shown by microbial samples taken from diseased coral species (Pantos et al., 2003; Richardson et al., 1998). These studies seem inconsistent with what was seen in *Heliofungia* as all individuals sampled in 2018 seemed healthy, with no obvious signs of disease. One consideration with respect to *Sphingomonas* in *Heliofungia* is

that the corals sampled in 2018 were collected from 'The Point' at OIRS as they were placed there after sampling in 2017. The dive site is at the end of the OIRS pipeline and is an area exposed to continuous boat traffic from researchers, tourists and fisherman. Therefore, perhaps *Sphingomonas* is present due to hydrocarbons in the water from the boats, similar to the Rubritalea in the mucus; however, further testing would be needed to validate this theory.

3.4.3.3 The endoderm

3.4.3.3.1 Core genera observed in the endoderm

The endoderm of *Heliofungia* showed *Endozoicomonas, Synechococcus* and *Ca. Actinomarina* to be core microbial genera (present in high abundance in both bleached and unbleached individuals) (Table 3.2). As these genera were deemed to be core with and without the Symbiodiniaceae and, have not been obligately associated with Symbiodiniaceae previously (Camp et al., 2020; Lawson et al., 2018), it is probable that they are host-associated or environmental opportunists. As a core genus, *Synechococcus* was highly abundant in both treatments but was doubled in abundance in the unbleached endoderm. Although the genus does not have an obligate relationship with Symbiodiniaceae, perhaps it thrives in an environment with higher levels of organic molecules derived from the photosymbionts (nitrogen and sulphur metabolites) (Moore et al., 2002). Furthermore, *Synechococcus* have been shown to assimilate and utilise dimethylsulfoniopropionate (DMSP) (Malmstrom et al., 2005) which is known to be given off by Symbiodiniaceae (Lawson et al., 2020; Matthews et al., 2020), explaining the greater abundance of *Synechococcus* in the unbleached endoderm. *Ca. Actinomarina* contains genes linked to F420 coenzyme which is heavily involved in redox reactions (López-Pérez et al., 2020). Redox reactions are an important part of photosynthesis (Allen et al., 1995) and mitigating the harmful reactive oxygen species (ROS) that can occur during bleaching (Lesser, 2011; Weis, 2008). Therefore, having bacteria in the endoderm known for redox reactions would be beneficial for the Symbiodiniaceae and homeostasis, particularly during a bleaching event when antioxidant defence is required (Jones et al., 1998; Warner et al., 1999), this hypothesis may also explain the higher abundance of *Ca. Actinomarina* seen when bleached (65% *vs.* 45%). Furthermore, F420 coenzyme is a flavin derivative and Symbiodiniacaea contain flavin proteins (Shimakawa et al., 2021; Shoguchi et al., 2021) making the endoderm a hospitable tissue for the bacteria.

3.4.3.3.2 Unique genera observed in the endoderm

The microbiome of the bleached endoderm may be influenced by the absence of the Symbiodiniaceae or even, the Symbiodiniaceae species. Littman et al (2010) demonstrated that *Acropora tenuis* colonies containing *Durusdinium sp*. had a larger increase in pathogenic *Vibrio* than *A. tenuis* colonies containing *Cladicopium sp.* during in-field bleaching. Three out five *Heliofungia* individuals used in this study were *Cladicopium sp.* dominant, one was *Durusdinium sp.* dominant and the other contained both (for results; see Chapter 4). The *Cladicopium sp.* genotypic group contained the lowest abundance of *Vibrio* (Figure S3), however this was not significant. Potentially, the Symbiodiniaceae genus in *Heliofungia* influences the microbiome during bleaching; however, a larger sampling size in individuals containing various symbiont species would be needed to elucidate this further. Another pathogenic genus was *Streptococcus*. *Streptococcus* has been found in shallow water *Acropora cervicornis* colonies but not species at lower depths (Godoy-Vitorino et al., 2017), perhaps, the bacteria prefer the warmer waters of the shallow reef and may be why it is so prevalent during bleaching. Another study found increased heat stress on *Pocillopora damicornis* led to an increase in pathogens (including *Streptococcus mutans*) (Wu et al., 2019); *Heliofungia* contained three species of *Streptococcus* (*intermedius, agalactiae* and *mutans*) all of which are pathogenic (Raabe & Shane, 2019; Whiley et al., 1992; Wu et al., 2019) identifying this unique genus as a negative constituent of the bleached endoderm. These pathogenic bacteria may explain the presence of *Pseudoalteromonas* and *Bacillus* in the bleached endoderm which, as discussed in the ectoderm may be present for their, indirect or direct, beneficial, antimicrobial capabilities. The other unique genus observed in the bleached endoderm of *Heliofungia* was the archaea *Halococcus. Halococcus* rhodopsin proteins that can generate ATP in anaerobic/anoxic environments if light is available (Post, 1977). The archaea have only been found in the microbiome of bleached *M. aequituberculata* and normal *Porites cylindrica* and analysis of *Halococcus* traits implied a fermentative and oxidative role in the corals (Beleneva et al., 2005). These findings match with what was assumed from archaea associated with *Acanthastrea*, *Favia* and *Fungia* spp. by Siboni et al (2008) as they identified sequences known for oxidation of ammonia and nitrogen recycling. As *Heliofungia* is also a fungiid, it is possible that the host is taking advantage of the capabilities of *Halococcus* when bleached as the bacteria had an abundance of 40% in the bleached endoderm and <1% when unbleached.

The presence of the Symbiodiniaceae is likely to affect the microbiome of the endoderm of *Heliofungia*, as shown by the changes in the microbiome of *A. digitifera* larvae following the addition of Symbiodiniaceae (Bernasconi et al., 2019). The unique genera observed in the unbleached endoderm included *Spirochaete, Sphingomonas, Enhydrobacter, Acinetobacter*

and *Bradyrhizobium. Bradyrhizobium* is known for being involved in nitrogen cycling and a symbiotic genus that is found commonly associated with plants (Bottomley, 1992; Kaneko et al., 2002; Ormeño-Orrillo & Martínez-Romero, 2019). The genus has also been observed in; *A. millepora*, *A. cervicornis, A. muricata, Favosites speciosa, Galaxea fascicularis, Platygyra lamellina, P. damicornis* and *P. lutea* (Godoy-Vitorino et al., 2017; Gong et al., 2020; Lema et al., 2012; K. A. Lema et al., 2014) and these studies associate *Bradyrhizobium* with diazotrophs or the Symbiodiniaceae. The abundance of *Bradyrhizopium* was <1% in bleached corals and 30% in unbleached, this follows previous studies results implying an association between the genus and Symbiodiniaceae. *Spirochaetes* are abundantly observed in coral studies, they have been linked to nitrogen cycling and carbohydrate metabolism (Cleary et al., 2021; Lawler et al., 2016; van de Water et al., 2016; Weiler et al., 2018), growing prolifically in the presence of cellobiose (cellulose degraded by other organisms) (Park et al., 2013). These factors, plus the abundance of *Spirochaetes* in the bleached (10%) and unbleached (90%) endoderm, suggest an association with the Symbidiniaceae. *Enhydrobacter* is known to break down cellulose (Premalatha et al., 2015), potentially into the cellobiose *Spirochaetes* need, which may explain their 10-fold increase in abundance in the unbleached endoderm. The genus has been observed in *Montipora capitata, Acropora muricata, S. pistillata* and *Mussismilia hispida* (Leite et al., 2018; Shore-Maggio et al., 2015; Yang et al., 2020). In *M. hispida*, *Enhydrobacter* and *Acinetobacter* have shown a co-abundance relationship (Shore-Maggio et al., 2015). The genera were suggested as part of the microbial community that act as proxies for pollution and *Acinetobacter* can break down pollutants (Wang & Wang, 2018). These findings fit with the high abundance of *Sphingomonas and Rubritalea* and the hypothesis that Heliofungia contains pollutant degrading bacteria (Busse et al., 1999; Kawai & Enokibara, 1996; White et al., 1996).

3.4.3.4 The gut

3.4.3.4.1 Core genera observed in the gut

The five most abundant core genera observed in the gut when bleached included; *Endozoicomonas, Sphingomonas, Spirochaeta, Vibrio* and *Pseudoalteromonas* and, when unbleached, *Synechoccus* replaced *Pseudoalteromonas* as it was more abundant than *Pseudoalteromonas* (Table 3.2). *Endozoicomonas* has been reported in several reef fish gut microbiomes (Gao et al., 2020; Parris et al., 2016) and its presence throughout *Heliofungia* made it unsurprising to see high levels in the gut. *Synechococcus* is likely present due to its role in DMSP cycling (Malmstrom et al., 2005) and the nutrients available to it in the gut. *Synechococcus* has also been hypothesised to aid with coral heterotrophic demands during

bleaching by utilising resources from diazotrophs (Meunier et al., 2019) which may be present in the gut, as observed in the guts of termites and copepods (Desai & Brune, 2012; Golichenkov et al., 2006; Scavotto et al., 2015). The metabolic roles of *Spirochaeta* make it a likely constituent of the gut (Hespell & Canale-Parola, 1970; Lim et al., 2019), particularly as it has been observed in the gastric cavities of: whelks (Yang et al., 2019), surgeonfish (Clements & Bullivant, 1991), sea urchins (Ketchum et al., 2021; Schwob et al., 2020; Yao et al., 2019) and oysters (King et al., 2012). *Sphingomonas* were also involved in whelk (Yang et al., 2019) and fish digestion (Hyun et al., 2021; Park & Kim, 2021; Zarkasi et al., 2017) (as well as hydrocarbon digestion (Chaudhary & Kim, 2016; He et al., 2017), as discussed earlier). Also, they can break down polyethylene (Kawai & Enokibara, 1996) so, could potentially be used to biodegrade microplastics that the corals have mistaken for prey and ingested (Hall et al., 2015). Also, *Pseudoalteromonas* can degrade the agar found in algae (Romanenko et al., 2003; Vera et al., 1998), this would be useful in the coral gut as algae may be drawn into the gut via the mucus or water column which may explain its presence in the gut microbiome of *T. coccinea*, a heterotrophic feeding coral (Engelen et al., 2018). Furthermore, *Pseudoalteromonas* is known for producing exopolysaccharides (EPS) which increase marine organisms survival rates (Qin et al., 2007; Saravanan & Jayachandran, 2008) These EPS, when digested, have probiotic capabilities and have been shown to improve the health of mammal guts (Angelin & Kavitha, 2020; Ruas-Madiedo et al., 2006), this may be why antimicrobial and antipathogenic properties have been observed in corals (Sa'adah & Sabdono, 2018; Shnit-Orland et al., 2012) but further testing would be needed to examine this. These factors in regard to *Pseudoalteromonas* make it seem an extremely useful bacteria to have present in the core gut microbiome, particularly during a stress event like bleaching.

3.4.3.4.2 Unique genera observed in the gut

Brevibacillus, Caedibacter, Algicola, Micrococcus and *NS10 marine group* were the five most abundant unique genera observed in the bleached gut (Figure 3.6). *Brevibacillus* is another genus known for being a hydrocarbon and polyethylene biodegrader (Badis, 2016; Panda et al., 2014) and as a probiotic (Desjardine et al., 2007; Sanders et al., 2003). The genus has been reported in sponges, soft and hard coral (Banakar et al., 2019; ElAhwany et al., 2015; Hong et al., 2009). The *Caedibacter* genus are known for living in symbiosis with the *Paramecium tetraurelia* (Preer Jr et al., 1974) and can be beneficial or parasitic, dependent on the hosts growth conditions (Schu & Schrallhammer, 2018). One study into *Caedibacter* has used comparative genomics of symbiont-free and -containing protists to show the bacteria causes upregulation of metabolic pathways and heat shock genes (Grosser et al., 2018), which would be useful for a bleached coral. However, further study would be needed to

determine if *Caedibacter* is associated with the host or purely environmental; similar genera (100% identity) have been found in the corals *Erythropodium caribaeorum, Gorgonia ventalina* and *O. faveolata* (Schrallhammer et al., 2018). *Algicola* is another bacterium known to be associated with algae but with respect to coral disease (Becker et al., 2021; Meyer et al., 2019) implying this genus is unlikely to be involved in coral digestion and is just utilising the niche the gut provides it. *NS10 marine group* may also not have a digestive role and instead may be a food source, as it was highly abundant in the bleached mucus. *Heliofungia* may be eating its mucus (Brown & Bythell, 2005) as an additional energy source when bleached as none of the same unique genera were observed between the mucus and gut when the corals were unbleached and Symbiodiniaceae present.

The top five abundant unique genera of the gut in the unbleached *Heliofungia* were different to what was observed when they were bleached (Figure 3.6). These results imply that the digestive microbiome has shifted between the two time points and this may be due to the reestablishment of symbiosis. All the unique genera in the unbleached gut have been linked with digestion or the production of nutrients. For example, *Cetobacterium,* the most abundant unique genus observed, is prominently known for being part of the digestive microbiome of fish (Bhute et al., 2020; Tan et al., 2019), making up as much as 87- 94% of the digestive microbiota (Larsen et al., 2014; Ramírez et al., 2018). The main role of *Cetobacterium* is to produce B_{12} for the fish (Tsuchiya et al., 2008); B_{12} has been observed in the gut of *G. fascicularis* and is likely due to the bacteria in the gut (Agostini et al., 2008; Agostini et al., 2009; Agostini et al., 2012). Agostini (2009) did not undertake sequencing and *Cetobacterium* has not been reported in *G. fascicularis*. However, *Fusobacteriaceae*, the family of *Cetobacterium*, is found in microbiome studies of *Galaxea* spp (Cai, Tian, et al., 2018; Cai, Zhou, et al., 2018; Cleary et al., 2020) and the genus itself has been reported in *Madrepora oculata, O. faveolata, Lophelia pertusa* and *P. asteroides* (Meistertzheim et al., 2016; Morrow et al., 2012), suggesting a link between the genus and B_{12} production in the coral gut. Also, with respect to nutrients, *OM27 clade* has been shown to transform high molecular weight DON to usable DON (Orsi et al., 2016). The genus has been found to be prevalent in the seawater surrounding reefs and the corals themselves (Apprill et al., 2021; Biagi et al., 2020; Damjanovic et al., 2020; Weber et al., 2019). *NS2b marine group*, which have been observed in *Acropora loripes* and *M. cavernosa* (Becker et al., 2021; Damjanovic et al., 2020), have been linked to a large range of macromolecule metabolism pathways and the degradation of algal by-products (Allen et al., 2020; Díez-Vives et al., 2019) which may explain their presence in the gut as phytoplankton being digested is probable however, whether this is in association with the host or the genus utilising the resources for themself is unknown.

Shewanella form syntrophic relationships with fermentative bacteria leading to a wider range of by-products they can utilise to produce many different end-products (Nealson & Scott, 2006). This could be an extremely useful feature of a bacteria in the gut microbiome and may explain why they have been found in such abundance in the gut of lampreys (Y. Li et al., 2021). They have been found in numerous corals; *A. muricata, A. tenuis, Cladiella* sp., *Favia* sp., *P. damicornis, Tubastrea faulkneri, Sarcophyton* sp. and *Sinularia* sp. (Ceh et al., 2012; Dobretsov et al., 2015; Sabdono & Radjasa, 2006; Shnit-Orland et al., 2010; M. Sweet et al., 2011), but currently there is no evidence to support if they have a gut-specific role similar to the lamprey. The genus is also known for its use in biotechnology for the bioremediation of several chemicals and pollutants, including oil spills (Joe et al., 2019; Martín-Gil et al., 2004), which fits with other genera identified in *Heliofungia*. Alike to *Shewanella, Salinisphaera,* a genus that has been reported in the digestive tract of whelks (Yang et al., 2019), also contains an alkane hydroxylase and is used as an oil degrader (Wang et al., 2010).

3.5 Concluding remarks

The surprising finding in this study was the number of genera present known for oil bioremediation, water sampling should be carried out around the dive site to see if oil is present. As well as sampling remote *Heliofungia* to determine whether they contain these genera. It was also observed that there were more unique genera when the corals were unbleached in comparison to bleached. Furthermore, similar to previous microniche studies on cnidarians (Bonacolta et al., 2020; Deines et al., 2020), splitting the coral into tissue and regions allowed a greater level of analysis into the coral microbiome and identified unique genera that would previously have been lost in the sequencing data of a whole polyp. The endoderm and gut genera were most enlightening with respect to the changes that occur in the microbiome with and without Symbiodiniaceae. However, one of the greatest challenges with microbiome studies is to determine whether the microbes present are associated with the host and symbionts or, whether it is purely environmental, future metagenomic studies should be carried out on the regions to answer this. The bacteria in the healthy unique microbiomes highlight species that may be important in health and should be considered in future BMC work. The analysis laid out in this thesis of the separate tissue layers and the gut is a novel approach which has considerable potential to answer new and existing research questions, which in turn could potentially impact mitigation strategies for rising surface sea temperatures.

Chapter 4: Differences in molecular response to bleaching between the ectoderm and endoderm

4.1 Introduction

Coral reefs are hyperdiverse marine ecosystems that support around 25% of known marine species (Fisher et al., 2015) and provide ecosystem services of immense value to coastal people in tropical regions worldwide (Lau et al., 2021; Lester et al., 2020). Coral bleaching is a significant global threat to the health and survival of coral reefs (Hoegh-Guldberg et al., 2018). Bleaching has become more prevalent over the last half century with at least five widespread bleaching events occurring on the Great Barrier Reef (Australia) in the past decade (Hughes & Kerry, 2017; Hughes, Kerry, et al., 2017; Sully et al., 2019). There is an urgent need to understand the molecular mechanisms within the host that underlie the bleaching response, mitigation and recovery (Suggett & Smith, 2020; Voolstra et al., 2015). However, practical challenges in working with corals as subjects of molecular biology experiments have meant that progress has been limited and current models previously used are impractical for certain aspects of study.

Much of what is currently known about the molecular mechanism of coral bleaching comes from 'omics studies on corals and cnidarian models such as the sea anemone *Aiptasia* (Cleves et al., 2020; Seneca et al., 2010; Tortorelli et al., 2020). Results from these studies suggest that excessive heat exposure leads to overproduction of reactive oxygen species (ROS) by the symbiont which in turn causes oxidative stress in the host (Figure 4.1) (Lesser, 2006; Smith et al., 2005). The damage in the host, resulting from excessive ROS, is thought to lead to an increasingly toxic internal environment in the host, and to mitigate this the Symbiodiniaceae are either expelled or engulfed, although it is unknown which party instigates the removal (Downs et al., 2002; Oakley & Davy, 2018; Smith et al., 2005). Corals undergoing oxidative stress present high levels of antioxidant enzymes and other protective proteins, presumably to combat the increasing ROS concentration (Downs et al., 2002).

Some recent studies have questioned the role of ROS as the primary trigger of dysbiosis (Szabó et al., 2020). This is due to results from these studies suggesting that heat-induced ROS does not severely damage the endosymbionts and that the PSI should have the necessary protective mechanisms to mitigate oxidative stress. Instead, researchers have proposed that symbiont expulsion may likely be due to secondary messenger cascades

leading to exocytosis and/or a metabolic stress response (Nielsen et al., 2018; Szabó et al., 2020). However, these theories are still inconclusive and further study on a comprehensive coral model and the consequences of dysbiosis is needed.

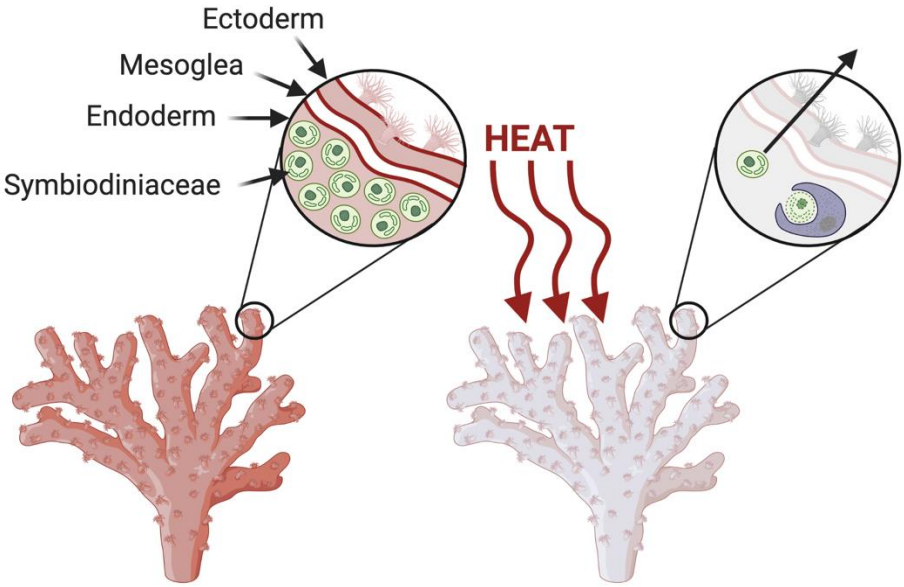


Figure 4.1: The two tissue layers of corals (ecto- and endoderm) split by the mesoglea, the Symbiodiniaceae reside in the endoderm. During heat stress the Symbiodiniaceae are either expelled or engulfed by the host, figure made with Biorender (https://biorender.com/).

In addition to the molecular processes thought to initiate the bleaching response, a range of other functional groups of genes are now thought to be associated with bleaching. This includes heat-shock proteins (HSPs), apoptotic machinery, antioxidant defence and host energy storage, all of which include genes that show consistent differential expression in association with bleaching. Multiple studies across a range of species, (Acroporiids, *Montastraea*, *Stylophora*, *Porites*), have shown that when whole coral fragments are exposed to heat stress, the expression of heat-shock proteins (HSPs) is consistently upregulated (DeSalvo et al., 2008; Louis et al., 2020; Seveso et al., 2020; Traylor-Knowles et al., 2017). Antioxidant defence proteins are commonly seen to be expressed alongside HSPs. When *A. millepora* was exposed to rising thermal stress in the lab four genes; HSP70, ferritin, Zn^{2+}-metalloprotease and MnSOD, were targeted and showed significant regulation changes in response to oxidative stress (Császár et al., 2009). Antioxidant gene defence is a host and Symbiodiniaceae response as multiple studies have shown both to increase expression under heat stress. Common responses include; ion superoxide dismutase, catalase and various peroxidases (Higuchi et al., 2008; Krueger, Fisher, et al., 2015; Krueger, Hawkins, et al., 2015). Another key group of genes seen to be enriched in response to bleaching and oxidative stress is that of apoptotic machinery. The transcriptome of *A. millepora* has been used to

identify pro- and anti-apoptotic gene repertoires in corals showing them to be conserved from corals to higher metazoans and therefore, likely to be vital in combating cellular stress (Moya et al., 2016). Furthermore, in *Stylophora* and *Pocillopora* colonies (amongst others) manipulative heat stress over varying time frames consistently demonstrated an upregulation of apoptosis-related genes (Kvitt et al., 2011; Li et al., 2020). It has been suggested that the speed at which the coral can respond to the onset of apoptosis is a key factor for coral recovery (Kvitt et al., 2011; Li et al., 2020; Maor-Landaw et al., 2014).

The processes behind bleaching are still unclear, particularly whether the host or symbiont initiates expulsion. Studies based on whole coral samples have proposed numerous theories without definitive conclusions (Alves Monteiro et al., 2020; Bayer et al., 2012; Liu et al., 2018; Vidal-Dupiol et al., 2009). Therefore, research has moved towards understanding the mechanisms behind symbiosis in the hope that defining this process will aid in uncovering what is lost during dysbiosis (Bernasconi et al., 2019; Byler et al., 2013; Wolfowicz et al., 2016). One area in particular relates to the host immune response and arrested phagocytosis. Mohamed et al (2016 & 2019) used RNAseq methods to identify differentially expressed gene clusters involved in symbiosis, the most prominent findings included; the use of immunosuppression, an early-arrested phagosome as the symbiosome in the host and an upregulation of metabolism genes in the symbionts (Mohamed et al., 2016; Mohamed et al., 2019). Also considering metabolism, how resilient the coral host is during and following a bleaching episode is likely to depend on the amount of energy stored by the coral (Anthony et al., 2009), how dependent that species is on autotrophy (Houlbreque & Ferrier-Pagès, 2009; Hughes & Grottoli, 2013) and whether it is able to catabolize lipids when under metabolic stress (Grottoli & Rodrigues, 2011). The studies to date have been undertaken on whole coral samples and have highlighted the importance of these functional gene groups in coral survival and how complex the bleaching response is.

One factor that limits the ability of most ‘omics’ studies to provide clear insights into the molecular mechanisms of bleaching is the likely heterogeneity of responses between different tissues and the fact that this is not captured by typical sampling techniques that homogenise entire polyps or colony fragments. The fact that the Symbiodiniaceae are restricted to the coral endoderm (also known as the gastrodermis) suggests that this host tissue region is likely to exhibit gene expression changes related to both bleaching itself and its proximate cause (heat stress) whereas the ectoderm, should respond largely to the stress alone. This distinction between tissue layers makes interpretation of studies on whole polyps difficult, however the majority of transcriptomics studies on heat stress and bleaching to date have used whole polyps (DeSalvo et al., 2010; Seneca & Palumbi, 2015; Thomas & Palumbi, 2017). This is

because the small, interconnected polyps that characterise most well-studied corals, such as *Acropora*, *Porites* and *Stylophora* species, render them unsuitable for investigation of tissue-specific responses (Kenkel et al., 2011; Maor-Landaw et al., 2014; Seneca et al., 2010). The mushroom coral, *Heliofungia actiniformis* (henceforth referred to as *Heliofungia*) offers a potential solution to this long-standing issue. *Heliofungia* individuals are large (up to 33 cm in diameter; (Bos & Hoeksema, 2015)) single polyps that have permanently extended tentacles and are unattached. Due to its size and outstretched tentacles, a dissection method developed by Richier (2003) for sea anemones can be applied to *Heliofungia*, allowing fractionation of the two tissue layers (ecto- and endoderm) (Moya et al., 2012; Richier et al., 2003).

The ability to separate the tissues of *Heliofungia* provides an opportunity to improve our understanding of bleaching by allowing the separate responses of tissues with (endoderm) and without (ectoderm) symbionts to be compared and contrasted. As far as we are aware, no studies to date have examined tissue-specific responses to coral bleaching. Here I propose that during a natural bleaching event, the greatest change in gene expression will be seen in the endoderm due to the presence, or lack of, Symbiodiniaceae and there will be a significant increase in molecular mechanisms related to immunity, apoptosis, and catabolism in *Heliofungia* with tissue-specific responses observable. Furthermore, separating the tissues will facilitate understanding into which genes are thermal stress-specific (shown by expression in both regions) and which are related to symbiosis/dysbiosis (expressed in the endoderm only). In this study, tissue separation of *Heliofungia* during the 2016 bleaching event will facilitate us to study these aims.

4.2 Methods

4.2.1 Sample collection

In April 2017 significant, widespread bleaching was observed throughout the coral reefs of the Palm Island group (Hughes & Kerry, 2017, Williamson et al., 2019) which included *Heliofungia*. During this event, five bleached individuals were collected from Little Pioneer Bay (Figure 4.2) and sampled on the 15th and 16th of April. After sampling, the individuals were returned beneath the marker buoy at The Point (Figure 4.2). On the 15th and 16th of April 2018 - one year after the bleaching event (Table 4.1) - sampling was conducted at The Point with the intention to re-collect the same five individuals that were sampled in 2017 (GBRMPA Permit No. G11/34573.1 and G17/39908.1).

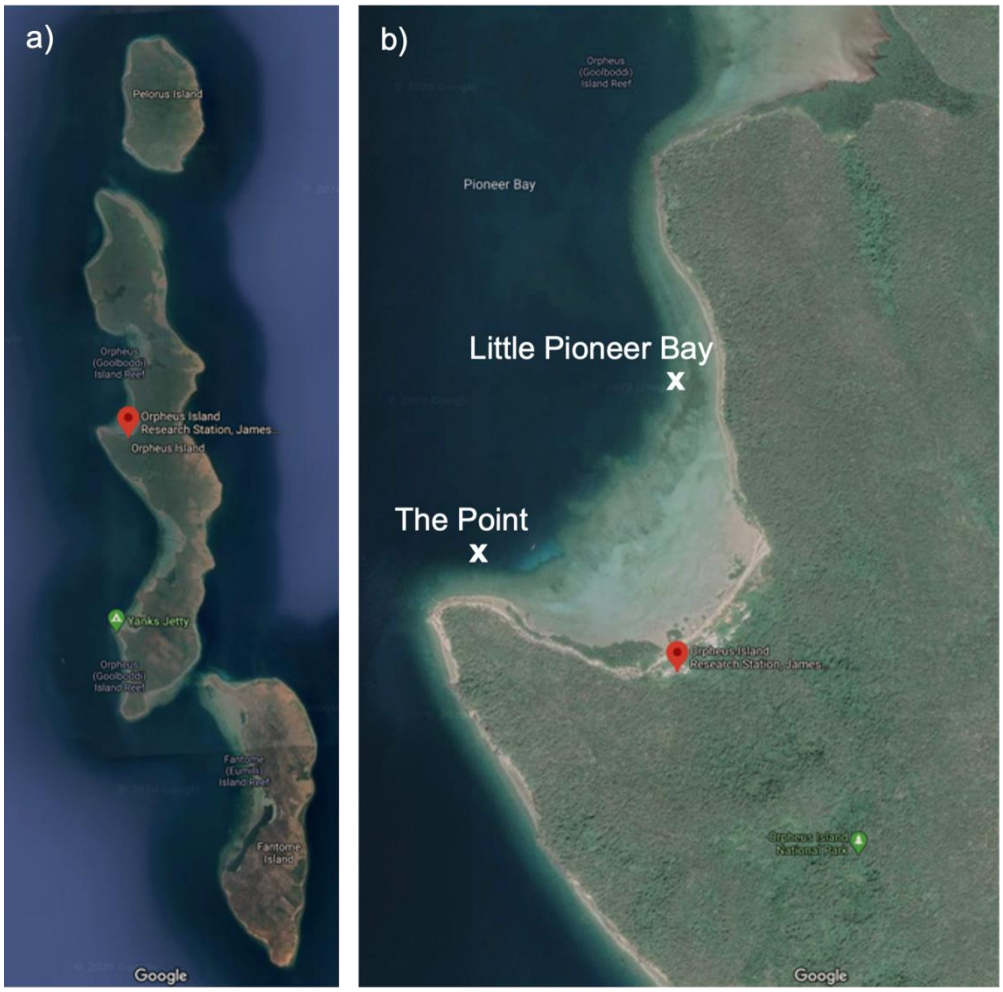


Figure 4.2: screenshots from Google Maps used to show the collection locations of the *Heliofungia* individuals. a) shows three of the islands in the Palm Island group (Orpheus Island is the middle island in, indicated by the red marker) and, b) is a close-up of Orpheus Island (red marker indicates Orpheus Island Research Station (OIRS)). Bleached specimens were collected in April 2017 from Little Pioneer Bay (b)) and placed under the marker buoy at The Point afterwards with the intention to re-sample them in April 2018 (b)).

Samples were collected by scuba and the five individuals were brought back to OIRS and placed in a raceway. The raceways were filled with filtered seawater brought in from the reef to match environmental parameters and tentacles were taken for dissection (methodology, see chapter 3). Dissection was performed on ice to reduce transcriptomic change and occurred during the same time window on consecutive days to decrease the effect of circadian rhythm on gene expression (see Table 4.1. for sampling timings). From each *Heliofungia* specimen (Figure 4.3), five tentacles were cut from each individual with sterilised scissors and then dissected to separate the ectoderm and the endoderm regions. Thus 25 ectoderm samples and 25 endoderm samples were taken in 2017 and again in 2018. The separated tissues were placed in sterile cryotubes and snap frozen in liquid nitrogen; they were then

stored at -80°C at JCU. This research was undertaken under the GBRMPA Permits No. G11/34573.1 and G17/39908.1.

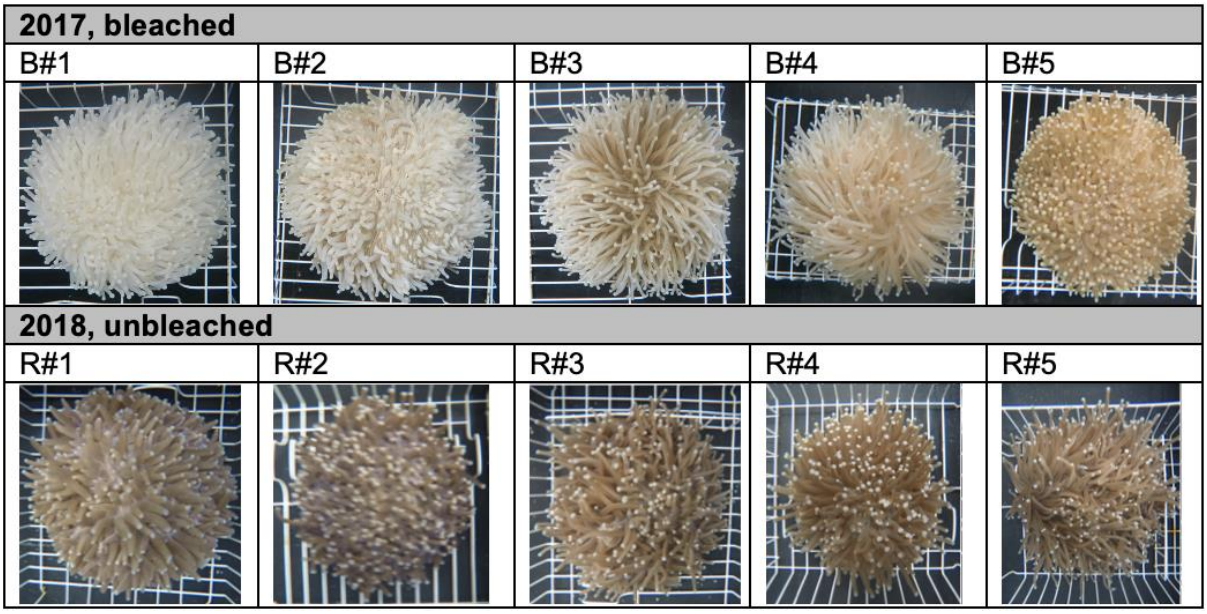


Figure 4.3: Middle row - the five *Heliofungia* sampled during the 2017 bleaching event. Bottom row - the five unbleached individuals sampled in April 2018.

Table 4.1. sampling time points for the tissue-specific host response to coral bleaching.

Sampling period	Date	Individual	Tissue	Time period
Bleached	15/04/17	All	Whole tentacle	16:00 - 16:30
	15/04/17	#1 & #4	Ecto- and Endoderm	16:30 – 18:00
	16/04/17	#2, #3 & #5	Ecto- and Endoderm	16:30 – 18:00
Unbleached	15/04/18	All	Whole tentacle	15:30 - 16:00
	15/04/18	#1 & #5	Ecto- and Endoderm	16:30 – 18:00
	16/04/18	#2, #3 & #5	Ecto- and Endoderm	16:30 – 18:00

4.2.2 RNA extraction

For each biological replicate and tissue a single RNA sample was prepared resulting in a total of 20 samples for sequencing. To ensure sufficient yield, ectoderm samples were prepared by pooling three technical replicates (taken from tentacles of the same colony) and for endoderm samples pools were made from two technical replicates. Homogenization was performed by adding 600µl of RLT buffer and half a tube of 1.4mm ceramic beads to the cryotube containing the sample, bead beating was undertaken at 5.0s (FastPrep-24) for three periods of 10s on and 15s rest. The 'RNeasy Mini Kit' (Qiagen) protocol for the 'Purification of Total RNA from Animal Tissues', including 'Appendix D', was followed with certain alterations (supplementary information). Spectrophotometry (Nanodrop 1000, ThermoFisher

Scientific) was used as an RNA quality check to ascertain there were no contaminants present, and the Qubit (ThermoFisher) determined each sample's quantity. Before sequencing, the samples were run in a gel using the Tapestation (2200, Agilent) to check that they met quality standards required for Illumina sequencing; 2-3µg of RNA per sample with a concentration of ≥100ng/µl, RIN value of ≥ 8.0 and an A260/280 ratio of 1.8 – 2.0.

4.2.3 Library preparation and sequencing

Samples were sent to the Australian Genome Research Facility (AGRF) for library preparation and sequencing. Sequencing libraries were prepared using the TruSeq stranded mRNA protocol with unique dual indices. Sequencing was performed on a single S2 flowcell of a NovaSeq 6000 resulting in a total of 708 million reads, which equated to approximately 35.4 million 100bp single-end reads per sample (+/- 8.61×10^5 or, on average 3.83 Gb +/- 0.08 Gb). AGRF performed base calling and demultiplexing and provided data as raw fastq files.

4.2.4 Sequencing QC and Alignment

Trimmomatic (v 0.38; (Bolger et al., 2014)) was used to trim adapters from the raw reads and fastQC (v 0.11.7; (Andrews, 2017)) was used to calculate basic read quality metrics for each sample. These fastqc reports were combined using multiqc (v 1.8; (Ewels et al., 2016)) package so all samples could be compared with one another and could be checked for any discrepancies. Following the quality control (qc) checks, a combined host and symbiont reference transcriptome database was created to be used for alignment and mapping of the trimmed reads. Reference genomes from representative genera of Symbiodiniaceae including *Cladocopium* and *Fugacium* (both from 'Reef Genomics', http://symbs.reefgenomics.org/download/), *Symbiodinium* (from 'Genbank', https://www.ncbi.nlm.nih.gov/genome/51771), *Durusdinium* (from https://www.ddbj.nig.ac.jp/index-e.html, TSA GAFP01000001) and *Brevioloum* (from OIST, https://github.com/iracooke/atenuis_wgs_pub/blob/master/08_metagenome.md) were combined with a reference transcriptome assembled from RNA sequencing of endoderm, ectoderm and skeleton fractions of *Heliofungia* (Moya et al., ~). Taxonomic identities were encoded into sequence names in this database using bioawk (v 20110810 (Li, 2017)). Bowtie2 (v 2.4.1, (Langmead & Salzberg, 2012)) was used to map reads to the database and samtools (v 1.7; (Li et al., 2009)) was used to split aligned reads into separate files based on the species corresponding to the primary alignment of each read.

4.2.5 Genotyping and relationship analysis

Genotyping was undertaken by adapting the Genome Analysis Toolkit (GATk, gatk4/v 4.1.7.0, (Poplin et al., 2018)) best practices pipeline for RNA-seq (https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-Workflows) to accomodate a non-model organism. Firstly, the fastq reads from *Heliofungia* were aligned to the draft reference genome (Cooke et al., in prep; see http://coralreefgenomes.jcu.edu.au/ for a genome browser) using STAR (v 2.5.4a; (Wang et al., 2013)). Picard (v 2.22.0, http://broadinstitute.github.io/picard) and the gatk package then merged the unmapped reads to the aligned ones and marked the duplicates present. These alignments then needed to be reformatted to account for the fact that RNA aligners are different to DNA ones by splitting the reads with skipped regions in the CIGAR (Compact Idiosyncratic Gapped Alignment Report) string and generating multiple supplementary alignments, account for mismatch overhangs and reassign the mapping qualities to match with the DNA properties; this was achieved using an additional fasta index (samtools/v 1.7, (Li et al., 2009)) and the gatk package function 'SplitNCigarReads'. Haplotype calling could then be undertaken using gatk's function 'HaplotypeCaller' which can call SNPs and indels together using a local *de-novo* haplotype assembly. HaplotypeCaller is better for more accurately calling regions with difficult aspects and indels which is necessary in an under-studied organism like *Heliofungia* (Poplin et al., 2018). The output from this pipeline can then all be merged into one dataset and input into R using the package vcfR. Packages poppr (v 2.9.2, (Kamvar et al., 2014)), ape (v 5.5, (Paradis & Schliep, 2019)), tidyverse (v 1.3.1, (Wölfer et al., 2014)) and vcfR (v 1.12.0, (Knaus & Grünwald, 2017)) were then used to create a distance matrix and phylogenetic tree of the different *Heliofungia* individuals.

4.2.6 Host and symbiont read separation

A significant GC shift was detected in fastQC (v 0.11.7, (Andrews, 2017)) analysis of the 2018 endoderm samples (Figure S4.1), where the unbleached endoderm samples had a higher GC content (54%) than the ectoderm and bleached endoderm (43%). This GC bias was likely due to the presence of the symbionts in the unbleached endoderm and disappeared after the removal of symbiont reads (see 'Sequencing QC and alignment') giving an average of 42% GC content for each sample. Similarly, the samples from the before-bleaching time point showed a GC content of 41% once the symbiont reads were removed.

4.2.7 Differential gene expression

Alignments were used to calculate transcript level read counts using RSEM (v 1.3.1; (Li & Dewey, 2011)). RSEM outputs were then imported and converted to gene level counts using the R package tximport. DESeq2 (v 1.26.0, (Love et al., 2014)) was then used to generate variance stabilised expression values suitable for exploratory analysis with PCA. Formal tests for differential expression between treatments were performed using the R package LIMMA (v 3.42.2, (Ritchie et al., 2015)). The function limma-trend was used to model gene expression differences between treatments and tissues whilst accounting for correlation between samples that came from the same genotype. This was done by fitting a model with treatment, tissue and their interaction as fixed effects while using the duplicateCorrelation function to account for genotype as a random effect. The gene counts were combined with a design matrix to create three coefficients/variables which include; the treatment group (bleached vs. unbleached corals), the tissue group (ectoderm vs. endoderm) and the interaction group (the combined effect of both tissue and treatment). The gene counts were filtered and normalised before undergoing differential expression analysis. Tables were produced for each group of significantly DEG (adjusted p = ≤0.05) consisting of gene ID, log-fold change, average expression, p value and adjusted p value.

4.2.8 Functional annotation of the *Heliofungia* transcriptome

Functional information was obtained for transcripts in the *Heliofungia* reference transcriptome (Moya et al in-prep) through a suite of homology-based analyses closely mirroring the Trinotate pipeline (https://trinotate.github.io). Files required for Trinotate included the Uniprot protein sequences obtained from Swissprot and the Pfam database. Additional to this, several cnidarian reference databases were incorporated into the annotation report via Trinotate; these came from previous work undertaken with or, in the Miller Lab and included sequences from a corallimorph, *Acropora millepora* and *Acropora digitifera* and also *Nematostella* taken from Sebe-Pedros (2018).

4.2.9 Gene ontology enrichment and pathway analysis

ClusterProfiler (v 3.14.3, (Yu et al., 2012)) was used to undertake gene ontology (GO) enrichment analysis. Tables from LIMMA (v 3.42.2, (Ritchie et al., 2015)) containing the significant Differentially Expressed Genes (DEG) from each group (treatment, tissue and

interaction) were imported to be used in analysis. Firstly, the annotation table was imported to make a vector of all the gene IDs and the GO IDs present in the dataset. This vector was separated out into three for each GO classifications; molecular function (MF), biological process (BP) and cellular component (CC). Another vector was created which included the GO IDs and the terms these IDs refer to (http://geneontology.org/docs/download-ontology/) and a final vector containing only one column of gene ID. The list of significant DEG from each group was split into positive or negative log-fold change and all three groups were merged creating a significant gene list for all groups. These vectors and lists were then used in the 'enricher' function in clusterProfiler to obtain GO enrichment.

4.3 Results and discussion

A key assumption in the experimental design for this study is that the primary difference between corals collected in 2018 vs. 2017 is their bleaching status. An attempt was made to control for genotype by collecting corals from underneath the marker buoy at The Point in April 2018 with the intention of resampling the same individuals sampled in April 2017; however as shown in Figure 4, this turned out not to be true for most individuals. Our subsequent analysis of bleached versus unbleached corals is therefore based on the assumption that gene expression differences between (mostly) independent samples of corals in 2017 versus 2018 represent differences due to bleaching. It is also important to note that although 2018 individuals had healthy populations of symbionts (unbleached) it is highly likely that this represents a state of recovery from bleaching. This is supported by the very high prevalence of bleaching in the Palm Island group in 2017 (Hughes & Kerry, 2017; Hughes, Kerry, et al., 2017; Williamson et al., 2019). In addition to this, bleaching surveys were undertaken around the Palm Island group in April 2017 and no unbleached *Heliofungia* individuals were sighted during these observational dives, making it highly likely that corals sampled in 2018 would have bleached in 2017 (Normile, 2017). All corals sampled in 2018 appeared to have recovered fully from the stress event (Figure 4.2).

Although it is clear that individuals sampled in 2017 and 2018 differed in their bleaching status (Figure 4.2) it is important to acknowledge the possibility that other confounding factors such as differences between sampling sites (little pioneer bay; 2017 vs The Point 2018) may have contributed to differences between these samples. While acknowledging these caveats we use terminology related to bleaching when referring to 2017 and 2018 samples in presenting our results. The difference between 2017 and 2018 samples will be referred to as the

"bleaching" effect, and we refer to 2017 and 2018 samples as "bleached" vs "unbleached" respectively.

4.3.1 Key factors affecting gene expression

Principal component analysis (PCA) was used to determine which factors had the strongest influence on the gene response of the 20 samples. When all the samples were compared, it was apparent that differences between tissue (ectoderm vs. endoderm) explained the majority of variation in gene expression. Samples fell into two distinct clusters along PC1 in Figure 4.4 which explains 66% of variation. The treatment (bleached vs unbleached) effect did not result in clear clusters but showed some structure in relation to PC2 (9% of variation). In addition to these factors of interest it was postulated that host genetics could be structuring gene expression and therefore, the samples were genotyped.

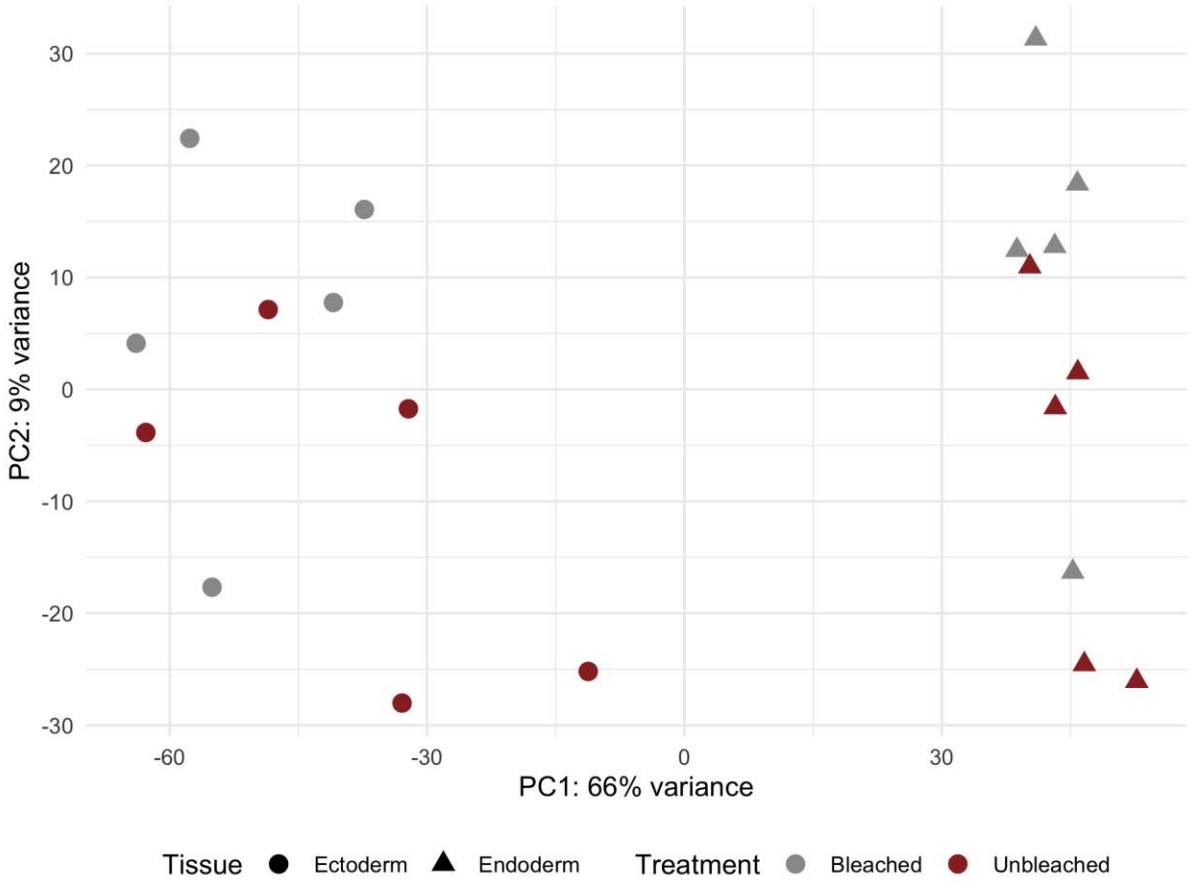


Figure 4.4: PCA plot showing the clustering effects placed on the differential gene expression of *Heliofungia* by the different tissue regions (shape) and during a bleaching event (grey) and one year after (red), n = 20.

Sample genotyping identified five distinct genetic groups (Figure 4.5). Two individuals sampled in 2017 belonged to their own group (a and c). Branch lengths within other groups were of a similar length to branches in groups a and c, indicating that whilst individuals in groups b, d and e are not the same, they are likely to be close relatives. Except for a single bleached individual in group e, it is unlikely that samples from 2017 were re-sampled in 2018.

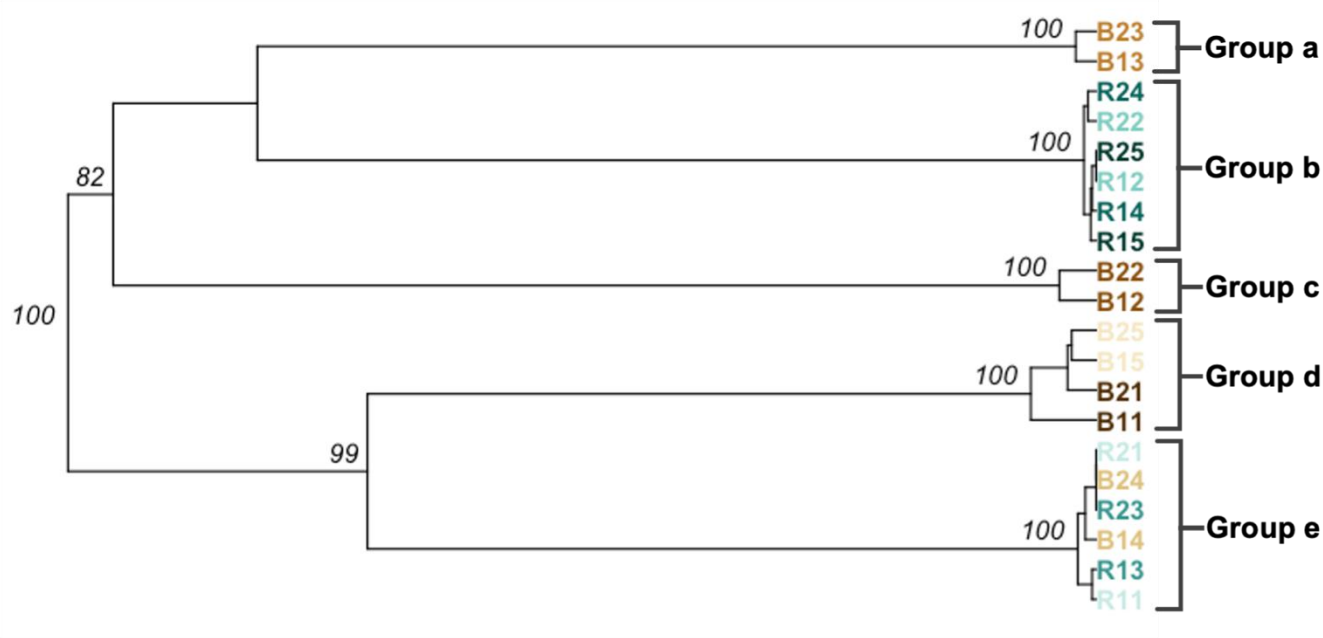


Figure 4.5: an upgma tree based on bitwise genetic distances between samples calculated using the aboot function from the poppr package in R. Bootstrap support values are shown for all nodes where support was >50%. The individuals are represented by a different colour; 'B' = 2017 and 'R' = 2018, the first digit stands for the particular tissue; 1 = ectoderm and 2 = endoderm and second digit is the replication number/the individual; 1 - 5 = the number of the individual sampled therefore, B23 is the bleached endoderm from individual number three.

Overall, however, the genetic analysis demonstrated unexpectedly high relatedness between separate individuals. To investigate whether this relatedness affected gene expression a PCA per tissue was plotted to see genotypic group and treatment effect (Figure 4.6). Treatment exhibited a clustering effect on the gene expression of *Heliofungia* as no overlap was seen between the two bleaching treatment groups (shown by colour), particularly in the endoderm. Importantly, the genotype group also appeared to influence gene expression with samples from the same group appearing clustered together.

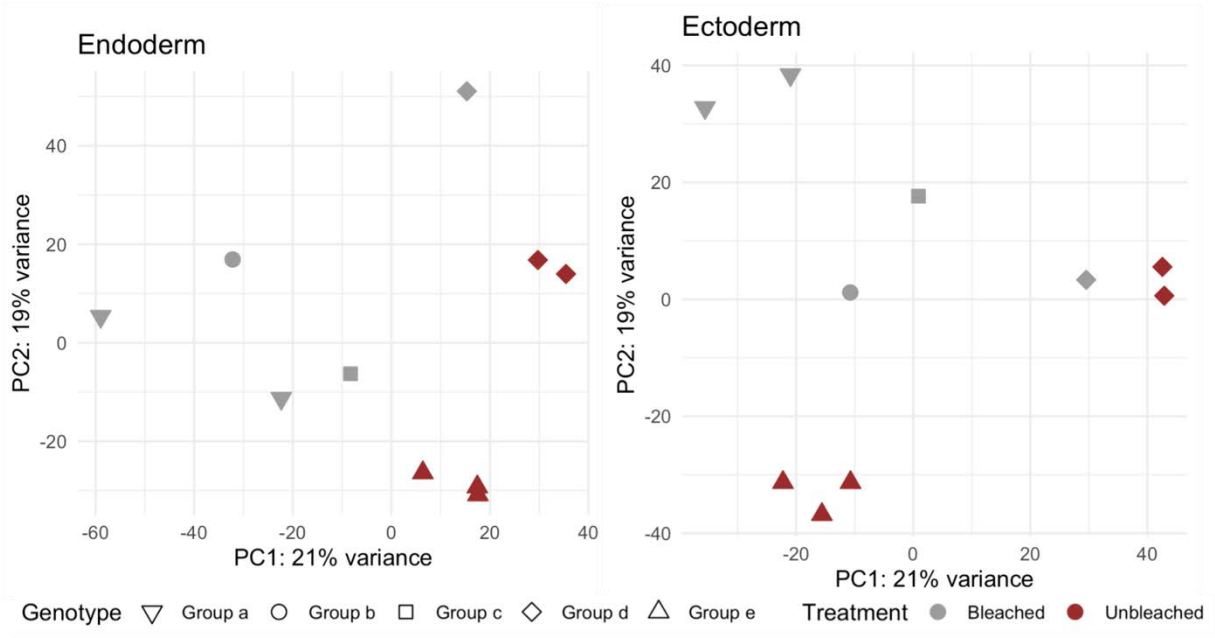


Figure 4.6: PCA of the gene expression of the ectoderm (left) and endoderm (right) of *Heliofungia* showing the clustering effect of bleaching treatment (grey = bleached, red = unbleached) and genotypic grouping (shape).

Bleaching studies focusing on intra-specific bleaching responses of corals (*Porites lobata* and *A. palmata*) showed that some individuals coped better than others due to their inbuilt defences and coping strategies (Barshis et al., 2010; Durante et al., 2019). A reciprocal transplant study using *P. lobata* showed that when colonies were switched between their usual locales (either a fluctuating back reef or stable fore reef) the most limiting factor, in regards to physiological response to stress, was the colonies genotype (Barshis et al., 2010). When considering a molecular response, it is imperative to know the study species genotype as assuming that replicates are biologically distinct can be problematic, particularly with corals as that can asexually reproduce. Identification of genetic groupings in this study meant that this could be accounted for in the differential expression analysis (via a random effect).

4.3.2 Genes differentially expressed

A total of 9608 genes were significantly differentially expressed between ectoderm and endoderm, while 1309 genes were differentially expressed between bleached and unbleached individuals. In addition, a smaller number of genes (156) were found to be differentially expressed due to an interaction between both tissue and bleaching. A summary of the number of significant DEG up- and down-regulated in the three groups is provided in Figure 4.7.

Direction of change (positive or negative log2 fold change) for each factor should be interpreted as follows: for the Tissue factor positive means higher in endoderm than ectoderm; for Treatment positive means higher in unbleached individuals than those sampled during bleaching; for the Interaction positive means higher than expected in unbleached endoderm than could be accounted for by independent tissue and bleaching factors alone. Genes in this interaction group are of particular interest with respect to tissue-specific effects of bleaching. The conceptual diagram (Figure 4.8) illustrates the difference between separate tissue and bleaching effects versus their interaction.

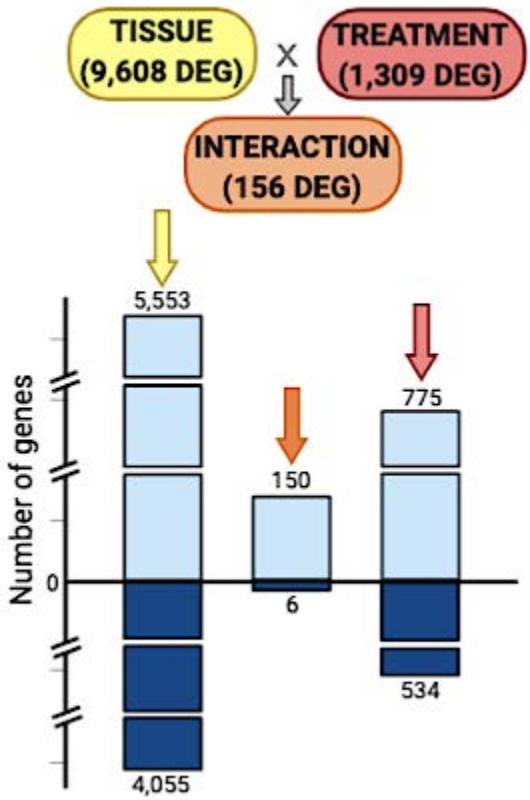


Figure 4.7: the number of significantly DEG (adjusted *p.* ≤0.05) in each experimental group; tissue = endoderm (up) *vs.* ectoderm (down), treatment = unbleached (up) *vs.* bleached (down) and, interaction = the combined effect of both treatment and tissue on gene expression, only expressed in the bleached endoderm (down) and expressed in all other treatment-tissue combinations (up). Integer above the bar represents the number of significant DEG in that set (created with biorender.com).

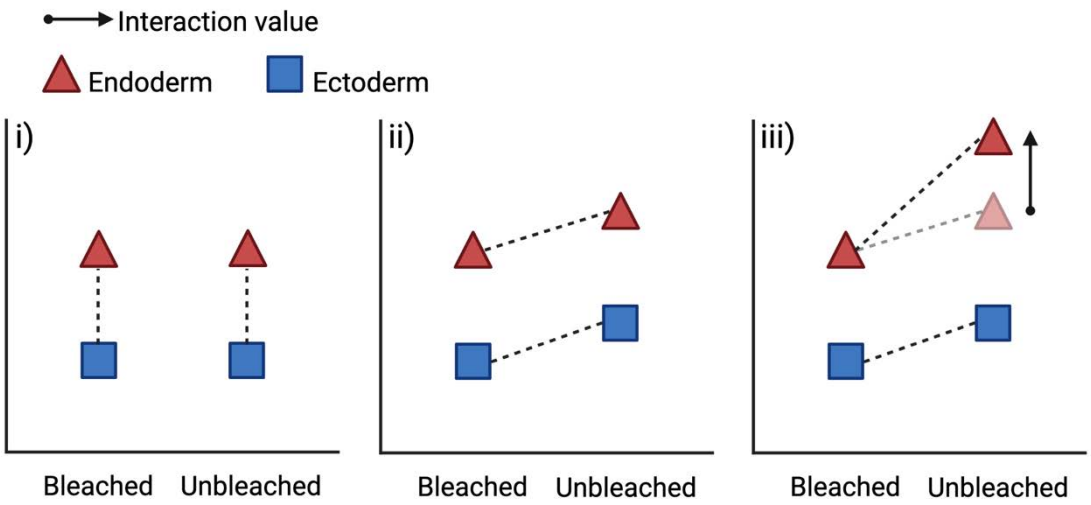


Figure 4.8. The change in expression relative to the norm of each grouping; genes in each group (i, ii and iii) showed a significant change in expression in relation to the baseline (change represented by dotted line). Plot i) represents the effect of tissue alone, plot ii) represents a gene showing both treatment and tissue effects acting independently and, iii) shows a gene from the interaction group, where there is a larger than expected change in expression in the endoderm than expected based on independent treatment and tissue effects - the black arrow represents the interaction value, figure made with Biorender (https://biorender.com/).

4.3.3 Differences within the gene expression of bleached and unbleached Heliofungia

4.3.3.1 GO terms enriched in the unbleached corals showing a higher expression than in the bleached group

Analysis of genes expressed at higher levels in unbleached individuals than in bleached samples led to recognition of nine enriched GO terms linked to cilia and the cytoskeleton (Figure 4.9). There were four processes related to development of the coral; ‘axenome assembly’, and reproduction; ‘flagellated sperm motility’ and ‘phosphatidylcholine biosynthetic process’. Phosphatidylcholine is a phospholipid commonly found in egg yolk and has been observed in coral oocytes (Lin et al., 2012; Pelech & Vance, 1984). These results imply that the individuals are post-stress as they are focusing on growth and reproduction, which is unlikely to occur during stress. Alternatively, phosphatidylcholine may play a role in oxidative stress mitigation due to its ability to alter membrane permeability facilitating transport of necessary lipids (Bachok et al., 2006; Smith et al., 2009; Tang et al., 2015). This would coincide with the terms ‘nucleoside diphosphate’ and ‘nucleoside kinase activity’ also enriched in the unbleached corals as these terms are strongly connected to stress response, immunity and growth. Finally, iron ion transport was enriched which could be due to the need for ferritin as a future protective mechanism or, as part of norepinephrine/epinephrine homeostasis

(Barshis et al., 2013; Tapryal & Mukhopadhyay, 2015; Wall et al., 2020). Reviewing the manual annotated genes showed several genes with roles linked to symbiosis recognition and homeostasis; von Willebrand factor, solute carriers and ion transporters (Motone et al., 2018; Reshef et al., 2008). Overall, the genes and GO terms gave the impression that unbleached *Heliofungia* is mainly focused on; development and growth, either for gametogenesis, organogenesis or body positioning; cilia structure, movement and maintenance and; metabolism and cellular cargo transport.

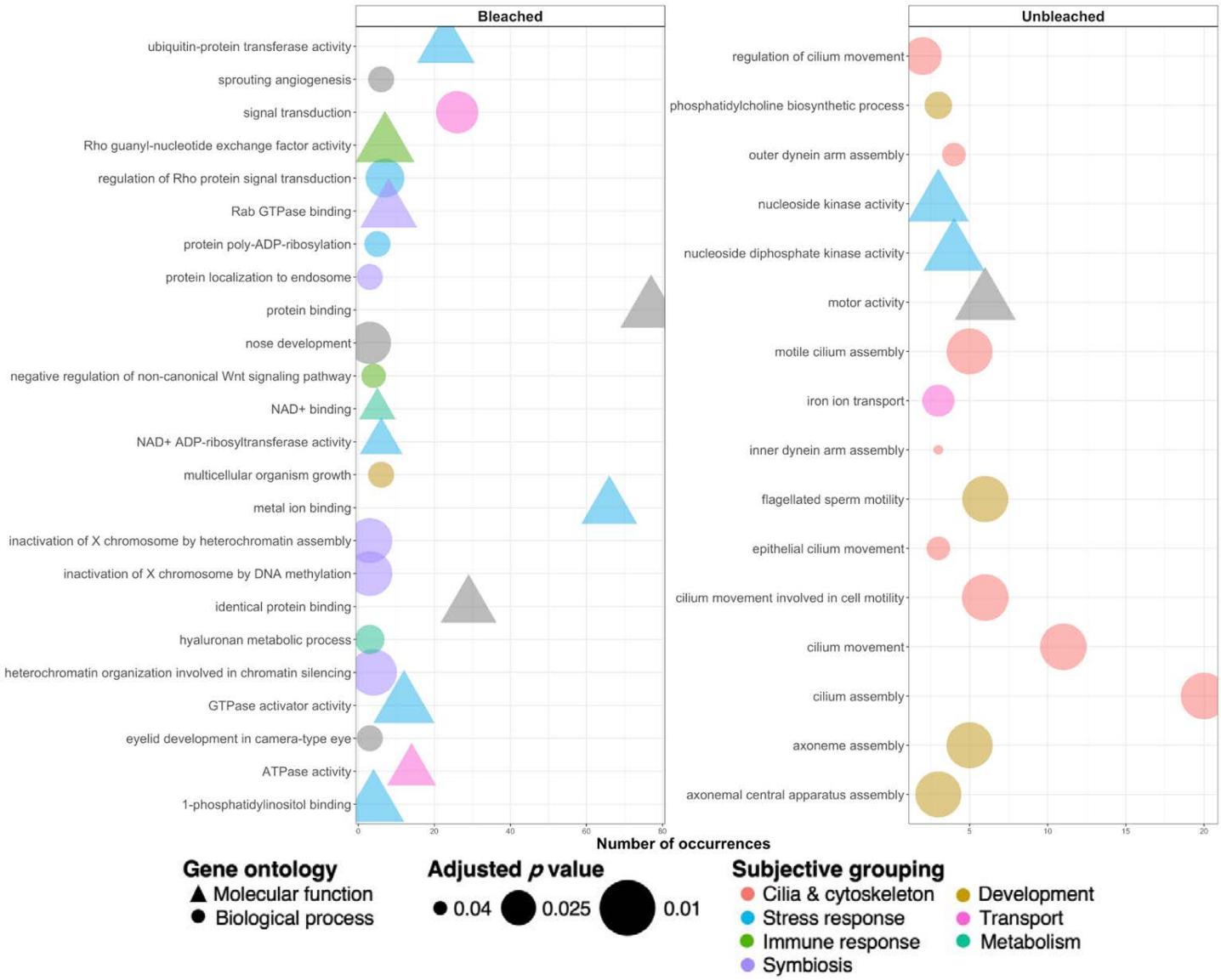


Figure 4.9. GO terms enriched in genes differentially expressed between bleached - April 2017 and unbleached - April 2018 *Heliofungia.* Left plot shows GO terms enriched in bleached corals and the right plot shows GO terms enriched in the unbleached samples. Shape of the point is based on whether the term was classified as a biological process (circle) or molecular function (triangle) and size represents the significance (adjusted *p* value < 0.05), larger being more significant. The terms were split into subjective functional groupings based on their associated terms and manual annotation in comparison to the coral literature, this is portrayed by different colours (please

note, some of the terms do cover multiple subjective groupings, the colour portrayed is the grouping that had the strongest linkages to the coral literature).

4.3.3.2 GO terms represented more highly in bleached corals in comparison to unbleached individuals

A total of 17 GO terms over-represented in bleached corals were subjectively associated with the integrated and oxidative stress response, cellular detoxification and pH balancing, apoptosis and UV protection. These results included metal ion binding, ubiquitin-protein transferase and negative regulation of the non-canonical Wnt signalling pathway which has been suggested to be involved in regulating oxidative stress and peroxide levels in cells (Dubreuil et al., 2020). Another unusual term that may have a stress connection is hyaluronan metabolism; hyaluronan is chiefly known for its role in soft connective tissue and coral exoskeleton (Goldberg, 2001; Laurent & Fraser, 1992; Watanabe et al., 2003) but recently hyaluronan metabolism been suggested to be key in maintaining homeostasis in the cytosol, mitigating endoplasmic reticulum stress and to have a strong anti-inflammatory role in mammals (Caon et al., 2021; Hascall et al., 2004; Hascall et al., 2014). As hyaluronan is known to interact with antiinflammatory cells and macrophages in mammals (Arenas Gomez et al., 2020; Rayahin et al., 2015) it would be useful to understand if it interacts with endosomes and phagosomes and therefore, may have a role in coral symbiosis. The rest of the genes and terms were linked to immune response, symbiosis and catabolism, many of these functions overlapped with one another. Intriguingly, an Arrestin domain-containing protein that is conserved from unicellular organisms to higher metazoans was found linked to a number of GO terms including; negative regulation of adenylate cyclase-activating adrenergic receptor signalling pathways, negative regulation of heat generation, beta-3 adrenergic receptor binding, temperature homeostasis, endosome, lysosome and protein transport. Arrestin is known for protein transport, downregulation of heat generation (essentially cooling), temperature homeostasis and in response to heat stress will trigger cell-surface adrenergic receptors to target endosomes for endocytosis (Habourdin et al., 2013). Therefore, Arrestin may have a role in coral dysbiosis, heat stress may lead to endocytosis to remove heat generators, ie. the Symbiodiniaceae, further analysis would be needed to validate this theory. Another gene homologue exhibited that may be related to bleaching, immunity and symbiosis was DMBT1 and has been positively regulated with bleaching survival (Wright et al., 2017). DMBT1 is a tumour suppressor gene that has ancient origins and has been cited in the literature as part of mitigating dysbiosis (Neubauer et al., 2016). DMBT1 has been linked to apoptosis suppression, mucosal and immune defence, cell fate decision and the remodelling of the cytoskeleton and epithelial differentiation for removal of harmful products via exocytosis

(Andrade Rodríguez, 2018; Neubauer et al., 2016). Considering the homologue had a 3.9 log fold change (the highest observed in the genes in the treatment group) one could assume it may have an important role in bleaching maintenance in *Heliofungia*. During bleaching *Heliofungia* has many significantly expressed genes and GO terms that are likely interconnected, ie. one gene is the initiator, another the mediator and one a receptor and so on, to winnow the 10k gene list down to the most important genes required in tissue-specific bleaching another grouping should be considered.

4.3.4 Tissue-specific genes that were differentially expressed between bleached and unbleached individuals

Relatively few of the genes that were differentially expressed between tissues were also differentially expressed between the bleached and unbleached samples (cases ii, and iii in Figure 4.8). Of the 4,055 genes with higher expression in the ectoderm and 5533 with higher expression in endoderm, only 291 and 158 respectively were significantly DE in response to bleaching (Figure. 4.10, Table S4.2). Overall, less than 2% of genes with tissue-specific expression also differed significantly in the bleached/unbleached comparison.

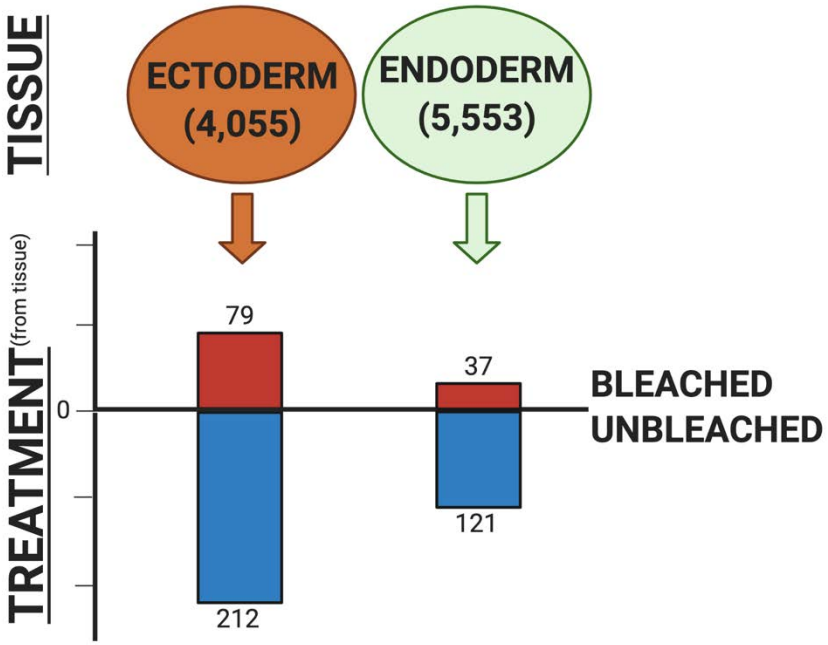


Figure 4.10: schematic representing the number of genes significantly differentially expressed in each region filtered by treatment (bleaching and unbleached). Out of the 4,055 DEG in the ectoderm, only 291 were also present in the treatments, 79 of these in the bleaching group and 212 in the unbleached group. In the endoderm, 5,553 genes were found to be DE but when filtered by treatment, this reduced the number to 158, 39 in the bleaching group and 121 in unbleached. Left bar represents the ectoderm and the right is the endoderm. Red bars (upregulated) exhibit the number of genes filtered from the specific region also found significant in bleaching and blue bars (downregulated) in the unbleached group, figure made with Biorender (https://biorender.com/).

As the Symbiodiniaceae reside in the endoderm, the 158 endoderm specific genes (Table S4.1) that responded to bleaching are of particular interest. Of these, only 37 had increased expression in bleached tissue whereas 121 had reduced expression. Eight of the 37 more highly expressed genes had potential functions in antioxidant defence and two were related to catabolism. HAL (histidine ammonia-lyase), which is involved in histidine catabolism (Langer et al., 1995) and PNPLA2 homologue (Patatin-like phospholipase) which is known for the catabolism of long chain fatty acids and lipid droplets to make energy in response to starvation and resource depletion and is commonly found in plants, including microalgae (X. Wang et al., 2015). The PNPLA2 protein has also been observed as part of the competition stress response of *A. millepora* to macroalgae (B.-N. Chen et al., 2020; Shearer et al., 2012). Patatin-like phospholipases have been found in the *N. vectensis* genome and identified in symbiotic *Aiptasia* (Nevalainen, 2008). In bleached corals, *Heliofungia* would no longer be gaining energy from its Symbiodiniaceae (Wooldridge, 2014) therefore, increasing PNPLA2 expression might be involved in catabolism of phospholipids to provide energy; as seen in *Lophelia pertusa* which underwent a six-month starvation experiment (Larsson et al., 2013). The results indicated that tissue composition is a good indicator for malnourishment due to

the reduction of tissue lipid content (phospholipids included) as they were broken down to feed the corals during starvation (Larsson et al., 2013). Overall, 11 out of 37 homologues expressed had a clear connection with oxidative stress response including one gene that was negatively so, TRIO (triple functional domain protein) has been known to be a positive regulator of apoptosis (B. Wang et al., 2015). Furthermore, silencing the TRIO gene through knockdown studies showed a significant decrease of expression of other harmful, apoptotic-associated genes like BCL-2 (B. Wang et al., 2015), a gene repertoire known to be conserved in *A. millepora* (Moya et al., 2016), potentially causing *Heliofungia* to perish rather than re-establish symbiosis.

4.3.4 Interaction between treatment and tissue in determining gene expression

The interaction dataset identifies genes where differences in expression between tissues and between years are greater than expected based on independent tissue and treatment effects. A positive interaction value represents a stronger bleaching response in the endoderm than in the ectoderm (Figure 4.11). In support of the hypothesis that the endoderm would have a greater change in gene expression between treatments, the vast majority of the 156 genes with a significant interaction had positive values (150, Figure 4.6).

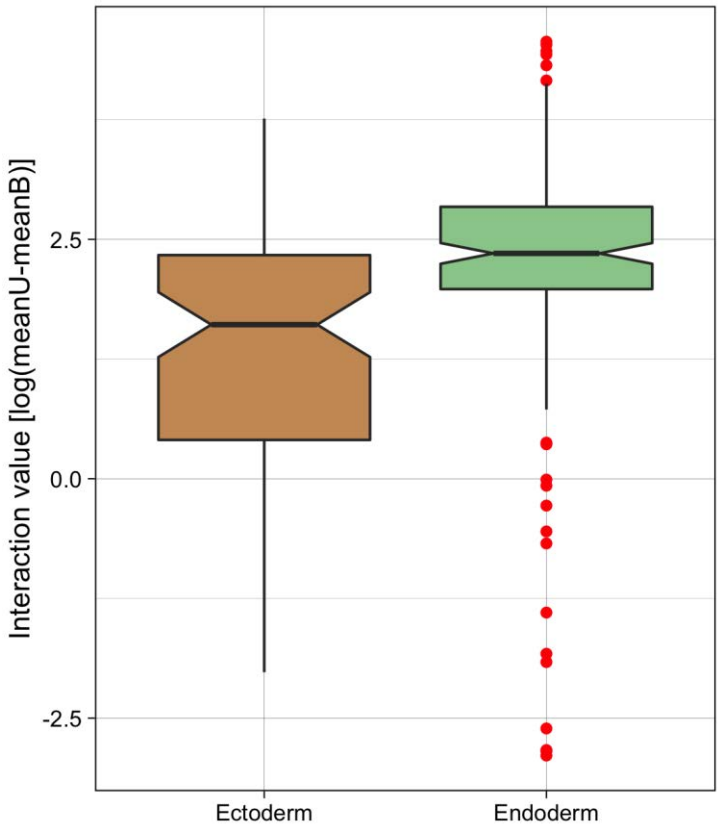


Figure 4.11: Tissue-specific bleaching effect for 156 genes with a significant interaction effect between tissue and bleaching. Bleaching effect (y-axis) is calculated for each gene in each tissue as the log mean difference in expression between bleached (B) and unbleached (U) samples.

The results show that bleaching has a stronger effect on the endoderm, than the ectoderm. The normalised counts of each significant DEG from the interaction group were plotted. From these plots, it became apparent that the 156 genes could be assigned to one of six qualitatively distinct expression patterns (labelled a – f, Figure 4.12) for the DEG in the interaction group. Note that the patterns shown in Figure 4.12 do not represent all possible patterns, but capture all patterns seen in the data.

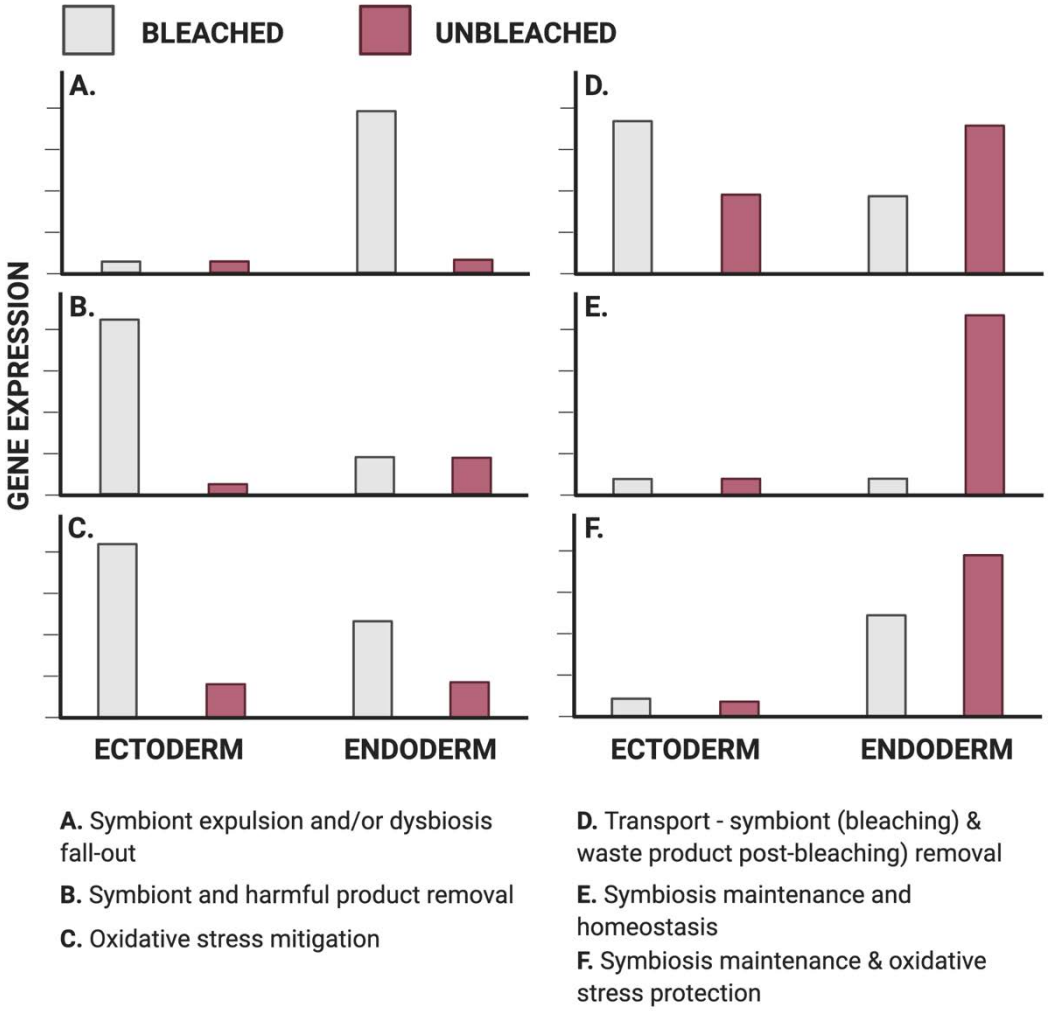


Figure 4.12: A schematic representing the six different groupings (a-e) based on the expression of the genes in the interaction dataset. The expression pattern observed may infer the biological roles these genes play and biological suggestions for each grouping are beneath the bar charts. Ectoderm is on the left of the bar chart and endoderm on the right, treatment is represented by colour; bleaching = grey and unbleached = red. The highest bar portrays the treatment tissue that had the largest increase in expression, the medium bar is the next highest expression, the small bar depicts a low background expression and the very small bar is genes that showed none to negligible expression in the treatment/tissues, figure made with Biorender (https://biorender.com/).

Of the 156 significant genes with a significant interaction value, only 26 had close homologues in other cnidarians and higher metazoans (based upon Trinotate's selection criteria). Combining the data from the gene plots and the annotation of the genes in the interaction group (Table 4.2) created an impression of the functions each of the gene expression patterns related to which could be summarised in Figure 4.12. Group b) represented genes expressed in the bleached ectoderm with roles relating to transporting harmful products, and potentially the symbionts, out of the cells. Group d) had a mixed response, with highest expression in the bleached ectoderm and unbleached endoderm, these genes also had functions linked to removing harmful byproducts and maintaining homeostasis particularly through ion transport and interacting with the symbionts. The gene expression of groups e) and f) were focused on

the endoderm with genes in e) showing a significant increase in expression in the unbleached endoderm only and, f) during both treatments. The genes in these groups are associated with symbiosis maintenance, stress protection, regulating homeostasis and the allomonal system - production of toxins for defence and prey capture.

Table 4.2: the gene homologues that could be annotated from the interaction set (34/156). The genes are split into their groups (a-f) dependent on their expression pattern (see Figure 10), with gene name and associated protein alongside.

Group	Gene	Associated protein
a)	pol	Retrovirus-related Pol polyprotein from transposon opus
	PXDNL	Peroxidasin-like protein
	Rfx6	DNA-binding protein RFX6 (Regulatory factor X 6)
	Sil1	Nucleotide exchange factor SIL1
	Slc22a3	Solute carrier family 22 member 3
b)	ChT	High affinity choline transporter 1
	HNF4G	Hepatocyte nuclear factor 4-gamma
c)	n/a	DAD domain-containing protein
	Micall2	MICAL-like protein 2
	Map3k1	Mitogen-activated protein kinase kinase kinase 1
	psiD	L-tryptophan decarboxylase
	Slc4a10	Sodium-driven chloride bicarbonate exchanger (Solute carrier family 4 member 10)
d)	Igsf23	Immunoglobin superfamily member 23
	Slc6a13	Sodium- and chloride-dependent GABA transporter 2
e)	Thap4	Thap domain-containing protein 4
	n/a	Glycoside hydrolase family 55 protein
	Stimate	Store-operated calcium entry (SOCE) regulator STIMATE (Transmembrane protein 110)

	CDC123	Cell division cycle protein 123-like
	Klhl30	Kelch-like protein 30
	n/a (*ptsG*)	Lung surfactant protein A
	VWF	Von Willebrand factor (vWF)
	RGpF	Rhamnan synthesis protein F
	Hst6st3	Heparan-sulfate 6-O-sulfotransferase 3
	n/a (*PREP1*)	Presequence protease 1 (chloroplastic/mitochondrial)
	Pso-o-1	Peptidase 1 (Mite group 1 allergen Pso o 1)
	LRAT	Lecithin retinol acyltransferase
	n/a (*Map3k2*)	Mitogen-activated protein kinase kinase kinase 2
	n/a	Fe2OG dioxygenase domain-containing protein
f)	n/a	Uncharacterized - Ten1 2 superfamily
	n/a	LIM domain-containing protein A-like isoform X3
	Cyp17a1	Steroid 17-alpha-hydroxylase/17,20 lyase (Cytochrome P450 17A1)
	CD63	CD63 antigen
	RPL40	Ubiquitin-60S ribosomal protein L40
	Tatdn1	Putative deoxyribonuclease TATDN1

Of the six gene groupings identified in Figure 4.12 and Table 4.2 the genes from groups a and c are of particular interest in relation to dysbiosis. Group a) contains genes that are highly expressed in the bleached endoderm with none to negligible expression elsewhere – related to expelling the Symbiodiniaceae and mitigating the effects of oxidative stress and dysbiosis. Group c) covered genes with high expression in the bleached ectoderm, medium-to-high expression in the bleached endoderm and low-to-no expression in the unbleached regions - heavily involved in stress response mitigation.

The group a) homologues (only significantly expressed in the bleached endoderm, Figure 4.13) included; Slc22a3, pol, Sil1, Rfx6, and PXDNL. The coral literature associates these genes to immune response, removal of harmful products/toxins, return of homeostasis and anti-apoptotic pathways in whole coral colonies (Kenkel et al., 2013; Ruiz-Jones & Palumbi, 2017; Tarrant et al., 2018; Traylor-Knowles et al., 2017; Yuyama et al., 2012); this is the first time the gene expression has been shown to be endoderm-specific. Slc22a3 is a solute carrier that has been observed in several corals as a heat stress biomarker and an organic ion transporter used in detoxification of tissues (Kenkel et al., 2014; Koepsell, 1998; Levy et al., 2016). Slc22a3 expression was practically undetectable in all tissues except the bleached endoderm where it is expressed 17-fold more highly than in the normal endoderm; these results suggest a significant need for detoxification in the endoderm once the coral has bleached and oxidative stress. Another suggested coral and symbiont heat stress biomarker is pol, a gene that produces retrovirus-related pol polyprotein (Chen et al., 2018; Durante et al., 2019; Traylor-Knowles et al., 2017). The bleached endoderm pol was expressed 10 times higher than the bleached ectoderm and around five times higher than the unbleached endoderm thus, implying a significant endodermal role in the bleaching response and this large increase in pol expression has been seen in other heat-stress experiments on corals (Garcia et al., 2013; Traylor-Knowles et al., 2017). Although not previously defined as an endoderm-specific response, increased pol polyprotein expression has been linked to holobiont response and that acute heat stress activates pol expression in Symbiodiniaceae (Chen et al., 2018; Daniels et al., 2015) therefore, it is not surprising to observe an endoderm-specific response in *Heliofungia*.

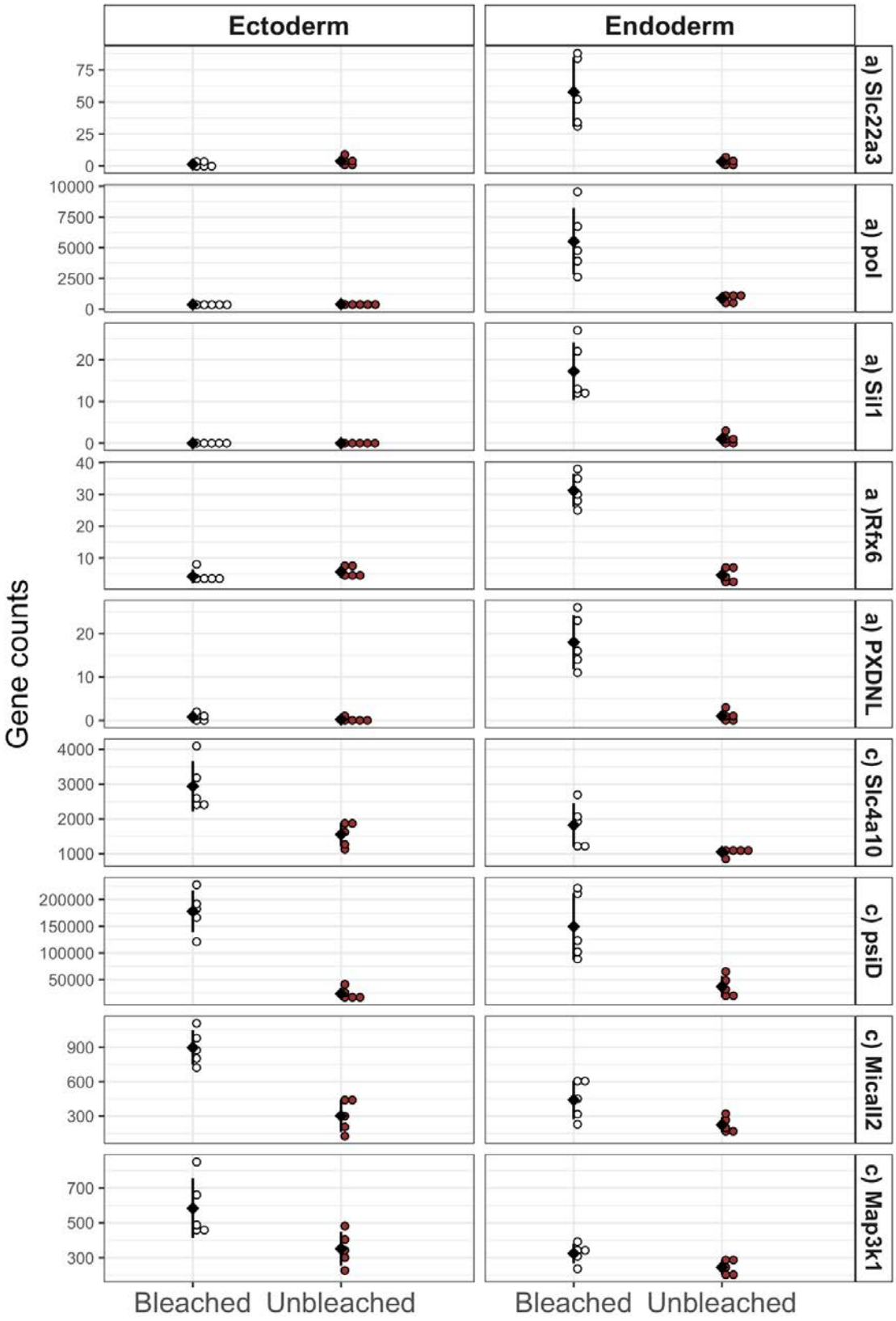


Figure 4.13: the gene counts for homologues from group a) and c). Group a) are only expressed significantly in the bleached endoderm and c), both bleached ectoderm and endoderm. Colour represents the treatment; white being bleached specimens and red unbleached. Ectoderm is displayed on the left and endoderm on the right, note changes in y axis.

Sil1 is known to take part in protein translocation, folding and as a HSP chaperone and in *Acropora hyacinthus* was identified as defence against environmental stress (Ruiz-Jones & Palumbi, 2017). HSPs are well known as a coral bleaching response (Baird, Bhagooli, et al., 2009; Fang et al., 1997) and their expression in only the bleached endoderm validates this theory. No expression of Sil1 was observed in the ectoderm therefore, this gene is likely endoderm-specific and has an important role in refolding degraded proteins and removing overly-damaged proteins from the cells in this tissue (Georgopoulos & Welch, 1993; Oakley & Davy, 2018). Proteins relating to protein folding were shown to be the second most differentially abundant in *Aiptasia* during a heat-shock experiment (Oakley et al., 2017) therefore, it was surprising to see a low raw count of Sil1 in the *Heliofungia* endoderm, perhaps sampling earlier during bleaching would have shown a higher abundance of Sil1 or, there are a number of other proteins that could supply similar roles. Once Sil1 has removed the harmful proteins from the endoderm they may be accumulated by a transport, exocytosis or endocytosis protein in the ectoderm for permanent removal which may explain the bleached ectoderm increases in homologues like ChT, Slc4a10 and Micall2 which are all known for these roles (Hayward et al., 2011; Rahajeng et al., 2010; Rocker et al., 2015).

RFX6 is known as a transcription factor for cell development and differentiation (Smith et al., 2010), potentially needed to alter cells during stress in the coral endoderm and, an RFX-type homologue has been described in *Pocillopora damicornis* with DNA binding and transcription functions (https://www.uniprot.org/uniprot/A0A3M6UB49). Whereas, PXDNL produce Peroxidasin-like proteins which in the coral bleaching literature are known for their roles in antioxidant defence and phagocytosis and as apoptotic mediators (Nuño, 2018; Pernice et al., 2011; Tisthammer et al., 2019; C. R. Voolstra et al., 2009). As PXDNL expression was detected only in the endoderm, this strengthens the idea that the main impact of bleaching is on the endoderm as antioxidant defence is a key mechanism in mitigating the harmful by-products caused from increased ROS concentration (Császár et al., 2009).

Group c) comprises the rest of the homologues expressed in the endoderm also increased in the bleached ectoderm (at a higher level). This response implies that they are needed in both regions during bleaching but in a greater quantity in the ectoderm. For example, Slc4a10 has previously been referenced as a cotransporter of Na^{+}/HCO^{3-} to regulate intracellular pH and specifically in cnidarian literature, as a stress response to both increased heat and pCO_2 (Rocker et al., 2015). Furthermore, it has also been linked to coral calcification and photosynthesis (Barott et al., 2015; Furla et al., 2000; Hemond et al., 2014; Zoccola et al.,

2015) as the tentacle tissues do not calcify and the endoderm has lost its photosynthetic counterpart, it is likely that Slc4a10 is working in a stress response role in group c).

The gene psiD has several potential roles; as a protective mechanism during psilocybin synthesis (a natural psychedelic), as a fast-oxidative stress response and signal for phagocytic macrophages (Shvedova et al., 2002) and, in cnidarian-literature, calcium-binding and regulation of growth rates and patterns (Bay et al., 2009; Isa, 1989). PsiD was shown to have the largest expression of the genes in group c) with a background expression in both regions when unbleached and, during bleaching, increased 7-fold and 5.5-fold in the ectoderm and the endoderm respectively. Due to the large expression of psiD during bleaching it is likely a fast oxidative stress response. Furthermore, as it signals phagocytic macrophages in mammals (Shvedova et al., 2002) this capability should be studied in corals also, as this may be a method that the Symbiodiniaceae are removed from the tissues which would explain the large increase in expression in both tissues during bleaching. Another gene that may be related to symbiont removal is Micall2. Micall2 homologues have been found in *S. pistillata* and *P. damicornis* with roles linked to metal ion binding, calponin-homology (calcium binding), Rab GTPase activity and endocytic recycling (https://www.uniprot.org/uniprot *S. pistillata* ascension: A0A2B4SSE8 and *P. damicornis* ascension: A0A3M6UVE2). MICAL is known for binding with Rab GTPases causing an increase in NADPH oxidase (NOX) in mammals (Esposito et al., 2019). NOX have been linked to several functions (Panday et al., 2015) and, when considering coral dysbiosis, the NOX observed here could be either; i) as a negative mechanism leading to greater ROS production (Hawkins et al., 2014) or, ii) as an immune response mechanism, stabilising phagosome pH and aiding in phagocytosis to remove harmful products or the symbionts themselves (Jancic et al., 2007). The second theory is particularly interesting considering NOX has recently been suggested to be localised to the symbiosome membrane of *Aiptasia* Symbiodiniceae during heat stress and activation of NOX is a key part of dysbiosis (Rice, 2020). For conclusive understanding, greater interpretation on the type of MICAL and NOX *Heliofungia* produce is needed however, Micall2 was expressed in all tissues throughout treatments but, it was expressed three and two times higher in the bleached ectoderm and endoderm compared to the normal. This data implies a significant bleaching role in both regions and, out of the 19 GO terms connected to this particular Micall2 homologue, 12 were related to trans-membranal endocytosis, three antioxidant defence and the others were undefined protein binding. Therefore, the Micall2 homologue observed here in *Heliofungia* seems to be involved in removing molecules (potentially the symbionts) from the endoderm to the ectoderm where they are either engulfed or expelled from the tissue.

Upregulation of Map3k1 leads to an increase in mitogen-activated kinase (MAPKs), proteins which have been heavily referenced in the coral-literature as having assumed roles in osmoregulation, signalling, stress response, apoptotic pathways and growth (De Keuckelaere et al., 2018; Mayfield et al., 2010; Seneca et al., 2010). Map3k1 has also been tentatively linked to the maintenance and establishment of algal-host symbiosis (Christian R Voolstra et al., 2009). In addition to this, in non-stressed *P. damicornis* an increase in MAPKs expression was linked to reduced Symbiodiniaceae photosynthesis. MAPK production is higher during the night as osmotic pressure in the host is reduced due to less osmolytes, organic compounds that impact fluid properties, generated by the symbionts (which are light-dependent). Less osmolytes mean a reduction in intracellular crowding (the cells have more space) therefore, there is more space available for more structurally complex proteins, like MAPKs, to be formed (Mayfield et al., 2010). Potentially there is a link between Symbiodiniaceae being expelled to create space for necessary, bulky proteins needed for survival as this may explain the large increase in Map3k1 expressed in the endoderm following symbiont loss. This theory would need to be studied further as Mayfield et al (2010) were not able to separate the coral tissues to observe how the difference in ecto- and endoderm expression affected results.

4.3.5 The Symbiodiniaceae present in Heliofungia

In addition to the numerous molecular mechanisms behind bleaching survival, Symbiodiniaceae species present is also an important consideration. The innate thermal tolerance, resource partitioning and oxidative stress response of the Symbiodiniaceae in question influences whether that species is better equipped to deal with bleaching events (Baker, 2004; Manzello et al., 2019; Silverstein et al., 2015; Thomas et al., 2019). Therefore, the reads from the *Heliofungia* individuals were mapped to reference Symbiodiniaceae genomes to see which species were present. As *Heliofungia* is more bleaching tolerant (it commonly recovers following dysbiosis) it was presumed it would contain *Durusdinium sp.* rather than *Cladicopium sp.* However, the results showed that the majority of the individuals contained *Cladicopium sp.* and only a few (B24, R21 and, R23) were associated with *Durusdinium sp.* (Figure 4.14).

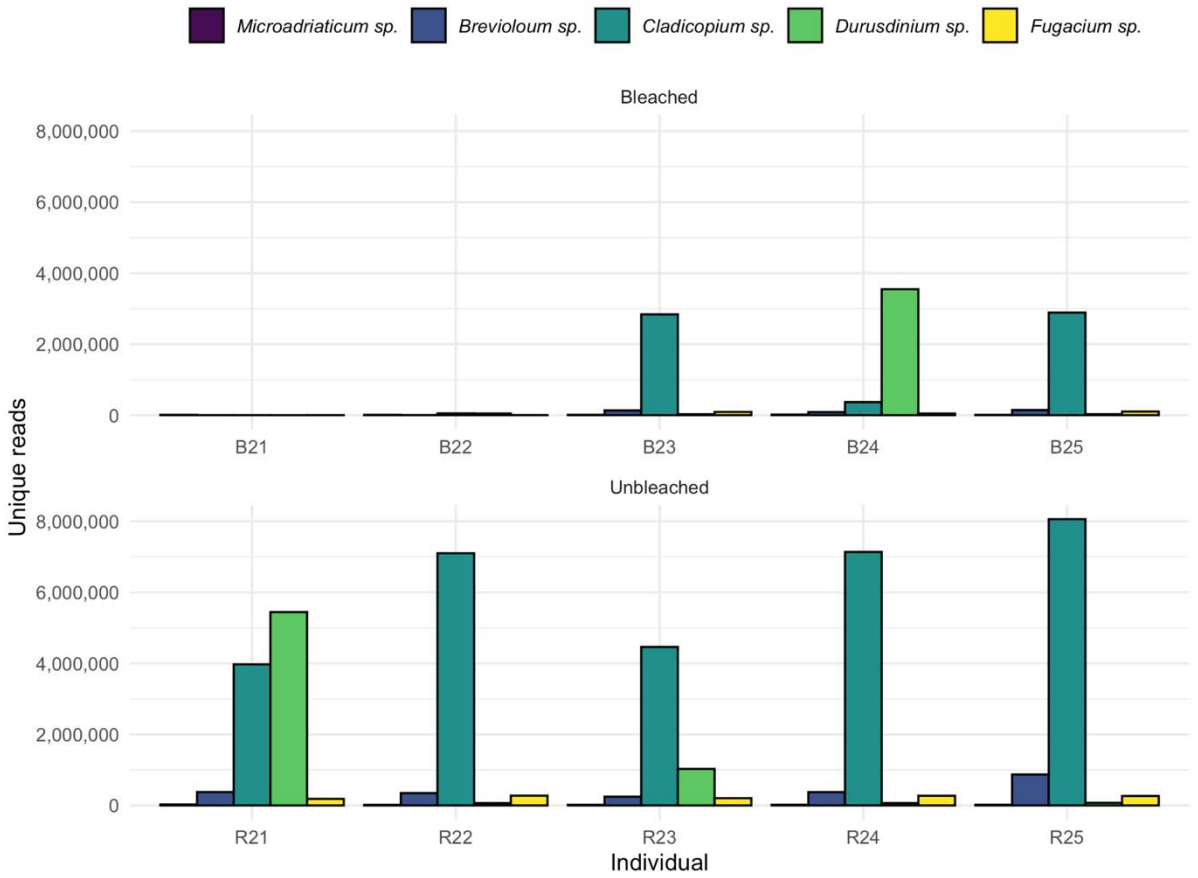


Figure 4.14: the number of unique reads for each Symbiodiniaceae genera in the individual *Heliofungia* samples, colour of bar represents the genus (see legend (top) for definition). Top plot is the endoderm samples from the five corals sampled in April 2017 (bleached) and bottom is the endoderm samples from the specimens collected in April 2018 (unbleached).

Symbiodiniaceae have been shown to have different thermal tolerances dependent on species (Silverstein et al., 2015) and Symbiodiniaceae from the D1 clade (currently known as *Durusdinium spp.*) are thought to generally be more thermally tolerant than are clade C (now known C*ladicopium spp.*), another common scleractinian associate (LaJeunesse et al., 2010). Symbiodiniaceae have species-specific responses to heat stress (Abrego et al., 2008) and previous studies have shown that after a bleaching event it is fairly common that corals will select for more heat-tolerant symbionts (Silverstein et al., 2015). Potentially, the mixed population of *Cladocopium* and *Durusdinium* in B24, R21 and R23 (Figure 4.13) could be due to them experiencing more severe bleaching in 2016 in comparison to the other individuals sampled. Alternatively, studies have shown coral species to have a predilection for a particular symbiont species and may reject a symbiont if it is not their preferred species (Mohamed et al., 2019; Tortorelli et al., 2020). Potentially, this predilection is coded within the coral genotype which may explain why only the samples from the genotypic grouping e (Figure 4.5 and B24, R21 and R23, Figure 4.14) showed to contain *Durisdinium sp.* as the rest were *Cladicopium*

sp. dominant. However, the degree to which coral will reject or accept particular symbionts is still under contention therefore, knowing how genotype influences this decision would be useful; further testing with a larger sample size would be needed to test this theory. If more stress tolerant symbionts are incompatible with particular coral species, restoration efforts involving flooding the reef with heat-tolerant symbionts is likely to be unsuccessful.

4.4 Concluding remarks

Considering tissue-specificity in this study has revealed new perspectives into coral biology and the bleaching response. The molecular bleaching data presented here are generally consistent with the literature. During bleaching there is a significant increase in expression of genes related to antiapoptosis, heat shock proteins, immunity, antioxidant defence, cellular repair and possibly also symbiosis communication. With respect to the removal of the Symbiodiniaceae, homologues of several bilaterian genes that may play roles in the engulfment or expulsion of the symbionts or, at minimum, mitigate the harmful ROS produced during oxidative stress. Knowing where the genes are expressed gives new insights into how they may be involved with bleaching and dysbiosis. For example, DMBT1-like genes being triggered to remodel the endoderm to facilitate the removal of the symbionts via adrenergic receptors and cytosis proteins (Andrade Rodríguez, 2018; Neubauer et al., 2016; Wright et al., 2017). Furthermore, the approach used here provides a platform to undertake further research on tissue-specific gene expression in response to stress.

From the results of the tissue and treatment groups it became apparent that oxidative stress and immune response were integral during bleaching and that these responses occur predominantly in the endoderm, as predicted at the outset. Utilising the interaction group (the genes significantly expressed when comparing both tissue and treatment) provided further support for this. The interaction group gives a more precise list of genes that are important in facilitating dysbiosis and may be useful in determining the trigger that leads to symbiont expulsion or engulfment. Unfortunately, one of the main aspects limiting interpretation of the results is the lack of annotation for coral genes and *Heliofungia* specifically, as nearly three quarters of the genes in the interaction group were either un-annotated or had no significant match in the database. Given the challenges to any kind of functional genetic analysis in corals, this situation is unlikely to change anytime soon.

However, despite these limitations, the interaction group identified in this study provided some intriguing results. Being able to localise known stress response genes to specific tissues may

permit more sophisticated modelling of the coral bleaching hypothesis. The expression of homologues of PXDNL and Sil1 in the bleached endoderm and of HNF4G and ChT in the bleached ectoderm suggests that, whilst the endoderm is the “front line” of oxidative stress and impaired symbiosis, the ectoderm may be involved in removing harmful by-products of damage and possibly the symbionts themselves. Furthermore, the genes expressed in both tissues during bleaching (group c), with a higher level in the ectoderm, may theoretically play a part in the instigation of dysbiosis. Micall2, psiD and Map3k1 are of particular interest due to their possible roles in endo- and exocytosis between the two tissues. Micall2 and Map3k1 are frequently mentioned in the bleaching literature however, no studies have been undertaken to date that observe how the response of these particular genes change in the different tissues and analysis of this may give a new outlook into the bleaching mechanism. Using the tissue separation technique of *Heliofungia* combined with better gene annotation and an understanding of protein size and structure could help clarify whether there is any weight behind the hypothesis that dysbiosis may occur to give the host space to produce necessary stress survival proteins. This may be an exciting avenue of research to follow in the bleaching field and further understanding of the roles psiD, Micall2 and Map3k1 play in dysbiosis would be advantageous.

The results from the normal, healthy endoderm have highlighted potential homeostasis genes in *Heliofungia* -and the abundance of proteins with transporter and/or transmembrane roles ferrying molecules around the cells. The group d) homologues (expressed in the bleached ectoderm and normal endoderm) should be considered for further investigation as, similar to the genes in group c), they may have an important role in symbiosis maintenance by removing Symbiodiniaceae during stress in the ectoderm and transporting products between the host and symbionts during normal functioning in the endoderm. These results again highlight the need for better genome annotations in corals as there are potentially many other genes missing from this group that are currently undescribed. Considering symbiosis communication, there were many other genes categorised in groups e) and f) (expressed in the normal endoderm only and, during both treatments in the endoderm respectively). These homologues, for example Hs6st3, Stimate, VWf, CD63 and RGpF, were all linked to GO terms pertaining to symbiosis and cytosis through different means. RGpF in particular would be interesting to study further to determine its role in *Heliofungia* due to its reference to symbiosis between plants and nitrogen-fixing bacteria and its role in phagocytosis (Ardissone et al., 2011; Shibata et al., 2009). As arrested phagosomes have been suggested as a key part in symbiosis establishment (Mohamed et al., 2016) knowing how RGpF may be involved in this may expand our understanding of symbiosis. Furthermore, explaining whether some of the homologues seen expressed in the normal endoderm are symbiont-associated may lead us

to better understand the processes lost through dysbiosis and what is missing that may lead to coral death.

It would be useful to undertake this experiment again but without the limitations mentioned earlier; the same corals and a larger sample size before, during and after bleaching. It is difficult to discern whether the stress protection and antioxidant defence homologues expressed in the normal endoderm are being frontloaded as a protective measure due to the 2016 bleaching event or, are normally expressed at low levels independent of previous stress events. The presence of significantly expressed homologues like RPL40, Cyp17a1 and CD63 which have a strong oxidative stress response role would be useful to study across timepoints to see how a bleaching treatment impacts their expression a year later. Also, it would be wise to genotype the corals before experimentation to determine whether expression patterns do share a genotypic link. Furthermore, how the genotype may affect the Symbiodiniaceae or, vice versa. Advancing our understanding of how genotype affects *Heliofungia* symbiont preference could have useful ramifications in the field as, using heat tolerant Symbiodiniaceae may be a useful way of bolstering a corals thermal resilience overall. However, if the coral is going to reject a symbiont species more suited to future climate scenarios due to its genotypic preference, then other modes of increasing Symbiodiniaceae-host thermal tolerance should be considered.

Overall, using *Heliofungia actiniformis* to gain insights into tissue-specific stress responses in corals has provided valuable groundwork into coral bleaching research. *Heliofungia* has the potential to allow a much greater understanding of how symbiosis works.

Chapter 5: General discussion

The research presented here is centred on the use of *Heliofungia actiniformis* as a novel coral model. This use is particularly focused on the tissue-specific analyses that have not been achieved in coral previously. The large size, individuality and morphology of *Heliofungia* make it a useful species to study molecular and microbial variation between different tissue layers, but also the finer details of how corals respond when they are bleached. This chapter will synthesise the results presented throughout this thesis, and further discuss the importance of the findings and how they can be advanced to aid in our understanding of coral biology and the threats they face in this changing world.

5.1 Knowledge gaps approached in this thesis

Coral bleaching continues to degrade highly significant reef ecosystems (Bridge et al., 2013; Moberg & Folke, 1999; Woodhead et al., 2019), and numerous studies have been undertaken into the mechanisms behind bleaching, what causes heat-induced dysbiosis between host and Symbiodiniaceae, and how it impacts the reef (Baird, Bhagooli, et al., 2009; Douglas, 2003; Lesser, 2011). However, current research has predominantly been focussed on the study of whole animals, larvae or Symbiodiniaceae (Camp et al., 2020; Chua et al., 2013; Newkirk et al., 2020; Rosic et al., 2011; Seneca et al., 2010). Findings from these studies have been useful in identifying important processes related to bleaching; however, since they typically do not localise the response to a specific body area, it is difficult to infer tissue-specific functions. This knowledge gap was identified at the commencement of this thesis and is particularly relevant in the bleaching response because the coral endoderm (the inner tissue layer) contains the Symbiodiniaceae. Many questions remain about the partnership between symbiont and tissue, for example, which member of the holobiont triggers the bleaching mechanism. In order to investigate this area, more groundwork was needed, which raised several questions: do corals contain tissue-specific microbiomes similar to what has been observed in other cnidarians and reef invertebrates (Bonacolta et al., 2020; Deines et al., 2020; Rossbach et al., 2019)? If so, how does bleaching alter the tissue microbiome? And lastly, does bleaching change the tissue-specific gene response of the host? The coral *Heliofungia actiniformis* was presented as a species that could be used to fill these knowledge gaps. A unique feature of this species, and its primary advantage as a model is its very large polyp size, making it uniquely suitable for dissection. As shown in this thesis, being able to separate the molecular and microbial response of the ectoderm and endoderm can provide

unique insights into coral biology. These lay the groundwork for further tissue-specific analysis, as planned in the introduction of this thesis.

5.2 Major conclusions and outcomes

5.2.1 Chapter 2

Chapter 2 contains bacterial community analyses across multiple sampling points over the years of 2016, 2017 and 2018. *Heliofungia* presented distinct α- and β-diversity between the five regions tested: mucus, acrosphere, ectoderm, endoderm and gut. This clear definition of individual tissue-level microbiomes of *Heliofungia* suggests a strong division of each tissue on the function of the specific microbiome. Although separate regional (all soft tissue, skeleton and mucus) microbiomes have been shown previously (Hadaidi et al., 2017; Hernandez-Agreda et al., 2016; Hussien et al., 2019), Chapter 2 presents the first coral-specific results of differentiated tissue-level and gut microbiomes, similar to what has been observed in the cnidarian models *Nematostella* and *Hydra* (Bonacolta et al., 2020; Deines et al., 2020). Key findings demonstrated that while Endozoiocmonadaceae was seen in all regions, it was most abundant in the ectoderm (≥90%). Previous studies on whole coral polyps have shown that various species of Endozoicomonadaceae are often dominant in coral microbiomes (Bayer et al., 2013; Bourne et al., 2016; Shiu & Tang, 2019), but this is the first study to demonstrate that they are concentrated in the ectoderm. In whole polyp studies, the high amount of Endozoicomonadaceae in the ectoderm likely masks the bacterial diversity of the other regions, resulting in a lower resolution in taxa across the tissues. This was also shown in work on *Nematostella* where *Spirochaeta* were concentrated in the capitulum, which would normally have been missed in whole body sampling (Bonacolta et al., 2021).

Overall, the endoderm showed greater α-diversity and more varied β-diversity than the ectoderm. The endoderm harboured a large abundance of Endozoicomonadaceae, in addition to many other abundant families. Furthermore, the dominant *Endozoicomonas* ASVs have not been reported previously and were different to previous coral isolated strains (W.-M. Chen et al., 2019; Pike et al., 2013; Sheu et al., 2017; Tandon et al., 2018; Yang et al., 2010). A number of families were identified that may be associated with the endoderm and, consequently, the Symbiodiniaceae. For example, Cyclobacteriaceae, Nitrinocolaceae and Rhodobacteriaceae have been linked to nutrient cycling and signalling (Mori et al., 2019; Rosenberg et al., 2014; Yang et al., 2015) which may utilise the osmolytes given off by the Symbiodiniaceae for their benefit.

Histology and fluorescent *in situ* hybridization (FISH) is a useful tool for understanding the coral tissues, their structure, and microbial localisation. Corals previously studied with FISH consisted of colonies with small polyps, making it difficult to easily demarcate the tissue regions. However, in *Heliofungia* there was a clear separation of the tissue regions and the mesoglea. The Symbiodiniaceae were closely packed in the endoderm edge, facing the ectoderm and mesoglea in the main tentacle trunk. In contrast, only small numbers of Symbiodiniaceae were present in the tip where the acrosphere and nematocysts are present. Furthermore, the *Endozoicomonas*-specific probes identified large aggregates of *Endozoicomonas* between the Symbiodiniaceae and coelentric cavity which contains seawater, nutrients and other bacteria (Herndl & Velimirov, 1985; Yuan et al., 2018). These slides imply a potential association between the Symbiodiniaceae and the bacteria (Matthews et al., 2020), where *Endozoicomonas* may be ferrying nutrients from the seawater to the Symbiodiniaceae, or transporting waste products from the Symbiodiniaceae to the seawater for removal (Yuan et al., 2018).

This thesis presents for the first time a detailed examination of the gut bacterial composition, which due to its size is generally difficult to sample. The gut contained the largest abundance of archaea out of all the regions, the dominant families being: Nitrosopumilaceae, Haloferaceae, Halomicrobiaceae and Halococcaceae. Several families (Spirochaeta, Fusobacteriaceae, Marinifilaceae and Marinilabiliaceae) were observed that have been found in gut microbiomes of other animals; surgeonfish (Miyake et al., 2015; Parata et al., 2020), sea cucumbers (Weigel, 2020), crabs (An et al., 2021) and sea urchins (Ketchum et al., 2021). As well as families with functions associated with digestion such as the breakdown of organic material and amino acids, nutrient cycling, and production of polysaccharides (Cho, 2014; Lilburn et al., 2001; Zeibich et al., 2019). The time of day did not significantly affect the microbiome composition; however, it was shown that the abundance of bacteria related to nitrogen fixation, chlorophenol degradation and sulphur metabolism were higher in the morning, and lignin degradation and denitrification in the afternoon.

5.2.2 Chapter 3

Chapter 3 compares the microbiomes of the *Heliofungia* tissues of the bleached population from April 2017, and a year later in April 2018 when the population was no longer bleached. It was expected that the endoderm and gut would be most affected by bleaching; the endoderm would lose its Symbiodiniaceae and associated microbes, which would have a knock-on effect on the gut due to the loss of autotrophic feeding, causing a larger requirement

on heterotrophy (Grottoli et al., 2006; Hughes & Grottoli, 2013). When *Heliofungia* was bleached, the highest abundance unique genera of the endoderm were linked to either disease (*Vibrio* and *Streptococcus*) (Kemp et al., 2018; Kushmaro et al., 2001; Wu et al., 2019), or genera known as being antimicrobial or probiotic (*Pseudoalteromonas* and *Bacillus*) (Elshaghabee et al., 2017; Nissimov et al., 2009). These results imply that as the endoderm is stressed, we see an increase in pathogenic bacteria trying to take advantage of this, but also an increase in beneficial bacteria to counter this. It was also interesting to see the archaea *Halococcus* in the bleached endoderm, as this genus can generate ATP in light and has oxidative and fermentative capabilities (Beleneva et al., 2005; Post, 1977), which could fill some of the niche roles lost with the Symbiodiniaceae. Understanding whether these beneficial microbes are truly associated with the endoderm would be the next steps needed to examine this further. Interestingly, nutrient cycling was the main role of bacteria found in the unbleached endoderm; particularly the use and breakdown of plant derivatives, potentially from the recovered Symbiodiniaceae (*Spirochaeta*, *Bradyrhizobium* and *Enhydrobacter*) (K. A. Lema et al., 2014; Park et al., 2013; Premalatha et al., 2015). These links between bacteria and Symbiodiniaceae imply a likely association with the tissue and its photoendosymbionts.

Caedibacter was shown to be unique to the bleached gut. With respect to bleaching, this genus is known to cause upregulation of heat shock genes and metabolic pathways of its host (Grosser et al., 2018). Both these factors would be extremely useful for the bleached *Heliofungia*; however, further testing would be needed to know if the genus is associated with the coral. Some genera were hypothesised to be a food source brought in from the mucus (as it was only abundant in the bleached mucus) that the coral can utilise when its autotrophic requirements cannot be met (Grottoli et al., 2018; Grottoli et al., 2006). Bacteria that may be antagonistic to one another were observed. For example, pathogenic genera that are likely taking advantage of the stressed niche (Becker et al., 2021; Meyer et al., 2019) and, genera known for being probiotic and aiding their hosts in mitigating disease (Desjardine et al., 2007; Sanders et al., 2003). Also, a genus that can biodegrade polyethylene and hydrocarbons (*Brevibacillus*) (Badis, 2016; Panda et al., 2014) which matches roles with two genera observed to be unique in the unbleached gut; *Shewanella* and *Salinsphaera*. Although different genera, the breakdown of hydrocarbons seemed to be a prominent role in the gut and may explain why so many links between genera were observed in the network analysis of the gut. *Cetobacterium* unique to the unbleached gut was interesting as they have been heavily associated with the gut and digestive microbiome of many fish species (Bhute et al., 2020; Larsen et al., 2014; Ramírez et al., 2018; Tan et al., 2019). Particularly with its ability to produce B_{12} for the fish (Tsuchiya et al., 2008) and work by Agostini et al (2008) demonstrating the coral gut contains high levels of B_{12}, which would need bacteria to produce this (Agostini

et al., 2008; Agostini et al., 2009; Agostini et al., 2012). *Cetobacterium* should be investigated further and also be considered in future BMC work as, during stress, corals will need bacteria known for healthy gut functioning to assist in heterotrophy.

5.2.3 Chapter 4

Chapter 4 investigated the transcriptomic response of the ectoderm and endoderm of bleached *Heliofungia* collected from the April 2017 bleaching event and unbleached individuals a year after the bleaching event. Observing the tissue-specific responses of genes known to be important in bleaching in whole polyps gives a new outlook and valuable groundwork into their role in bleaching. Most importantly, observing genes that were solely expressed at high levels in the bleached endoderm link these genes to potential instigators and pathways of bleaching. For example, RGpF, which is known to be involved in phagocytosis and modulating symbiosis between nitrifying bacteria and plants (Ardissone et al., 2011; Shibata et al., 2009), was significantly expressed in the bleached endoderm, implying it may have a role in the symbiosis of coral and Symbiodiniaceae. Furthermore, the high expression of DMBT1 which has been hypothesised to be linked to symbiosis maintenance and mitigating apoptosis during stress (Andrade Rodríguez, 2018; Neubauer et al., 2016; Wright et al., 2017). In addition to this, genes known to ferry toxic by-products from the coral (Kenkel et al., 2014; Mayfield et al., 2010; Seneca et al., 2010) were highly expressed in the bleached ectoderm and endoderm, with low to no expression in unbleached tissues. Therefore, these genes may be involved in the removal process of Symbiodiniaceae, once bleaching is triggered. Also, this model could be used to better identify differentially expressed pathways between the tissues as some genes that were highly expressed in the endoderm (for example, protein chaperones (Georgopoulos & Welch, 1993; Oakley & Davy, 2018; Ruiz-Jones & Palumbi, 2017)), had a synergistic gene partner highly expressed in the bleached ectoderm (genes known to collect and remove damaged proteins (Hayward et al., 2011; Rahajeng et al., 2010; Rocker et al., 2015)).

5.3 Synthesis

These chapters highlight the importance of studying genetic and microbial responses on a tissue level. New observations have been made using this process as well as a better idea of gene and microbe function with respect to location. The research presented in this thesis is the groundwork for explaining the functional roles of each region of the metaorganism in finer detail.

5.3.1 The ectoderm

The ectoderm seems to be predominantly involved in transport, physical protection and signalling, which is reflected in the host transcriptomics and microbial community results. There was a strong enrichment of neuronal GO terms in the ectoderm that suggested a larger role in neurogenesis than the endoderm (Chapter 4). This might reflect a sensory role for neurons in the ectoderm, which is the tissue layer that faces the environment and is particularly important for a free-living, mobile coral (Borel-Best & Hoeksema, 1987). In addition to the acrosphere that utilises neurons as part of the discharge system (Bos & Hoeksema, 2015; Marlow et al., 2009; Martindale & Hejnol, 2009), the ectoderm demonstrated further roles in host protection as there were Neuropeptide FF receptors and a gene that is homologous to one found in cone shells involved in toxin production (Li et al., 2019; Robinson & Norton, 2014). Furthermore, bacteria known for protection against bacterial infection (*Bacillus* and *Pseudoalteromonas*) were prominent in the ectoderm, particularly as unique genera when the corals were bleached. These genetic findings match what was observed in the FISH imagery of the tissue (Chapter 2) as the nematocysts were condensed tightly at the tip of the ectoderm and would require genes for toxin production. Specific transport genes were expressed in the ectoderm (Ferguson & Blakely, 2004; Zoccola et al., 2015), which matched with certain nutrient transport genes observed in *Endozoicomonas* (Neave et al., 2017; Tandon et al., 2020). Although there have not been any coral ectoderm studies previously, work in *Hydra* has proposed the ectoderm to be a physicochemical barrier between host and environment, as shown by damage to the ectoderm leading to bacterial infection (Augustin & Bosch, 2010; Bosch, 2013). For the first time, host genes and microbes are observed in the coral ectoderm, with clear roles in host protection and transport, giving the impression that these may be working together, or at least complement one another, for a common goal. However, further work would be needed to understand this.

5.3.2 The endoderm

The role of the endoderm is much more complex than the ectoderm; however, similar to the ectoderm, commonality was observed between the genetic and microbial findings in the tissue. The roles that stood out the most in the endoderm were related to immunity and symbiosis, processes that have already been associated with each other in the coral literature (Kvennefors et al., 2010; Mohamed et al., 2016; Mohamed et al., 2019). Immunity seemed to be a strong role observed in the endoderm. Bacteria with suggested probiotic roles and involvement in immune response signalling (for example, NF-κB signalling) were present (Hashem et al., 2019; Shnit-Orland et al., 2012; Weigel, 2020; Yang et al., 2015). These

bacterial findings corresponded to the trends in hosts genes and corresponding GO terms (when bleached and unbleached), known for their role in immune response (including stimuli linked with NF-κB signalling; cytokines, ultraviolet irradiation and viral antigens) (Ben-Neriah & Karin, 2011; Cunning et al., 2018; Pham et al., 2004). Also, genes previously believed to be solely involved in immune function, these are now believed to be involved in symbiont acquisition and maintenance (Mohamed et al., 2016; Christian R Voolstra et al., 2009). Bacteria linked to the Symbiodiniaceae were reported in the endoderm (Lawson et al., 2018; Matthews et al., 2020), which are known for producing molecules the Symbiodiniaceae could utilise or vice-versa. The core genera in the endoderm, although not likely solely Symbiodiniaceae associated since they were present in bleached and unbleached corals, contained characteristics that would complement the roles of the Symbiodiniaceae, including DMSP breakdown, nitrogen cycling and antioxidant defence (López-Pérez et al., 2020; Malmstrom et al., 2005), and were in higher abundance when unbleached. The high abundance of genera known for nitrogen fixation and living in symbiosis with other algae (Bottomley, 1992; Kaneko et al., 2002; Park et al., 2013) was promising and may be linked to symbiosis maintenance through the products they supply to the Symbiodiniaceae, or making the endoderm a more hospitable tissue for them to inhabit. Overall, both tissues showed strong links in function between the host response and microbiome present, this is an avenue of research that should be studied further.

5.4 Future directions

5.4.1 Biotechnology - the acrosphere

During the numerous dissections and working at an in-depth level with *Heliofungia* the acrosphere was isolated as its own region. Most corals have their nematocysts throughout their ectoderm and it seemed uncharacteristic that *Heliofungia* would have them condensed to the tentacle tip however, although not tested, two papers had previously described the region (Bos & Hoeksema, 2015; Hoeksema, 2014). Isolating the acrosphere and classifying its microbiome are the first steps in characterising the region as its own unique microniche and opens doors for future work. Samples were only taken across four time points rather than the full six however, the acrosphere was shown to harbour α- and β-diversity which clusters most closely to the endoderm. From the isolation of this region, the acrosphere has already had proteins identified that may contain novel biotechnological properties (Schmidt et al., 2020). In addition to proteomics, gene expression data should be acquired as this may advance our understanding of the coral defence system and identify novel toxins. With more

time, it would be enlightening for the acrosphere to undergo RNAseq and observe the transcriptomics of the region and determine the most prolific proteins in the region.

5.4.2 Coral imaging

We have shown that the coral Heliofungia presents undeniable qualities to improve our understanding of coral biology through coral imaging. Several avenues could be taken in future studies, including; the use of genera-specific fluorescent probes towards Nitrinocolaceaea or Spirocheataceae, to investigate whether these genera are present in the ecto- or endoderm aggregates observed in Heliofungia tissues or associated with the Symbiodiniaceae (as shown by the sequencing results in Chapter 2). However, one of the biggest factors limiting the development of probes and sequencing work is the large number of unknown ASVs. More studies towards isolating bacteria as well as metagenomic studies are needed to improve our understanding of bacteria identification and function. To overcome this challenge, live-FISH (Batani et al., 2019) could be used in *Heliofungia* to tag specific unknown bacteria in the cells and isolate them without the challenges that come with cultivating bacteria (selecting correct media, selecting correct bacteria etc.).

The FISH and histology images presented in Chapter 2 demonstrate the high clarity that is available while working with Heliofungia. However, this could be developed further with respect to observing microbe movement and tagging nutrients to view their transport pathway. There are new techniques arising that involve live imaging of bacteria and proteins within animals (Chaconas et al., 2021; Geng & Pertsinidis, 2021; Genshaft et al., 2021; Munck et al., 2021). These methods are challenging, particularly when working with a less-studied animal, but repeated testing utilising various probes, multiple slides, and trying different laser wavelengths could produce valuable results. One of the limiting factors working with FISH is time; it takes a long time to search through the slides, adjust the wavelengths, find the right area to scan and magnify further whilst also trying to reduce the exposure of the slide to the laser. One way this could be improved is utilising digital pathology with a machine learning annotation, which is a new technique that is showing promising results in medicine. This technique has been particularly useful in radiology and cancer detection, where areas of soft tissue are scanned and a machine learning algorithm is applied to segment out healthy tissue and organs in a 3D space and identify tumours (Madabhushi & Lee, 2016). Digital pathology is also being applied to bacteria, where it is used to identify pathogens in animals and plants. There are also hopes to be able to use this technique to identify the bacteria themselves (Poornima et al., 2019; Sajedi et al., 2020). To be successful, the images must be of good

resolution, which is achievable with *Heliofungia* and would be an interesting technique to apply to microbiome studies in coral.

Nutrient transfer between the Symbiodiniaceae and *Endozoicomonas* could also be viewed by tagging the nutrients with stable isotope and mass spectrometry, which have had previous success in corals (Tanaka et al., 2015; Wangpraseurt et al., 2015). Taking another technique from medicine, it may be possible to build, or grow, an ecto-endoderm chamber using organoids (Drost & Clevers, 2018). If one could grow the tissue membrane between the regions in a chamber, nutrients and bacteria could be added to each side of the chamber and transport between the two could be reported using sensors. Overall, there are several different techniques, some newly developed for medicine, that could be applied to continue the work achieved in this thesis and could lead to a greater understanding of coral biology and the microbiome.

5.4.3 Symbiodiniaceae

For a better understanding of the endoderm, more information is needed on the Symbiodiniaceae present. As *Heliofungia* was majoritively *Claudicopium* spp. dominant (Chapter 4) it would be extremely useful to sequence the microbiome of this Symbiodiniaceae and to compare the microbiome of the *Heliofungia* endoderm (Chapter 2 and 3) and *Claudicopium*. This comparison would allow us to identify which microbes are linked with the Symbiodiniaceae and which with the host. Furthermore, mass spectrometry and stable isotope labelling could be applied to the Symbiodiniaceae to see the nutrients passed between host, microbes and Symbiodiniaceae (Tanaka et al., 2015; Wangpraseurt et al., 2015). This would give a better understanding of the bacteria present in the endoderm and the genes seen to be expressed relating to nutrient transfer, which are in fact interacting with the Symbiodiniaceae. In the endoderm microbiome work it was noted that certain bacteria are known for having a co-abundance with one another in corals (*Enhydrobacter* and *Acinetobacter* (Shore-Maggio et al., 2015)), with more time available, it would be interesting to study if this occurs with other genera. Particularly interesting would be *Enhydrobacter* and *Spirochaeta*, as they make and break down cellulose, respectively, and were both present when *Heliofungia* was unbleached and the Symbiodiniaceae had returned. *Synechococcus* and *Candidatus Actinomarina* should also be studied further, as these were both seen to be core genera in the endoderm but at higher levels with Symbiodinaceae. Isolation and sequencing of the genomes of these species with comparative analysis of the *Cladicopium* sp. genome could be enlightening in determining whether Symbiodiniaceae and the bacteria share roles in the endoderm.

5.4.4 Digestive studies

The results here present the first account of the coral digestive microbiome that was sampled with relative ease. With more time, many more questions could be asked. Of particular interest, would be to clarify if diel cycle does impact the microbiome which could be answered by taking more sampling time points over several days. This would be an even stronger study if the number of 'unknown' ASVs could be reduced, potentially utilising some of the techniques mentioned above. As well as water sampling from the gut and immediate surrounding to test for nutrient and pollutant levels. Previously, microelectrodes, microsensors and small capillaries have been used to test the gastric cavity of corals (Agostini et al., 2012; Cai et al., 2016) which is arduous. With *Heliofungia*, it would be simple to place sensors in the gut to test for nutrient levels and gradients, as well as using capillaries or syringes to extract the liquid from the cavity for proteomics, transcriptomics and 16S sequencing. Furthermore, sampling the gut for RNAseq, in corals studied previously the mouth size would limit the amount of RNA one could get from a polyp however, with *Heliofungia* this could be achievable, and samples could be taken from the liquid in the gut as well as the tissue that makes up the gut wall. Comparing host genetics, the microbiome or even metagenomics in *Heliofungia* could advance our understanding of coral digestion and heterotrophy exponentially.

5.4.5 Advancing bleaching understanding

It would be useful to re-run the collection of samples but adding additional timepoints to build a more nuanced picture of the mechanisms involved and answer questions that arose during the research. The corals should be genotyped beforehand and kept in separate holding tanks. Originally corals were not tagged as drilling a hole in them would alter gene expression and even cause death. Also, they were not expected to move as far as they did between the sampling points in April, August and November. Corals could be set up in their own tanks in a lab for a bleaching experiment or pens created underwater. Sampling in-field gives a greater idea of real environmental responses; however, the control created in the lab gives a much more direct approach. More sampling points should be brought in as it would be useful to see if changes occur in the Symbiodiniaceae species present and observe their gene expression during bleaching. In this experiment, reads were generated from the unbleached time point for the Symboidiniaceae; however, there were very few reads that could be compared from the bleached time point. Therefore, sampling pre-bleaching and then every 6 six hours during the bleaching process and recovery would add a greater depth to results here. As well as likely identify the bleaching trigger and whether it is host or symbiont initiated. Furthermore, as in this research, sampling for the ecto- and endoderm microbiome during this bleaching

experimentation would give a closer inspection of how the tissue-specific microbiome is altered as the Symbiodiniaceae leave and the host becomes more stressed. It would be noteworthy to run the experiment with three groups; control, bleaching stress and bleaching stress with potentially beneficial microorganisms. Taxa highlighted in this thesis suggested that contain beneficial properties could be cultured and *Heliofungia* inoculated with this mix to see if this diminishes the bleaching response, reduces the number of pathogens present when bleached and alters the gene response. If possible, it would be noteworthy to add in proteomics work and observe if the genes seen to be upregulated are producing the proteins hypothesised, giving a much more accurate understanding of the bleaching pathways and knock-on effects on coral biology.

5.5 Concluding remarks

In conclusion, the work in this thesis filled important knowledge gaps with respect to tissue-specific data in corals. The ectoderm and endoderm tissues were shown to harbour their own unique microbiome and their genes respond differently when bleached and unbleached. New insights into the coral gut were achieved by successfully sequencing the microbial community within. The acrosphere, a bundle of nematocysts located at the tip of each tentacle in Heliofungia, was isolated for the first time and its specific microbiome was described. This research lays valuable groundwork for understanding the coral bleaching response further, which can be built upon to answer the questions raised in this thesis.

Appendix A: Chapter 2

Table S2.1: Primers used in sequencing for bacterial and archaea ASVs (Robbins et al., 2021; Robbins et al., 2019). The forward mix is made up of a ratio of 2a:b:c:d.

CCCG	Illumina linker	Primer (5'-3')	Full sequence	Bases	TM [°C] (50µM NaCl)	Seq SpecTM [°C] (50µM NaCl)	GC [%]
iTAG_803Fa	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	TTAGATACCCTGGTAGTC	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGATACCCTGGTAGTC	51	67.9	46.7	49
iTAG_803Fb	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	TTAGATACCCSGGTAGTC	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGATACCCSGGTAGTC	51	68.7	49.6	51
iTAG_803Fc	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	TTAGATACCCYHGTAGTC	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGATACCCYHGTAGTC	51	67.7	46.2	48.7
iTAG_803Fd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	TTAGAGACCCYGGTAGTC	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGAGACCCYGGTAGTC	51	69	51.0	52
iTAG_803Mix	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	TTAGAKACCCBNGTAGTC	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGAKACCCBNGTAGTC				
iTAG_1392wR	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	ACGGGCGGTGWGTRC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGGGCGGTGWGTRC	49	70.9	57.1	58.1

Table S2.2: Sequences and dyes used for FISH, ratio used for EUB338iii was 1:1:1 and for ENDOZ 1:1.

Name	Probe	Sequence	Dye
NONEUB	NONEUB	5'-ACATCCTACGGGAGGC-3'	Atto 550
EUB338iii	EUB338i	5'-GCTGCCTCCCGTAGGAGT-3'	Atto 550
	EUB338ii	5'-GCAGCCACCCGTAGGTGT-3'	
	EUB338iii	5'-GCTGCCACCCGTAGGTGT-3'	
NONEUB	NONEUB	5'-ACATCCTACGGGAGGC-3'	Atto647N
ENDOZ	Endozoi663	5'-GGAAATTCCACACTCCTC-3'	Atto647N
	Endozoi736	5'-GTCAGTGTCAGACCAGAG-3'	

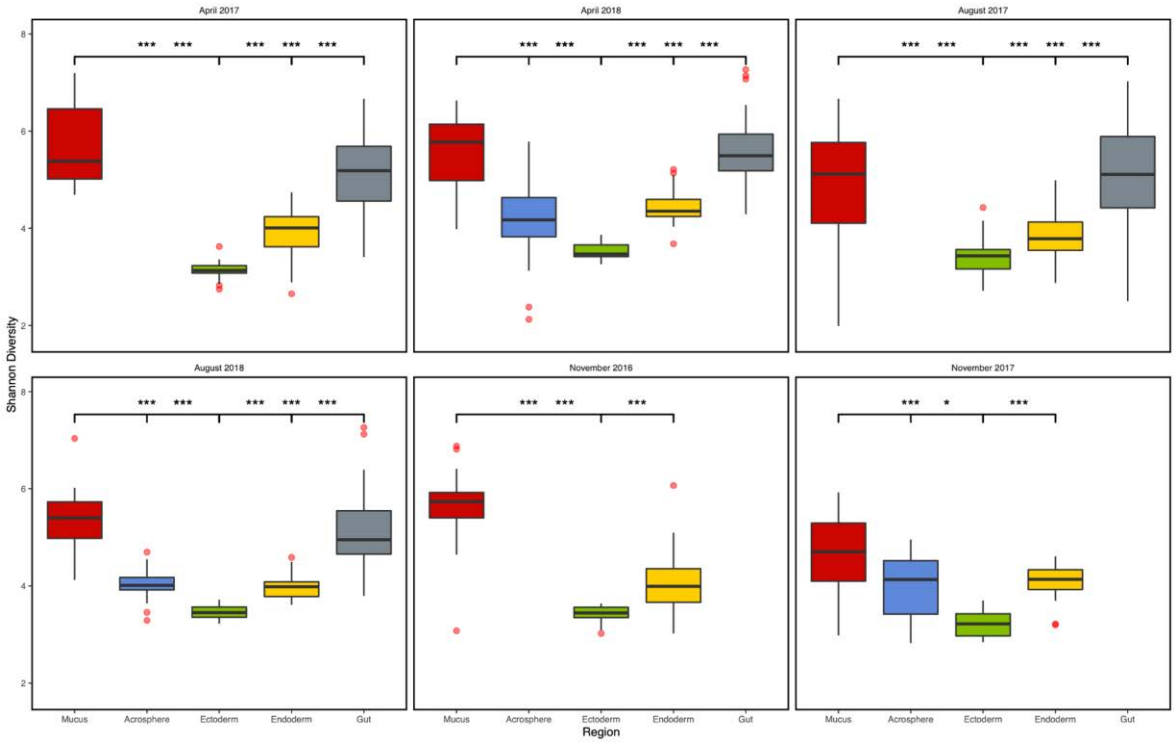


Figure S2.1: The α-diversity (shannon diversity) seen in each of the five tissue regions (mucus - red, acrosphere - blue, ectoderm - green, endoderm - yellow and gut - grey). Significance of comparison shown above (*** = *p* value > 0.0005, ** = *p* value > 0.005).

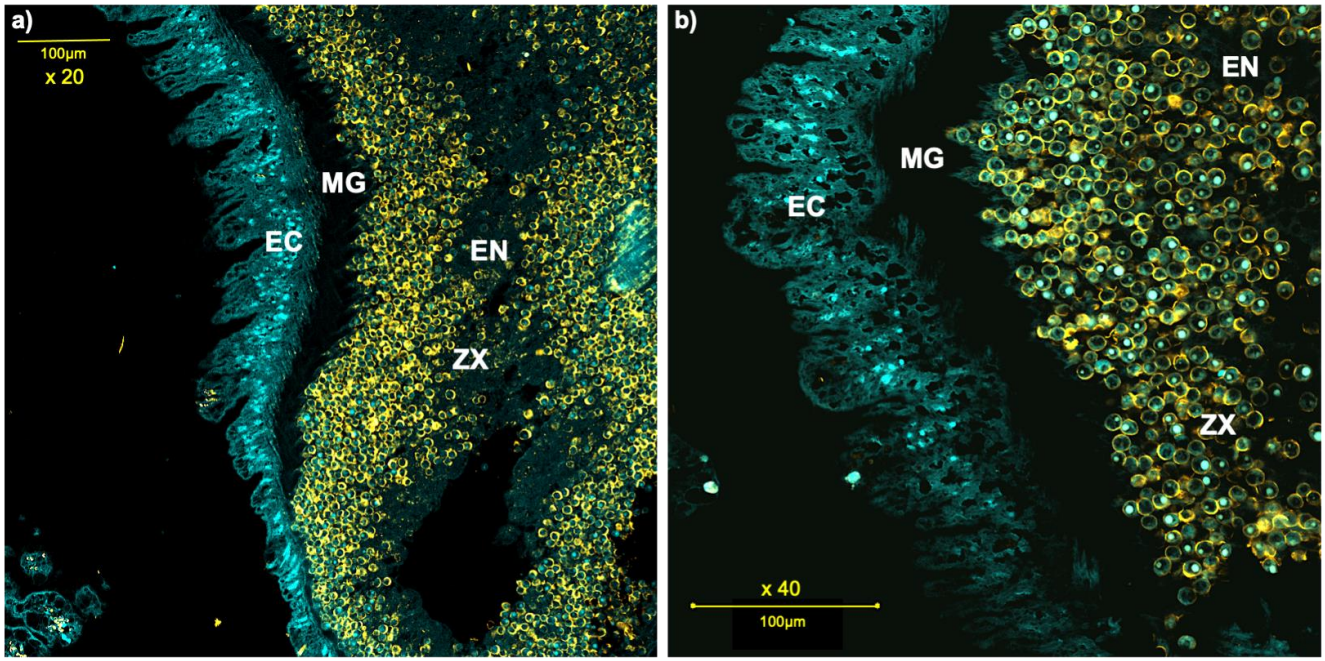


Figure S2.2: NONEUB images of *Heliofungia* showing the ectoderm (Ec), mesoglea (Mg) and naturally fluorescing Symbiodiniaceae (Zx) in the endoderm (En), image a) is at 20x magnification and b) at 40x.

Appendix b: Chapter 3

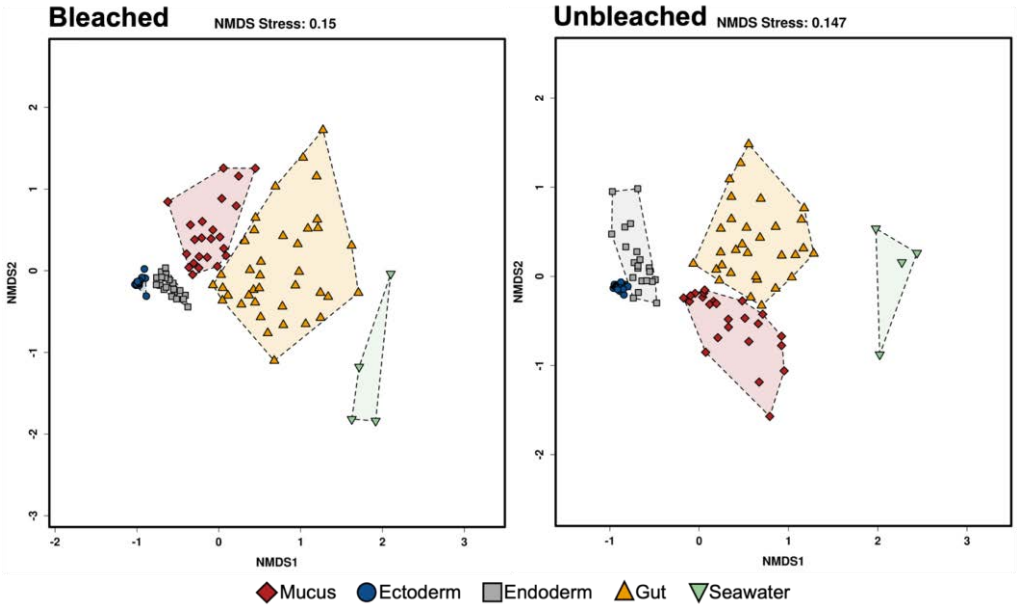


Figure S3.1: NMDS plot (Bray-Curtis dissimilarity) showing the β-diversity of each region (bottom legend) and the seawater samples taken in 2017 (bleached) and 2018 (unbleached).

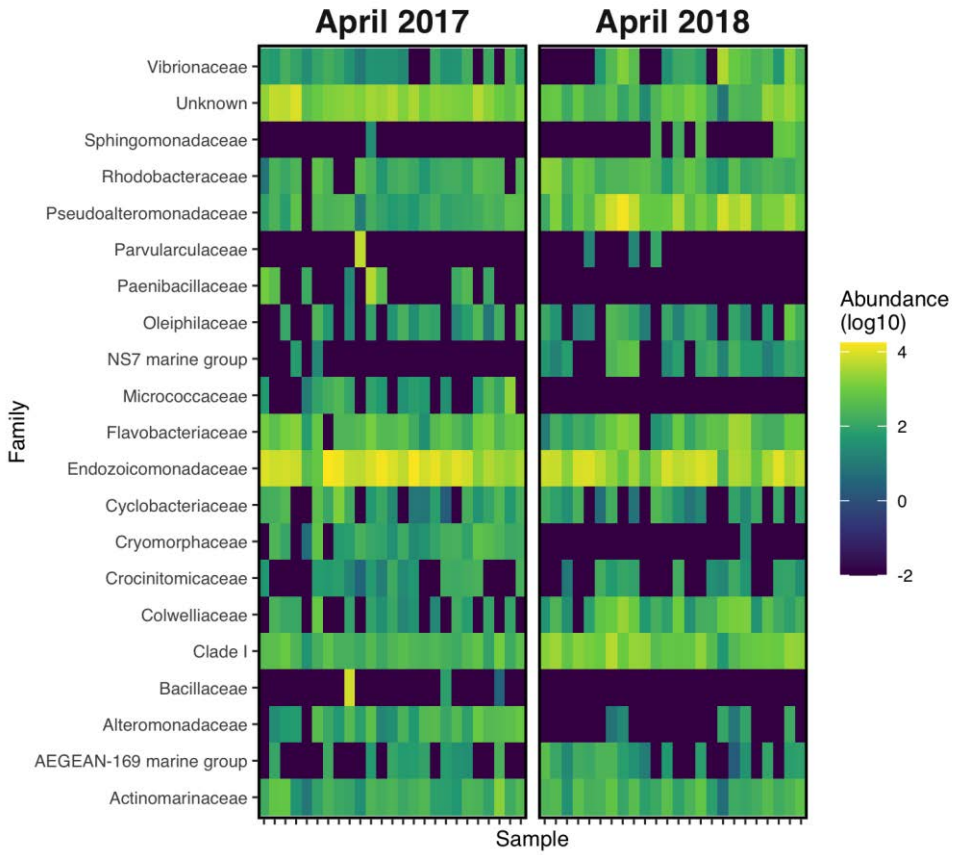


Figure S3.2: heatmap showing top 20 microbial families present in the mucus and how these changed between the bleaching event in April 2017 and a year after when recovered in April 2018. Five technical repeats were taken per mucus per individual (five at each time point).

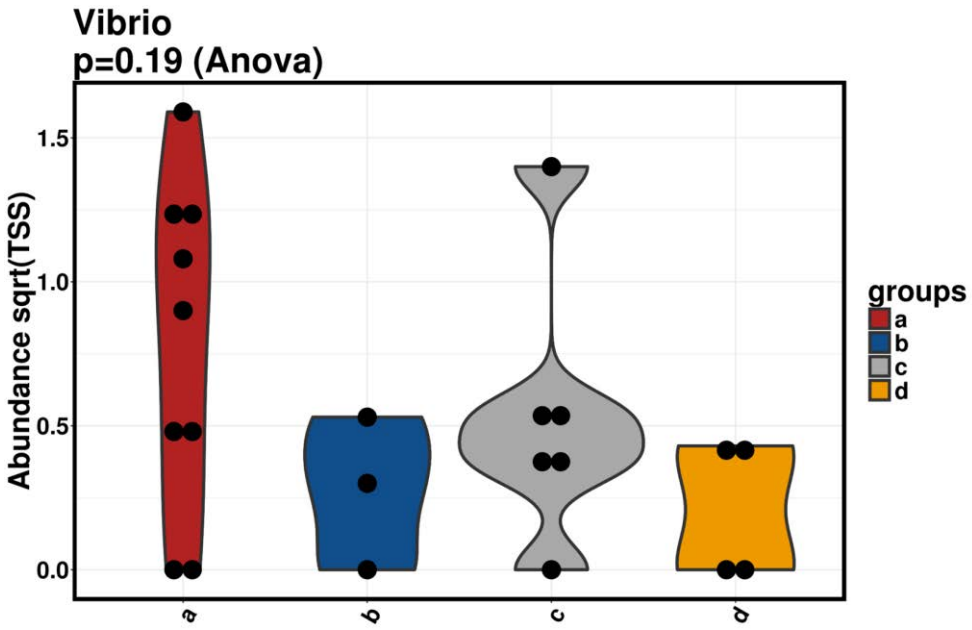


Figure S3.3: Abundance of *Vibrio* in the endoderm when *Heliofungia* was bleached. Groups a - c include individuals containing *Durusdinium* sp. Symbiodiniaceae and d contains *Cladicopium* sp.

Table S3.1: all genera reported to be core, following Calypso genome nets (Zakrzewski et al., 2017) core/unique analysis, in the regions of *Heliofungia* when bleached and unbleached.

Genus	2017 abundance	2018 abundance	2017 occurrence	2018 occurrence
Mucus				
Unclassified	5.429	4.907	1	1
Endozoicomonas	5.179	4.271	1	1
uncultured_bacterium	3.028	3.737	1	1
uncultured	1.635	1.016	1	1
Candidatus_Actinomarina	0.934	1.033	1	1
Pseudoalteromonas	0.893	2.762	1	1
Vibrio	0.79	1.256	1	0.96
NS5_marine_group	0.717	0.836	0.88	1
Synechococcus_CC9902	0.68	1.713	1	1
Alteromonas	0.667	0.343	0.83	0.71
Oleiphilus	0.562	0.588	0.83	0.83
Thalassotalea	0.558	1.31	0.79	1
unidentified_marine_bacterioplankton	0.485	0.739	0.92	0.96
Streptococcus	0.477	0.2	0.71	0.5
Micrococcus	0.475	0.265	0.62	0.54

Tenacibaculum	0.458	0.451	0.71	0.67
Crocinitomix	0.416	0.516	0.75	0.83
NS4_marine_group	0.382	0.317	0.62	0.71
Acinetobacter	0.376	0.172	0.67	0.5
Algicola	0.364	1.148	0.75	0.92
Staphylococcus	0.345	0.252	0.46	0.54
Fluviicola	0.344	0.337	0.71	0.58
Marinoscillum	0.344	0.437	0.5	0.75
bacterium_WHC712	0.342	0.282	0.58	0.71
Clade_Ib	0.312	0.734	0.75	0.92
Sphingomonas	0.312	0.536	0.54	0.67
Aureispira	0.308	0.395	0.46	0.62
marine_metagenome	0.282	0.347	0.62	0.75
Sva0996_marine_group	0.281	0.149	0.62	0.54
OM27_clade	0.265	0.4	0.58	0.75
Amphritea	0.257	0.213	0.5	0.46
OM60NOR5_clade	0.245	0.289	0.71	0.62
Methylobacterium	0.222	0.38	0.42	0.75
Lentisphaera	0.195	0.363	0.5	0.62
Oleispira	0.18	0.249	0.42	0.54
Ectoderm				
Endozoicomonas	9.901	9.856	1	1
Unclassified	0.645	0.7	1	1
uncultured_bacterium	0.396	0.81	0.89	1
Endoderm				
Endozoicomonas	7.772	7.518	1	1
Unclassified	4.765	4.089	1	1
uncultured_bacterium	1.405	1.655	0.96	1
uncultured	0.497	0.293	0.91	0.58
Synechococcus	0.337	0.814	0.65	0.83
Candidatus_Actinomarina	0.287	0.238	0.65	0.46
Gut				
Unclassified	6.867	6.15	1	1
Endozoicomonas	2.945	3.029	1	1
Sphingomonas	1.999	1.598	0.92	0.94
Pseudoalteromonas	1.424	0.937	0.95	0.74
Spirochaeta_2	1.32	1.566	0.95	0.91
uncultured	1.307	1.175	1	1
Vibrio	1.207	1.135	0.85	0.89
uncultured_bacterium	1.088	1.28	1	1
Synechococcus	1.009	1.897	0.92	1
Candidatus_Actinomarina	0.644	0.92	0.9	0.97
Clade_Ib	0.547	0.645	0.72	0.83
Thalassotalea	0.526	0.478	0.67	0.63
Ekhidna	0.476	0.304	0.62	0.43
NS5_marine_group	0.471	0.947	0.64	0.97
uncultured_candidate_division_CABI_bacterium	0.436	0.588	0.62	0.77

unidentified_marine_bacterioplankton	0.387	0.584	0.59	0.86
Crocinitomix	0.331	0.469	0.51	0.77
OM60NOR5_clade	0.251	0.403	0.46	0.74
Tenacibaculum	0.25	0.262	0.49	0.51
NS4_marine_group	0.218	0.414	0.46	0.77
Sva0996_marine_group	0.214	0.333	0.46	0.57
Oleiphilus	0.177	0.164	0.44	0.43
Marinoscillum	0.156	0.398	0.44	0.74
Fluviicola	0.152	0.452	0.41	0.77
marine_metagenome	0.145	0.348	0.41	0.71

Table S3.2: all genera reported to be unique when *Heliofungia* was bleached or unbleached, in each region, created using Calypso genome nets (Zakrzewski et al., 2017) core/unique analysis.

Genus	Treatment	**2017 abundance**	**2018 abundance**	**2017** occurrence	**2018 occurrence**
Mucus					
NS10_marine_group	Bleached	0.684	0.071	0.88	0.17
Aestuariibacter	Bleached	0.662	0.105	0.83	0.29
Parvularcula	Bleached	0.374	0.105	0.67	0.29
Bacillus	Unbleached	0.365	0.12	0.33	0.42
Caedibacter_taeniospiralis_group	Bleached	0.352	0.042	0.5	0.12
Rubritalea	Bleached	0.317	0.163	0.54	0.29
HTCC5015	Bleached	0.288	0.037	0.5	0.21
uncultured_gamma_proteobacterium	Bleached	0.266	0.084	0.54	0.25
Oleibacter	Bleached	0.252	0.09	0.5	0.25
Oceanospirillum	Bleached	0.246	0.12	0.58	0.25
Chryseobacterium	Bleached	0.24	0.027	0.42	0.08
Ekhidna	Bleached	0.238	0.123	0.42	0.29
Corynebacterium_1	Bleached	0.23	0.09	0.46	0.29
Candidatus_Omnitrophus	Bleached	0.225	0.074	0.58	0.29
Ruegeria	Bleached	0.225	0.102	0.42	0.21
Thalassolituus	Bleached	0.208	0.054	0.58	0.08
Haemophilus	Bleached	0.2	0.07	0.42	0.12
uncultured_organism	Bleached	0.199	0.058	0.42	0.25
Enhydrobacter	Unbleached	0.171	0.486	0.25	0.67
uncultured_bacterium_HF0200_39L23	Unbleached	0.15	0.239	0.29	0.67
NS2b_marine_group	Unbleached	0.142	0.27	0.33	0.71
Salinisphaera	Unbleached	0.129	0.188	0.25	0.5
metagenome	Unbleached	0.115	0.178	0.33	0.46
Spirochaeta_2	Unbleached	0.115	0.259	0.38	0.58
Aliikangiella	Unbleached	0.114	0.247	0.33	0.71
Litoribrevibacter	Unbleached	0.114	0.337	0.29	0.71
Woeseia	Unbleached	0.104	0.165	0.29	0.42

uncultured_marine_group_II_euryarchaeote_HF10_15G04	Unbleached	0.087	0.168	0.38	0.46
Arcobacter	Unbleached	0.083	0.272	0.21	0.58
uncultured_Vibrio_sp.	Unbleached	0.077	0.138	0.29	0.42
Psychrosphaera	Unbleached	0.065	0.605	0.17	0.79
uncultured_Oceanospirillales_bacterium	Unbleached	0.053	0.17	0.17	0.42
Marinomonas	Unbleached	0.045	0.244	0.17	0.5
uncultured_gamma_proteobacterium_HF0070_25G02	Unbleached	0.042	0.196	0.12	0.5
Pleionea	Unbleached	0.025	0.171	0.08	0.5
Phalacroma_mitra	Unbleached	0.016	0.134	0.08	0.46
Reinekea	Unbleached	0.015	0.241	0.04	0.54
Bradyrhizobium	Unbleached	0.006	0.207	0.04	0.46
Litoricola	Unbleached	0	0.269	0	0.58
Thiomicrorhabdus	Unbleached	0	0.285	0	0.58
Ectoderm					
Pseudoalteromonas	Bleached	0.221	0.035	0.58	0.13
Vibrio	Bleached	0.202	0.013	0.58	0.09
Bacillus	Bleached	0.162	0.03	0.47	0.13
Spirochaeta	Unbleached	0.101	0.189	0.05	0.65
uncultured	Bleached	0.086	0.102	0.47	0.39
Sphingomonas	Unbleached	0.056	0.418	0.21	0.91
Kistimonas	Unbleached	0.05	0.28	0.21	0.65
Synechococcus	Unbleached	0.021	0.239	0.11	0.65
Enhydrobacter	Unbleached	0	0.199	0	0.57
Endoderm					
Vibrio	Bleached	0.544	0.107	0.74	0.25
Pseudoalteromonas	Bleached	0.465	0.279	0.7	0.12
Bacillus	Bleached	0.415	0.015	0.61	0.04
Halococcus	Bleached	0.393	0.04	0.74	0.08
Streptococcus	Bleached	0.359	0.066	0.48	0.12
Allorhizobium Neorhizobium Pararhizobium Rhizobium	Bleached	0.287	0.032	0.57	0.08
Acuticoccus	Bleached	0.281	0	0.57	0
Alcanivorax	Bleached	0.258	0	0.48	0
Alteromonas	Bleached	0.228	0.063	0.52	0.17
Sphingomonas	Unbleached	0.139	1.206	0.26	1
Acinetobacter	Unbleached	0.138	0.416	0.39	0.42
Enhydrobacter	Unbleached	0.076	0.805	0.22	0.88
Spirochaeta	Unbleached	0.066	2.119	0.17	0.88
Methylobacterium	Unbleached	0.063	0.41	0.17	0.58
Bradyrhizobium	Unbleached	0.013	0.331	0.04	0.42

Gut					
Brevibacillus	Bleached	0.822	0.005	0.41	0.03
Caedibacter_taeniospiralis_group	Bleached	0.511	0.039	0.72	0.17
Algicola	Bleached	0.481	0.32	0.67	0.34
Micrococcus	Bleached	0.48	0.108	0.79	0.26
NS10_marine_group	Bleached	0.443	0.028	0.56	0.11
Alteromonas	Bleached	0.384	0.101	0.62	0.26
Thalassolituus	Bleached	0.305	0.004	0.49	0.03
Acinetobacter	Bleached	0.279	0.127	0.46	0.34
Staphylococcus	Bleached	0.273	0.102	0.49	0.23
Parvularcula	Bleached	0.206	0.064	0.41	0.2
Aestuariibacter	Bleached	0.192	0.009	0.54	0.06
Corynebacterium_1	Bleached	0.182	0.148	0.41	0.2
Pseudomonas	Bleached	0.179	0.045	0.46	0.14
Aureispira	Unbleached	0.156	0.332	0.26	0.63
NS2b_marine_group	Unbleached	0.153	0.42	0.38	0.63
OM27_clade	Unbleached	0.149	0.494	0.31	0.77
Salinisphaera	Unbleached	0.133	0.441	0.23	0.8
Tetraselmis_cordiformis	Unbleached	0.13	0.454	0.28	0.74
Methylobacterium	Unbleached	0.113	0.535	0.26	0.77
uncultured_bacterium_HF0200_39L23	Unbleached	0.106	0.232	0.26	0.49
Enhydrobacter	Unbleached	0.092	0.248	0.21	0.49
Shewanella	Unbleached	0.039	0.881	0.1	0.46
Phalacroma_mitra	Unbleached	0.028	0.197	0.13	0.46
Cetobacterium	Unbleached	0.026	1.5	0.1	0.57
Rubripirellula	Unbleached	0.02	0.192	0.1	0.49
Pseudonocardia	Unbleached	0.02	0.163	0.05	0.4
uncultured_marine_group_II_euryarchaeote_HF10_15G04	Unbleached	0.019	0.157	0.08	0.4
Thiomicrorhabdus	Unbleached	0	0.278	0	0.66
Rubricoccus	Unbleached	0	0.162	0	0.46

Table S3.3: Potential functions observed in the core and unique bacteria in the bleached and unbleached regions of *Heliofungia*, for a list of genera present see table 1 and figure 6. Negative attributes are highlighted in red, roles related to bleaching survival are in green.

Region	Bleached		Unbleached	
	Core	Unique	Core	Unique
Mucus	- bleaching (*Vibrio*) - disease (*Vibrio, Pseudoalteromonas*) - redox reactions (*Ca. Actinomarina*) - degradation of organic matter (*NS5*) - antimicrobial (*Pseudoalteromonas*) - Exopolysaccharide production (*Pseudoalteromonas*) - nutrient cycling (*Endozoicomonas*)	- prefer warmer temperatures (*NS10*) - disease (*Aestuariibacter*) - biofilm (*Aestuariibacter*) - probiotic consortium for reef clean-ups (*Parvularcula*) - bleaching related (*Rubritalea*)	- breakdown of complex compounds (*Thalassotalea*) - nutrient cycling (*Thalassotalea, Synechococcus, Endozoicomonas*) - antimicrobial (*Pseudoalteromonas, Synechococcus*) - Exopolysaccharide production (*Pseudoalteromonas*) - disease (*Vibrio, Pseudoalteromonas*) - bleaching (*Vibrio*)	- antimicrobial and pathogen control (*Psychrosphaera*) - sulphur oxidisation (*Thiomicrorhabdus*) - disease (*Acrobacter*) - hypoxic reefs (*Acrobacter*) - cellulose breakdown (*Enhydrobacter*) - oil degradation (*Enhydrobacter*)
Ectoderm	- nutrient transfer and acquisition (*Endozoicomonas*) - host health (*Endozoicomonas*)	- disease (Vibrio, *Pseudoalteromonas*) - antibacterial and pathogen control (*Pseudoalteromonas, Bacillus*) - Exopolysaccharide production (*Pseudoalteromonas*) - probiotic (*Bacillus*) - starch hydrolysis (*Bacillus*)	- nutrient transfer and acquisition (*Endozoicomonas*) - host health (*Endozoicomonas*)	- nitrogen assimilation (*Kistimonas*) - nitrogen fixation (*Kistimonas, Synechococcus, Spirochaeta*) - breakdown of complex carbohydrates (*Spirochaeta, Enhydrobacter*) - hydrocarbon breakdown (*Sphingomonas*) - DMSP cycle (*Synechococcus*) - disease (*Sphingomonas*)
Endoderm	- DMSP cycle (*Synechococcus*) - nutrient cycling (*Synechococcus*) - redox reactions (*Ca. Actinomarina*) - heterotrophy (*Synechococcus*)	- bleaching (*Vibrio*) - disease (*Vibrio, Streptococcus, Pseudoalteromaons*) - antimicrobial and probiotic (*Pseudoalteromonas, Bacillus*) - Exopolysaccharide production (*Pseudoalteromonas*) - starch hydrolysis (*Bacillus*)	- DMSP cycle (*Synechococcus*) - nutrient cycling (*Synechococcus*) - redox reactions (*Ca. Actinomarina*) - heterotrophy (*Synechococcus*)	- Nitrogen cycling (*Bradyrhizopium, Spirochaeata*) - Plant symbiont (*Bradyrhizopium*) - breakdown of complex carbohydrates (*Spirochaeta, Enhydrobacter*) - oil, pollutants and microplastic degradation (*Enhydrobacter,*

		- fermentation and oxidation (*Halococcus*)		*Acinetobacter, Sphingomonas*) - disease (*Sphingomonas*)
Gut	- DMSP cycle (*Synechococcus*) - nutrient cycling (*Synechococcus, Spirochaeata*) - heterotrophy (*Synechococcus*) - breakdown of complex carbohydrates (*Spirochaeta*) - oil, pollutants and microplastic degradation (*Sphingomonas*) - Agar degradation (*Pseudoalteromonas*) - Exopolysaccharide production (*Pseudoalteromonas*) - disease (*Sphingomonas, Pseudoalteromonas*) - antimicrobial (*Psudoalteromonas*)	- oil, pollutants and microplastic degradation (*Brevibacillus*) - upregulation of metabolism & heat shock genes (*Caedibacter*) - disease (*Algicola*) - prefer warmer temperatures (*NS10*) - antibacterial (*Micrococcus*)	- DMSP cycle (*Synechococcus*) - nutrient cycling (*Synechococcus, Spirochaeata*) - heterotrophy (*Synechococcus*) - breakdown of complex carbohydrates (*Spirochaeta*) - oil, pollutants and microplastic degradation (*Sphingomonas*) - Agar degradation (*Pseudoalteromonas*) - Exopolysaccharide production (*Pseudoalteromonas*) - disease (*Sphingomonas, Pseudoalteromonas*) - antimicrobial (*Psudoalteromonas*)	- digestive microbiome of fish or other marine inverts (*Cetobacterium, Om27 clade, NS2b marine group*) - B12 production (*Cetobacterium*) - macromolecule metabolism (*Om27 clade, NS2b marine group*) - redox reactions (*Shewanella*) - disease (*Shewanella*)

Appendix c: Chapter 4

For methods, RNA extraction:

- Keep everything on ice until addition of DNase (buffers RW1/RPE work best at room temp) inc. place columns/bead tubes on ice prior to addition of samples.
- Add β-Mercaptoethanol to Buffer RLT Plus (10µl per 1ml) prior to extractions (one-month shelf life with β-ME so aliquot for the number of samples you need (+1) and keep fresh).
- Ignore 'Optional step 10'.
- Re-elute with eluate from step 11 for 12 – Ectoderm elute into 20µl, endoderm 35µl.
- Set up collection tubes, extra eppendorfs needed beforehand, label and on ice ready for samples – you want to make the process as fast and smooth as possible.
- UV everything beforehand (make sure to rotate pipettes/tube packs intermittently) and RNAse zap eliminator spray the shizz out of everything and your workspace.
- Better to do multiple extractions with less samples, than doing one extraction, all samples at once to reduce degradation of RNA.

Homogenization:

Trialled *(see additional info/lab book for breakdown)* - tube pestle and crushing liquid nitrogen, bead-beating - range of speeds, number of beads, duration (of beating and resting).

Best for tissue regions =

- Pool two samples for endoderm and three for ectoderm (add 600ul of RLT buffer to cryo tube, mix with pipette, pipette into second tube, mix with pipette, pipette into bead tube)
- ½ tube of ceramic beads 1.4mm (green lid)
- 5.0 m/s on FastPrep-24
- 10s on, 15s rest x 3
- Continue with RNeasy minikit protocol (next step centrifuge for 3 mins at 14,000rpm)

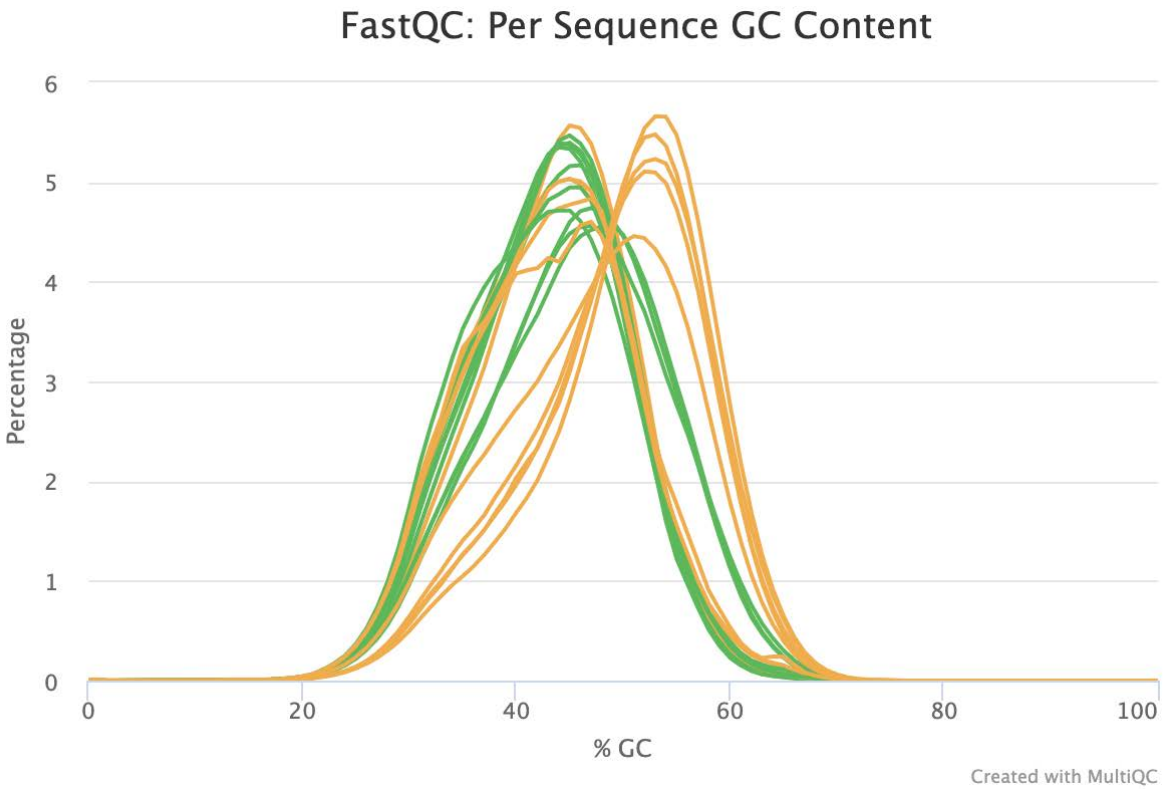


Figure S4.1. GC shifts in samples, samples containing Symbiodiniaceae are the five on the right with a higher GC content (~55%).

Table S4.1. Genes reported with a significant log fold-change in the treatment groups, filtered by tissue

Bleached by tissue	logFC	blast hit		Unbleached by tissue	logFC	blast hit
Ectoderm	-1.58	.		Endoderm	2.47	HES4B_XENLA
Ectoderm	-1.86	.		Endoderm	2.67	HRH2_MOUSE
Ectoderm	-1.51	.		Endoderm	5.07	ITAM_HUMAN
Ectoderm	-2.57	.		Endoderm	3.02	KCP_HUMAN
Ectoderm	-2.88	.		Endoderm	3.11	LGR5_RAT
Ectoderm	-1.85	.		Endoderm	2.25	MEOX1_DANRE
Ectoderm	-1.89	.		Endoderm	1.85	MEOX1_PANTR
Ectoderm	-3.51	.		Endoderm	1.38	MET27_HUMAN
Ectoderm	-1.86	.		Endoderm	4.06	MMP2_BOVIN
Ectoderm	-1.48	.		Endoderm	5.55	MMP25_MOUSE
Ectoderm	-1.94	.		Endoderm	2.03	MOT10_DANRE
Ectoderm	-2.15	.		Endoderm	3.58	NLRC3_HUMAN
Ectoderm	-1.54	.		Endoderm	1.99	NPFF2_RAT
Ectoderm	-1.92	.		Endoderm	1.92	NPFF2_RAT
Ectoderm	-3.15	.		Endoderm	1.48	NRX3A_HUMAN
Ectoderm	-1.6	.		Endoderm	1.57	NS1BA_DANRE
Ectoderm	-1.56	.		Endoderm	1.59	PEAM1_ARATH
Ectoderm	-2.08	.		Endoderm	2.04	PISD_CRIGR

Ectoderm	-1.76	.		Endoderm	2.75	PK1L2_MOUSE
Ectoderm	-2	.		Endoderm	1.53	PLAC8_BOVIN
Ectoderm	-1.61	.		Endoderm	1.48	PLMT_YEAST
Ectoderm	-1.09	.		Endoderm	1.78	POL_BAEVM
Ectoderm	-4.21	.		Endoderm	2.22	PPN_DROME
Ectoderm	-3.61	.		Endoderm	1.88	REXO4_YEAST
Ectoderm	-1.52	.		Endoderm	2.79	SOX7_XENLA
Ectoderm	-2.05	.		Endoderm	1.25	SYT15_HUMAN
Ectoderm	-3.15	.		Endoderm	2.72	TBX15_HUMAN
Ectoderm	-3.22	.		Endoderm	4.1	TIMP3_CHICK
Ectoderm	-1.94	.		Endoderm	1.58	UBP36_MOUSE
Ectoderm	-1.16	.		Endoderm	1.43	USOM7_ACRMI
Ectoderm	-1.08	.		Endoderm	2.1	VWCE_HUMAN
Ectoderm	-0.86	.		Endoderm	2.43	ZCH24_HUMAN
Ectoderm	-2.03	.				
Ectoderm	-2.1	.		Ectoderm	1.16	.
Ectoderm	-2.48	.		Ectoderm	1.31	.
Ectoderm	-2.23	.		Ectoderm	1.9	.
Ectoderm	-2.3	.		Ectoderm	1.6	.
Ectoderm	-1.41	AA2AR_CANLF		Ectoderm	1.59	.
Ectoderm	-0.64	ACAP2_MOUSE		Ectoderm	1.11	.
Ectoderm	-1.93	ACO11_MOUSE		Ectoderm	0.91	.
Ectoderm	-1.17	ARRD3_BOVIN		Ectoderm	1.31	.
Ectoderm	-2.38	BMP1_MOUSE		Ectoderm	1.44	.
Ectoderm	-2.31	CAHM6_RAT		Ectoderm	0.91	.
Ectoderm	-3.53	COCA1_CHICK		Ectoderm	0.92	.
Ectoderm	-1.75	CTR2_CHICK		Ectoderm	1.97	.
Ectoderm	-1.84	ELK1_RAT		Ectoderm	1.43	.
Ectoderm	-0.9	END4_BACTN		Ectoderm	1.05	.
Ectoderm	-0.67	GRP1_XENTR		Ectoderm	1.14	.
Ectoderm	-0.91	GXLT1_MOUSE		Ectoderm	1.24	.
Ectoderm	-1.01	IGLO5_HUMAN		Ectoderm	1.85	.
Ectoderm	-0.69	IVD_CAEEL		Ectoderm	1.41	.
Ectoderm	-0.96	MALT1_HUMAN		Ectoderm	1.76	.
Ectoderm	-1.16	MFRP_HUMAN		Ectoderm	1.05	.
Ectoderm	-1.57	MILK2_MOUSE		Ectoderm	2.38	.
Ectoderm	-2.3	MRP4_HUMAN		Ectoderm	0.82	.
Ectoderm	-2.6	MRP4_HUMAN		Ectoderm	1.55	.
Ectoderm	-2.16	MRP4_HUMAN		Ectoderm	1.63	.
Ectoderm	-2.17	MRP4_HUMAN		Ectoderm	1.71	.
Ectoderm	-2.24	MRP6_HUMAN		Ectoderm	1.52	.
Ectoderm	-1.47	MSHA_RHOE4		Ectoderm	1.27	.

Ectoderm	-1.61	MTNN_DESPS		Ectoderm	1.02	.
Ectoderm	-0.61	NA		Ectoderm	0.89	.
Ectoderm	-1.71	NCF1C_HUMAN		Ectoderm	1.03	.
Ectoderm	-0.78	NPAL2_MOUSE		Ectoderm	1.49	.
Ectoderm	-2.69	PLPP2_MOUSE		Ectoderm	1.27	.
Ectoderm	-2.06	PRDM6_MOUSE		Ectoderm	1.39	.
Ectoderm	-1.26	PTPRF_RAT		Ectoderm	1.63	.
Ectoderm	-0.64	S4A10_BOVIN		Ectoderm	1.37	.
Ectoderm	-1.24	SDK1_MOUSE		Ectoderm	0.96	.
Ectoderm	-2.05	SPD2A_HUMAN		Ectoderm	0.94	.
Ectoderm	-1.94	TEN3_DANRE		Ectoderm	1.07	.
Ectoderm	-1.89	TENX_HUMAN		Ectoderm	0.97	.
Ectoderm	-1.28	UVR8_ARATH		Ectoderm	1.08	.
Ectoderm	-1.08	WHRN_MOUSE		Ectoderm	0.7	.
Ectoderm	-2.31	Y2800_NOSS1		Ectoderm	0.93	.
Ectoderm	-1.2	YR571_MIMIV		Ectoderm	0.71	.
Ectoderm	-2.23	ZNFX1_HUMAN		Ectoderm	0.94	.
Ectoderm	-1.78	ZNFX1_HUMAN		Ectoderm	1.05	.
Ectoderm	-2.2	ZNFX1_MOUSE		Ectoderm	1.41	.
				Ectoderm	1.13	.
Endoderm	-1.36	.		Ectoderm	1.12	.
Endoderm	-1.64	.		Ectoderm	0.81	.
Endoderm	-1.7	.		Ectoderm	0.74	.
Endoderm	-1.19	.		Ectoderm	0.78	.
Endoderm	-1.23	.		Ectoderm	0.86	.
Endoderm	-0.84	.		Ectoderm	0.63	.
Endoderm	-1.54	.		Ectoderm	1.53	AKA14_RAT
Endoderm	-1.69	.		Ectoderm	1.03	ANMY1_MOUSE
Endoderm	-1.49	.		Ectoderm	0.95	ARF_DUGJA
Endoderm	-1.04	CPSF1_HUMAN		Ectoderm	1.36	ARMC3_HUMAN
Endoderm	-1.76	DCL1_NEUCR		Ectoderm	1.56	ARMC4_HUMAN
Endoderm	-1.78	FIG4_HUMAN		Ectoderm	1.27	ARMD1_BOVIN
Endoderm	-0.93	HCFC1_HUMAN		Ectoderm	1.16	AT1B1_CAEEL
Endoderm	-1.23	HUTH_MOUSE		Ectoderm	1.32	BBOF1_XENLA
Endoderm	-1.1	LAR_DROME		Ectoderm	2.24	C070B_XENLA
Endoderm	-1.19	MARF1_MOUSE		Ectoderm	1.06	CAPSL_MOUSE
Endoderm	-2.33	MYRF_HUMAN		Ectoderm	0.89	CATR_SCHDU
Endoderm	-1.45	NA		Ectoderm	1.7	CB073_BOVIN
Endoderm	-0.87	NA		Ectoderm	1.24	CBCO1_HUMAN
Endoderm	-1.45	P52K_HUMAN		Ectoderm	0.96	CBPC6_MOUSE
Endoderm	-1.88	PDE5_CAEEL		Ectoderm	1.47	CC151_BOVIN
Endoderm	-1.47	PLPL2_BOVIN		Ectoderm	0.93	CC169_XENLA

Endoderm	-0.92	RBM19_HUMAN		Ectoderm	1.64	CC173_HUMAN
Endoderm	-0.85	RHG21_MOUSE		Ectoderm	1.22	CCD40_HUMAN
Endoderm	-1.41	SDK2_HUMAN		Ectoderm	1.02	CCD63_BOVIN
Endoderm	-1.01	ST32B_MOUSE		Ectoderm	1.03	CD047_XENLA
Endoderm	-1.14	SUCO_HUMAN		Ectoderm	1.11	CDK20_DANRE
Endoderm	-1.01	SYNRG_HUMAN		Ectoderm	1.78	CE049_HUMAN
Endoderm	-1.51	THAP1_SALSA		Ectoderm	1.43	CETN1_BOVIN
Endoderm	-1.05	TIF1A_HUMAN		Ectoderm	1.3	CF251_HUMAN
Endoderm	-0.96	TRIO_MOUSE		Ectoderm	1.62	CF299_XENLA
Endoderm	-0.87	TTC17_DANRE		Ectoderm	1.32	CFA44_MOUSE
Endoderm	-1.42	V162_FOWPN		Ectoderm	1.15	CFA47_HUMAN
Endoderm	-2.34	VP13B_MOUSE		Ectoderm	0.96	CFA52_HUMAN
Endoderm	-0.95	XPOT_HUMAN		Ectoderm	1.08	CFA69_PAPAN
Endoderm	-0.81	Y2161_MYCVP		Ectoderm	1.78	CG057_RAT
Endoderm	-1.6	ZNFX1_MOUSE		Ectoderm	0.62	CH076_HUMAN
Endoderm	4.51	.		Ectoderm	1.46	CI116_SALSA
Endoderm	2.3	.		Ectoderm	0.86	COA6_BOVIN
Endoderm	2.62	.		Ectoderm	0.6	COX7C_PAPHA
Endoderm	1.16	.		Ectoderm	1.21	CP100_HUMAN
Endoderm	1.69	.		Ectoderm	1.05	DCDC2_RAT
Endoderm	1.82	.		Ectoderm	1.08	DCLK1_RAT
Endoderm	3.92	.		Ectoderm	1.13	DLRB2_BOVIN
Endoderm	4.63	.		Ectoderm	1.34	DNAL1_XENLA
Endoderm	1.76	.		Ectoderm	1.19	DNAL4_MOUSE
Endoderm	1.94	.		Ectoderm	0.92	DRC2_MOUSE
Endoderm	5.94	.		Ectoderm	1.82	DRC3_MOUSE
Endoderm	1.67	.		Ectoderm	1.3	DRC4_HUMAN
Endoderm	2.96	.		Ectoderm	1.4	DRC5_HUMAN
Endoderm	2.9	.		Ectoderm	1.29	DRC6_HUMAN
Endoderm	2.11	.		Ectoderm	1.62	DRC8_MOUSE
Endoderm	2.15	.		Ectoderm	0.81	DYH2_MOUSE
Endoderm	0.67	.		Ectoderm	1.01	DYI3_HELCR
Endoderm	1.07	.		Ectoderm	1.16	DYL1_HELCR
Endoderm	2.94	.		Ectoderm	1.19	DYLT1_HUMAN
Endoderm	4.4	.		Ectoderm	1.42	EFC10_MOUSE
Endoderm	6.28	.		Ectoderm	1.9	EFCB1_XENLA
Endoderm	1.13	.		Ectoderm	1.77	EFHC2_DANRE
Endoderm	2.89	.		Ectoderm	1.29	ENKUR_HUMAN
Endoderm	2.51	.		Ectoderm	1.22	ERIP6_HUMAN
Endoderm	2.17	.		Ectoderm	2.03	F221B_MOUSE
Endoderm	2.33	.		Ectoderm	1.29	FBW10_HUMAN
Endoderm	2.18	.		Ectoderm	0.7	FKB1A_XENLA

Endoderm	2.28	.		Ectoderm	1.29	HAAF_LIMPO
Endoderm	0.75	.		Ectoderm	1.34	HARB1_RAT
Endoderm	1.9	.		Ectoderm	1.39	HEAT4_HUMAN
Endoderm	2.84	.		Ectoderm	1.08	HYDIN_HUMAN
Endoderm	2.37	.		Ectoderm	0.78	ICK_RAT
Endoderm	1.86	.		Ectoderm	1.37	IDLC_STRPU
Endoderm	2.24	.		Ectoderm	0.7	IFT46_DANRE
Endoderm	2.97	.		Ectoderm	1.41	ING1_MOUSE
Endoderm	2.53	.		Ectoderm	0.86	IQCAL_HUMAN
Endoderm	2.38	.		Ectoderm	1.14	KAD7_HUMAN
Endoderm	1.83	.		Ectoderm	1.58	KAD8_HUMAN
Endoderm	2.1	.		Ectoderm	1.07	KAD9_HUMAN
Endoderm	2.4	.		Ectoderm	0.95	LR2BP_XENLA
Endoderm	2.35	.		Ectoderm	1.56	LRC23_MOUSE
Endoderm	2.11	.		Ectoderm	1.35	LRC63_MOUSE
Endoderm	1.61	.		Ectoderm	1.43	MDH1B_BRAFL
Endoderm	2.11	.		Ectoderm	0.96	MORN2_BOVIN
Endoderm	2.25	.		Ectoderm	1.33	MORN3_XENLA
Endoderm	2.55	.		Ectoderm	1.65	PACRG_MOUSE
Endoderm	1.78	.		Ectoderm	1.16	POL4_DROME
Endoderm	1.66	.		Ectoderm	1.1	PPR36_HUMAN
Endoderm	1.64	.		Ectoderm	2.34	PXDN_HUMAN
Endoderm	2.08	.		Ectoderm	1.15	RGS22_HUMAN
Endoderm	1.34	.		Ectoderm	1.22	ROP1L_XENLA
Endoderm	2.21	.		Ectoderm	2.18	RSPH1_MOUSE
Endoderm	2.84	.		Ectoderm	1.83	RSPH9_DANRE
Endoderm	2.43	.		Ectoderm	1.32	SAM15_MACFA
Endoderm	2.31	.		Ectoderm	1.27	SFP4_BOVIN
Endoderm	3.99	.		Ectoderm	2.52	SPAS1_HUMAN
Endoderm	2	.		Ectoderm	1.61	SPG16_HUMAN
Endoderm	2.49	.		Ectoderm	1.49	SPG17_MOUSE
Endoderm	2.07	.		Ectoderm	1.32	SPT17_MOUSE
Endoderm	3.28	.		Ectoderm	0.81	STAR7_HUMAN
Endoderm	1.88	.		Ectoderm	0.91	TC1D2_HUMAN
Endoderm	2.18	.		Ectoderm	2.27	TEX36_HUMAN
Endoderm	1.41	.		Ectoderm	1.31	TEX43_BOVIN
Endoderm	1.47	.		Ectoderm	1.72	TEX45_HUMAN
Endoderm	2.26	.		Ectoderm	1.63	TEX47_HUMAN
Endoderm	2.02	.		Ectoderm	1.92	THEGL_MOUSE
Endoderm	2.7	.		Ectoderm	0.83	TMM26_HUMAN
Endoderm	2.01	.		Ectoderm	1.47	TRIM3_RAT
Endoderm	1.06	.		Ectoderm	1.38	TTC16_MACFA

Endoderm	3.04	.		Ectoderm	0.93	TTL10_RAT
Endoderm	3.27	AA3R_BOVIN		Ectoderm	1.27	TXIP1_MOUSE
Endoderm	1.9	ADRB1_XENLA		Ectoderm	1.04	UCK2_HUMAN
Endoderm	2.61	AGRG6_DANRE		Ectoderm	1.18	VAFNB_MOUSE
Endoderm	2.28	ARHB_XENLA		Ectoderm	1.26	VASH2_HUMAN
Endoderm	2.86	ATS2_MOUSE		Ectoderm	0.95	WDR93_MOUSE
Endoderm	2.05	BARH1_DROME		Ectoderm	0.96	XRCC6_CHICK
Endoderm	1.49	BARH1_DROME		Ectoderm	1.11	Y4080_AZOC5
Endoderm	3.13	BP10_PARLI		Ectoderm	0.69	ZNF93_HUMAN
Endoderm	2.35	CBH_CLOPE				
Endoderm	2.41	CBPA2_RAT				
Endoderm	2.27	COTL1_RAT				
Endoderm	1.66	DEFI6_HUMAN				
Endoderm	1.27	DHE3_BACTN				
Endoderm	1.62	GALR1_RAT				
Endoderm	2.23	GBX1_HUMAN				
Endoderm	0.79	GRB14_HUMAN				

Table S4.2: The annotated genes from the interaction group and their response in the tissue regions and treatments, proteins and gene names, families and domains, subcellular location, gene function, protein function and cnidarian-literature reference.

Protein name	Group	Gene name	Protein families	Domain	Sub-cellular location	Function gene	Function protein	Cnidarian hit
Retrovirus-related Pol polyprotein from transposon opus	a	pol	Pol polyprotein	Reverse transcriptase. Integrase catalytic	Nucleus.	Catalytic activity, *retrovirus-related (quick) response to heat stress (Traylor-Knowles, Rose et al. 2017), retrotransposable elements in response to disease (Garcia, Gregoracci et al. 2013)	#heat-activated retrotransposons (Chen, Cui et al. 2018), *# (Durante, Baums et al. 2019)	*Symbiodinium microadriaticum, A. palmata, S. fitti*
Nucleotide exchange factor SIL1	a	Sil1	Sil1 family	Signal	Endoplasmic reticulum (ER) lumen.	Protein translocation and folding, HSP chaperone, *defence against environmental stress (Ruiz-Jones and Palumbi 2017)	Adaptive response to mitigate ER stress (Siegenthaler, Pareja et al. 2017, Stevens, Black et al. 2017), *defence against environmental stress (Ruiz-Jones and Palumbi 2017)	*A. hyacinthus*
DNA-binding protein RFX6 (Regulatory factor X 6)	a	rfx6	RFX family	RFX-like. RFX DNA-binding	Nucleus.	Transcription factor for cell development and differentiation (Smith, Qu et al. 2010)	*DNA binding &transcription factor activity (https://www.uniprot.org/uniprot/A0A3M6UB49)	*P. damicornis*
Peroxidasin-like protein	a	PXDNL	Peroxidase family, XPO subfamily	LRRNT. LRRCT. Ig-like C2-type 1 - 4. VWFC.	Secreted. Cytoplasm.	Selectively degrades certain mRNAs. *stress antioxidant response (NUÑO 2018), *extracellular matrix (ECM) formation (Tarrant, Payton et al. 2018)	*ECM organiser, defence pathways, phagocytosis (Barshis, Ladner et al. 2013, DeLeo, Herrera et al. 2018), *oxidative stress response (Voolstra, Schnetzer et al. 2009, Tisthammer, Timmins-Schiffman et al. 2019), *apoptotic mediator (Pernice, Dunn et al. 2011)	*Pocillopora verrucose, Nematostella vectensis, A.hyacinthus, Paramuricea biscaya, Montastraea faveolata, A.millepora, Porites lobata*
Solute carrier family 22 member 3 (Organic cation transporter 3)	a	Slc22a3	Major facilitator superfamily, Organic cation transporter family	n/a	Membrane. Multi-pass membrane protein.	Organic cation transporter. Potential important role in disposition of neurotoxins and transmitters. General detoxification of tissues (Koepsell 1998, Vialou, Amphoux et al. 2004)	*Heat stress biomarker (Yuyama, Harii et al. 2012, Kenkel, Meyer et al. 2013, Kenkel, Sheridan et al. 2014), organic-ion transporter (Levy, Karako-Lampert et al. 2016)	*Stylophora pistillta, Acropora millepora, A. tenuis, Porites astreoides*
MICAL-like protein 2	c	Micall2	n/a	Calponin-homology (CH).	Cell membrane. Cell junction.	Cytoskeleton reorganisation, endocytic recycling, moves products to plasma membrane, epithelial cell	Cytoskeleton regulator (Giridharan and Caplan 2014), endocytotic trafficking (Rahajeng, Giridharan et al. 2010) and ER stress (Cai, Arikkath et al.	*S. pistillata, P. damicornis*

				LIM zinc-binding. bMERB.	Peripheral membrane protein. Recycling endosome. Cell projection. Cytoplasm, cytoskeleton & cytosol.	differentiation/spreading. *DNA translation and calponin-homology	2016), upregulation of ROS (Deng, Wang et al. 2016).	
Sodium-driven chloride bicarbonate exchanger (Solute carrier family 4 member 10)	c	Slc4a10	Anion exchanger family	n/a	Basolateral & apical cell membrane; Multi-pass membrane protein. Cell projection, dendrite & axon. Perikaryon. Cell junction, synapse, pre- & postsynapse.	Cotransporter of Na+/HCO3- to regulate intracellular pH, required in retinal cells (hence GO term for retina?). *Coral calcification (expressed particularly in tips) (Hemond, Kaluziak et al. 2014, Barott, Venn et al. 2015) and biomineralization (Zoccola, Ganot et al. 2015)	*Stress response to increased heat and pCO2 (Rocker, Noonan et al. 2015). *Calcification and photosynthesis (Furla, Galgani et al. 2000).	*A. palmata, S. pistillata, A. yongei, A. millepora*
Mitogen-activated protein kinase kinase kinase 1	c	Map3k1	Protein kinase superfamily, STE Ser/Thr protein kinase family, MAP kinase kinase kinase subfamily	Protein kinase.	n/a	Key component in the protein kinase signal transduction cascade (ERK/JNK pathway activator). *Involved in signalling and apoptotic pathways (De Keuckelaere, Hulpiau et al. 2018). *Osmoregulation (Mayfield, Hsiao et al. 2010). *Mutations in Map3k1 can cause tumours in mammals similar to malformations in coral (Ben-Neriah and Karin 2011)	*Regulators of cell volume changes (greater expression at night as (theoretically) due to lower osmotic presssue (less osmolytes being produced from Symbiodinium)) (Putnam, Mayfield et al. 2013). Stress response and growth related (Liew, Zoccola et al. 2018). *Potential key link to establishing and maintaining host-algal symbiosis, immunity and apoptosis (Voolstra, Schwarz et al. 2009). Stress-related, potential coral biomarker, in-field bleaching (Seneca, Forêt et al. 2010, Drigotas, Affolter et al. 2013, Sun, Chen et al. 2013).	*A. digitifera, P. damicornis, Seriatopora hystrix, S. pistillata, A. palmata, M. faveolata, platygyra carnosus and more*
L-tryptophan decarboxylase	c	psiD	Phosphatidylserine decarboxylase family	n/a	n/a	L-tryptophan decarboxylase. Mediator of psilocybin biosynthesis (natural psychedelic), may be a protective mechanism.	*Calcium binding (Isa 1989). *Regulates growth patterns and rates (Bay, Nielsen et al. 2009). Quick oxidative stress response, potential recognition signal for phagocytic macrophages (Shvedova, Tyurina et al. 2002).	*A. hebes, A. millepora*
Hepatocyte nuclear factor 4-gamma	b	HNF4G	Nuclear hormone receptor family, NR2 subfamily	NR LBD.	Nucleus.	Transcription factor, *roles in development and cell physiology (Putnam, Srivastava et al. 2007, Reitzel and Tarrant 2009), *nuclear receptors (NR)(Grasso, Hayward et al. 2001, Reitzel, Pang et al. 2011) for development, metamorphosis, homeostasis and metabolism. *Developmental NR (Ball, Hayward et al. 2002)	*Development and cell physiology (Putnam, Srivastava et al. 2007, Reitzel and Tarrant 2009), *NR (Mehr, DeSalle et al. 2013)	*N. vectensis, Mnemiopsis leidyi, Pleurobrachia pileus, A. millepora, Faviids*
High-affinity choline transporter 1	b	ChT	Sodium:solute symporter family	n/a	Membrane. Multi-pass membrane protein.	Imports choline from the extracellular with high affinity, Na+ and Cl- dependent	*acetylcholine expression and synthesis (Horiuchi, Kimura et al. 2003). Transport protein (Hayward, Hetherington et al. 2011).	*Radinathus crispus, Dendronepthya habereri*
Immunoglobin superfamily member 23	d	IGSF23	Ig-like fold	Ig-like. Signal. Transmembrane. Transmembrane helix.	Membrane. Integral component of membrane.	Receptor in immune response pathways.	*Protein family – developing nervous systems, larvae to planulae (Polato, Vera et al. 2011). *Cell signalling and immune response (Poole and Weis 2014)	*A. palmata, Acroporids, Pocilloporids*
Sodium- and chloride-dependent GABA transporter 2	d	Slc6a13	Sodium:neurotransmitter symporter family; SLC6A13 subfamily	Transmembrane. Transmembrane helix	Cell membrane. Multi-pass membrane protein.	Transporter of Na-dependent GABA, taurine and B-alanine. Regulator of terminating GABA signalling via GABA uptake.	*Transporter protein, regulated by symbiosis and go-between for host-algae, also response to acidification, heat stress and development (Ganot, Moya et al. 2011, Hayward, Hetherington et al. 2011, Moya, Ganot et al. 2012, Lehnert, Mouchka et al. 2014, Bertucci, Forêt et al. 2015)	*A. millepora, Anemonia viridis, Aiptasia, A. gemmifera, N. vectensis*
Rhamnan synthesis protein F	e	rgpF	n/a	n/a	n/a	Transferase.	Transferring glycosyl groups.	
Ubiquitin-60S ribosomal protein L40	e	RPL40	Ubiquitin family; Eukaryotic ribosomal protein eL40 family	Ubiquitin-like.	Cytoplasm. Nucleus.	Dependent on attachment molecule, numerous roles from protein degradation, cell signalling and kinase activation.	*potential housekeeping role (Le Goff, Ganot et al. 2016), *regeneration (Pasten, Ortiz-Pineda et al. 2012), activation of protein kinases and signalling (frontloaded in case Map3k1 needed?).	*Corallium rubrum, Scleronephthya gracillimum*
Putative deoxyribonuclease TATDN1	e	Tatdn1	Metallo-dependent hydrolases superfamily, TatD-type hydrolase family	n/a	Nucleus.	Putative deoxyribonuclease.	Deoxyribonucleases - *in toxins (Sher, Knebel et al. 2005, Ponce, Brinkman et al. 2016)	*Hydra, scyphozoa,*

Heparan-sulfate 6-O-sulfotransferase 3	e	Hs6st3	Sulfotransferase 6 family	n/a	Membrane. Single-pass type II membrane protein.	Sulphation enzyme, catalyses the transfer of sulphate.	Heparan-sulfate binding proteins (known as the HS interactome) are ancient molecules invoved in numerous processes from cell migration, attachment, differentiation, morphogenesis, organogenesis, metabolism, inflammation and invasion (Xu and Esko 2014).	*Hydra*
von Willebrand factor (vWF)	e	VWF	n/a	VWFD 1 - 4. TIL 1 - 4. VWFA 1 - 3. VWFC 1 - 3. CTCK.	Secreted. Secreted, extracellular space & matrix.	Maintains hemostasis and structure. Also, coagulation factor chaperone to injury site (dissection? but would expect to be in bleaching also?).	*Allogeneic rejection, self-non-self rejection (in relation to symbiosis), immunity (Oren, Amar et al. 2010). *Mucus-related (Jatkar 2009, Bythell and Wild 2011).#adhesion, motility and differentiation (Reshef, Ron et al. 2008). Injury mitigation and infection due to oxidative stress (Motone, Takagi et al. 2018). Vitellogenesis (Imagawa, Nakano et al. 2004)	*S. pistillata (ectodermal layer), Anthopleura elegantissima, N. vectensis, Vibrio shiloi, A. tenuis, Favites chinensis*
Thap domain-containing protein 4	e	Thap4	Thap	Coiled coil. Zinc-finger. THAP-type.	Secreted. Secreted, extracellular space & matrix.	Protein coding, DNA and heme binding, transcription factor and protein homodimerization.	*Transcription-related (Hayward, Hetherington et al. 2011, Lin, Wang et al. 2018)	*Stylophora pistillata, Galaxea fascicularis*
Lecithin retinol acyltransferase	e	LRAT	H-rev107 family	n/a	ER membrane. Single-pass membrane protein. Rough ER. Endosome, multivesicular body. Cytoplasm, perinuclear region.	Makes retinyl esters, a form of vitamin A storage and also necessary for rhodopsin chromophores and cone photopigments (eye GO terms? maybe in ye evolutionary olde times of cnidaria its just for photoreceptive pigments?).	*Retinol esterification (Theodosiou, Laudet et al. 2010, Albalat, Brunet et al. 2011, Liegertová 2016).	*N. vectensis, Tripedalia cystophora*
Kelch-like protein 30	e	klhl30	n/a	BTB. BACK.	n/a	Protein-protein interactions, substrate adaptors for specific ligase.	*Part of the complex that responds to ROS to coordinate transcription of detoxifying, antioxidant and cell survival genes (Kensler, Wakabayashi et al. 2007, Gacesa, Dunlap et al. 2015, Doonan, Hartigan et al. 2019)	*A. digitifera, hydra vulgaris*
CD63 antigen	e	CD63	Tetraspanin (TM4SF) family	n/a	Cell membrane. Multi-pass membrane protein. Lysosome membrane. Late endosome membrane. Endosome, multivesicular body. Melanosome. Secreted, extracellular exosome. Cell surface.	Important role in numerous cellular signalling cascades leading to activation of numerous processes; integrin signalling, cell survival, reorganisation of cytoskeleton, cell adhesion, migration and spreading, activates other compounds, internal regulation. intracellular vesicle transport, compound trafficking and, immunity.	*Related to the cell cycle, cytoskeleton and endo-exophagocytosis response (affected by bleaching) (Ricaurte, Schizas et al. 2016). *Tetraspanin related to development (Iguchi 2007, Iguchi 2014) and *many other roles in physiology, cell biology, cell-cell and cell-matrix interactions, cancer regulators and pathogenesis (Huang, Yuan et al. 2005)(thap4 next option was an ADAM gene which is linked to tetraspanins/devo. CDs). *Endocytosis-related (suggested CD63 is blocked during symbiosis uptake to increase transport to golgi, lysosomes and/or endosome-derived vacuoles) (Oakley, Ameismeier et al. 2016).	*A. palmata, A. millepora, Acroporiids, Hydra magnipapillata, Aiptasia*
Steroid 17-alpha-hydroxylase/17,20 lyase (Cytochrome P450 17A1)	e	CYP17A1	Cytochrome P450 family	n/a	Membrane.	Converts hormones into steroids. *Stress survival response to heat and potential interaction with host-algae (Rosic, Pernice et al. 2010)	*Cellular response to stress, manipulation and excretion of toxic compounds (potential biomarker) (Rougée, Downs et al. 2006). *Protection from high oxygen (ROS) (Gassman and Kennedy 1992) and heat stress (Voolstra, Schnetzer et al. 2009)	*P. damicornis, Favia fragum, M. faveolata, A. millepora*
Store-operated calcium entry (SOCE) regulator STIMATE (Transmembrane protein 110)	e	Stimate	STIMATE family	n/a	ER membrane. Multi-pass membrane protein.	SOCE regulator at endoplasmic reticulum and plasma membrane junctions, helps maintain and reorganise ER-PM junctions. *Protein degradation and amino acid scavenging	*Major Ca2+ influx pathway in nonexcitable and excitable cells, regulator of Ca2+ homeostasis and, exocytosis (Cai, Wang et al. 2015). *Part of immune response pathway (Song, Hu et al. 2013). *Replenisher of intracellular calcium stores after depletion (Florn 2014).	*Erythropodium caribaeorum, N. vectensis, Porites rus, Pavona maldivensis*
Peptidase 1 (Mite group 1 allergen Pso o 1)	e		Peptidase C1 family	n/a	Secreted.	Probable protease (thiol).	*Thiol-specific antioxidant enzymes (a.k.a peroxiredoxins (Prxs)) relate to stress tolerance and nearshore/intertidal corals had a higher abundance (Tisthammer, Timmins-Schiffman et al. 2019).	*Porites lobata*
Cell division cycle protein 123-like	e	CDC123	SmpB family	N-acetyltransferase. Transmembrane. Transmembrane helix. GNAT.	Integral component of membrane	Cell division and N-acetyltransferase activity.	*Heat-stress response (DeSalvo, Sunagawa et al. 2010)	*A. palmata*

The 130 sequences that were unannotated were run through NCBI blast, NCBI conserved domain, pfam and uniport blast. Matches that had an E value of >1e-03 were consolidated into the table beneath (Table S4.3). All of these additional genes were expressed highest in the unbleached endoderm and only one (DAD domain-containing protein) showed a higher expression in the bleached ectoderm.

Table. S4.3: significant DEG in the *Heliofungia* interaction group found via manual, individual sequence annotation. Proteins discovered by NCBI conserved domain search are identified with an * otherwise, results come from Uniprot blast.

EctoRvsEndoR	Gene name	Protein name	Function & process	Domain	Family	E-value
Endo	AK812 - SmicGene3064	Putative peptidase C1-like protein	Calcium ion binding, cysteine-type peptidase activity, proteolysis	EF hand, thiol protease His	peptidase C1	2e-11
Endo	Pdam - 00014509	DAD domain-containing protein	.	DAD, transmembrane, helical	.	3.3e-142
Endo	SAMN04488052 - 102504	Putative ABC transport system permease protein	.	Signal	.	2.9e-3
Endo	PTSG - 06887	Ras-1	GTPase activity, GTP binding, nucleotide binding, signal transduction	Transmembrane, transmembrane helix, C-type lectin, coiled coil	Ras, p-loop, small GTPase, galactose binding	4.2e-8
Endo	EMWEY - 00049950	Zinc finger (C2H2 type) protein, putative	.	Coiled coil	.	2.7e-27
Endo		*Transporter of divalent cations	.	.	*MIT CorA-like super family	*1.01e-6
Endo	AK812 - SmicGene18190	Uncharacterised, * Telomere-capping protein	Single-stranded DNA binding	Transmembrane, transmembrane helix, Ten1	*Ten1 2 super family	1.3e-9, *1.79e-8
Endo	Map3k2	Mitogen-activated protein kinase kinase kinase 2	Protein kinase activity, ATP binding, kinase activity, protein phosphorylation, phosphorylation	Fibronectin type-III, protein kinase	*PKc-like super family	3.5e-17, *4.3e-7
Endo	AK812 - SmicGene5793	Fe2OG dioxygenase domain-containing protein	oxidoreductase activity, oxidation reduction process	Fe2OG dioxygenase, signal	FE2OG-OXY, Oxoglu/Fe-dep dioxygenase	9.4e-16
Endo		*Silent information regulator 2	.	*Sir2 superfamily	.	*9.38e-3
Endo	LOC106603129	LIM domain-containing protein A-like isoform X3	.	Coiled coil	*PRK10681 super family	*1.31e-3
Endo	NCTC12022 - 02672	Rsbr N terminal - 100% similarity identity protein	.	* DUF4116	.	3.e-3, *2.02-7

		= chaperone protein DnaJ 1 (HSP40 chaperone)				
Endo	PREP1	Presequence protease 1, chloroplastic/mitochondrial	Metal ion binding, catalytic activity, peptidase activity, proteolysis	M16C_associated, coiled coil	.	5.5e-22
Endo	OIDMADRAFT - 183060	Glycoside hydrolase family 55 protein	Hydrolase activity	Pectate_lyase_3, signal	*PAT1 super family	*1.43e-3

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