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Feasibility of bacterial probiotics for mitigating coral bleaching

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Thesis submitted in total fulfilment of the requirements of the degree of
Doctor of Philosophy

September 2020

School of BioSciences
The University of Melbourne

Declaration

This is to certify that:

1. This thesis comprises only of my original work towards the PhD, except where indicated in the preface.
2. Due acknowledgements have been made in the text to all other material used.
3. The thesis is under 100,000 words, exclusive of tables, bibliographies, and appendices.

Signed:

Date: 11 September 2020

General abstract

Given the increasing frequency of climate change driven coral mass bleaching and mass mortality events, intervention strategies aimed at enhancing coral thermal tolerance (assisted evolution) are urgently needed in addition to strong action to reduce carbon emissions. Without such interventions, coral reefs will not survive. The seven chapters in my thesis explore the feasibility of using a host-sourced bacterial probiotic to mitigate bleaching starting with a history of reactive oxygen species (ROS) as a biological explanation for bleaching (**Chapter 1**). In part because of the difficulty to experimentally manipulate corals post-bleaching, I use Great Barrier Reef (GBR)-sourced *Exaiptasia diaphana* as a model organism for this system, which I describe in **Chapter 2**. The comparatively high levels of physiological and genetic variability among GBR anemone genotypes make these animals representatives of global *E. diaphana* diversity and thus excellent model organisms. The 'oxidative stress theory for coral bleaching' provides rationale for the development of a probiotic with a high free radical scavenging ability. In **Chapter 3**, I construct a probiotic comprised of *E. diaphana*-associated bacteria able to reduce oxidative stress by neutralizing free radicals such as ROS. I identified six strains with high free radical scavenging ability belonging to the families Alteromonadaceae, Rhodobacteraceae, Flavobacteriaceae, and Micrococcaceae. In parallel, I established a "negative" probiotic consisting of closely related strains with poor free radical scavenging capacities. The application of this probiotic to mitigate the negative impacts of exposure to a simulated heat wave was tested in **Chapter 4**. There was no evidence for improved thermal tolerance in *E. diaphana*. Changes in the relative abundance of anemone-sourced *Labrenzia* provided evidence for its integration in the *E. diaphana* microbiome. Uptake of other probiotic members was inconsistent and probiotic members did not persist in the anemone microbiome over time. Consequently, the failure of the probiotic inoculation to confer improved thermal tolerance may have been due to the absence of probiotic bacteria for the full duration of the experiment. Importantly, there were no apparent physiological impacts on the holobiont following inoculation, thus showing that shifting the abundance of native anemone microbiome members was not detrimental to holobiont

health. Further, I found no evidence for an increase in ROS in the *E. diaphana* holobiont when it was exposed to heat. Some of the most compelling evidence in support of the 'oxidative stress theory of coral bleaching' comes from three published studies that expose corals, cultures of their algal endosymbiont, or *E. diaphana* to exogenous antioxidants during thermal stress. To confirm that ROS is the main driver behind thermal bleaching in *E. diaphana*, I replicated these previous experiments with novel methods that allowed a more accurate quantitation of ROS, and found that dosing with exogenous antioxidants (mannitol and ascorbate plus catalase) mitigates bleaching in *E. diaphana*, with no correlation between bleaching and increased ROS (**Chapter 5**). A serendipitous finding was that the *E. diaphana* bacterial community diversity can be rapidly reduced when anemones are reared in sterile seawater, making this model suitable for testing the efficacy of microbial restructuring strategies (**Chapter 6**). Taken together, the work from my PhD has shown that ROS scavenging varies among anemone-associated bacteria and that a high ROS-scavenging probiotic can be developed. Further, my findings have unveiled several main knowledge gaps that need to be filled before probiotics can be implemented, including administration strategies and choice of probiotic bacteria that maximise the maintenance of probiotic communities over time and a direct measurements of ROS in bleaching corals (**Chapter 7**).

Preface

Three of the seven chapters of this thesis (Chapters 2, 3, and 6) have been submitted to peer-review journals and are either published or under review. Chapters 4-5 have been written in publication format for future submission. Details of contribution for each manuscript are outlined below.

Chapter 2 (published in *Symbiosis* on 29 January 2020)

Dungan A., Hartman L., Tortorelli G., Belderok R., Lamb A., Pisan L., McFadden G., Blackall L., van Oppen M. (2020), *Exaiptasia diaphana* from the Great Barrier Reef: a valuable resource for coral symbiosis research, *Symbiosis* **80**: 195-206. DOI: 10.1007/s13199-020-00665-0

All authors contributed to conceptual development and the final edited version of the manuscript. AD, GT, RB, and LP collected data, AD, LH, and AL performed data analysis, and AD and LH wrote the first draft of the manuscript. AD contributed to approximately 60% of this work.

Chapter 3 (published as a preprint on *bioRxiv* on 3 July 2020; submitted to *Microbial Biotechnology* on 21 August 2020)

Dungan A., Bulach D., Lin H., van Oppen M., Blackall L. (2020), Development of a free radical scavenging probiotic to mitigate oxidative stress in cnidarians, *bioRxiv*. DOI: 10.1101/2020.07.02.185645 (Submitted to *Microb. Biotechnol.*)

AD, MvO, and LB conceived and designed the study. AD performed the sampling and sample processing. AD, DB, and HL completed bioinformatic analyses. AD wrote the first draft. All authors edited and approved the final manuscript. AD contributed with ~75% of the work.

Chapter 4 (in progress for *Journal of Applied Microbiology*)

Dungan A., Hartman L., Blackall L., van Oppen M., (2020), Assessment of a ROS-targeted bacterial probiotic aimed at improving thermal tolerance in the coral model, the sea anemone *Exaiptasia diaphana*. (in prog. *J. Appl. Microbiol.*)

AD, LH, MvO and LB conceived and designed the study. AD and LH performed the sampling, sample processing and bioinformatic analyses. AD and LH wrote the first draft. All authors edited and approved the final manuscript. AD completed 51% of this work.

Chapter 5 (in progress for *Global Change Biology*)

Dungan A., Maire J., Perez-Gonzalez A., Blackall L., van Oppen M., (2020), Lack of evidence for the oxidative stress theory of bleaching in the sea anemone, *Exaiptasia diaphana*. (in prog. *Glob. Chang. Biol.*)

AD, LB, and MvO conceived and designed the study. AD and JM performed the sampling, sample processing and analyses. APG assisted with flow cytometry. AD wrote the first draft. All authors edited and approved the final manuscript. AD contributed with ~70% of the work.

Chapter 6 (submitted to *Frontiers in Marine Science* on 25 August 2020)

Dungan A., van Oppen M., Blackall L., (2020), Short-term exposure to sterile seawater reduces bacterial community diversity in the sea anemone, *Exaiptasia diaphana*. (Submitted to *Front. Mar. Sci.*)

AD, MvO, and LB designed and conceived the experiments. AD conducted the experiment, collected, analysed, and interpreted the data. MvO and LLB contributed reagents, materials, and analysis tools. AD wrote the first draft of the manuscript. All authors read and approved the final manuscript. AD completed 70% of the work.

Other relevant publications during candidature:

Blackall L., **Dungan A.**, Hartman L., van Oppen M., (2020), Probiotics for corals. *Microbiol. Aust.*, 41(2), 100-104.

Ng E-L., Silk Y.L., **Dungan A.**, Colwell J., Ede S., Lwanga E.H., Geissen V., Blackall L., Chen D., (2020), Forest soil microbiome altered by microplastic pollution. (Submitted to *J. Hazard. Mater.*)

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Chapter 1

1.1 Coral bleaching

The Great Barrier Reef (GBR) is the largest biologically formed structure on Earth, covering 350,000 km², an area roughly the size of Japan. The GBR has been valued at \$56 billion AUD for the economic, social, and icon resources it provides, which includes 64,000 jobs and an annual contribution of \$6.4 billion AUD to the Australian economy (O'Mahony et al., 2017). In addition to its large economic value, the GBR is rich in species abundance and diversity. The coral architects deposit calcium carbonate skeletons that serve as the structural foundation of the reef. The thousands of species, including over 400 species of hard coral, that call the reef home make it one of the most unique and complex ecosystems in the world (Fisher et al., 2015). Ultimately, the value of the GBR, and all coral reefs for that matter, is priceless; there is no replacement for the loss of these ecosystems. Yet, despite great contributions that coral reefs provide, we are quickly destroying these habitats, primarily due to climate change.

Corals and other reef organisms have been dying, largely due to climate change related environmental stressors, such as increased sea surface temperatures (SSTs) (Hughes et al., 2017b; Stuart-Smith et al., 2018). Coral reefs are made up of colonies of individual coral animals, or polyps. Each polyp and their associated skeleton lives in symbiotic association with other organisms, including bacteria, protists, fungi, archaea, and viruses (Blackall et al., 2015; Ainsworth et al., 2017; Huggett and Apprill, 2019; Ricci et al., 2019). These microbial associates contribute to the overall health of this complex host-microbe association, or holobiont (Rohwer et al., 2002). A noted eukaryote in the coral holobiont is the algal endosymbiont, a dinoflagellate of the family Symbiodiniaceae. In this mutualistically beneficial relationship, corals provide a safe haven and inorganic nutrients, while the Symbiodiniaceae meet most of the corals energy needs through the transfer of photosynthate (Muscatine et al., 1981; Yellowlees et al., 2008; Tremblay et al., 2014), in an

otherwise nutrient poor seawater environment. The relationship between the coral host and Symbiodiniaceae breaks down during the biological process of coral bleaching.

Coral bleaching is the loss of Symbiodiniaceae from the coral host tissues described through a variety of mechanisms including exocytosis, host cell detachment and host cell apoptosis. (Weis, 2008). A spike in SST during unusually hot summers triggers coral bleaching (Hughes et al., 2018a; Hoegh-Guldberg et al., 2019). Bleaching is often preceded by a reduction in photosynthetic capacity (Fitt et al., 2001; Takahashi et al., 2004; Venn et al., 2006), and/or photosynthetic efficiency (Takahashi et al., 2004; Bouchard and Yamasaki, 2008). Because intracellular Symbiodiniaceae translocate photosynthetically fixed carbon to the coral host, any one of these measurable changes in the Symbiodiniaceae community is detrimental to the coral holobiont. Coral bleaching has been one of the most significant contributors to declining coral cover globally (Hughes et al., 2017b; Stuart-Smith et al., 2018). Since 2015, half of the corals on the GBR, that is 1 billion corals, have died as a result of thermal stress induced bleaching (Meyer, 2018).

Given this dramatic loss of coral from human induced climate change but the optimism for developing assisted evolution strategies to mitigate bleaching (van Oppen et al., 2015; Anthony et al., 2017; Damjanovic et al., 2017; Peixoto et al., 2017; van Oppen et al., 2017; Rosado et al., 2018; van Oppen et al., 2018; Epstein et al., 2019a; Buerger et al., 2020), the goal of this thesis is to address the feasibility of bacterial probiotics to mitigate coral bleaching. In this chapter, I aim to explore the biological impacts of elevated SST on cnidarian holobionts, specifically looking at the photosynthetic machinery of their algal endosymbiont. Further, I will show precedent for assisted evolution strategies, such as probiotics, to mitigate these biological impacts and rescue corals during thermal bleaching events.

1.2 Elevated ROS appears to be the smoking gun

There are several hypotheses detailing the mechanisms driving bleaching (Weis, 2008; Cunning and Baker, 2012; Wiedenmann et al., 2012; Wooldridge, 2013), with a common theme being the overproduction and toxic accumulation of reactive oxygen species (ROS) from the algal symbiont. Experiments and observations have developed and refined the “oxidative stress theory of coral bleaching,” (Downs et al., 2002; Czienski et al., 2019) whereby accumulation of ROS lead to signalling cascades and in turn expulsion of Symbiodiniaceae from the coral host (Smith et al., 2005; Weis, 2008; Davy et al., 2012; Suggett and Smith, 2019). However, much of the information known connecting ROS to coral bleaching is indirect due to the transient nature of these molecules and challenges in acquiring quantitative data.

ROS are a normal product of photosynthesis; the light triggered splitting of water molecules in the oxygen evolving complex (OEC) and subsequent transfer of electrons from photosystem II (PSII) to photosystem I (PSI) generates ROS in chloroplasts (Fig. 1-1; Trubitsin et al., 2014; Szabó et al., 2020). Singlet oxygen ($^1\text{O}_2$) can be formed via energy transfer when ground-state molecular oxygen interacts with chlorophyll triplets. This ROS is produced either in the reaction centre of the PSII complex or in the light-harvesting antenna, and reacts with proteins, lipids and pigments to induce photo-oxidative damage to the photosynthetic apparatus (Krieger-Liszkay, 2005). Rehman et al. (2016) documented the light-dependent intracellular production of $^1\text{O}_2$ in Symbiodiniaceae cultures. The major site of superoxide ($\text{O}_2^{\bullet-}$) production is the thylakoid membrane-bound primary electron acceptor of PSI (Szabó et al., 2020). This $\text{O}_2^{\bullet-}$ is further converted to hydrogen peroxide (H_2O_2) within the chloroplast mostly by superoxide dismutase (SOD) (Asada, 1987). Hydroxyl radicals ($\text{OH}^\bullet$) are produced in the Haber-Weiss or Fenton reactions through the interaction of H_2O_2 and $\text{O}_2^{\bullet-}$ or directly from H_2O_2 in the presence of transition metals (Halliwell and Gutteridge, 1990).

Photosynthetic organisms have had to evolve efficient strategies to cope with the accumulation of these potentially toxic compounds that are integral components of

photosynthesis. As such, the ROS content in cells depends on the relative rates of ROS generation and neutralization by antioxidant enzymes and low molecular weight antioxidants. The Mehler Ascorbate Peroxidase (MAP) pathway has evolved to detoxify $O_2^{\bullet-}$ using SOD by reducing it to H_2O_2 (Szabó et al., 2020). H_2O_2 can be further reduced by ascorbate peroxidase, thioredoxin, or catalase to water and oxygen. Since the photosynthetic electron transport chain extends from the water-splitting OEC of PSII to the water forming reaction of the MAP process, this is known as the water-water cycle. Non-enzymatic antioxidants, such as carotenoids (Venn et al., 2006), ascorbic acid, glutathione, tocopherols, and flavonoids are also active in the chloroplast lumen and stroma, as well as the Symbiodiniaceae cytosol, reducing $OH^{\bullet}$ to water and oxygen. The MAP pathway is the basis of some coral bleaching models (Jones et al., 1998; Wooldridge, 2009).

Significantly higher cellular ROS concentrations have been observed in some, but not all, Symbiodiniaceae species exposed to elevated temperatures (Lesser, 1996; Suggett et al., 2008; McGinty et al., 2012; Roberty et al., 2015; Goyen et al., 2017; Wietheger et al., 2018). Extracellular $^1O_2$ production is enhanced in Symbiodiniaceae cultures under elevated temperatures (Rehman et al., 2016). ROS production during elevated temperature in symbiotic cnidarians has not been as extensively reported. Marty-Rivera et al. (2018) found significantly higher ROS levels for the coral *Porites astreoides* and the coral model, *Exaiptasia diaphana*, after a short term (4d) heat stress. There was significantly higher production of $O_2^{\bullet-}$ during the summer months for *Pocillopora capitata* (Liñán-Cabello et al., 2010).

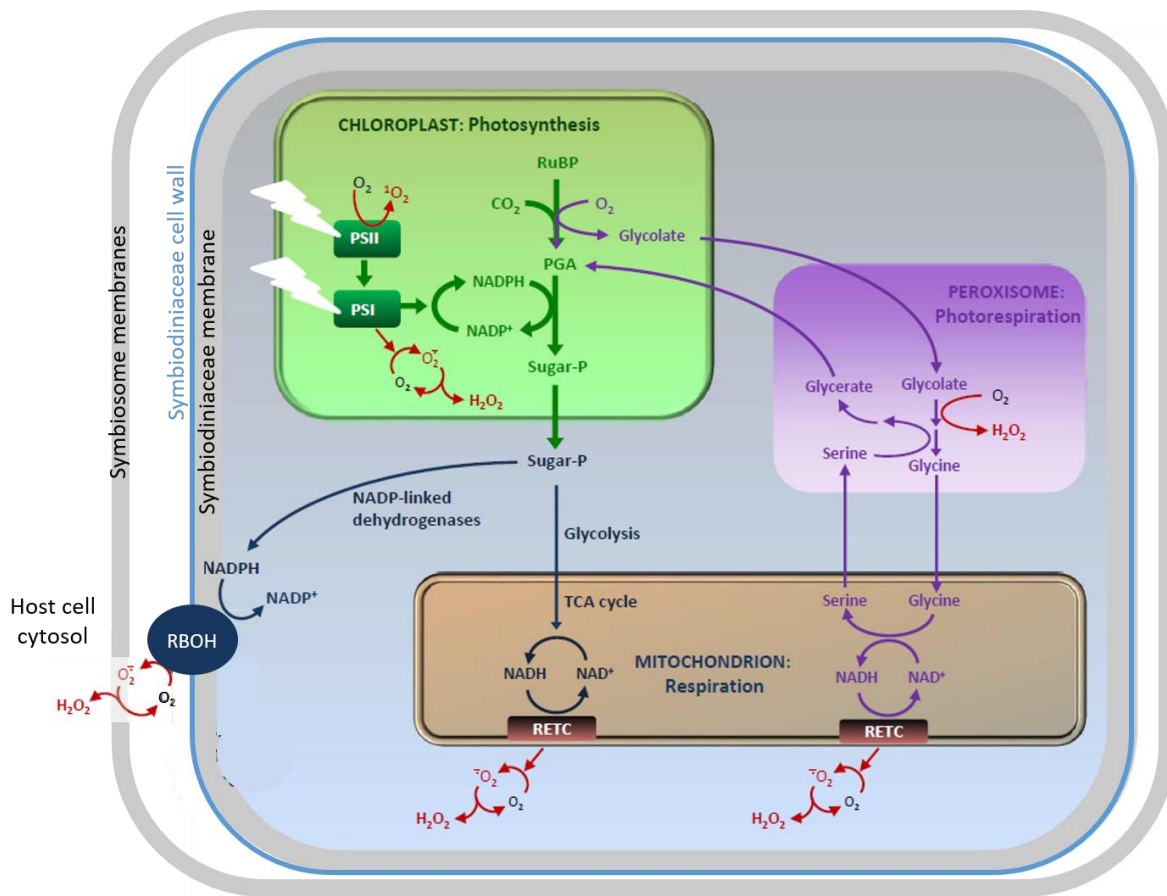


Figure 1-1 Normal production of ROS (red) from photosynthesis (green box), photorespiration (purple box), and cellular respiration (brown box). PGA, 3-phosphoglycerate. PSI/II, photosystem I/II. RBOH, respiratory burst oxidase homologue. RETC, respiratory electron transport chain. RuBP, ribulose 1,5 bisphosphate. Sugar-P, sugar phosphate. Adapted from Noctor et al. (2018) and Szabó et al. (2020).

As a proxy for elevated ROS, increased activity or production of antioxidants has been shown under elevated temperatures in Symbiodiniaceae cultures (Lesser, 1996; McGinty et al., 2012; Roberty et al., 2015) and for cnidarian holobionts (Lesser et al., 1990; Downs et al., 2002; Higuchi et al., 2008), although these responses are not necessarily correlated with a bleaching susceptibility phenotype (Hawkins et al., 2015; Krueger et al., 2015b). Gierz et al. (2017) found that exposure to elevated temperature increased the expression of genes associated with oxidative stress in Symbiodiniaceae cultures, including SOD and catalase peroxidase.

Once generated, ROS can trigger the oxidation of essential photosynthetic molecules, such as the D1 protein in PSII (Lesser, 1996; Wang et al., 2011; Mathur et al., 2014), thylakoid

membranes (Tchernov et al., 2004; Roberty et al., 2016), and enzymes of the Calvin-Benson cycle (Asada, 1987; Lesser and Farrell, 2004), thereby interfering with the supply of fixed carbon to the holobiont (Lesser, 2004). Increased abundance of ROS in the chloroplast also reduces the biosynthesis of chlorophyll (Takahashi et al., 2008; Roberty et al., 2016).

The D1 protein in the PSII apparatus plays a central role in the primary processes of charge separation and stabilization in PSII (Schmitt et al., 2014). Repair rates for the D1 protein are also negatively influenced by excess ROS (Nishiyama et al., 2006; Schmitt et al., 2014). Thus, photoinhibition occurs when the rate of photodamage to PSII exceeds the rate of its repair. A moderate increase in temperature accelerates photoinhibition, primarily through inhibition of the repair process (Baird et al., 2009; Ragni et al., 2010; Hill et al., 2011). Damage to or inhibition of the D1 protein is directly connected to decreases in maximum quantum yield (F_v/F_m) (Ragni et al., 2010). Studies consistently show that the stability of F_v/F_m is directly correlated with bleaching tolerance in both cnidarians (Voolstra et al., 2020) and Symbiodiniaceae cultures (Ragni et al., 2010; Goyen et al., 2017; Dang et al., 2019b).

The addition of exogenous scavengers of ROS suggests a role for oxidative stress in the inhibition of photosynthesis and corresponding bleaching by elevated temperatures. Exposure to exogenous antioxidants can reduce host ROS levels and bleaching in thermally stressed corals and coral models (Lesser, 1997; Marty-Rivera et al., 2018). In addition, the photosynthetic performance of thermally stressed Symbiodiniaceae has been shown to improve and net ROS to decrease within hours of receiving a single antioxidant dose (Lesser, 1996; Motone et al., 2020). In a recent study, heat evolved Symbiodiniaceae strains increased bleaching tolerance during thermal stress when inoculated in *Acropora tenuis* larvae with a concomitant decrease in secreted ROS (Buerger et al., 2020).

Together these data provide strong support for a significant role of ROS in the bleaching response, which may directly (by triggering messenger systems to induce bleaching) or indirectly (through cellular damage) be involved in mass bleaching.

1.3 Heat stress has biological consequences beyond ROS production

Elevated SST or heat stress, has several biological consequences to photosynthetic organisms beyond the production of ROS (Fig. 1-2; Farooq et al., 2016). In the marine environment, the solubility of CO₂ decreases with increasing temperature. The Type II ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO), the major enzyme in the Calvin-Benson cycle, present in Symbiodiniaceae has lower affinity for CO₂ versus O₂. Thus, this lower bioavailability of CO₂ has direct consequences on the rate of carboxylation, translating to reduced photosynthetic output (Wooldridge, 2009). Additionally, Type II RuBisCO is heat sensitive and becomes impaired under temperature conditions that cause coral bleaching (Jones et al., 1998; Lilley et al., 2010). Impairment of RuBisCO can lead to inhibition of the electron transport between PSII and PSI (Szabó et al., 2017), thus reducing photophosphorylation rates, as well as induce a loss of PSII function (Bhagooli, 2013; Hill et al., 2014), and promote intracellular ROS formation (Rehman et al., 2016). In plants, moderately high temperatures impair the activation of RuBisCO by its catalytic chaperone, RuBisCO activase (RCA), which becomes the primary cause of the decrease in photosynthesis in response to elevated temperature (Farooq et al., 2016; Perdomo et al., 2017). In Symbiodiniaceae culture and in nubbins of the coral *Pocillopora damicornis*, administering potassium cyanide, which inhibits CO₂ fixation by binding to RCA and preventing its release from RuBisCO, reduced photosynthetic performance, and led to bleaching (Hill et al., 2014). However, the presence of a RCA in Symbiodiniaceae has not yet been confirmed (Lilley et al., 2010). Notably, potassium cyanide also causes inhibition of plastoquinone-oxidoreductase, ascorbate peroxidase, and the scavenging of hydroxyl radicals (Hill et al., 2014). Thus, the bleaching response by *P. damicornis* may have been promoted by ROS formation.

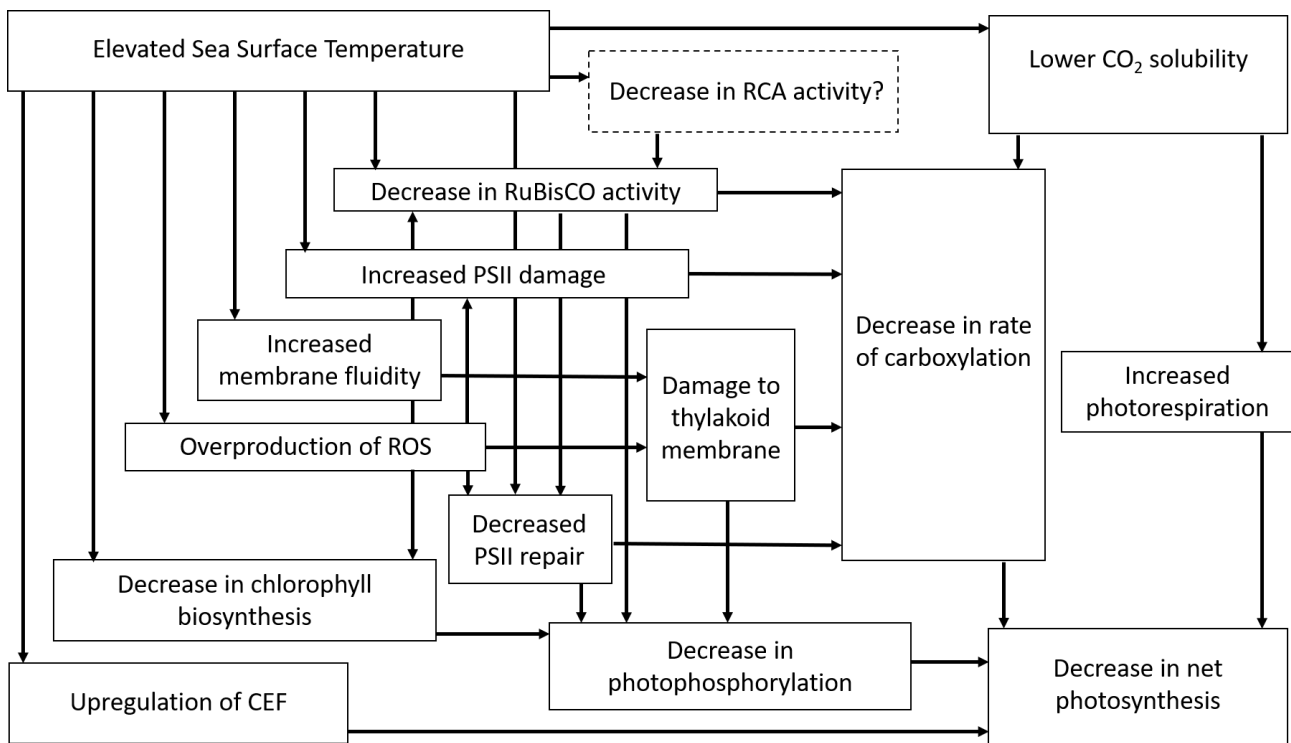


Figure 1-2 Diagram showing the cascade of effects that elevated sea surface temperature can have on Symbiodiniaceae photosynthetic machinery, ending with a decrease in net photosynthesis. Adapted from Farooq et al. (2016). RuBisCO=ribulose 1,5-bisphosphate carboxylase oxygenase, RCA=Rubisco activase, PSII=photosystem II, ROS=reactive oxygen species, CEF=cyclic electron flow. The dashed line indicates that the presence of an RCA in Symbiodiniaceae has not yet been confirmed.

Regardless of the mechanism, inhibition of RuBisCO leads to increased NADPH levels (due to the decreased consumption of NADPH by the damaged carbon fixation mechanisms), which has its own cascade of effects. Higher NADPH concentrations translate to a higher proportion of reduced ferredoxin blocking the electron acceptor side of PSI and thus impair photo-oxidation of P700, the primary electron donor of PSI (Szabó et al., 2017; Szabó et al., 2020). This can create a back log of shifted equilibria toward reduced pools of plastoquinone (Lutz et al., 2015) and direct the photosynthetic machinery to adjust from non-cyclic to cyclic electron flow (CEF) (Szabó et al., 2020). The CEF pathway may be activated due to an accumulation of NADPH when CO₂ fixation is impaired in Symbiodiniaceae, which could occur during thermal stress through the inactivation of RuBisCO (Lilley et al., 2010). CEF can be induced with heat stress in Symbiodiniaceae culture, albeit at a strain specific rate (Aihara et al., 2016; Dang et al., 2019b). Because upregulation of CEF decreases the reduction of NADP⁺ to NADPH, a necessary electron

donor for the Calvin-Benson cycle, there may be reductions in photosynthate production.

Damage to PSII, specifically at the D1 protein, is increased under elevated temperatures (Warner et al., 1999; Lesser and Farrell, 2004; McGinley et al., 2012), though the mechanism is not clear. As photodamage is considered a normal part of photosynthesis (Aro et al., 1993), this damage may be associated with increases in enzymatic activity (Iglesias-Prieto et al., 1992) that are driving increases in photosynthetic rates for Symbiodiniaceae during periods of elevated SST (Castillo and Helmuth, 2005; Hoadley et al., 2016). Alternatively, ROS can damage PSII in plant cells (Mathur et al., 2014), which may translate to Symbiodiniaceae as well. The damaged PSII is normally repaired, but this process is sensitive to heat (Takahashi et al., 2004; Takahashi et al., 2009) and excess ROS (Nishiyama et al., 2006), which gives rise to reductions in PSII efficiency – a metric noted for predicting bleaching susceptibility (Takahashi et al., 2008; Dang et al., 2019b; Voolstra et al., 2020). Heat stress can also inhibit biosynthesis of chlorophyll in Symbiodiniaceae cultures (Takahashi et al., 2008), resulting a decline of photosynthetic capacity, though not all Symbiodiniaceae species experience decreases in chlorophyll content with elevated temperatures (Krueger et al., 2015b).

An increase in SST can affect the stability of thylakoid membranes (Hill et al., 2009; Díaz-Almeyda et al., 2011), preceding the physiological damage to which cells actually respond (Downs et al., 2013). The thylakoid membrane can also be damaged by ROS (Tchernov et al., 2004); damage to the membranes integrity can disturb PSI, PSII, and the OEC, while simultaneously inducing the production of ROS, and a weakening of the proton concentration gradient that drives ATPsynthase (Farooq et al., 2016). Lower ATP availability, in turn, affects RuBisCO regeneration, thus limiting the rate of CO₂ fixation (Perdomo et al., 2017).

1.4 Precedent for bacterial probiotics

Mass bleaching events, that is extensive coral bleaching over 10s, 100s, or even 1000s of kilometres, as occurred on the Great Barrier Reef (GBR) in 1998, 2002, 2016, 2017, and now 2020, leads to increased mortality of corals. At present there is no biological treatment that can minimize coral bleaching in the field. Management priorities for coral reefs must move beyond documenting their declines and toward investigating potential approaches for mitigating coral bleaching, such as the application of coral probiotics.

Naturally occurring commensal prokaryotic species inhabit the surfaces and internal compartments of nearly every organism on Earth (Johnson and Klaenhammer, 2014). In humans, it is understood that the consumption of allochthonous beneficial bacteria can have a positive influence on general health via commensal interactions between the host and bacteria. These beneficial bacteria, known as probiotics, are defined by the World Health Organization as “live microorganisms, which when administered in adequate amounts, confer a health benefit upon the host” (Araya et al., 2002). Whilst the large portion of probiotic research remains in the medical field, the paradigm of probiotic research is rightfully shifting towards understanding the role of beneficial bacteria in other systems. Probiotics are now being used in aquaculture (Verschuere et al., 2000), agriculture (Lugtenberg and Kamilova, 2009), and recently in maintaining the health of coral.

The “Coral Probiotic Hypothesis” (Reshef et al., 2006) postulates that the coral microbiome can evolve or modify during changing environmental conditions more rapidly than the coral host itself, leading to adaptation of the coral holobiont. The concept of microbiome modification via probiotic application to “change relevant characteristics” (Rosenberg and Zilber-Rosenberg, 2011) or mitigate disease in corals (Bourne et al., 2009; Teplitski and Ritchie, 2009) started appearing in coral literature in the past decade. These concepts have been supported by studies that have successfully used probiotics on corals or the coral model, *E. diaphana* (Chapter 2), to minimize the effects of stressors during short-term aquarium experiments (Alagely et al., 2011; dos Santos et al., 2015; Rosado et al., 2018) and those that reported changes in health with antibiotic usage (Gilbert et al., 2012; Mills et al.,

2013; Glasl et al., 2016). In a first experiment, inoculation of the anemone *E. diaphana* (Weis et al., 2008) with Alphaproteobacteria were able to inhibit disease progression caused by the white pox pathogen (Alagely et al., 2011). As a bioremediation strategy, inoculation of adult coral with bacteria that were highly effective in degrading petroleum hydrocarbons minimized the effects of petroleum on coral health and reduced impact on the coral's native microbiome (dos Santos et al., 2015). Another study showed that inoculation with a selection of bacterial candidates reduced sensitivity of adult coral fragments to elevated temperatures (Rosado et al., 2018). Through the manipulation of beneficial coral microbes (Peixoto et al., 2017; Sweet et al., 2017), via the probiotic approach, the coral holobiont could become more resilient to other types of stress, such as prolonged high temperature (van Oppen et al., 2015).

1.5 Thesis objectives and aims

Given the extensive evidence on the involvement of ROS in the cnidarian bleaching response and the interdisciplinary support for probiotic efficacy, my thesis explores the feasibility of using a host-sourced bacterial probiotic to mitigate bleaching. Based on the 'oxidative stress theory for coral bleaching' (Downs et al., 2002), I develop an *E. diaphana*-sourced probiotic able to reduce oxidative stress by neutralizing free radicals such as ROS (Chapter 3). In parallel, I established a "negative" probiotic consisting of closely related strains with poor free radical scavenging capacities. The application of this probiotic to mitigate the negative impacts of exposure to a simulated heat wave was tested in Chapter 4. Further, I explore the oxidative stress theory of coral bleaching following exogenous antioxidant dosing regimens that have previously been established (Lesser, 1996; 1997; Marty-Rivera et al., 2018) with newly created methods of tracking secreted ROS for *E. diaphana* when exposed to elevated temperatures (Chapter 5). Along the way, I tested a method to reduce the *E. diaphana* bacterial community (Chapter 6). This thesis provides valuable insights into strategies for developing coral probiotics, the feasibility for their transmission to a coral model host, and their persistence through time. Nonetheless, my findings have unveiled main knowledge gaps that need to be filled before probiotics can be implemented, including administration strategies and choice of probiotic bacteria that

maximize the maintenance of probiotic communities over time, as well as data from direct measurements of ROS in bleaching corals (Chapter 7).

I believe that the application of a coral probiotic specifically tailored to address coral bleaching by neutralizing ROS (Chapter 3&4) could provide hope for the future of coral reefs. I also understand that this form of intervention may not work alone, but could benefit by pairing with other strategies such as enhancing coral resistance and resilience using other assisted evolution approaches such as assisted gene flow, hybridization and experimental evolution of the algal symbionts (Omori, 2011; Young et al., 2012; Rinkevich, 2014; van Oppen et al., 2015; van Oppen et al., 2017; Chan et al., 2018; Morikawa and Palumbi, 2019; Buerger et al., 2020; Quigley et al., 2020). Climate warming will continue even with the most drastic reductions in greenhouse gas emissions, thus, additional interventions such as coral probiotics present an alternative that could lead to relief from coral bleaching in real time.

Chapter 2

***Exaiptasia diaphana* from the Great Barrier Reef: a valuable resource for coral symbiosis research**

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2.1 Abstract

The sea anemone, *Exaiptasia diaphana*, previously known as *Exaiptasia pallida* or *Aiptasia pallida*, has become increasingly popular as a model for cnidarian-microbiome symbiosis studies due to its relatively rapid growth, ability to reproduce sexually and asexually, and symbiosis with diverse prokaryotes and the same microalgal symbionts (family Symbiodiniaceae) as its coral relatives. Clonal *E. diaphana* strains from Hawaii, the Atlantic Ocean, and Red Sea are now established for use in research. Here, we introduce Great Barrier Reef (GBR)-sourced *E. diaphana* strains as additions to the model repertoire. Sequencing of the 18S rRNA gene confirmed the anemones to be *E. diaphana* while genome-wide single nucleotide polymorphism analysis revealed four distinct genotypes. Based on *Exaiptasia*-specific inter-simple sequence repeat (ISSR)-derived sequence characterized amplified region (SCAR) marker and gene loci data, these four *E. diaphana* genotypes are distributed across several divergent phylogenetic clades with no clear phylogeographical pattern. The GBR *E. diaphana* genotypes comprised three females and one male, which all host *Breviolum minutum* as their homologous Symbiodiniaceae endosymbiont. When acclimating to an increase in light levels from 12 to 28 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, the genotypes exhibited significant variation in maximum quantum yield of Symbiodiniaceae photosystem II and Symbiodiniaceae cell density. The comparatively high levels of physiological and genetic variability among GBR anemone genotypes make these animals representative of global *E. diaphana* diversity and thus excellent model organisms. The addition of these GBR strains to the worldwide *E. diaphana* collection will contribute to cnidarian symbiosis research, particularly in relation to the climate resilience of coral reefs.

2.2 Introduction

The Great Barrier Reef (GBR) contains abundant and diverse biota, including more than 300 species of stony corals (Fabricius et al., 2005), making it one of the most unique and complex ecosystems in the world. In addition to its tremendous environmental importance, the GBR's social and economic value is estimated at \$A56 billion, supporting 64,000 jobs and injecting \$A6.4 billion into the Australian economy annually (O'Mahony et al., 2017).

Coral reef waters are typically oligotrophic, but stony corals thrive in this environment and secrete the calcium carbonate skeletons that create the physical structure of the reef. Corals achieve this through their symbiosis with single-celled algae of the family Symbiodiniaceae that reside within the animal cells and provide the host with most of its energy (Muscatine and Porter, 1977). Additional support is provided by communities of prokaryotes, and possibly by other microbes such as viruses, fungi and endolithic algae living in close association with coral. This entity, comprising the host and its microbial partners, is termed the holobiont (Rohwer et al., 2002). During periods of extreme thermal stress, the coral-Symbiodiniaceae relationship breaks down. Stress-induced cellular damage creates a state of physiological dysfunction, which leads to separation of the partners and, potentially, death of the coral animal. This process, 'coral bleaching', is a substantial contributor to coral cover loss globally (Baird et al., 2009; De'ath et al., 2012; Eakin et al., 2016; Hughes et al., 2017b; Hughes et al., 2018b).

Model systems are widely used to explore research questions where experimentation on the system of interest has limited feasibility or ethical constraints. Established model systems, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have played crucial roles in the progress of understanding organismal function and evolution in the past 50 years (Davis, 2004). The coral model *Exaiptasia diaphana* was formally proposed by Weis et al. (2008) as a useful system to study cnidarian endosymbiosis and has since achieved widespread and successful use.

E. diaphana is a small sea anemone (≤ 60 mm long) found globally within temperate and tropical marine shallow-water environments (see Grajales and Rodriguez, 2014 for detailed geographic distribution references); this global distribution is likely influenced by introduction of the species to new regions via human transport. The species is cryptic and typically found in highly shaded habitats such as marinas, rocky rubble, and in isolated fouling communities (Schlesinger et al., 2010), preferring calm and protected waters. Originally positioned taxonomically within the genus *Aiptasia*, *E. diaphana* and twelve other *Aiptasia* species were combined into a new genus, *Exaiptasia* (Grajales and Rodriguez, 2014). Although *Exaiptasia pallida* was proposed as the taxonomic name for the

twelve synonymized species, the International Commission on Zoological Nomenclature (ICZN) ruled against this because the species epithet *diaphana* (Rapp, 1829) predated *pallida* (Verrill, 1864) and therefore had precedence according to the Principle of Priority (ICZN, 2017).

E. diaphana was first used to study cellular regeneration (Blanquet and Lenhoff, 1966), with its systematic use in the study of cnidarian-algal symbioses dating back to 1976 when the regulation of *in hospite* Symbiodiniaceae density was explored (Steele, 1976). Since then, studies using *E. diaphana* have focused on the onset, maintenance, and disruption of symbiosis with Symbiodiniaceae (Belda-Baillie et al., 2002; Fransolet et al., 2014; Bucher et al., 2016; Hillyer et al., 2017; Cziesielski et al., 2018). *E. diaphana* has also been used in studies of toxicity (Duckworth et al., 2017; Howe et al., 2017), ocean acidification (Hoadley et al., 2015), disease and probiotics (Alagely et al., 2011), and cnidarian development (Chen et al., 2008; Grawunder et al., 2015; Carlisle et al., 2017). Key differences between shallow water corals and *E. diaphana* are the absence of a calcium carbonate skeleton, the constant production of asexual propagates, the preference for low ($< 100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) light, and the greater ability to survive bleaching events in the latter. These features allow researchers to use *E. diaphana* to investigate cellular processes that would otherwise be difficult with corals, such as those that require survival post-bleaching to track re-establishment of eukaryotic and prokaryotic symbionts. Further, adult anemones can be fully bleached without eliciting mortality, which is often difficult for corals, thus providing a system for algal reinfection studies independent of sexual reproduction and aposymbiotic larvae (Tortorelli et al., 2019). Given the ecological differences between *E. diaphana* with reef building corals, we caution against direct comparisons between the two, specifically in the context of tolerances and responses to climate change. However, we feel strongly that *E. diaphana* will be crucial to the development of tools to document and understand responses to climate change as well as in trialling strategies to mitigate the effects of climate change.

Three strains of *E. diaphana* currently dominate the research field as models for coral research. Strain H2 (female) was originally collected from Coconut Island, Hawaii, USA

(Xiang et al., 2013), CC7 (male) from the South Atlantic Ocean off North Carolina, USA (Sunagawa et al., 2009b) and RS (female, pers. comm., but see Schlesinger et al. (2010)) collected from the Red Sea at Al Lith, Saudi Arabia (Cziesielski et al., 2018). The majority of *E. diaphana* resources have been developed from the clonal line, CC7, including reproductive studies (Grawunder et al., 2015), transcriptomes (Sunagawa et al., 2009b; Lehnert et al., 2012; Lehnert et al., 2014), and an anemone genome sequence (Baumgarten et al., 2015). Prior work evaluating multiple *E. diaphana* strains has shown variation in sexual reproduction (Grawunder et al., 2015), Symbiodiniaceae specificity (Thornhill et al., 2013; Grawunder et al., 2015), and resistance to thermal stress (Bellis and Denver, 2017; Cziesielski et al., 2018). Because these strains may have been distributed via anthropogenic vectoring such as ballast waters, fouling of ships, aquaculture, or the aquarium trade (Thornhill et al., 2013), it is plausible that they are not native to where they were collected from. However, efficient clonal propagation has led to their establishment in research laboratories around the globe with strains tracked by genetic markers. Australian contributions to these efforts have been hampered as researchers have not had access to the established strains with import permits for alien species difficult to secure.

The aim of the present work is to add GBR representatives to the existing suite of *E. diaphana* model strains, though it is unknown whether GBR populations of *E. diaphana* are native to the GBR or have been introduced from other regions. We describe the establishment and maintenance of four genotypes, including their provenance, taxonomy, histology, physiology, and Symbiodiniaceae symbiont type. The results highlight characteristics that make GBR-sourced *E. diaphana* a valuable extension to this coral model, such as the physiological and genetic variability between genotypes, which is more representative of a global population. This foundation information will be useful to international researchers interested in using the GBR-sourced animals in their research and will give Australian researchers access to this valuable model.

2.3 Methods

2.3.1 Anemone acquisition and husbandry

Several pieces of coral rubble bearing anemones were obtained from holding tanks in the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS) in Townsville and sent to Swinburne University of Technology, Melbourne (SUT) in late 2014. Additional anemones from the SeaSim were sent to the Marine Microbial Symbiont Facility (MMSF) at the University of Melbourne (UoM) in early 2016. Most material in the SeaSim tanks originates from shallow water coral reef ecosystems of the central GBR; therefore, this is the likely origin of the anemones. Because these anemones were not part of a specific culture collection at AIMS, but rather 'pests', their specific collection location and habitat is unknown with the likely possibility that this population came from multiple sites on the GBR. The SUT and UoM populations were consolidated at MMSF in early 2017 where they were segregated according to single polynucleotide polymorphism (SNP) analysis groupings (see below). Anemones were grown in 4 L polycarbonate tanks in reverse osmosis (RO) water reconstituted Red Sea Salt™ (RSS) at ~34 parts per thousand (ppt), and incubated without aeration or water flow at 26°C under lighting of 12-20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (light emitting diode - LED white light array) on a 12h:12h light:dark cycle in a walk-in incubator. This species requires very low light levels, such as provided in shaded or deep reef habitats; conversely, shallow unshaded GBR coral habitats can average 1400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ over the course of a day (Bainbridge, 2017). Anemones were fed *ad libitum* with freshly hatched *Artemia salina* (brine shrimp, Salt Creek, UT, USA) nauplii twice weekly. Tanks were cleaned each week after feeding by loosening algal debris with water pressure applied through disposable plastic pipettes, removing algal biomass, and complete water changes. When cleaning, ~25% of the anemones were cut into 2-6 fragments to promote population expansion through regeneration of the tissue fragments into whole anemones. Every third week, all anemones were transferred to clean tanks.

2.3.2 Anemone identity and genotyping

Anemone identity was determined by Sanger sequencing of the 18S rRNA gene. Genomic

DNA (gDNA) was extracted from twelve whole anemones following the protocol described by Wilson et al. (2002), modified with a 15 min incubation in 180 μ M lysozyme, and 30 s bead beating at 30 Hz (Qiagen Tissue-Lyser II) with 100 mg of sterile glass beads (Sigma G8772). The 18S rRNA genes were PCR amplified from all anemone samples using external Actiniaria-specific 18S rRNA gene primers 18S_NA, 5'-TAAGCACTTGTCTGTGAAACTGCGA-3' and 18S_NB, 5'-TAAGCACTTGTCTGTGAAACTGCGA-3' (Grajales and Rodriguez, 2016) with 0.5 U 2x Mango Mix (Bioline), 2 μ L of DNA template, 0.2 μ M of each primer, and nuclease-free water up to 25 μ L. PCR conditions consisted of initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s followed by a final extension at 72°C for 5 min. PCR products were purified with an ISOLATE II PCR and Gel Kit (Bioline, BIO-52059) according to the manufacturers guidelines, and supplied to the Australian Genome Research Facility (AGRF) for Sanger sequencing with the external primers and four internal primers: 18S_NL, 5'-AACAGCCCGGTCTAGTAACACG-3', 18S_NC 5'-AATAACAATACAGGGCTTTTCTAAGTC-3', 18S_NY 5'-GCCTTCCTGACTTTGGTTGAA-3', and 18S_NO 5'-AGTGTATTGGATGACCTCTTTGGC-3'. The raw 18S reads were aligned in Geneious (v 10.0.4) (Kearse et al., 2012) to produce the near-complete 18S rRNA gene sequence, which was evaluated by BLASTn (Altschul et al., 1990) to identify the anemones.

For more specific genotyping, DNA was extracted as described above from 23 whole anemones or their tentacles and sent to Diversity Arrays Technology Pty Ltd (Canberra, Australia) for DArT next-generation sequencing (DArTseq). DArTseq combines complexity reduction and next generation sequencing to generate genomic data with a balance of genome-wide representation and coverage (Cruz et al., 2013). Complexity reduction was achieved by using restriction endonucleases to target low-copy DNA regions. These regions were then sequenced using Illumina HiSeq2500 (Illumina, USA) with an average read depth exceeding 20x. The data were processed by DArT Pty Ltd to remove poor quality sequences and to ensure reliable assignment of sequences to samples. DArTsoft14 was then used to identify SNPs at each locus as homozygous reference, homozygous alternate or heterozygous for each individual (Melville et al., 2017). Monomorphic loci, loci with <100% reproducibility or missing values were removed in the R package, dartR, to improve the quality of and reduce linkage within the dataset; this reduced the dataset from

8288 loci to 1743 loci (R Core Team, 2013; Gruber et al., 2017).

Euclidean distances between individual anemones, based on differences in the allele frequencies at each of the SNP loci, were calculated from the reduced SNP dataset in *dartR*, then viewed as a histogram and printed into a matrix in RStudio. Although the genetic distance between individuals of the same genotype should be zero, small differences may occur due to sequencing errors and somatic mutations. The genetic distance between individuals of different genotypes will be larger than that within individuals. Therefore, the genetic distances among individuals from several genotypes should form a bi- or multi-modal distribution; one peak with a relatively small mean represents genetic distances between pairs of individuals of the same genotype, another represents inter-genotypic distances with a larger mean. The inter-genotypic distribution can be multi-modal because different pairs of genotypes can differ by different amounts. Note that two samples from the same individual were genotyped to determine methodological error rates and verify the baseline for clonality. A principal coordinates analysis was performed and plotted in *dartR* to visualize the genotype assignments.

To compare the phylogenetic relationship of the GBR-sourced anemones with the previously described clonal lines, we used a set of four *Exaiptasia*-specific inter-simple sequence repeat (ISSR)-derived sequence characterized amplified region (SCAR) markers developed by Thornhill et al. (2013) and an additional six *Exaiptasia*-specific gene loci (Bellis and Denver, 2017). gDNA was extracted from five animals of each genotype, as described above, and amplified with each of the four SCAR marker (3, 4, 5, and 7) and gene primer pairs (Thornhill et al., 2013; Bellis and Denver, 2017). PCR solutions for each marker contained 12.5 μ L MyTaq HS Mix polymerase (Bioline), 1 μ L of DNA template, 0.4 μ M of each primer, and nuclease-free water up to 25 μ L. Thermocycling consisted of an initial denaturation at 94°C for 1.5 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min followed by a final extension at 72°C for 5 min. Amplified products were purified and sequenced in forward and reverse directions at AGRF. Sequences were aligned and edited in Geneious version 2019.2. Substantial non-specific binding of the SCAR marker 7 primers generated unusable sequence data. Further, the forward and reverse sequences

from two anemone genotypes that were heterozygous for two or more indels at different points in the sequences of SCAR markers 3 (anemone AIMS1) and 4 (anemones AIMS2-4) could not be aligned and this prohibited the inclusion of those loci in the analyses. Therefore, only sequence data from SCAR marker 5 was used for phylogenetic analyses. For the six *Exaiptasia*-specific gene loci, AIPGENE19577 and Atrophin-1-interacting protein 1 (AIP1) contained heterozygous indels or non-specific binding of the forward primer, therefore four of the six gene loci were used for phylogenetic analysis.

For each genotype, the SCAR marker 5 sequences and each of the six *Exaiptasia*-specific gene loci of the clonal replicates were aligned and a consensus sequence was generated. For the SCAR marker, the consensus sequences were aligned with twelve reference sequences from Thornhill et al. (2013) and three experimental anemone sequences (Grawunder et al., 2015), while the *Exaiptasia*-specific gene sequences were aligned with five reference sequences from Bellis and Denver (2017). Each alignment was used to create a phylogenetic tree using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000) in MEGA X (Kumar et al., 2018). Topology, branch length, and substitution rate were optimized, and branch support was estimated by bootstrap analysis of 1000 iterations.

2.3.3 GBR anemone gender determination

Over a period of two months, several anemone individuals from each genotype were reared to a pedal disk diameter of ~7 mm. One animal per genotype was anesthetized in a 1:1 solution of 0.37 M magnesium chloride and filter-sterilized RSS (fRSS) for 30 min, dissected and viewed under a stereo microscope (Leica MZ8) to confirm the presence of gonads. When gonads were observed, one animal per genotype was anesthetized as described above, fixed in 4% paraformaldehyde in 1x PBS (pH 7.4) overnight, washed three times in 1x PBS and processed by the Biomedical Histology Facility of The University of Melbourne. Samples were embedded in paraffin and 7 µm transverse sections were cut (see Table S2-1 for histological processing). Slides were stained with hematoxylin and eosin (H&E) and viewed under a compound microscope (Leica DM6000B).

2.3.4 Symbiodiniaceae

The ITS2 region of Symbiodiniaceae gDNA was analysed by metabarcoding to determine the Symbiodiniaceae species associated with the GBR-sourced anemones. gDNA extracted from three animals of each genotype, as described above, was amplified in triplicate with the ITS-Dino (forward; 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAATTGCA-GAACTCCGTG-3') (Pochon et al., 2001) and its2rev2 (reverse; 5'-GTCTCGTGGGCTCGGAGA-TGTGTATAAGAGACAGCCTCCGCTTACTTATATGCTT-3') (Stat et al., 2009) primers modified with Illumina adapters. PCRs included 10 µL MyTaq HS Mix polymerase (Bioline), 1 µL of DNA template, 0.25 µM of each primer, and nuclease-free water up to 20 µL. Thermocycling consisted of an initial denaturation at 95°C for 3 min, 35 cycles at 95°C, 55°C and 72°C for 15 sec each, and 1 cycle at 72°C for 3 min. Library preparation on pooled triplicates and Illumina MiSeq sequencing (2x250 bp) was performed by the Ramaciotti Centre for Genomics at the University of New South Wales, Sydney. Raw, demultiplexed MiSeq read-pairs were joined in QIIME2 v2018.4.0 (Bolyen et al., 2019). Denoising, chimera checking, and trimming was performed in DADA2 (Callahan et al., 2016). The remaining sequences were clustered into operational taxonomic units (OTUs) at 99% sequence similarity using closed-reference OTU picking in vsearch (Rognes et al., 2016). A taxonomic database adapted from Arif et al. (2014) was used to seed the OTU clusters and for taxonomic classification.

2.3.5 Anemone and Symbiodiniaceae physiological properties

Physiological assessments were performed on anemones maintained in a healthy, unbleached state for over two years. Three of the four anemone genotypes named AIMS2, AIMS3, and AIMS4, with oral disk diameters of 3-4 mm were transferred from the original holding tanks in the walk-in incubator and randomly distributed between three replicate (by genotype) 250 mL glass culture containers containing RSS. Anemones of this size were chosen as they were considered sexually immature (Muller-Parker et al., 1990; Grawunder et al., 2015) and therefore variability in sexual development or gonadal reserves were unlikely to influence the results. AIMS1 was excluded from long-term assessment as it had poor survival at densities required for the analyses.

Glass culture containers were transferred from the walk-in incubator to experimental growth chambers (Taiwan HiPoint Corporation model 740FHC) fitted with red, white, and infrared LED lights. Initial light levels were set at $12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (HiPoint HR-350 LED meter) to correspond with the walk-in incubator conditions and ramped up to $28 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ over a period of 72 h on a 12 h:12 h light:dark cycle to approximate experimental conditions reported in *E. diaphana* literature (Fig. S2-1; Fransolet et al., 2014; Hoadley and Warner, 2017). While the commonly used *E. diaphana* strains (CC7, H2, RS) are often maintained at $>50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (pers. comm.), the GBR strains appeared healthier, with extended bodies and open tentacles, at lower light intensities. The anemones were maintained in RSS, fed *A. salina* nauplii and cleaned as described above. The pH, salinity and temperature of the water and applied light levels were monitored thrice weekly and were stable over time: 8.14 ± 0.02 , 35.0 ± 0.04 ppt, $25.77 \pm 0.07^\circ\text{C}$ and $28.08 \pm 0.12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively. Culture containers were placed on a single incubator shelf and randomly rearranged after each clean to minimize confounding by position.

The maximum dark adapted quantum yield (F_v/F_m) of photosystem II (PSII) of *in hospite* Symbiodiniaceae provides an indication of photosynthetic performance and is useful as a bio-monitoring tool (Howe et al., 2017). F_v/F_m is measured as the difference in fluorescence produced by PSII reaction centres when either saturated by intense light (F_m) or in the absence of light (F_o), versus F_m (i.e. $(F_m - F_o)/F_m$, or F_v/F_m). Symbiodiniaceae F_v/F_m was measured weekly over nine weeks for each culture container after 4 hr into the incubator light cycle and 30 mins dark adaption using imaging pulse amplitude modulated (iPAM) fluorometry (IMAG-MAX/L, Waltz, Germany). Settings for all measurements were: saturating pulse intensity 8, measuring light intensity 2 with frequency 1, actinic light intensity 3, damping 2, gain 2. Average F_v/F_m values for each dish were calculated from readings taken on three to five anemones (Fig. S2-2).

Anemones from each culture container were individually homogenized in a sterile glass homogenizer in 1 mL fRSS, and 100 μL of homogenate was collected and stored at -20°C for total protein measurement. The remaining 900 μL of homogenate was centrifuged at

5000 $\times g$ for 5 min at 4°C to pellet the Symbiodiniaceae while leaving the anemone cells in suspension. A volume of 100 μL of supernatant was collected and stored at -20°C for host protein measurement. The pelleted Symbiodiniaceae were twice washed with 500 μL fRSS and centrifuged at 5000 $\times g$ for 5 min at 4°C, and the final pellet resuspended in 500 μL fRSS and stored at -20°C for Symbiodiniaceae counts. Triplicate cell counts (cells mL^{-1}) were completed within 48 hrs of sample collection on a Countess II FL automated cell counter (Life Technologies) and normalized to host protein (mg mL^{-1}). This process was repeated 15 times over the course of nine weeks for a total of 45 replicates per genotype.

Samples for protein analysis stored at -20°C (see above) were used within one month of collection to determine total and host protein (mg mL^{-1}) by the Bradford assay (Bradford, 1976) against bovine serum albumin standards (Bio-Rad 500-0207). Readings were taken at 595 nm (EnSpire microplate reader MLD2300).

F_v/F_m values and Symbiodiniaceae cell densities were assessed for genotype-specific responses. All variables were tested for normality and homoscedasticity prior to parametric analyses, which were completed in R (v 3.6.0). Genotypic responses of F_v/F_m were compared using a linear model in the R package nlme (Pinheiro et al., 2017) to evaluate independent and interaction relationships between the factors of genotype and time. F-statistics were obtained using the analysis of variance (ANOVA) function, nlme, and pairwise *post hoc* analyses were performed using the glht function in the R package multcomp (Hothorn et al., 2016) with Tukey's correction for multiple comparisons. Symbiodiniaceae cell densities were pooled across time by genotype and analysed with a one-way ANOVA with a *post hoc* Tukey test to determine pairwise significance.

2.4 Results and Discussion

2.4.1 Anemones

All nucleotide data presented here can be found on GenBank under BioProject number PRJNA576020. The assembled and aligned consensus (by genotype) 18S rRNA gene sequences covered 1586 bp with one nucleotide mismatch between the genotypes.

BLASTn against the NCBI database identified all samples as either *Aiptasia pulchella* or *Exaiptasia pallida*. *A. pulchella* has been synonymised with *E. pallida* (Grajales and Rodriguez, 2014). However, since *E. diaphana* has precedence (ICZN, 2017), all samples were designated *E. diaphana*.

Four SNP genotypes of *E. diaphana*, which were of GBR-origin but AIMS-sourced, were identified and designated AIMS1, AIMS2, AIMS3 and AIMS4 (Fig. 2-1). The clonal distribution of Euclidean distances was identified (range of 0-9.61) and pairs of individuals with Euclidean distances within this distribution were inferred to have the same genotype (Fig. S2-3, Table S2-2).

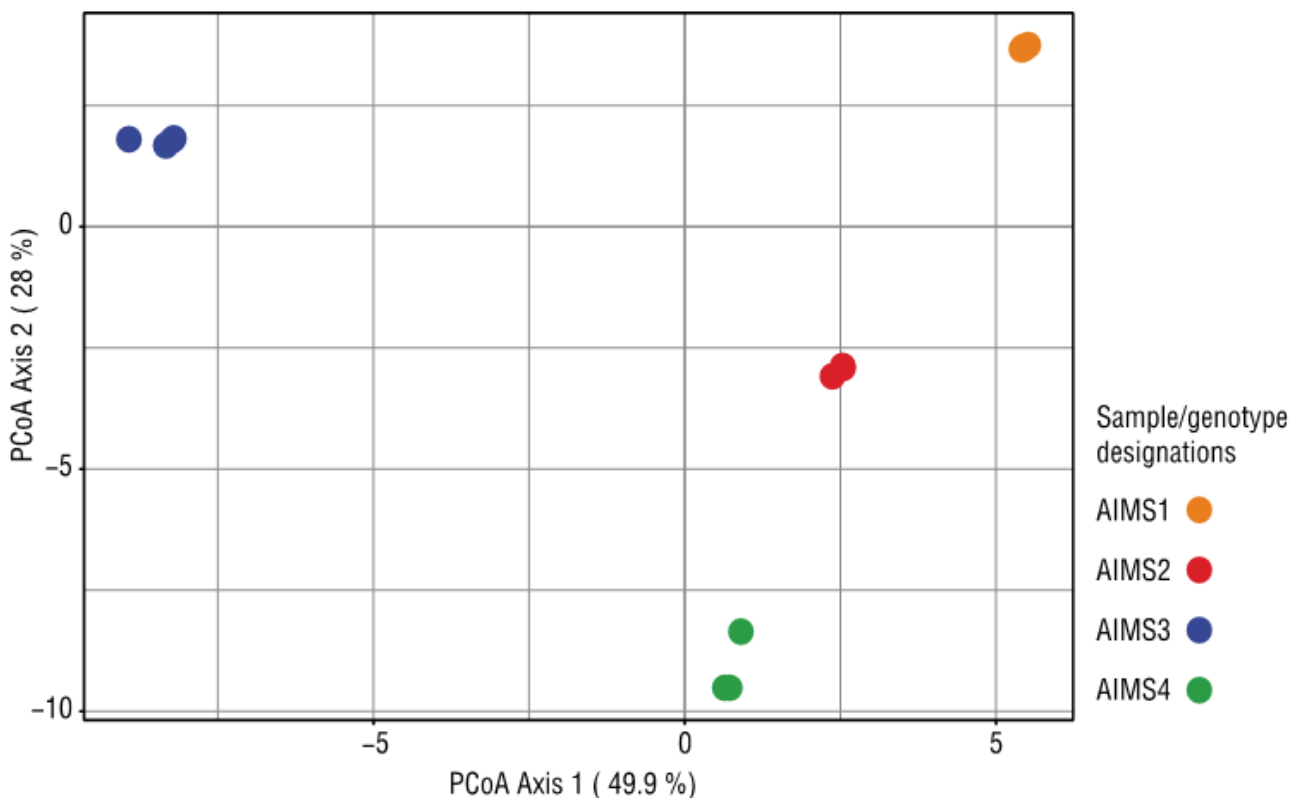


Figure 2-1 PCoA ordination of *Exaiptasia diaphana* genotypes based on Euclidean genetic distance measurements of SNP data using allele frequencies within individuals to calculate genetic distance between them (AIMS1, n=8; AIMS2, n=5; AIMS3, n=7; AIMS4, n=3). Individuals of the same genotype overlap in the plot due to their high similarity.

Using phylogenetic analysis of our data, previously described SCAR marker 5, and the *Exaiptasia*-specific gene sequence data (Thornhill et al., 2013; Grawunder et al., 2015; Bellis and Denver, 2017), we placed the GBR anemones into a phylogeographical context. The alignment of SCAR5 sequences was 706 bp long and contained 19 variable nucleotide

positions (Fig. 2-2), while the concatenated *Exaiptasia*-specific gene sequences were 3276 bp long with 92 variable nucleotide positions (Fig. 2-3).

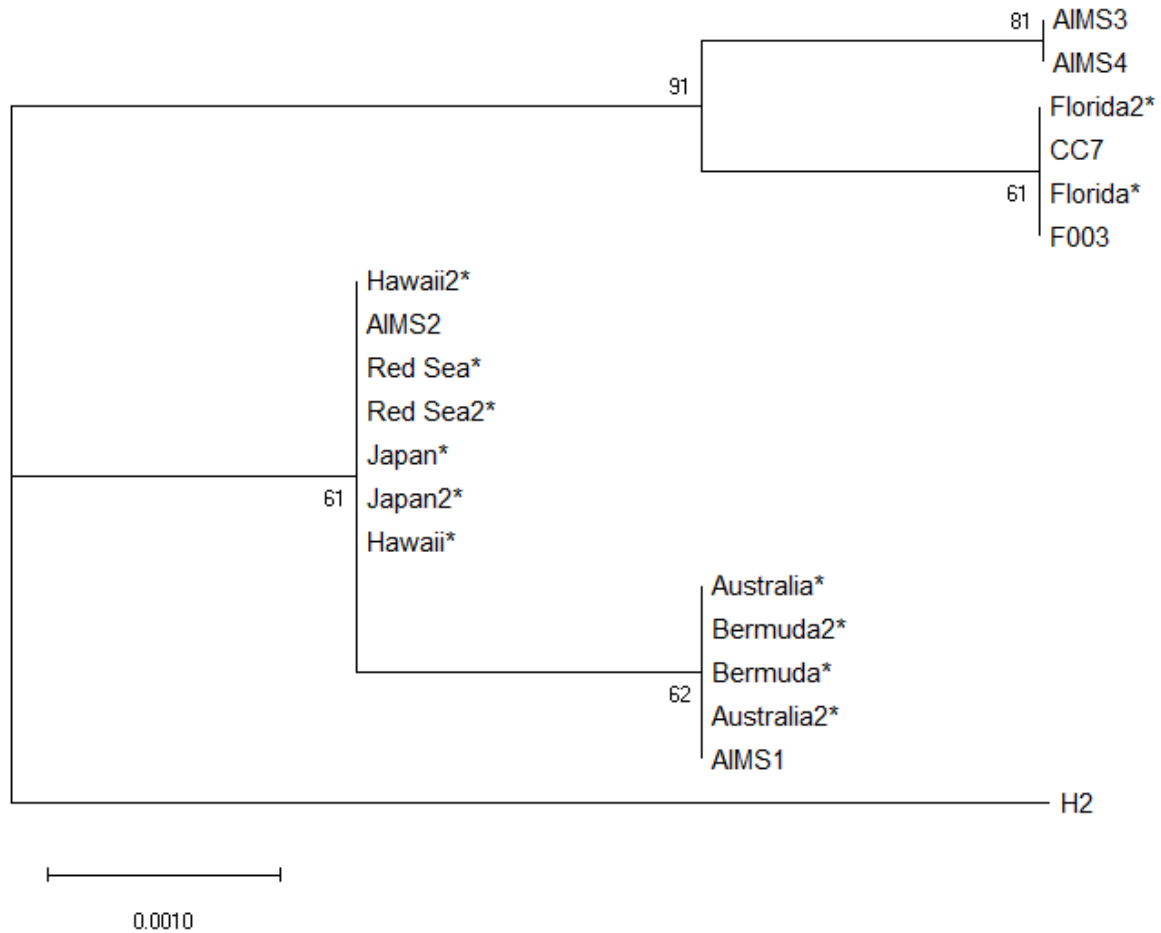


Figure 2-2 The phylogenetic relationships of the four GBR *E. diaphana* compared to other conspecific anemones sampled across the globe inferred from SCAR marker 5 using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood is shown with bootstrap values next to the nodes. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 19 *E. diaphana* sequences and 706 nucleotide positions were included. Sequences were from our *E. diaphana* genotypes (AIMS1-4), globally distributed *E. diaphana* from Thornhill et al. (2013), indicated with an asterisk, and from experimental genotypes CC7, H2, and F003 (Grawunder et al., 2015).

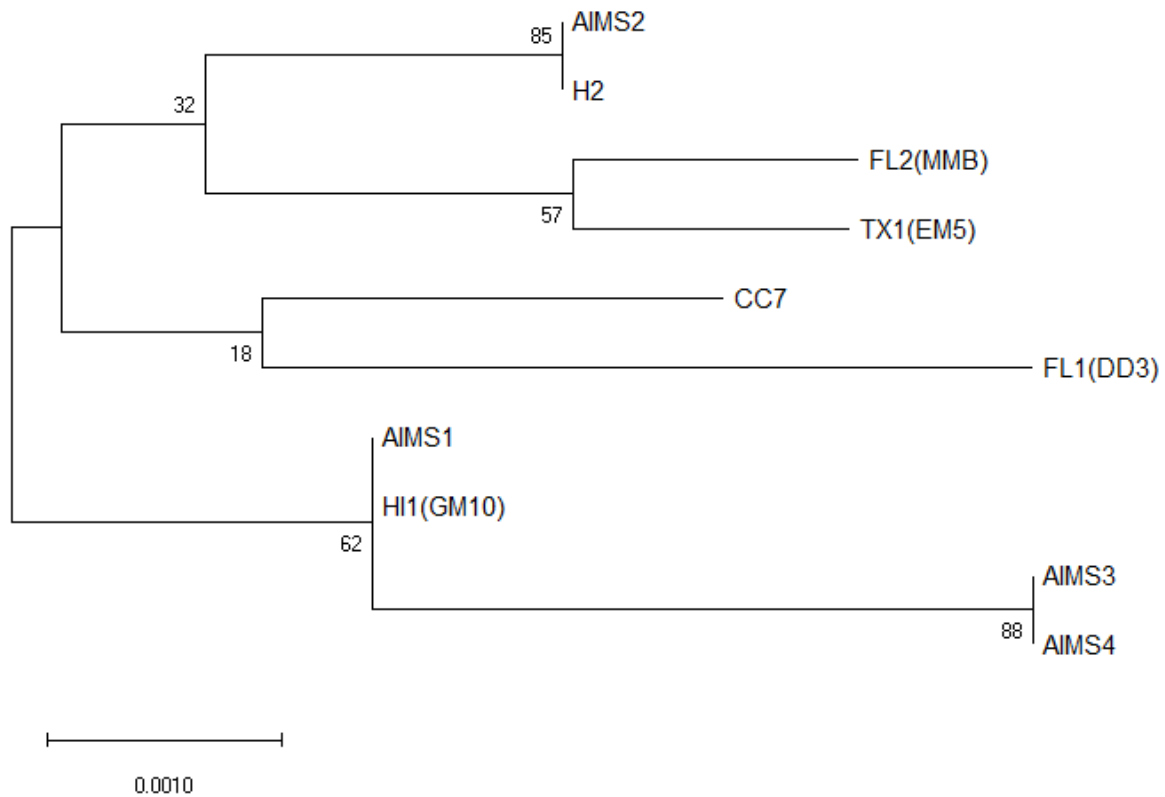


Figure 2-3 The phylogenetic relationships of the four GBR *E. diaphana* (AIMS1-4) compared to other experimental anemones using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000) on six concatenated *Exaiptasia*-specific gene sequences (Bellis and Denver, 2017). Data from Bellis and Denver (2017) was downloaded from GenBank (accession numbers KU847812-KU847847); tree branches are named as strain name followed by the alternative strain name in parentheses. Bootstrap values are shown next to the nodes. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 10 *E. diaphana* sequences and 3267 nucleotide positions were included.

Based on SCAR marker data, *E. diaphana* is regarded as a single pan-global species with two distinct genetic lineages: one from the USA Atlantic coast, and a second consisting of all other *E. diaphana* worldwide (Thomhill et al., 2013). The SCAR5 allele sequenced for genotype AIMS1 is identical with that from anemones originally collected off Heron Island, Australia and Bermuda, while that of AIMS2 is similar to (two base pair differences, both with an ambiguous base) samples from Hawaii, Japan and the Red Sea. AIMS3 and AIMS4 are most closely related to anemones from Florida and North Carolina, USA and are identical to one another in their SCAR5 sequence.

Similar to the findings by Bellis and Denver (2017), our data from the *Exaiptasia*-specific gene sequences (Fig. 2-3) show that, while anemone strains are genetically distinct, there is not a strong phylogenetic separation between individuals collected from distant geographic locations. Again, AIMS1-4 show genetic variation, with AIMS1 and AIMS2 clustering with anemone strains originating from Coconut Island, Hawaii, USA which were collected independently in 1979 and early 2000's, respectively. Corresponding with the SCAR5 loci data, AIMS3-4 have near identical sequences in the sequenced regions (differences at two heterozygous sites). Because the available data for SCAR5 and the *Exaiptasia*-specific gene targets are not from all the same individuals, except for CC7 and H2, comparing between the two is not feasible. However, given the larger number of alignment positions and variable sites in the gene regions with better PCR results, we suggest that researchers use the primers presented in Bellis and Denver (2017) for future comparisons between *E. diaphana* used in experiments. The diversity that is revealed by genome-wide SNP analysis (Fig. 2-1) is not apparent with these six *Exaiptasia*-specific gene sequences, suggesting that there are more informative loci not yet published for *E. diaphana* genotyping.

There are several possible explanations for the AIMS1-4 anemones to be spread throughout the phylogenetic trees. First, due to small sample sizes at all sampling locations, only a subset of the alleles has been sampled and location-specific alleles may have been missed. Second, it is conceivable that the GBR is the source of all other *E. diaphana* populations and founder effects mean that other geographic locations have *E. diaphana* that represent only some of the diversity. Third, it is possible that the GBR *E. diaphana* was a distinct lineage, and the GBR has since been invaded by *E. diaphana* from other lineages, or the GBR lineage has been introduced elsewhere. Introductions over such vast spatial scales may have occurred via ship ballast water or attached to ships hulls in fouling biomass, which is notorious for transporting marine life and introducing invasive animals and plants, or via the aquarium trade or marine farms. Irrespective of the cause, the genetic variation across the four GBR genotypes is more representative of global diversity than a single localized population. Because strain-specific responses to environmental variables have been observed among *E. diaphana* strains (Bellis and Denver,

2017; Cziesielski et al., 2018) it is critical to conduct experiments, such as those regarding climate change and symbiosis, with a diverse set of individuals, much like the diversity presented by the GBR-sourced *E. diaphana*.

2.4.2 Symbiosis with Symbiodiniaceae

According to a global survey by Thornhill et al. (2013), Pacific Ocean *E. diaphana* (e.g., H2) associate exclusively with the Symbiodiniaceae species *Breviolum minutum* (formerly ITS2 type *Symbiodinium* Clade B, sub-clade B1 (LaJeunesse et al., 2018)). Symbiodiniaceae sequences from the four GBR *E. diaphana* genotypes were almost exclusively *Breviolum minutum* (>99.6%, Tortorelli et al., 2019), thus concurring with Thornhill et al. (2013). Two as-yet unnamed sequence types of *Breviolum* (previously known as *Symbiodinium* sub-clades B1i and B1L (LaJeunesse et al., 2018)) were also identified in *E. diaphana*. Their very low relative abundance suggests they are either intragenomic variants or rare strains.

Stable endosymbiotic relationships between corals and Symbiodiniaceae are vital for sustaining coral reef ecosystems; this symbiotic relationship is the focus of much coral reef research. Interestingly, while the GBR anemones are genetically diverse, all host *B. minutum* as their sole Symbiodiniaceae. Given this, we may be able to investigate the symbiotic mechanisms of the host-algal relationship and answer questions about this symbiosis that are not restricted by anemone strain. Having a single Symbiodiniaceae species as the homologous symbiont could have negative consequences to research investigations as the mechanisms of the host-algal relationship revealed by GBR-sourced *E. diaphana* may not be universal for other types of Symbiodiniaceae.

2.4.3 Sex of *E. diaphana*

E. diaphana lacks obvious gender defining morphological features, but gonad development is related to size (Chen et al., 2008). Partially developed oocytes with germinal vesicles (*i.e.*, the nucleus of an oocyte arrested in prophase of meiosis I) were observed in histological slides in animals of AIMS1, AIMS3 and AIMS4 and Stage V spermaries were observed in AIMS2 (Fig. 2-4). In Stage V, the spermary is made up of a

mass of spermatozoa with their tails facing in the same direction. In this advanced stage the spermatozoa are capable of fertilization (Fadlallah and Pearse, 1982; Goffredo et al., 2012). While most AIMS1 anemones grew to only 5 mm in pedal disk diameter, gonad development was still observed. Differences between male and female gonads were present but were macroscopically cryptic. Male gonads have been observed to be smaller and lighter coloured than female gonads in *E. diaphana* (Grawunder et al., 2015), and this was also the case in the GBR-sourced females (AIMS1, AIMS3 and AIMS4) and male (AIMS2) *E. diaphana*.

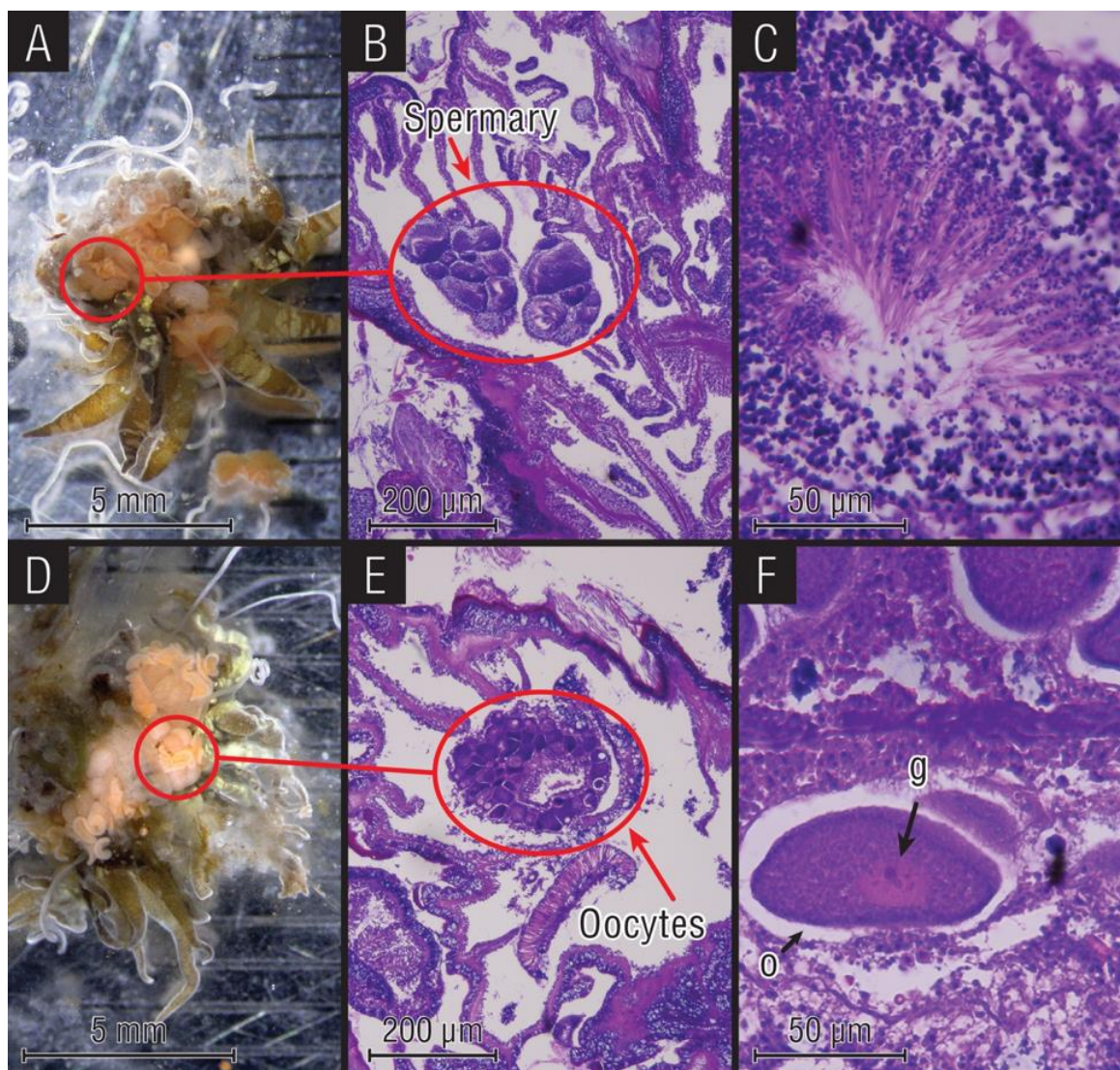


Figure 2-4 (A) Dissected male anemone with developing gonads (AIMS2, ~1 cm pedal disk diameter); (B) H&E stained tissue section, with stage 5 spermary; (C) Increased magnification of stage 5 spermary; (D) dissected female anemone with developed gonads (AIMS4, ~1 cm pedal disk diameter); (E) H&E stained tissue section with oocytes; (F) increased magnification of female gonad with developing oocyte (o) and germinal vesicle (g).

2.4.4 *E. diaphana* and Symbiodiniaceae physiology

Throughout the nine-week evaluation period all *E. diaphana* maintained a healthy appearance, including *in hospite* Symbiodiniaceae, tentacle extension, active feeding of *A. salina* nauplii, and asexual propagation. Average Symbiodiniaceae cell densities (normalized to host protein) were significantly different between genotypes (one-way ANOVA ($F_{(3,200)}=3.985$, $p=0.00872$). All anemones hosted $\sim 10^6$ Symbiodiniaceae cells mg^{-1} host protein, which is comparable to densities reported for other lab cultured model *E. diaphana* systems (Hoadley et al., 2015; Hawkins et al., 2016b; Rädcker et al., 2018) and is similar to Symbiodiniaceae cell densities found in scleractinian corals (Cunning and Baker, 2014; Ziegler et al., 2015; Kenkel and Bay, 2018). Pairwise comparisons revealed a significantly reduced Symbiodiniaceae cell density in AIMS2 (mean $\pm$ SE; $7.17 \times 10^6 \pm 2.73 \times 10^5$ per mg host protein, $n=66$) compared to AIMS3 (mean $\pm$ SE; $8.46 \times 10^6 \pm 2.96 \times 10^5$ per mg host protein, $n=66$, $p=0.018$).

All genotypes experienced a nearly identical drop in F_v/F_m after the light intensity was increased from 12 to 28 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, from an average of 0.53 on day 0, to an average of 0.40 by day 21 (Fig. 2-5). However, by day 36 the F_v/F_m values had returned to initial levels.

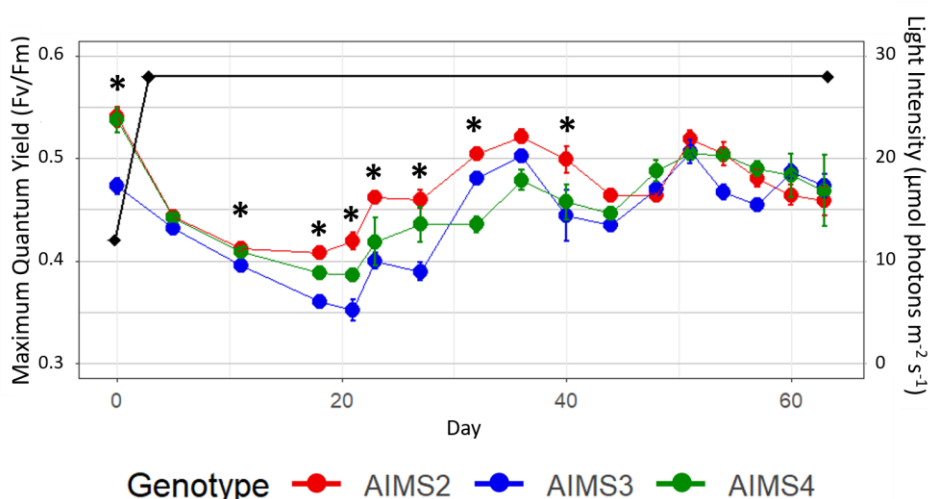


Figure 2-5 F_v/F_m measurements for anemones AIMS2, AIMS3 and AIMS4 over a 63-day period. Anemones were initially exposed to light levels (black line) of 12 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (12:12 light:dark cycle), which were then increased to 28 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ over a 72 h period. Day 0 marks the start of light ramping. The anemones took ~ 35 d to recover their maximum quantum yield due to the increase in light exposure although Symbiodiniaceae densities remained largely constant. Asterisks indicate significant differences in pairwise comparisons between genotypes at given time points (Table S2-3)

Changes in environmental variables, such as light intensity, are known to influence photobiological behaviour of Symbiodiniaceae (Wangpraseurt et al., 2014; Hoadley and Warner, 2017). A change in light intensity can alter photosynthetic efficiency as measured by F_v/F_m (Hoadley and Warner, 2017). GBR anemone genotypes AIMS2, AIMS3 and AIMS4 took 36 d to recover from this environmental change and return to photosynthetic efficiencies recorded at $12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Such information is critical for planning and conducting symbiosis studies. Most of the statistical differences between the genotypes occurred between days 19-28 (Table S2-3) when F_v/F_m was lowest. Once acclimated at day 36, average F_v/F_m values for all genotypes were not significantly different (mean $\pm$ SE; 0.479 ± 0.003 , $n=80$), except for day 40 where the F_v/F_m of AIMS2 was significantly higher than AIMS3 ($p=0.0068$). Light levels used in this experiment are far below those in open shallow reef habitats occupied by reef-building corals on the GBR (Bainbridge, 2017), but rather represent light levels that occur in highly shaded or deep reef slope habitats. As such, the photobiology responses of this anemone are different from those of reef-building corals, and *E. diaphana* will likely experience extreme physiological stress if exposed to the light levels experienced by most shallow reef corals.

As all the GBR-sourced anemones harbour *Breviolum minutum* as their homologous symbiont type, it is not unexpected that the different genotypes would have similar maximum F_v/F_m values. Furthermore, similar F_v/F_m values have been reported for other anemones hosting homologous *B. minutum* (Hawkins et al., 2016b; Hillyer et al., 2017). However, it is noteworthy that AIMS2 is not only able to recover its photosynthetic efficiency quicker from changing light levels with a milder dip in F_v/F_m values (Fig. 2-5), but also hosts significantly fewer Symbiodiniaceae cells mg^{-1} host protein compared to AIMS3. Reductions in the maximum quantum yield of photosystem II (PSII) (F_v/F_m) is observed in the early phases of natural bleaching events (Gates et al., 1992; Franklin et al., 2004) and the ability of AIMS2 to maintain a higher efficiency of PSII photochemistry during changing environmental conditions could translate into higher thermal tolerance (Suggett et al., 2008; Ragni et al., 2010; Goyen et al., 2017).

These individuals are genetically diverse based on SNP genotyping (Fig. 2-1) and phylogenetic analysis (Fig. 2-2, Fig. 2-3); the phenotypic feature of AIMS2 being more robust to increasing light levels compared to AIMS3 and AIMS4 could be a host genotypic effect. There is evidence that genetic variation of *E. diaphana* may influence holobiont response to heat stress, though this hypothesis has only been tested on anemone strains hosting different Symbiodiniaceae species (Bellis and Denver, 2017; Czieleski et al., 2018) or after experimentally bleaching anemones and inoculating with new heterologous algal cells (Perez et al., 2001). As we have four GBR-sourced *E. diaphana* genotypes with inherent genetic variability and all contain *B. minutum* as their homologous symbiont, we will be able to explore the roles of host and symbiont in the bleaching response.

An alternative possibility is that the Symbiodiniaceae community diversity of the GBR-sourced anemones may be hidden under the resolution of the ITS2 sequences used in this experiment, which are driving the differences in photosynthetic efficiency. Distinct strains of a given Symbiodiniaceae species can have different susceptibilities to thermal stress (Ragni et al., 2010; Howells et al., 2012; Hawkins et al., 2016a), with evidence that these variations in thermal optima can drive host-Symbiodiniaceae interactions (Hawkins et al., 2016a). Thus, varying rates of recovery of F_v/F_m among Symbiodiniaceae strains (Fig. 2-5) could provide a mechanism for the emergence of novel and potentially resilient cnidarian-Symbiodiniaceae associations in a rapidly warming environment.

Another explanation for the physiological differences between AIMS2 and AIMS3 is through algal cell density moderation by the host. It is thought that the coral host controls Symbiodiniaceae densities through nitrogen limitation (Falkowski et al., 1993), although the mechanisms are not well understood (Davy et al., 2012). During temperature stress, higher densities of Symbiodiniaceae have been implicated in increasing the susceptibility of corals to bleaching, potentially as a result of the higher reactive oxygen species production relative to corals' antioxidant capacity (Cunning and Baker, 2012). Altogether, our data suggest that AIMS2, which hosted fewer algal symbionts and recovered from increased light conditions faster than AIMS3, may be more resilient to thermal stress, while AIMS3 could be more susceptible to bleaching with AIMS4 as an intermediate.

2.5 Conclusions and future directions

The study of *E. diaphana* anemones of GBR origin described here provide further information on phenotypic and genetic variation within this species and complements data on the more widely used *E. diaphana* strains CC7 and H2. The four genotypes in our collections capture a level of genetic diversity previously observed in animals from different oceans and are therefore a hugely valuable addition to the model collections. While a direct comparison between *E. diaphana* and reef building corals may not be relevant given the extent of the ecological differences between them, a comprehensive characterization of *E. diaphana* strains enhances and broadens the potential of this model system for climate change research in corals, particularly - but not exclusively - for Australian researchers. Future research on this collection should focus on characterization of associated prokaryotes to explore the value of these animals as models for coral-prokaryote symbiotic interactions. Moreover, development of axenic (germ-free) or gnotobiotic (with a known microbial community) cultures of *E. diaphana* would greatly enhance research into cnidarian-prokaryotic interactions. Such cultures could be used to test the influence of native and non-native microbiota on holobiont performance, and the ability of probiotic inocula to support animal health during stress (Alagely et al., 2011; Damjanovic et al., 2017; Rosado et al., 2018).

2.6 Acknowledgements

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2.7 Supplementary material

Table S2-1 Histological processing steps used to serially dehydrate anemone tissue after fixation but prior to embedding. This protocol is modified from Carlisle et al. (2017).

Process	Solution	Duration
Rinsing	Deionized water	15 min
	Running tap water	4 hr
Dehydration	50% ethanol	50 min
	70% ethanol	Overnight
	90% ethanol	50 min
	100% ethanol I	50 min
	100% ethanol II	50 min
	100% ethanol III	50 min
Clearing	1:1 100% ethanol:Xylene	50 min
	Xylene I	50 min
	Xylene II	50 min
Embedding	Histoplast paraffin wax I	1 hr
	Histoplast paraffin wax II	1 hr
	Histoplast paraffin wax III	1 hr
	Histoplast paraffin wax IV	1 hr

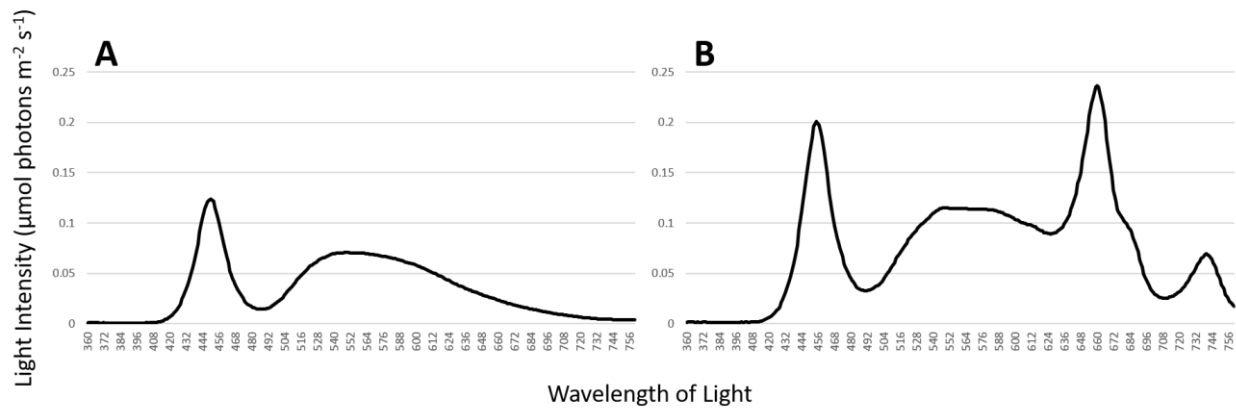


Figure S2-1 Spectra of light intensities (HiPoint HR-350 LED meter) from the A) walk-in incubator where the GBR-sourced stock anemones are held (fitted with white LEDs only) and B) the experimental growth chambers (Taiwan HiPoint Corporation model 740FHC) fitted with red, white, and infrared LED lights.

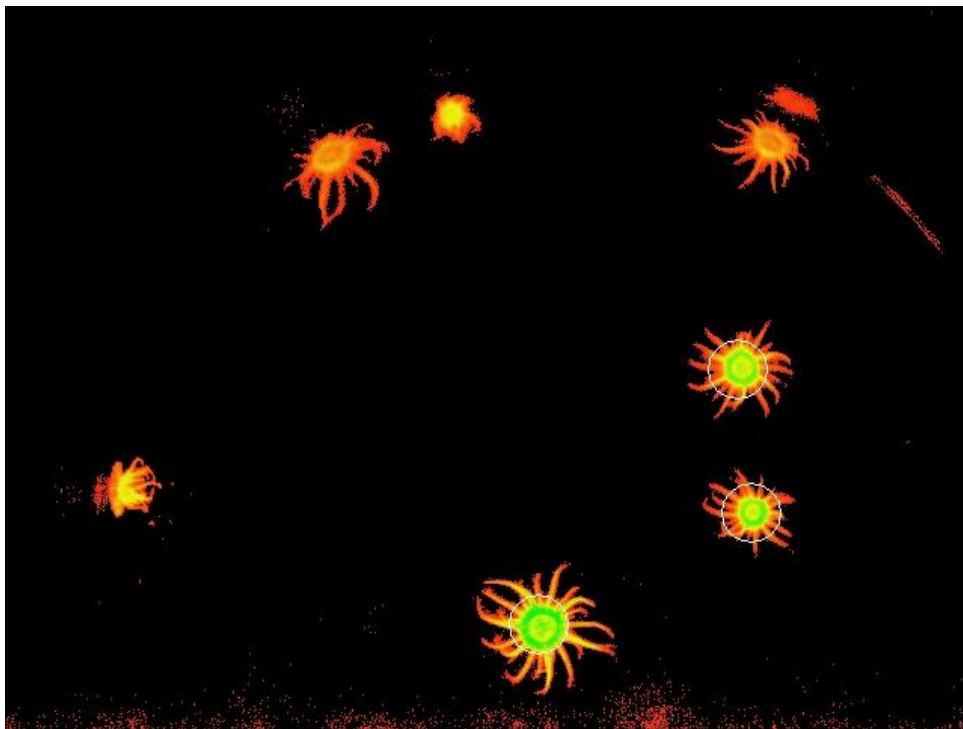


Figure S2-2 Areas of interest (AOIs) are shown as white circles surrounding the central body of the anemones and including the proximal portion of the tentacles. A minimum of three anemones per jar were selected, avoiding individuals on the sides that were out of focus and could skew the data. F_0 values ranged from 0.100 to 0.300. No scale available.

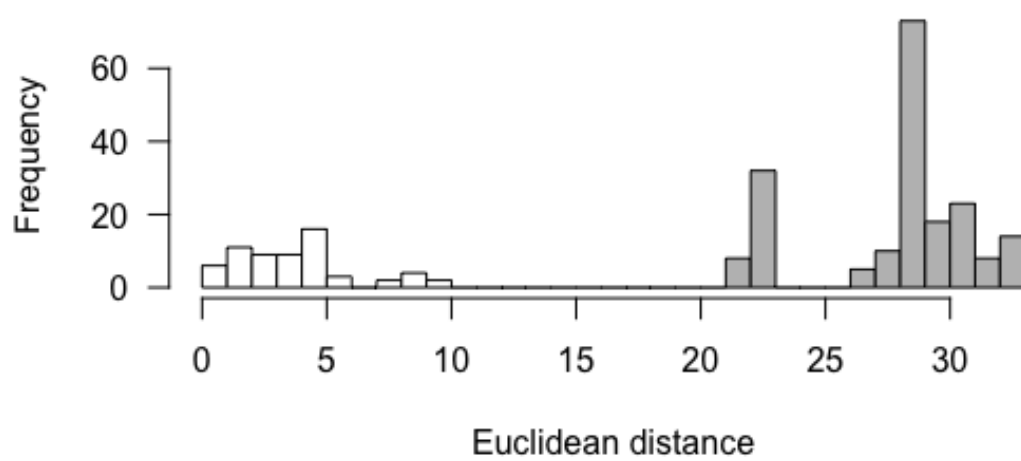


Figure S2-3 Histogram of Euclidean distances among *Exaiptasia diaphana* individuals. The inferred clonal (range of 0-9.61) and inter-genotypic distributions are shown in white and grey, respectively.

Table S2-2 Euclidean distance matrix between *E. diaphana* individuals. Individuals are arranged by genotype (AIMS1-4) and distances within and outside the clonal distribution are shown in white and grey, respectively. Name codes: Ed = *Exaiptasia diaphana* sent from AIMS, S= *Exaiptasia diaphana* originally from AIMS but held at Swinburne University of Technology (with I, II, A, B, and C as individuals)

Genotype	<u>AIMS1</u>								<u>AIMS3</u>								<u>AIMS2</u>				<u>AIMS4</u>			
Individual	Ed.01	Ed.02	Ed.03	Ed.07	Ed.08	Ed.09	Ed.12	Ed.21	Ed.06	Ed.11a	Ed.11b	Ed.14	Ed.17	Ed.18	Ed.19	Ed.10	Ed.20	Ed.22	SC	SII	SA	SB	SI	
Ed.01	0.00	3.76	1.67	4.22	0.50	0.71	2.90	2.12	28.28	28.29	29.48	28.31	28.87	28.29	28.32	22.11	22.12	21.96	21.92	22.16	30.66	30.80	28.97	
Ed.02	3.76	0.00	3.69	5.45	3.72	3.72	4.18	4.42	28.64	28.65	29.57	28.67	29.21	28.66	28.69	22.22	22.42	22.12	22.05	22.33	30.37	30.56	28.56	
Ed.03	1.67	3.69	0.00	4.37	1.59	1.81	2.90	2.63	28.32	28.33	29.46	28.35	28.90	28.34	28.38	22.11	22.15	21.99	21.94	22.18	30.67	30.82	29.01	
Ed.07	4.22	5.45	4.37	0.00	4.18	4.18	5.41	4.74	28.63	28.64	29.65	28.66	29.12	28.63	28.69	22.30	22.43	22.25	22.14	22.37	30.54	30.75	28.86	
Ed.08	0.50	3.72	1.59	4.18	0.00	0.50	2.84	2.05	28.28	28.29	29.47	28.30	28.87	28.28	28.32	22.10	22.12	21.96	21.91	22.16	30.65	30.80	28.97	
Ed.09	0.71	3.72	1.81	4.18	0.50	0.00	2.79	1.99	28.27	28.28	29.47	28.30	28.86	28.28	28.31	22.12	22.14	21.98	21.93	22.17	30.67	30.81	28.97	
Ed.12	2.90	4.18	2.90	5.41	2.84	2.79	0.00	3.44	28.67	28.68	29.87	28.70	28.96	28.67	28.72	22.25	22.27	22.15	22.11	22.44	30.90	31.07	29.09	
Ed.21	2.12	4.42	2.63	4.74	2.05	1.99	3.44	0.00	28.32	28.32	29.60	28.35	28.87	28.31	28.35	22.32	22.34	22.18	22.13	22.36	30.51	30.66	28.74	
Ed.06	28.28	28.64	28.32	28.63	28.28	28.27	28.67	28.32	0.00	1.73	8.67	1.00	4.43	1.50	1.67	28.16	28.23	27.95	27.91	28.15	32.61	32.61	31.51	
Ed.11a	28.29	28.65	28.33	28.64	28.29	28.28	28.68	28.32	1.73	0.00	8.82	0.00	4.43	0.50	1.33	28.20	28.26	27.99	27.96	28.21	32.61	32.60	31.50	
Ed.11b	29.48	29.57	29.46	29.65	29.47	29.47	29.87	29.60	8.67	8.82	0.00	8.68	9.61	8.66	9.07	29.47	29.89	29.19	29.10	29.43	32.73	32.84	31.43	
Ed.14	28.31	28.67	28.35	28.66	28.30	28.30	28.70	28.35	1.00	0.00	8.68	0.00	4.43	1.00	1.67	28.20	28.27	27.99	27.96	28.21	32.63	32.63	31.53	
Ed.17	28.87	29.21	28.90	29.12	28.87	28.86	28.96	28.87	4.43	4.43	9.61	4.43	0.00	4.59	4.86	28.72	28.87	28.46	28.47	28.72	32.71	32.73	31.40	
Ed.18	28.29	28.66	28.34	28.63	28.28	28.28	28.67	28.31	1.50	0.50	8.66	1.00	4.59	0.00	1.24	28.17	28.23	27.97	27.93	28.18	32.63	32.61	31.50	
Ed.19	28.32	28.69	28.38	28.69	28.32	28.31	28.72	28.35	1.67	1.33	9.07	1.67	4.86	1.24	0.00	28.12	28.13	27.97	27.93	28.17	32.70	32.63	31.58	
Ed.10	22.11	22.22	22.11	22.30	22.10	22.12	22.25	22.32	28.16	28.20	29.47	28.20	28.72	28.17	28.12	0.00	4.15	3.66	3.14	4.43	30.51	30.69	26.88	
Ed.20	22.12	22.42	22.15	22.43	22.12	22.14	22.27	22.34	28.23	28.26	29.89	28.27	28.87	28.23	28.13	4.15	0.00	4.37	4.04	5.03	30.40	30.56	26.84	
Ed.22	21.96	22.12	21.99	22.25	21.96	21.98	22.15	22.18	27.95	27.99	29.19	27.99	28.46	27.97	27.97	3.66	4.37	0.00	2.09	3.88	30.22	30.45	26.68	
SC	21.92	22.05	21.94	22.14	21.91	21.93	22.11	22.13	27.91	27.96	29.10	27.96	28.47	27.93	27.93	3.14	4.04	2.09	0.00	3.47	30.22	30.43	26.67	
SII	22.16	22.33	22.18	22.37	22.16	22.17	22.44	22.36	28.15	28.21	29.43	28.21	28.72	28.18	28.17	4.43	5.03	3.88	3.47	0.00	29.69	29.88	26.27	

Table S2-3: Pairwise comparisons between genotypes by sampling day from a linear mixed effects model with genotype and sampling day as fixed effects and anemone jar as a random effect. Significant differences are noted in **bold**; $\alpha = 0.05$.

Sampling Day	Genotype	Mean	SE	lower.CL	upper.CL	contrast	estimate	SE	df	t.ratio	p.value
0	2	0.541	0.01202	0.517	0.565	2-3	0.067667	0.01701	60	3.979	0.0005
0	3	0.473	0.01202	0.449	0.497	2-4	0.003333	0.01701	60	0.196	0.979
0	4	0.537	0.01202	0.513	0.561	3-4	-0.06433	0.01701	60	-3.783	0.001
5	2	0.441	0.00721	0.427	0.456	2-3	0.006964	0.00992	60	0.702	0.7634
5	3	0.434	0.00682	0.421	0.448	2-4	-0.00335	0.00992	60	-0.338	0.9391
5	4	0.445	0.00682	0.431	0.458	3-4	-0.01032	0.00964	60	-1.07	0.5361
11	2	0.412	0.00491	0.402	0.422	2-3	0.016889	0.00694	60	2.433	0.0466
11	3	0.395	0.00491	0.385	0.405	2-4	0.003278	0.00694	60	0.472	0.8846
11	4	0.409	0.00491	0.399	0.419	3-4	-0.01361	0.00694	60	-1.961	0.131
18	2	0.408	0.00491	0.398	0.418	2-3	0.0477	0.00694	60	6.871	<.0001
18	3	0.36	0.00491	0.351	0.37	2-4	0.020478	0.00694	60	2.95	0.0124
18	4	0.388	0.00491	0.378	0.398	3-4	-0.02722	0.00694	60	-3.921	0.0007
21	2	0.421	0.00831	0.404	0.437	2-3	0.068978	0.01175	60	5.869	<.0001
21	3	0.352	0.00831	0.335	0.368	2-4	0.034306	0.01175	60	2.919	0.0135
21	4	0.386	0.00831	0.37	0.403	3-4	-0.03467	0.01175	60	-2.95	0.0124
23	2	0.463	0.00831	0.447	0.48	2-3	0.063478	0.01175	60	5.401	<.0001
23	3	0.4	0.00831	0.383	0.417	2-4	0.044639	0.01175	60	3.798	0.001
23	4	0.419	0.00831	0.402	0.435	3-4	-0.01884	0.01175	60	-1.603	0.2523
27	2	0.461	0.00831	0.445	0.478	2-3	0.071811	0.01175	60	6.11	<.0001
27	3	0.39	0.00831	0.373	0.406	2-4	0.025473	0.01175	60	2.167	0.0852
27	4	0.436	0.00831	0.419	0.453	3-4	-0.04634	0.01175	60	-3.943	0.0006
32	2	0.508	0.01155	0.485	0.531	2-3	0.021037	0.01634	60	1.288	0.4075
32	3	0.487	0.01155	0.463	0.51	2-4	0.06324	0.01634	60	3.871	0.0008
32	4	0.444	0.01155	0.421	0.467	3-4	0.042203	0.01633	60	2.584	0.0323
36	2	0.523	0.01398	0.495	0.551	2-3	0.014644	0.01813	60	0.808	0.6998
36	3	0.509	0.01155	0.485	0.532	2-4	0.035513	0.01813	60	1.959	0.1314
36	4	0.488	0.01155	0.465	0.511	3-4	0.02087	0.01633	60	1.278	0.4132

40	2	0.502	0.01155	0.479	0.525	2-3	0.051704	0.01634	60	3.165	0.0068
40	3	0.45	0.01155	0.427	0.473	2-4	0.035907	0.01634	60	2.198	0.0797
40	4	0.466	0.01155	0.443	0.489	3-4	-0.0158	0.01633	60	-0.967	0.6003
44	2	0.467	0.01155	0.444	0.49	2-3	0.025704	0.01634	60	1.574	0.2649
44	3	0.441	0.01155	0.418	0.464	2-4	0.01124	0.01634	60	0.688	0.7713
44	4	0.455	0.01155	0.432	0.478	3-4	-0.01446	0.01633	60	-0.886	0.6515
48	2	0.467	0.01155	0.444	0.49	2-3	-0.0093	0.01634	60	-0.569	0.837
48	3	0.476	0.01155	0.453	0.499	2-4	-0.03009	0.01634	60	-1.842	0.1648
48	4	0.497	0.01155	0.474	0.52	3-4	-0.0208	0.01633	60	-1.273	0.4157
51	2	0.522	0.01155	0.499	0.545	2-3	0.009371	0.01634	60	0.574	0.8346
51	3	0.513	0.01155	0.489	0.536	2-4	0.008907	0.01634	60	0.545	0.8493
51	4	0.513	0.01155	0.49	0.536	3-4	-0.00046	0.01633	60	-0.028	0.9996
54	2	0.508	0.01155	0.485	0.531	2-3	0.034371	0.01634	60	2.104	0.0975
54	3	0.473	0.01155	0.45	0.496	2-4	-0.00443	0.01634	60	-0.271	0.9604
54	4	0.512	0.01155	0.489	0.535	3-4	-0.0388	0.01633	60	-2.375	0.0534
57	2	0.484	0.01155	0.461	0.507	2-3	0.022704	0.01634	60	1.39	0.3526
57	3	0.461	0.01155	0.438	0.484	2-4	-0.01509	0.01634	60	-0.924	0.6274
57	4	0.499	0.01155	0.476	0.522	3-4	-0.0378	0.01633	60	-2.314	0.0615
60	2	0.467	0.01155	0.444	0.49	2-3	-0.02596	0.01634	60	-1.589	0.2581
60	3	0.493	0.01155	0.47	0.516	2-4	-0.02476	0.01634	60	-1.516	0.2909
60	4	0.492	0.01155	0.469	0.515	3-4	0.001203	0.01633	60	0.074	0.997
63	2	0.461	0.01155	0.438	0.484	2-3	-0.01796	0.01634	60	-1.1	0.5181
63	3	0.479	0.01155	0.456	0.502	2-4	-0.01609	0.01634	60	-0.985	0.589
63	4	0.477	0.01155	0.454	0.5	3-4	0.00187	0.01633	60	0.114	0.9928

Chapter 3

Development of a free radical scavenging probiotic to mitigate oxidative stress in cnidarians

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3.1 Abstract

Corals are colonized by symbiotic microorganisms that exert a profound influence on the animal's health. One noted symbiont is a single-celled alga (from the family Symbiodiniaceae), which provides the coral with most of its fixed carbon. Thermal stress furthers the production of reactive oxygen species (ROS) during photosynthesis. ROS can both damage and inhibit the repair of the algal symbiont's photosynthetic machinery, beginning a positive feedback loop for the toxic accumulation of ROS. If not scavenged by the antioxidant network, ROS may trigger a signalling cascade ending with the coral host and algal symbiont disassociating in a process known as bleaching. We used *Exaiptasia diaphana*, which is a model for corals, to construct a probiotic comprised of *E. diaphana*-associated bacteria able to neutralize free radicals such as ROS. We identified six strains with high free radical scavenging ability belonging to the families Alteromonadaceae, Rhodobacteraceae, Flavobacteriaceae, and Micrococcaceae. In parallel, we established a "negative" probiotic consisting of genetically related strains with poor free radical scavenging capacities. From the bacterial whole genome sequences, we focused on genes that may contribute to bleaching prevention roles. In particular, the occurrence of key pathways that are known to influence ROS in each of the strains has been inferred from the genome sequences and is reported here.

3.2 Introduction

Coral reefs are among the most biologically and economically valuable ecosystems on Earth (Cesar et al., 2003; Alder et al., 2006; Fisher et al., 2015). While they cover less than 0.1% of the ocean floor (Spalding and M. Grenfell, 1997), coral reefs support fisheries, tourism, pharmaceuticals and coastal development with a global value of \$8.9 trillion "international \$" per year (de Groot et al., 2012). Corals and other reef organisms have been dying, largely due to anthropogenic influences such as climate change (Hughes et

al., 2017b; Stuart-Smith et al., 2018), which has led to an increased frequency, intensity and duration of summer heat waves that cause coral bleaching (Hughes et al., 2018a; Hoegh-Guldberg et al., 2019).

The coral holobiont, which is the sum of the coral animal and its associated microbiota, including algae, fungi, protozoans, bacteria, archaea and viruses (Rohwer et al., 2002), is an ecosystem engineer. By secreting a calcium carbonate skeleton, the reef structure rises from the ocean floor, forming the literal foundation of the coral reef ecosystem. The success of corals to survive and build up reefs over thousands of years (Devlin-Durante et al., 2016) is tightly linked to their obligate yet fragile symbiosis with endosymbiotic dinoflagellates of the family Symbiodiniaceae (Glynn, 1996).

Intracellular Symbiodiniaceae translocate photosynthetically fixed carbon to the coral host (Muscantine and Porter, 1977; Tremblay et al., 2014) in exchange for inorganic nutrients and location in a high light environment with protection from herbivory (Venn et al., 2008; Yellowlees et al., 2008). During periods of thermal stress, the relationship between the coral host and their Symbiodiniaceae can break down, resulting in a separation of the partners and significantly, a fixed carbon shortage for the host. This phenomenon, 'coral bleaching', is devastating to the host and detrimental to the reef system. The ecosystem-wide effects of bleaching on the coral include reduced skeletal growth and reproductive activity, a lowered capacity to shed sediments, and an inability to resist invasion of competing species and diseases. Severe and prolonged bleaching can cause partial to total colony death, resulting in diminished reef growth, the transformation of reef-building communities to alternate, non-reef building community types, bioerosion and ultimately the disappearance of reef structures (Glynn, 1996).

There are several hypotheses detailing the mechanisms driving bleaching (Weis, 2008; Cunning and Baker, 2012; Wiedenmann et al., 2012; Wooldridge, 2013), with a common

theme being the overproduction and toxic accumulation of reactive oxygen species (ROS) from the algal symbiont. ROS are a normal product of photosynthesis; the light triggered splitting of water molecules in the oxygen evolving complex (OEC) and subsequent transfer of electrons from photosystem II (PSII) to photosystem I (PSI) generates ROS in chloroplasts (Trubitsin et al., 2014). Additionally, photodamage, particularly to PSII, is a normal part of the photosynthetic light cycle, with mechanisms available to efficiently repair this damage (Aro et al., 1993). Heat stress affects the fluidity and integrity of the thylakoid membrane, disturbing PSI, PSII, and the OEC, while simultaneously inducing the production of ROS (Farooq et al., 2016). Elevated ROS levels can both increase damage to (Mathur et al., 2014), and inhibit the repair of the D1 protein in the PSII apparatus (Warner et al., 1999; Nishiyama et al., 2006), and in a positive feedback mechanism, excess ROS is generated. Once generated, ROS can trigger the oxidation of essential photosynthetic molecules, such as thylakoid membranes (Tchemov et al., 2004) and enzymes of the Calvin-Benson cycle (Lesser and Farrell, 2004), thereby interfering with the supply of fixed carbon to the holobiont (Lesser, 2004). Once damaged, Symbiodiniaceae are no longer able to maintain their role in the symbiotic relationship with corals and separate from the host tissue via *in situ* degradation, exocytosis, host cell detachment, host cell apoptosis or host cell necrosis (Weis, 2008).

Probiotics are preparations of viable microorganisms that are introduced to a host to alter their microbial community in a way that is beneficial to the system. Microbiome engineering through the addition of probiotics has been postulated as a key strategy to facilitate adaptation to changing environmental conditions by enhancing corals with the metabolic capabilities of the introduced probiotic bacterial strains (van Oppen et al., 2015; Damjanovic et al., 2017; Peixoto et al., 2017; van Oppen et al., 2017; Damjanovic et al., 2019c; Epstein et al., 2019a). The differences in the bacterial species composition of

healthy and thermally stressed corals (Vega Thurber et al., 2009; Mouchka et al., 2010; Sunagawa et al., 2010; Littman et al., 2011; Epstein et al., 2019b; Pootakham et al., 2019) and the coral model *Exaiptasia diaphana* (Plovie, 2010; Ahmed et al., 2019; Hartman et al., 2019) suggest a role for microbiome engineering in cnidarian health. A disruption to the bacterial community of *Pocillopora damicomis* with antibiotic treatment diminished the resilience of the holobiont during thermal stress, whereas intact microbial communities conferred resilience to thermal stress and increased the rate of holobiont recovery after bleaching events (Gilbert et al., 2012). The relative stability of coral-associated bacterial communities has also been linked to coral heat tolerance; for instance, the bacterial community of heat sensitive *Acropora hyacinthus* corals shifted when transplanted to thermal stress conditions, whereas heat-tolerant *A. hyacinthus* corals harboured a stable bacterial community (Ziegler et al., 2017).

In recent years, researchers have begun to explore the use of probiotics in corals and the model organism for corals, *E. diaphana*. To inhibit the progression of white pox disease, caused by pathogenic *Serratia marcescens*, an Alphaproteobacteria cocktail containing several *Marinobacter* isolates was applied to *E. diaphana* (Alagely et al., 2011). These introduced strains were able to inhibit both biofilm formation and swarming of *S. marcescens*, which halted disease progression. The *Marinobacter*-based probiotic was deemed effective as anemones exposed to both the cocktail and pathogen survived after seven days, while anemones in the *S. marcescens* control treatment died. A bacterial consortium native to the coral *Mussismilia harttii* was selected to degrade water-soluble oil fractions (dos Santos et al., 2015). This bioremediation strategy reduced the negative impacts of oil on *M. harttii* health and accelerated the degradation of petroleum hydrocarbons (dos Santos et al., 2015). Coral microbiomes have also been manipulated through addition of a consortium of native or seawater derived bacteria to the surface of *P. damicomis* to mitigate the effects of

thermal stress (Rosado et al., 2018). The results from this study suggest the consortium was able to partially mitigate coral bleaching. Our goal was to identify bacterial strains suitable for use in a probiotic to mitigate the effects of thermal stress in *E. diaphana*. Given the potential role of ROS in the bleaching process, our focus was to select diverse *E. diaphana*-sourced bacterial strains with free radical scavenging (FRS) properties while avoiding potential pathogens. Antioxidant properties were measured using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is reduced in the presence of an antioxidant molecule, undergoing a colour change from a violet to a colourless solution.

3.3 Results

3.3.1 Diversity of culturable bacteria associated with *E. diaphana*

A total of 842 isolates were obtained from four genotypes of Great Barrier Reef (GBR)-sourced *E. diaphana*. There were no significant differences in bacterial colony forming units (CFUs) between the four genotypes, regardless of growth medium, with $5.9\text{--}10.3 \times 10^3$ CFUs per anemone on Reasoner's 2A agar (R2A) and $6.3\text{--}10.4 \times 10^3$ CFUs per anemone on marine agar (MA) ($p > 0.05$). Partial 16S rRNA gene sequences (~1000 bp) were used to identify the closest matches from GenBank using the Basic Local Alignment Search Tool (BLASTn). In total there were 109 species in 64 genera, 27 families and six phyla (Fig. 3-1). The most abundant genera were *Alteromonas*, *Labrenzia*, and *Ruegeria* (Table 3-1). Gram-positive bacteria comprised 23 species, including *Microbacterium* (31 isolates) and *Micrococcus* (28 isolates). Eight genera were found to be associated with all four genotypes (Table 3-1); these eight genera made up 59.4% of all *E. diaphana*-associated bacterial isolates.

Table 3-1 Bacterial genera associated with all four genotypes of GBR-sourced *E. diaphana* (AIMS1-4).

Genus	Class	No. of species	AIMS1	AIMS2	AIMS3	AIMS4	*Total Isolates
<i>Alteromonas</i>	Gamma-proteobacteria	6	10	52	24	30	116
<i>Labrenzia</i>	Alpha-proteobacteria	4	11	10	26	38	85
<i>Marinobacter</i>	Gamma-proteobacteria	4	7	25	10	13	55
<i>Muricauda</i>	Flavobacteriia	1	16	11	10	5	42
<i>Roseovarius</i>	Alpha-proteobacteria	3	9	12	5	6	32
<i>Ruegeria</i>	Alpha-proteobacteria	3	29	8	5	39	81
<i>Shimia</i>	Alpha-proteobacteria	2	2	3	9	40	54
<i>Vibrio</i>	Gamma-proteobacteria	3	3	7	31	15	56

*521 out of the 842 isolates obtained from the four *E. diaphana* genotypes.

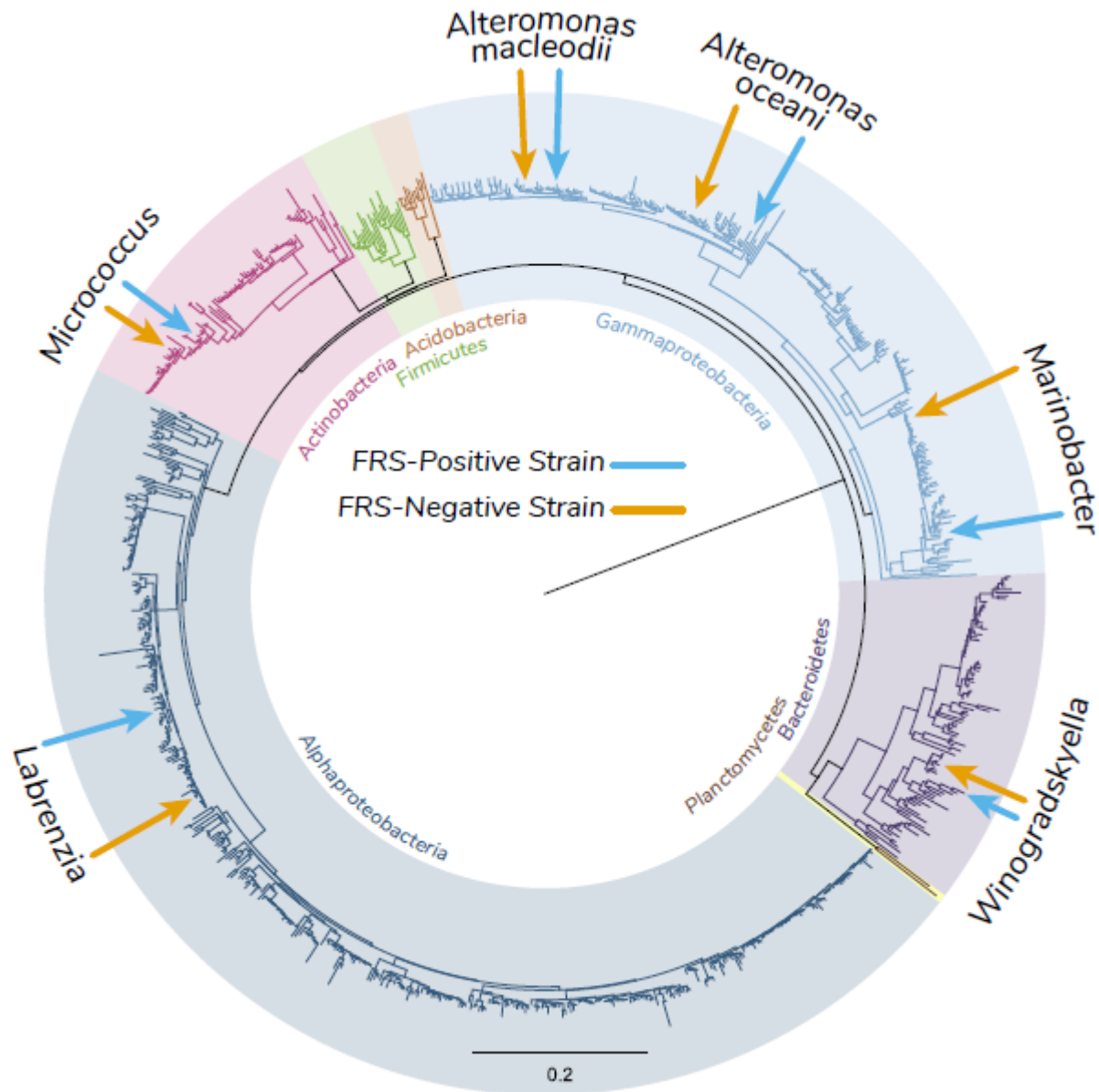


Figure 3-1 Neighbour-Joining tree showing an overview of the phylogenetic relationship between the 842 *E. diaphana*-associated bacterial isolates inferred using partial 16S rRNA sequences. These isolates covered six phyla indicated by shading over the tree with Proteobacteria split into the classes Gammaproteobacteria and Alphaproteobacteria. The positions of selected probiotic strains are highlighted by arrows with blue arrows indicating the high FRS strains and orange arrows indicating low FRS strains.

3.3.2 Bacterial probiotic selection

Of the original 842 isolates, 709 were screened for their ability to scavenge free radicals, divided into positive (144), weakly positive (121), and negative (444). Ninety-eight strains representing eight families and 18 genera were then quantitatively assessed for FRS. There was no clear pattern of FRS capacity at the family level (Fig. 3-2) with strain-specific responses evident. Probiotic members were selected by choosing *E. diaphana*-associated bacteria, where conspecific or congeneric pairs of strains displayed a high ("positive") and low ("negative") FRS ability (Fig. 3-2, Table 3-2). Of the 12 selected probiotic members, seven were catalase positive and five were catalase negative (Table 3-2). In each probiotic set (i.e., high or low FRS strains), none of the selected isolates showed antagonistic activity against one other as evidenced by the absence of any zone of inhibition and growth from each combination of isolates on a plate.

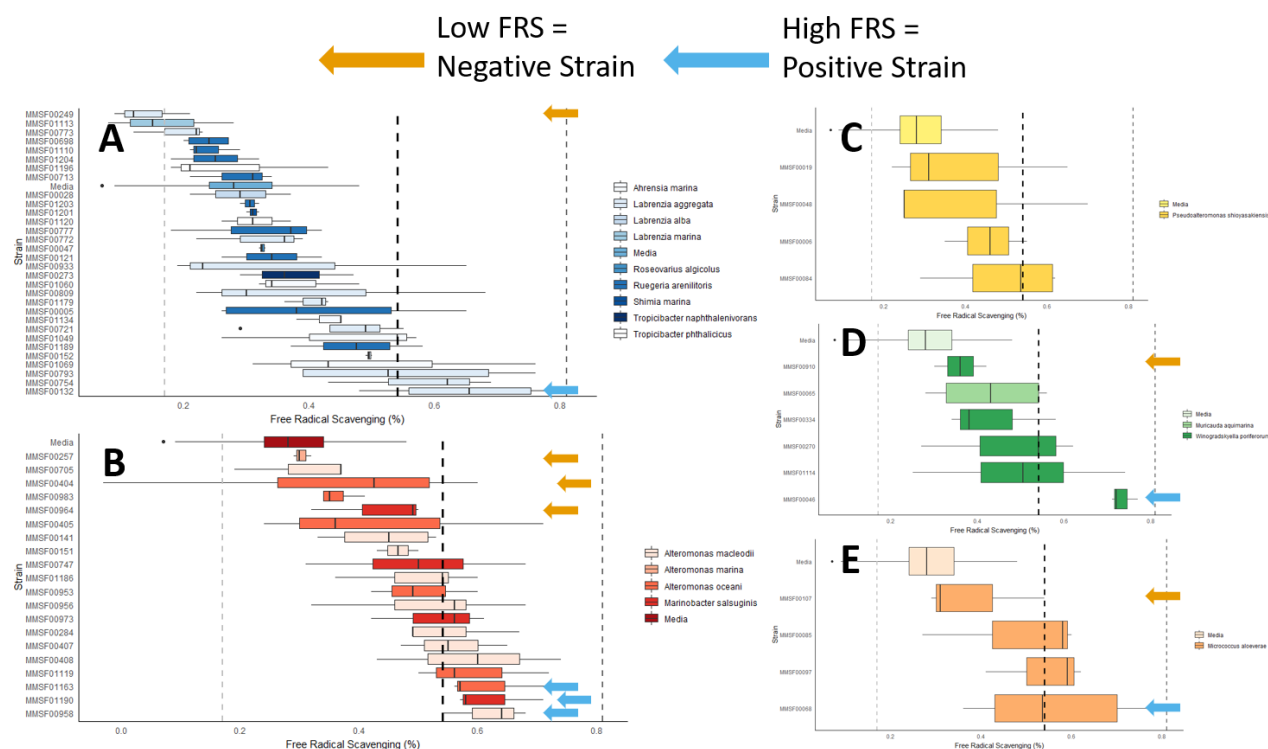


Figure 3-2 Quantitative FRS ability of *E. diaphana*-associated bacterial isolates, separated by Family. Families with high relative abundance among all cultured bacteria (Rhodobacteraceae – A, Alteromonadaceae – B, Pseudoalteromonadaceae – C, Flavobacteriaceae – D, and Micrococcaceae – E) were separately analysed to identify strains with a high FRS ability (blue arrows) and a corresponding conspecific or congeneric strain with a low FRS ability (orange arrows). In each panel, the light dashed vertical line on the left represents the mean FRS of a 0.025% (w/v) ascorbic acid standard, the middle dark dashed vertical line is the mean FRS for 0.05% (w/v) ascorbic acid standard, and the far right dashed line is the mean FRS of the 0.075% (w/v) ascorbic acid standard.

Table 3-2 Overview of probiotic high FRS bacterial strains and conspecific/congeneric low FRS control strains. All sequence data can be found under BioProject# PRJNA574193. References to each probiotic candidate at the Genus level are identified in the last three columns.

Strain	Bacteria species (Phylum or Class)	#FRS (% $\pm$ SE)	*Positive vs Negative	*Strain vs Growth Medium	Catalase	<i>E. diaphana</i> Literature	Coral Literature	Probiotic Literature
MMSF01163 (n=3)	<i>Alteromonas oceani</i> (Gammaproteobacteria)	61.7 $\pm$ 5.2	0.065	0.004	Negative	(Binsarhan, 2016; Röthig et al., 2016; Herrera et al., 2017)	(Chiu et al., 2012; Ceh et al., 2013; Rothig et al., 2017; Damjanovic et al., 2019c)	(Riquelme et al., 1997; Kesarcodi- Watson et al., 2010; Hai, 2015; Haryanti et al., 2017)
MMSF00404 (n=4)		35.5 $\pm$ 13.7		0.571	Negative			
MMSF00958 (n=3)	<i>Alteromonas macleodii</i> (Gammaproteobacteria)	62.0 $\pm$ 4.2	0.175	0.017	Negative			
MMSF00257 (n=3)		30.3 $\pm$ 0.9		0.431	Positive			
MMSF00132 (n=8)	<i>Labrenzia aggregata</i> (Biebl et al., 2007) (Alphaproteobacteria)	53.6 $\pm$ 8.7	0.016	0.101	Negative	(Binsarhan, 2016)	(Littman et al., 2011; Chiu et al., 2012)	(Mancuso et al., 2015)
MMSF00249 (n=3)		14.0 $\pm$ 3.6		0.103	Positive			
MMSF01190 (n=3)	<i>Marinobacter salsuginis</i> (Gammaproteobacteria)	62.0 $\pm$ 4.5	0.041	<0.0001	Negative	(Binsarhan, 2016; Brown et al., 2017; Herrera et al., 2017)	(Littman et al., 2009; Sharp et al., 2012; Rothig et al., 2017)	(Alagely et al., 2011)
MMSF00964 (n=3)		43.7 $\pm$ 5.8		0.020	Positive			
MMSF00068 (n=6)	<i>Micrococcus luteus</i> (Actinobacteria)	56.3 $\pm$ 7.3	0.210	0.002	Positive	(Binsarhan, 2016)	(Kellogg et al., 2013)	(El-Rhman et al., 2009; Osman et al., 2010; Hai, 2015)
MMSF00107 (n=3)	<i>Micrococcus yunnanensis</i> (Actinobacteria)	38.0 $\pm$ 8.0		0.306	Positive			
MMSF00046 (n=3)	<i>Winogradskyella poriferorum</i> (Lau et al., 2005) (Bacteroidetes)	73.3 $\pm$ 1.9	0.284	0.010	Negative	^	(dos Santos et al., 2015; Franco et al., 2018)	NA
MMSF00910 (n=3)		36.0 $\pm$ 3.5		0.174	Negative			

Mean R2A FRS (% $\pm$ SE) was $27.2 \pm 2.3\%$ (n=12) and it was catalase negative.

^ While there are no instances of *Winogradskyella* specifically identified in current *E. diaphana* literature, there are several Flavobacteriaceae that are not resolved to the genus level from metabarcoding data (Plovie, 2010; Röthig et al., 2016; Herrera et al., 2017; Ahmed et al., 2019).

* Indicates p values for pairwise comparisons from respective one-way analysis of variance (Tukey HSD) or Kruskal-Wallis rank sum test (Dunn test) with **bold** values representing significant differences; $\alpha = 0.05$.

3.3.3 Comparative genomics

As part of the characterization of the 12 isolates (six positive and six conspecific or congeneric negative FRS isolates), a draft genome sequence was determined for each isolate. A summary of the data and metrics for the draft genome sequences is presented in Table S3-1. The diversity of the six pairs of isolates is indicated by the %G+C range (35% to 72%) and genome size (2.4 Mb to 6.8 Mb). Each isolate pair was classified as the same species, according to 16S rRNA gene sequence identity, except the *Micrococcus* strains. Isolates MMSF00068 (high FRS strain) and MMSF00107 (low FRS strain) are classified as *Micrococcus luteus* and *M. yunnanensis*, respectively. These isolates have the smallest genomes among the isolates (2.43 Mb and 2.48 Mb, respectively) and the highest %G+C being 72.8% and 72.4%, respectively.

Core genome comparison provides an overview of the genetic relationship between the conspecific or congeneric isolate pairs. A wide range of genome variation between pairs was observed, which ranged from ~190,000 single nucleotide polymorphisms (SNP) differences between the *Alteromonas oceanii* strains, to fewer than five core genome SNPs between the *Labrenzia aggregata* isolates (Table S3-2). This is not a comprehensive estimate of isolate identity, e.g. the genome sequences of *Winogradskyella poriferorum* isolates MMSF00046 and MMSF00910 are nearly identical with fewer than ten pairwise SNP differences in the core genome, but there are accessory genome differences.

3.3.4 Genes of interest

The annotated genome sequences of each selected probiotic member were searched for key genes relevant to FRS capabilities (Table S3-3, Table S3-4). Dimethyl sulfoniopropionate (DMSP) cleavage to dimethyl sulfide (DMS) was identified by presence of one or more of the DMSP lyase genes; *dddP*, *dddD*, *dddL*, *dddW*, and *dddQ*. Only *L. aggregata* strains contained DMSP lyase genes (*dddP* and *dddL*) in their whole genome sequences. DMSP biosynthesis was identified by the presence of *dsyB*, which is the only described gene for an enzyme in the DMSP biosynthesis pathway. The *cobP* gene was used as an indicator for the presence of the dynamic vitamin B₁₂ pathway, which contains 27

genes (Table S3-4). Again, only the *L. aggregata* isolates contained *cobP*. Catalase positive strains were identified by the presence of *katG*; all positive and negative FRS strains contained *katG* except the *Micrococcus* spp., in which *katA* and *katE* were detected.

3.3.5 16S rRNA gene copy number

The 16S rRNA gene copy numbers of the 12 draft genomes were estimated using a read depth approach (Table S3-1). The copy numbers were similar within pairs of isolates, in which the pair of *A. macleodii* isolates (MMSF00257 and MMSF00958) contained the most copies (5.15 and 4.79, respectively), corresponding with copy numbers in a published closed genome of *A. macleodii* (Gonzaga et al., 2012). *W. poriferorum* isolates (MMSF00046 and MMSF00910) contained the fewest copies (1.03 and 0.77, respectively).

3.4 Discussion

The 842 *E. diaphana* bacterial isolates reported here comprise 109 species from 64 genera and six phyla. Using metabarcoding, studies of microbiomes associated with *E. diaphana* have revealed a similar diversity at the phylum level for *E. diaphana* sourced from the GBR (Hartman et al., 2019; Hartman et al., 2020), Hawaii (strain H2; Herrera et al., 2017), Pacific and Caribbean (Brown et al., 2017), Atlantic (strain CC7; Röthig et al., 2016) and Red Sea (Ahmed et al., 2019), as well as stony corals (Blackall et al., 2015). Thus, our culture collection of *E. diaphana* bacterial isolates capture the diversity of the *E. diaphana*-associated microbiome. The *E. diaphana* probiotics generated in this study were assembled from 12 isolated bacterial strains selected based on their FRS ability since free radical production, specifically ROS, is relevant in coral bleaching. The broader culture collection contains bacteria with a wide range of FRS capacity, and our probiotic comprises greater bacterial diversity compared to others (Alagely et al., 2011; Rosado et al., 2018).

The consistent and frequent reporting of our probiotic bacterial genera in *E. diaphana* and coral studies (Table 3-2) suggests these bacteria likely have key functions in cnidarian holobionts. Among these potential functions are the production of the antioxidant DMSP and the breakdown of DMSP to other antioxidants (Sunda et al., 2002). *L. aggregata* has

been reported to produce DMSP in the absence of any methylated sulfur compounds with *dsyB* identified as the first DMSP biosynthesis gene in any organism (Curson et al., 2017). The *dsyB* gene was found in the genome sequences of both high and low FRS *L. aggregata* strains (Table S3-3). Many *E. diaphana*-sourced bacterial species, specifically relatives of our selected probiotic members, are implicated in the degradation of DMSP to DMS (*Alteromonas* spp., (Raina et al., 2009); *Labrenzia* spp., (Hatton et al., 2012)). *dddP* codes for the enzyme responsible for cleaving DMSP to DMS and acrylate and was used (from the Prokka annotation) as an indicator of a DMSP degradation genotype. Only the *L. aggregata* isolates contained genes responsible for DMSP degradation (Table S3-3).

Carotenoids are among the strongest antioxidants and are highly reactive against both ROS and free radicals (Fiedor et al., 2005; Asker et al., 2007; Shindo et al., 2007; Fiedor and Burda, 2014; Flórez et al., 2015). Carotenoids are lipid-soluble pigments, and in bacteria they give an orange-yellow hue to colonies. Two of the five selected probiotic genera produce orange/yellow colonies (*Winogradskyella*, *Micrococcus*), and there is evidence of carotenoid production by marine Flavobacteriaceae (Shindo et al., 2007; Gammone et al., 2015) and *Micrococcus* (Mohana et al., 2013). A marine Flavobacteriaceae (strain GF1) was found to produce the potent antioxidant carotenoid zeaxanthin that protected Symbiodiniaceae from thermal and light stress (Motone et al., 2020).

Vitamin B₁₂ is a cofactor involved in the production of the amino acid methionine, which is needed to synthesize every protein and in diverse metabolic pathways including generation of the antioxidants glutathione and DMSP (Croft et al., 2005). Vitamin B₁₂ is synthesized by many heterotrophic bacteria (Raux et al., 2000). Genomic evidence suggests that Symbiodiniaceae have lost the capacity to synthesize vitamin B₁₂ (Matthews et al., 2020), which is in agreement with other work showing that free-living Symbiodiniaceae rely on bacterial symbionts for this important cofactor (Agostini et al., 2009). The genes involved in the biosynthesis of vitamin B₁₂ have been found in coral-associated bacteria, specifically *L. aggregata* cultured from the Caribbean coral, *Orbicella faveolata* (Smith, 2018). Eighteen genes, including the *cobP* gene (Raux et al., 2000), are present in each of *L. aggregata* isolates (MMSF00132 and MMSF00249), suggesting both are capable of

vitamin B₁₂ biosynthesis. None of the other sequenced isolated had genes related to vitamin B₁₂ biosynthesis, except in the *Marinobacter salsuginis* isolates, where a *cobO* gene was detected, strongly indicating that none of the remaining ten isolates were capable of vitamin B₁₂ biosynthesis (Table S3-4).

Bacteria have developed highly specific mechanisms to protect themselves against oxidative stress, in particular using enzymatic antioxidants such as catalase, peroxidase and superoxide dismutase (Imlay, 2018). It has been suggested that increasing the *in hospite* concentration of catalase in the coral holobiont by the application of a probiotic with catalase-positive organisms, could possibly minimize the impact of thermal stress by neutralizing hydrogen peroxide (H₂O₂) (Peixoto et al., 2017). Here we tested all probiotic candidates for catalase production using a standard H₂O₂ assay (Taylor and Achanzar, 1972). Catalase participates in cellular antioxidant defence by enzymatically breaking down H₂O₂ to H₂O and O₂. Hydroperoxidase I (HPI, *katG*) is a bifunctional enzyme with both catalase and peroxidase activity that present during aerobic growth and is transcriptionally controlled at different levels, while hydroperoxidase II (HPII, *katE*) is a monofunctional catalase and induced during stationary phase (von Ossowski et al., 1991; Cabiscol et al., 2000). The phenotypically determined catalase positive strains (MMSF00249, *L. aggregata*; MMSF00257, *A. macleodii*; MMSF00964, *M. salsuginis*) had a catalase-peroxidase gene (HPI, *katG*); but all strains had at least one *katG*, *katA* (catalase), or *katE* (catalase HPII) (Table S3-3). The lack of correlation between genotype and phenotype in relation to catalase activity may be associated with the culture conditions used or the bacterial growth phase during the catalase assay. The inconsistency between the catalase and FRS ability (in many cases high FRS isolates were catalase negative while the low FRS strain was catalase positive), justifies our approach of using the phenotype of FRS ability, instead of using the presence of catalase genes as a proxy for FRS.

A critical characteristic in the selection of probiotic members is the maintenance and proliferation of the inoculated bacteria in the host over time (Hai, 2015) and their potential transmission to the next generation. There is evidence that corals release bacteria with their offspring such as *Alteromonas* (Ceh et al., 2013), Flavobacteriaceae (Ceh et al., 2013),

Rhodobacteraceae (Damjanovic et al., 2020), and *Marinobacter* (Sharp et al., 2012). While broadcast spawning corals do not appear to transfer their bacterial symbionts with their gametes (vertical transfer) (Sharp et al., 2010), the brooding coral *Porites astreoides* transmits some bacteria vertically to planulae with two bacterial taxa (*Roseobacter* clade-associated bacteria and *Marinobacter* spp.) consistently and stably associating with juvenile *P. astreoides* (Sharp et al., 2012). *Marinobacter* potentially has antioxidant properties (Sharp et al., 2015), while *Roseobacter* spp. might be beneficial in facilitating larval settlement (Webster et al., 2004). If adult corals stably associate with inoculated probiotic candidates like *Marinobacter*, *Alteromonas*, and *Winogradskyella*, they may be passed on to offspring and thus have a long-term positive impact on these individuals.

The probiotic members were chosen from a highly diverse pool of *E. diaphana*-sourced bacterial isolates. While the selected probiotic members are phylogenetically diverse, potentially promising probiotic bacteria in the culture collection were omitted based on our selection criteria. For example, *Ruegeria* spp. were reported to breakdown DMSP and participate in denitrification (Smith, 2018). *Muricauda* isolates had high FRS abilities, but they did not grow consistently in the selected medium and therefore were excluded from the consortium. *Muricauda* isolates have genes for denitrification (Smith, 2018), can breakdown DMSP (Hatton et al., 2012), and produce potent carotenoids (Prabhu et al., 2014) that can mitigate thermal and light stresses in Symbiodiniaceae cultures (Motone et al., 2020). *Muricauda* will be included in future probiotic evaluations.

Interactions among members of the microbiota associated with marine animals are undoubtedly complex. Results presented in this manuscript show that pure cultured bacteria from *E. diaphana* can scavenge free radicals, albeit at a strain-specific rate. Inoculation with high FRS strains could be beneficial to the host under high oxidative stress conditions, such as those that contribute to coral bleaching. Outlined here is the start of a complex process to identify, evaluate, and select durable and useful probiotics that can buffer the coral host against climatic variation. Conspecific or congeneric pairs of bacteria provide an opportunity to determine the genetic basis for measured phenotypic differences between the pairs. An essential element of future work will be to investigate

the stability of the phenotypic differences observed and this stability may be reflected in the nature of the genetic differences between the pairs of strains.

3.5 Methods

3.5.1 Isolation of bacterial isolates

GBR origin *E. diaphana* were maintained in the laboratory at 26°C (Dungan et al., 2020b) and used to isolate probiotic candidates. Sixteen individuals from each of four *E. diaphana* genotypes (AIMS1-4) were collected using sterile disposable pipets and gently transferred to filter-sterilized (0.2 µm) reverse osmosis (RO) water reconstituted Red Sea Salt™ (Red Sea; RSS) at ~34 parts per thousand (ppt) salinity (fRSS). After 30 min, each anemone was transferred to a sterile glass homogenizer with 1 mL of fRSS. Each homogenate was used to prepare serial dilutions from 10⁻¹ to 10⁻⁴. From each dilution, 50 µL was spread plated onto three replicate plates each of MA (Difco™ Marine Agar 2216) and R2A (CM0906, Oxoid) supplemented with 40 g L⁻¹ RSS to 34 ppt salinity and incubated at 26°C. After one week, CFU counts were completed. Individual bacterial isolates were sub-cultured to purification from plates with <100 CFUs onto the initial isolation medium. All purified bacterial isolates were resuspended in 40% glycerol, aliquoted into 1.2 mL cryotubes and stored at -80°C.

3.5.2 Identification of *E. diaphana*-sourced isolates

Colony PCR with the universal bacterial primers 27f (5' – AGAGTTTGATCMTGGCTCAG – 3') and 1492r (5' – TACGGYTACCTTGTTACGACTT – 3') (Lane, 1991) was used to generate 16S rRNA gene amplicons from each isolate. Briefly, cells from each pure culture were suspended in 20 µL Milli-Q water and denatured at 95°C for 10 min. The suspension was then centrifuged at 2,000 x *g* at 4°C for two minutes and the supernatant was used as the DNA template for PCR amplification. The PCR was performed with 20 µL Mango Mix™ (Bioline), 0.25 µM of each primer and 2 µL of DNA template in a final volume of 40 µL. The thermal cycling protocol was as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension of 10 min at 72°C. Amplicons were

purified and Sanger sequenced on an ABI sequencing instrument by MacroGen Inc. (Seoul, South Korea) or by the Australian Genome Research Facility using the 1492r primer. Trimmed high quality read data from each isolate was used for presumptive identification by querying the 16S rRNA gene sequences via BLASTn. For some isolates the near-complete 16S rRNA gene sequence was determined by sequencing with additional primers (27f, 357f (5'-CCTACGGGAGGCAGCAG-3', (Muyzer et al., 1993)), 926f (5'-CCGTCAATTCMT-TTRAGTTT-3', (Lane et al., 1985)), 519r (5'-GWATTACCGCGGCKGCTG-3', (Muyzer et al., 1993)), 926r (5'-AACTRAAAMGAATTGACGG-3', (Lane et al., 1985)), and 1492r). The six reads for each isolate were aligned using Geneious Prime 2019.1.2 (<https://www.geneious.com>) via the Geneious global alignment default settings with automatic determination of read direction. From this alignment, a consensus sequence for the 16S rRNA gene was constructed based on the frequency of a base and its quality (from chromatogram data) in each alignment column. The consensus sequence length for each of the six isolate pairs varied from 1352 to 1495 nucleotides. GenBank accession numbers for sequences are shown in Table 3-1.

3.5.3 Qualitative free radical scavenging

DPPH is a stable free radical that is purple in its oxidized state but becomes white-yellow if reduced by antioxidants, and has been used to identify FRS marine bacteria (Takao et al., 1994; Velho-Pereira et al., 2015). To qualitatively assess *E. diaphana*-associated bacteria isolates for FRS ability, a sterile Whatman #1 filter paper was gently pressed against fresh (2-4 days old) colonies from a streak plate. Plates (with filter paper) were then incubated overnight at 26°C. The following day, filter papers were removed with forceps, allowed to dry in a fume hood for 30 min, and 500 µL of a 0.2 mM DPPH (Cat# D9132, Sigma-Aldrich) solution in methanol was applied with a pipette over individual colonies. As a positive control, a few drops of 0.1% (w/v) L-ascorbic acid (Cat# A7631, Sigma-Aldrich) were placed on a separate filter. The response of each isolate to DPPH was recorded within 3 min of DPPH application; a positive response was recorded if a white-yellow halo appeared around individual colonies within 1 min, a weak positive response was assigned to strains that had a halo form between 1 and 3 min after DPPH application, and a negative response

was listed for strains that failed to form a halo (Fig. 3-3). Approximately 700 isolates were screened using the qualitative DPPH assay.

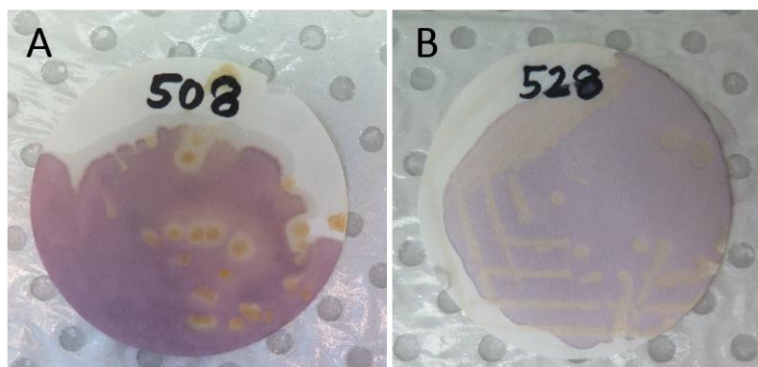


Figure 3-3 DPPH is a stable free radical that is purple in its oxidized state. When reduced by an antioxidant, a white-yellow halo will appear around individual bacteria colonies (A), this was qualitatively deemed a positive response. Isolates that did not have a halo around colonies within 3 min of DPPH application were deemed negative (B).

3.5.4 Quantitative free radical scavenging assay

To quantitatively assess the FRS ability, select isolates were grown in R2A broth (Table S3-5). Volumes of 50 mL of autoclaved medium were added to sterile 250 mL Erlenmeyer flasks and each flask was inoculated with an isolate colony grown from a R2A or MA plate culture. Cultures were grown with shaking (150 rpm; Ratek orbital incubator) at 37°C for 48 h. Sterile uninoculated R2A at 34 ppt was used as a control. A minimum of three replicate cultures were grown per isolate. After 48 h, the optical density of each culture was measured at 600 nm (OD600, CLARIOstar PLUS, BMG Labtech), and the cultures (including negative medium controls) were centrifuged at 3000 x *g* at 4°C for 30 min (Allegra X-12R) to pellet the bacterial cells. The cell-free supernatants (CFSs) were collected, frozen at -80°C, freeze dried (Alpha 1-4 LDplus, Martin Christ), and stored under inert gas in a dark, dry environment until analysis. Antioxidants were extracted from the CFSs by resuspending at 50 mg mL⁻¹ in 100% methanol, sonicating (Branson 2510) for 5 min, then centrifuging at 3000 x *g* at 4°C for 5 min. Quantitative DPPH assays were run by creating a 1:1 solution of 0.2 mM DPPH in methanol and CFS extract to a final volume of 1 mL, vortexing, and incubation in the dark at room temperature for 30 min. Samples were then vortexed briefly, and three 300 µL replicates of each sample were transferred into a well of a 96 well plate. Decolorization of DPPH was determined by measuring the decrease in absorbance at

517 nm (EnSpire 2300 plate reader, Perkin Elmer), and the FRS activity was calculated according to the formula, % DPPH scavenging activity = (Control – Sample) / Control × 100, where, Control is the absorbance of the DPPH control (1:1 0.2 mM DPPH:methanol), and Sample is the absorbance of CFS extract in DPPH. All samples were measured against a 100% methanol blank. Positive controls consisting of 0.01 - 0.001% (w/v) L-ascorbic acid were run on each 96-well plate. FRS activity ranged from 0-90%.

3.5.5 Catalase assay

The pelleted cells from above were resuspended in 2 mL fRSS and 500 µL H₂O₂ giving a final concentration of 16 mM. If bubbles appeared, the organism was considered catalase positive. If there were no bubbles, the organism was classified as catalase negative.

3.5.6 Inhibition testing

Each paired set of high and low FRS strains were inoculated crosswise along the middle of MA plates to test for antagonism. Plates were kept at 26°C and monitored daily for up to 7 days for antagonistic activity by documenting the presence or absence of both inoculated isolates and if there was a zone of inhibition between them.

3.5.7 Phylogenetic analysis

All partial 16S rRNA gene sequences (842) were aligned with reference sequences (72) of closely related organisms using Geneious Prime 2019.1.2 (<https://www.geneious.com>). This alignment was used to construct a neighbour-joining phylogenetic tree using the Jukes-Cantor method. Maximum-likelihood dendrograms were generated with bootstrap values of 1000.

3.5.8 Whole genome sequence analysis

Positive FRS strains along with conspecific or congeneric negative FRS strains were selected for genome sequencing; in total, six pairs of isolates were sequenced. Genomic DNA was isolated from a single colony using a JANUS Chemagic Workstation and Chemagic Viral DNA/RNA kit (PerkinElmer). Libraries were prepared with the Nextera XT

DNA sample preparation kit (Illumina). Readsets were produced using the Illumina sequencing platform (Instrument: Illumina NextSeq 500, 150 bases, paired-end) and the whole genome shotgun (WGS) method. Read depth coverage was approximately 100 times assuming a genome size of 4 M bases.

Illumina readsets for each isolate were assembled using Skesa (Souvorov et al., 2018) and the draft genome sequence annotated using Prokka (Seemann, 2014). A genome sequence based taxonomic classification for each isolate was determined using Kraken2 (Wood and Salzberg, 2014) with the Genome Taxonomy Database (GTDB; Parks et al., 2020) as the curated genomic data source. Classification was primarily based on the genome sequence of related isolates (within the relevant species where possible), which were obtained from GenBank. In situations where genomes of taxonomically relevant individuals were available, a species level classification was possible. Where available, closed genome sequences from GenBank were used for comparative genomics analysis. For each of the six pairs of isolates, core genome (i.e. genes shared between the isolate pair) comparisons were performed, as implemented in Nullarbor (<https://github.com/tseemann/nullarbor>), with phylogenies inferred using SNP differences. Genes for DMSP synthesis and degradation, vitamin B₁₂ synthesis, and catalase were identified from the annotated genome sequence (GFF format) produced by Prokka; specific genes were identified by both name and Refseq accession number.

The 16S rRNA gene copy number of the 12 draft genomes was predicted by the 16Stimator pipeline (Perisin et al., 2016). Briefly, all the 12 genomic assemblies were submitted to the rapid annotations using subsystems technology (RAST) server (Brettin et al., 2015), and the positions of 16S rRNA and a set of single-copied housekeeping genes (Table S3-6) were extracted from the RAST annotations. The clean read sets were mapped back to the corresponding genomic assemblies by Bowtie 2 (Langmead and Salzberg, 2012) to determine the read depth of each position. Finally, the 16S copy number of each isolate was calculated by dividing the median depth of 16S gene by the median depth of the single-copied housekeeping genes after the read depths were calibrated by the model parameters provided by 16Stimator.

3.5.9 Statistical analysis

CFU counts were analysed in R (v3.6.2, R Core Team, 2019) by first checking the assumptions of equal variance and homogeneity. An analysis of variance test was used to detect differences in the mean number of bacterial colonies from each anemone genotype by solid growth media (R2A or MA). A one-way analysis of variance (one-way ANOVA; Chambers and Hastie, 1992) was used to determine if there were significant differences between FRS abilities of selected positive (high FRS), negative (low FRS), and media controls, and pairwise comparisons were performed using Tukey's HSD (Miller, 1981; Yandell, 2017). Each probiotic pair and media control were tested to determine if data met the assumptions of normality and homoscedasticity. If either assumption was violated, the non-parametric Kruskal-Wallis rank sum test (Holland and Wolfe, 1973) was used with a Dunn test (Dunn, 1964) for multiple comparisons (p-values adjusted with the Benjamini-Hochberg method (Ferreira and Zwinderman, 2006)) with the R package "FSA" (Ogle, 2017).

3.6 Acknowledgements

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3.7 Data availability

WGS raw reads are freely available in the Sequence Read Archive under BioProject PRJNA574193; the complete data set is listed in Table S3-1.

3.8 Supplementary material

Table S3-1 Isolate Genome Sequence Data Summary. Strains are presented as high FRS (grey) followed by low FRS (white). 16S rRNA gene presumptive identity is derived from the NCBI classification of near-complete 16S rRNA gene sequences. *We were unable to determine the 16S rRNA copy number of isolate MMSF00068 due to the presence of contaminant sequences in the WGS.

Isolate	Genus Level Classification	Genus % Confidence	Family	Family % Confidence	SRA Accession	Sample Accession
MMSF00257	<i>Alteromonas</i>	96.65	Alteromonadaceae	96.74	SRR10186803	SAMN12851724
MMSF00958	<i>Alteromonas</i>	92.93	Alteromonadaceae	92.98	SRR10186806	SAMN12851731
MMSF01163	<i>Alteromonas</i>	9.28	Alteromonadaceae	12.01	SRR10186808	SAMN12851721
MMSF00404	<i>Alteromonas</i>	8.15	Alteromonadaceae	10.49	SRR10186805	SAMN12851732
MMSF00132	<i>Labrenzia</i>	90.9	Rhodobacteraceae	90.91	SRR10186800	SAMN12851727
MMSF00249	<i>Labrenzia</i>	90.83	Rhodobacteraceae	90.83	SRR10186799	SAMN12851728
MMSF00964	<i>Marinobacter</i>	59.99	Alteromonadaceae	60.1	SRR10186802	SAMN12851725
MMSF01190	<i>Marinobacter</i>	65.77	Alteromonadaceae	65.85	SRR10186797	SAMN12851730
MMSF00068	<i>Micrococcus</i>	71.81	Micrococcaceae	72.88	SRR10186807	SAMN12851722
MMSF00107	<i>Micrococcus</i>	83.18	Micrococcaceae	84.87	SRR10186798	SAMN12851729
MMSF00046	<i>Winogradskyella</i>	49.63	Flavobacteriaceae	49.72	SRR10186801	SAMN12851726
MMSF00910	<i>Winogradskyella</i>	54.44	Flavobacteriaceae	54.53	SRR10186804	SAMN12851723

Isolate	16S rRNA gene presumptive identity	Confidence	Reads	Total Bases	G+C%	Avg. Read Length	Max Read Length	Avg Quality
MMSF00257	<i>Alteromonas macleodii</i>	1.00	2233080	334079592	44.7	149	151	30.9
MMSF00958	<i>Alteromonas macleodii</i>	1.00	2971718	443870758	44.6	149	151	31.9
MMSF01163	<i>Alteromonas oceani</i>	0.83	2757340	412741366	48.7	149	151	33.7
MMSF00404	<i>Alteromonas oceani</i>	0.83	2067800	306005114	48.7	147	151	33.3
MMSF00132	<i>Labrenzia aggregata</i>	0.98	3758944	557529936	59.2	148	151	31.1
MMSF00249	<i>Labrenzia aggregata</i>	0.98	3333098	497032699	59.3	149	151	30.7

MMSF00964	<i>Marinobacter salsuginis</i>	1.00	1713198	256635737	57.1	149	151	31
MMSF01190	<i>Marinobacter salsuginis</i>	1.00	3239568	483874048	57.1	149	151	33.1
MMSF00068	<i>Micrococcus luteus</i>	0.99	2658898	398496031	72.4	149	151	30.3
MMSF00107	<i>Micrococcus yunnanensis</i>	0.99	2323654	348617190	72.8	150	151	31.9
MMSF00046	<i>Winogradskyella poriferorum</i>	0.59	1930584	285978916	35	148	151	31.8
MMSF00910	<i>Winogradskyella poriferorum</i>	0.59	3939880	579510587	35.6	147	151	33.9

Isolate	Est. Read Coverage	Contigs in Draft Genome	Bases in Draft Genome	Min Contig	Avg Contig	Max Contig	N50	16S rRNA gene copy number
MMSF00257	69	136	4831263	515	35523	210048	72973	5.15
MMSF00958	94	42	4732026	620	112667	580903	303003	4.79
MMSF01163	75	100	5507488	526	55074	394030	155957	4.18
MMSF00404	51	111	6014142	502	54181	256548	93725	3.97
MMSF00132	82	34	6792087	855	199767	1019681	302139	3.57
MMSF00249	73	44	6791310	851	154347	1286212	294458	3.02
MMSF00964	56	41	4588310	512	111910	932206	291012	3.67
MMSF01190	110	40	4404819	529	110120	769441	404351	3.21
MMSF00068	160	316	2484978	509	7863	78799	12670	*
MMSF00107	143	501	2435904	515	4862	42365	7717	1.75
MMSF00046	83	104	3446386	553	33138	299826	78549	1.03
MMSF00910	168	50	3456468	553	69129	355406	162623	0.77

Table S3-2 Pairwise comparison of the genome sequences between the pairs of isolates

NCBI Classification	Type strain	Core Genome	SNP difference
<i>Alteromonas macleodii</i>	ATCC 27126	85%	~60000
<i>Alteromonas oceani</i>	S35	60%	~190000
<i>Labrenzia aggregata</i>	IAM12614	55%	5
<i>Marinobacter salsuginis</i>	SD14B	15%	~120000
<i>Micrococcus</i> spp.	NCTC2665	70%	~35000
<i>Winogradskyella poriferorum</i>	NA	NA	10

Table S3-3 Search outcomes for genes of interest

Isolate	NCBI Classification	Gene	Gene Classification	Contig	Start	End	Strand
MMSF00132	<i>Labrenzia aggregata</i>	<i>dddL</i>	positive	Contig_16_56.34	104067	104756	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>dddL</i>	positive	Contig_18_45.1167	104045	104734	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>dddP</i>	positive x2 (Copy 1)	Contig_6_57.2063	50787	52046	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>dddP</i>	positive x2 (Copy 2)	Contig_6_57.2063	650583	651881	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>dddP</i>	positive x2 (Copy 1)	Contig_17_46.7174	317316	318575	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>dddP</i>	positive x2 (Copy 2)	Contig_17_46.7174	917112	918410	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>dsyB</i>	positive	Contig_32_56.2994	210397	211419	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>dsyB</i>	positive	Contig_4_46.0396	460215	461237	+
MMSF00046	<i>Winogradskyella poriferorum</i>	<i>katG</i>	positive	Contig_15_55.0851	16430	18661	+
MMSF00068	<i>Micrococcus luteus</i>	<i>katG</i>	ND; katA & katE detected	NA	NA	NA	NA
MMSF00107	<i>Micrococcus yunnanensis</i>	<i>katG</i>	ND; katA & katE detected	NA	NA	NA	NA
MMSF00132	<i>Labrenzia aggregata</i>	<i>katG</i>	positive	Contig_6_57.2063	425873	428044	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>katG</i>	positive	Contig_17_46.7174	692402	694573	-
MMSF00257	<i>Alteromonas macleodii</i>	<i>katG</i>	positive	Contig_101_46.6864	22130	24502	+
MMSF00404	<i>Alteromonas oceani</i>	<i>katG</i>	positive x2 (Copy 1)	Contig_26_39.8992	70873	73035	-
MMSF00404	<i>Alteromonas oceani</i>	<i>katG</i>	positive x2 (Copy 2)	Contig_52_41.1626	55994	58225	-
MMSF00910	<i>Winogradskyella poriferorum</i>	<i>katG</i>	positive	Contig_39_101.483	25621	27852	+
MMSF00958	<i>Alteromonas macleodii</i>	<i>katG</i>	positive	Contig_21_66.072	22086	24458	+
MMSF00964	<i>Marinobacter salsuginis</i>	<i>katG</i>	positive	Contig_25_36.8412	50391	52565	-
MMSF01163	<i>Alteromonas oceani</i>	<i>katG</i>	positive x2 (Copy 1)	Contig_46_63.2119	54248	56479	-
MMSF01163	<i>Alteromonas oceani</i>	<i>katG</i>	positive x2 (Copy 2)	Contig_86_59.9301	369720	371882	-
MMSF01190	<i>Marinobacter salsuginis</i>	<i>katG</i>	positive	Contig_22_84.8909	7780	9954	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobA</i>	positive	Contig_6_57.2063	552920	553741	-

MMSF00249	<i>Labrenzia aggregata</i>	<i>cobA</i>	positive	Contig_17_46.7174	819449	820270	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobB</i>	positive	Contig_6_57.2063	551611	552930	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobB</i>	positive	Contig_17_46.7174	818140	819459	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobC</i>	positive	Contig_6_57.2063	842748	843392	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobC</i>	positive	Contig_17_46.7174	1109277	1E+06	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobD</i>	positive x2	Contig_26_56.6932	208207	209184	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobD</i>	positive x2	Contig_26_56.6932	209194	210195	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobD</i>	positive x2	Contig_15_47.504	84229	85230	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobD</i>	positive x2	Contig_15_47.504	85240	86217	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobH</i>	positive	Contig_6_57.2063	545294	545950	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobH</i>	positive	Contig_17_46.7174	811823	812479	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobI</i>	positive	Contig_6_57.2063	547369	548139	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobI</i>	positive	Contig_17_46.7174	813898	814668	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobK</i>	positive	Contig_6_57.2063	555025	555786	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobK</i>	positive	Contig_17_46.7174	821554	822315	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobL</i>	positive	Contig_6_57.2063	546203	547417	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobL</i>	positive	Contig_17_46.7174	812732	813946	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobM</i>	positive	Contig_6_57.2063	550028	550801	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobM</i>	positive	Contig_17_46.7174	816557	817330	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobN</i>	positive	Contig_26_56.6932	201965	205723	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobN</i>	positive	Contig_15_47.504	88701	92459	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobO</i>	positive	Contig_26_56.6932	205720	206358	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobO</i>	positive	Contig_15_47.504	88066	88704	-
MMSF00964	<i>Marinobacter salsuginis</i>	<i>cobO</i>	positive	Contig_25_36.8412	168373	168930	+
MMSF01190	<i>Marinobacter salsuginis</i>	<i>cobO</i>	positive	Contig_21_84.3861	85977	86534	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobP</i>	positive	Contig_26_56.6932	200255	200806	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobP</i>	positive	Contig_15_47.504	93618	94169	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobQ</i>	positive	Contig_26_56.6932	206715	208205	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobQ</i>	positive	Contig_15_47.504	86219	87709	-

MMSF00132	<i>Labrenzia aggregata</i>	<i>cobU</i>	positive	Contig_24_56.1446	78969	79994	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobU</i>	positive	Contig_13_45.8339	40345	41370	+
MMSF00132	<i>Labrenzia aggregata</i>	<i>cobV</i>	positive	Contig_24_56.1446	77990	78862	-
MMSF00249	<i>Labrenzia aggregata</i>	<i>cobV</i>	positive	Contig_13_45.8339	39366	40238	-
MMSF00132	<i>Labrenzia aggregata</i>	<i>cbiD</i>	positive	Contig_6_57.2063	553941	555035	+
MMSF00249	<i>Labrenzia aggregata</i>	<i>cbiD</i>	positive	Contig_17_46.7174	820470	821564	+
MMSF00964	<i>Marinobacter salsuginis</i>	<i>cbiO</i>	positive	Contig_36_38.8121	420124	420720	+

Table S3-4 Summary of vitamin B₁₂ biosynthesis pathway genes. A "+" indicates the presence of the gene in the respective isolate, whereas a "-" represents the absence of that gene. Genes in red were not found in any isolate.

Isolate	Genus Level Classification	CobP as indicator	Gene Count	<i>CobA</i>	<i>CobB</i>	<i>CobC</i>	<i>CobD</i>	<i>CobE</i>	<i>CobF</i>	<i>CobG</i>
MMSF01163	<i>Alteromonas</i>	-	0	-	-	-	-	-	-	-
MMSF00257	<i>Alteromonas</i>	-	0	-	-	-	-	-	-	-
MMSF00958	<i>Alteromonas</i>	-	0	-	-	-	-	-	-	-
MMSF00404	<i>Alteromonas</i>	-	0	-	-	-	-	-	-	-
MMSF00132	<i>Labrenzia</i>	+	18	+	+	+	+ x2	-	-	-
MMSF00249	<i>Labrenzia</i>	+	18	+	+	+	+ x2	-	-	-
MMSF00964	<i>Marinobacter</i>	-	2	-	-	-	-	-	-	-
MMSF01190	<i>Marinobacter</i>	-	1	-	-	-	-	-	-	-
MMSF00068	<i>Micrococcus</i>	-	0	-	-	-	-	-	-	-
MMSF00107	<i>Micrococcus</i>	-	0	-	-	-	-	-	-	-
MMSF00910	<i>Winogradskyella</i>	-	0	-	-	-	-	-	-	-
MMSF00046	<i>Winogradskyella</i>	-	0	-	-	-	-	-	-	-

Isolate	Genus Level Classification	CobH	CobI	<i>CobJ</i>	CobK	CobL	CobM	CobN	CobO	CobP	CobQ
MMSF01163	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-
MMSF00257	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-
MMSF00958	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-
MMSF00404	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-
MMSF00132	<i>Labrenzia</i>	+	+	-	+	+	+	+	+	+	+
MMSF00249	<i>Labrenzia</i>	+	+	-	+	+	+	+	+	+	+
MMSF00964	<i>Marinobacter</i>	-	-	-	-	-	-	-	+	-	-
MMSF01190	<i>Marinobacter</i>	-	-	-	-	-	-	-	+	-	-
MMSF00068	<i>Micrococcus</i>	-	-	-	-	-	-	-	-	-	-
MMSF00107	<i>Micrococcus</i>	-	-	-	-	-	-	-	-	-	-
MMSF00910	<i>Winogradskyella</i>	-	-	-	-	-	-	-	-	-	-
MMSF00046	<i>Winogradskyella</i>	-	-	-	-	-	-	-	-	-	-

Isolate	Genus Level Classification	<i>CobP</i>	<i>CobQ</i>	<i>CobR</i>	<i>CobS</i>	<i>CobT</i>	<i>CobU</i>	<i>CobV</i>	<i>CobW</i>	<i>CobX</i>	<i>CobY</i>	<i>CobZ</i>	<i>Cbi</i>
MMSF01163	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00257	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00958	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00404	<i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00132	<i>Labrenzia</i>	+	+	-	-	-	+	+	-	-	-	-	cbiD
MMSF00249	<i>Labrenzia</i>	+	+	-	-	-	+	+	-	-	-	-	cbiD
MMSF00964	<i>Marinobacter</i>	-	-	-	-	-	-	-	-	-	-	-	cbiO
MMSF01190	<i>Marinobacter</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00068	<i>Micrococcus</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00107	<i>Micrococcus</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00910	<i>Winogradskyella</i>	-	-	-	-	-	-	-	-	-	-	-	-
MMSF00046	<i>Winogradskyella</i>	-	-	-	-	-	-	-	-	-	-	-	-

Table S3-5 Composition of R2A broth adjusted to suit marine bacteria. Final pH = 7.2 +/- 0.2 at 26°C. R2A broth was made by suspending 43.12 g of combined reagents in 1 L of MilliQ water, dissolving the medium completely, and sterilization by autoclaving at 121°C for 15 min. Final salinity ~34 ppt.

Component	grams L ⁻¹	Supplier
Casein acid hydrolysate	0.500	Cat#C0501, Sigma-Aldrich
Yeast extract	0.500	Cat#LP0021, Oxoid
Proteose peptone	0.500	Cat#211684, ThermoFisher
Dextrose	0.500	Cat#G360, PhytoTech Laboratories
Starch, soluble	0.500	Cat#AJA526, Univar
Dipotassium phosphate	0.300	Cat#P3786, Sigma-Aldrich
Magnesium sulfate	0.024	Cat#M2643, Sigma-Aldrich
Sodium pyruvate	0.300	Cat#P2256, Sigma-Aldrich
Red Sea Salt™	40.00	Cat#R11065, Red Sea

Table S3-6 Single-copy housekeeping genes extracted from the RAST annotations

Gene	Function	NCBI
16S	SSU rRNA ## 16S rRNA, small subunit ribosomal RNA	16S ribosomal RNA
23S	LSU rRNA ## 23S rRNA, large subunit ribosomal RNA	23S ribosomal RNA
fusA	Translation elongation factor G	elongation factor G
gyrB	DNA gyrase subunit B (EC 5.99.1.3)	DNA gyrase subunit B
gyrA	DNA gyrase subunit A (EC 5.99.1.3)	DNA gyrase subunit A
pyrG	CTP synthase (EC 6.3.4.2)	CTP synthetase
lepA	Translation elongation factor LepA	GTP-binding protein LepA
recA	RecA protein	recA protein
recG	ATP-dependent DNA helicase RecG (EC 3.6.4.12)	ATP-dependent DNA helicase RecG
rpoB	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)	DNA-directed RNA polymerase subunit beta
rpoD	RNA polymerase sigma factor RpoD	RNA polymerase sigma factor RpoD
atpD	ATP synthase beta chain (EC 3.6.3.14)	F0F1 ATP synthase subunit beta
ppk	Polyphosphate kinase (EC 2.7.4.1)	polyphosphate kinase
polA	DNA polymerase I (EC 2.7.7.7)	DNA polymerase I
pheSa	Phenylalanyl-tRNA synthetase alpha chain (EC 6.1.1.20)	phenylalanyl-tRNA synthetase subunit alpha
pheSb	Phenylalanyl-tRNA synthetase beta chain (EC 6.1.1.20)	phenylalanyl-tRNA synthetase subunit beta
ileS	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	isoleucyl-tRNA synthetase
leuS	Leucyl-tRNA synthetase (EC 6.1.1.4)	leucyl-tRNA synthetase
aspS	Aspartyl-tRNA synthetase (EC 6.1.1.12)	aspartyl-tRNA synthetase
alaS	Alanyl-tRNA synthetase (EC 6.1.1.7)	alanyl-tRNA synthetase
argS	Arginyl-tRNA synthetase (EC 6.1.1.19)	arginyl-tRNA synthetase
hisS	Histidyl-tRNA synthetase (EC 6.1.1.21)	histidyl-tRNA synthetase
valS	Valyl-tRNA synthetase (EC 6.1.1.9)	valyl-tRNA synthetase
srp1	SSU ribosomal protein S1p	30S ribosomal protein S1
srp2	SSU ribosomal protein S2p (SAe)	30S ribosomal protein S2
srp3	SSU ribosomal protein S3p (S3e)	30S ribosomal protein S3
srp4	SSU ribosomal protein S4p (S9e)	30S ribosomal protein S4
srp5	SSU ribosomal protein S5p (S2e)	30S ribosomal protein S5
srp6	SSU ribosomal protein S6p	30S ribosomal protein S6
srp7	SSU ribosomal protein S7p (S5e)	30S ribosomal protein S7
srp8	SSU ribosomal protein S8p (S15Ae)	30S ribosomal protein S8
srp9	SSU ribosomal protein S9p (S16e)	30S ribosomal protein S9
srp10	SSU ribosomal protein S10p (S20e)	30S ribosomal protein S10
srp11	SSU ribosomal protein S11p (S14e)	30S ribosomal protein S11
srp12	SSU ribosomal protein S12p (S23e)	30S ribosomal protein S12
srp13	SSU ribosomal protein S13p (S18e)	30S ribosomal protein S13
srp14	SSU ribosomal protein S14p (S29e) @ SSU ribosomal protein S14p (S29e), zinc-independent	30S ribosomal protein S14

srp15	SSU ribosomal protein S15p (S13e)	30S ribosomal protein S15
srp16	SSU ribosomal protein S16p	30S ribosomal protein S16
srp17	SSU ribosomal protein S17p (S11e)	30S ribosomal protein S17
srp18	SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent	30S ribosomal protein S18
srp19	SSU ribosomal protein S19p (S15e)	30S ribosomal protein S19
srp20	SSU ribosomal protein S20p	30S ribosomal protein S20
srp21	SSU ribosomal protein S21p	30S ribosomal protein S21
lrp1	LSU ribosomal protein L1p (L10Ae)	50S ribosomal protein L1
lrp2	LSU ribosomal protein L2p (L8e)	50S ribosomal protein L2
lrp3	LSU ribosomal protein L3p (L3e)	50S ribosomal protein L3
lrp4	LSU ribosomal protein L4p (L1e)	50S ribosomal protein L4
lrp5	LSU ribosomal protein L5p (L11e)	50S ribosomal protein L5
lrp6	LSU ribosomal protein L6p (L9e)	50S ribosomal protein L6
lrp7	LSU ribosomal protein L7/L12 (P1/P2)	50S ribosomal protein L7
lrp8	LSU ribosomal protein L8p	50S ribosomal protein L8
lrp9	LSU ribosomal protein L9p	50S ribosomal protein L9
lrp10	LSU ribosomal protein L10p (P0)	50S ribosomal protein L10
lrp11	LSU ribosomal protein L11p (L12e)	50S ribosomal protein L11
lrp12	LSU ribosomal protein L12p	50S ribosomal protein L12
lrp13	LSU ribosomal protein L13p (L13Ae)	50S ribosomal protein L13
lrp14	LSU ribosomal protein L14p (L23e)	50S ribosomal protein L14
lrp15	LSU ribosomal protein L15p (L27Ae)	50S ribosomal protein L15
lrp16	LSU ribosomal protein L16p (L10e)	50S ribosomal protein L16
lrp17	LSU ribosomal protein L17p	50S ribosomal protein L17
lrp18	LSU ribosomal protein L18p (L5e)	50S ribosomal protein L18
lrp19	LSU ribosomal protein L19p	50S ribosomal protein L19
lrp20	LSU ribosomal protein L20p	50S ribosomal protein L20
lrp21	LSU ribosomal protein L21p	50S ribosomal protein L21
lrp22	LSU ribosomal protein L22p (L17e)	50S ribosomal protein L22
lrp23	LSU ribosomal protein L23p (L23Ae)	50S ribosomal protein L23
lrp24	LSU ribosomal protein L24p (L26e)	50S ribosomal protein L24
lrp25	LSU ribosomal protein L25p	50S ribosomal protein L25
lrp26	LSU ribosomal protein L26p	50S ribosomal protein L26
lrp27	LSU ribosomal protein L27p	50S ribosomal protein L27
lrp28	LSU ribosomal protein L28p	50S ribosomal protein L28
lrp29	LSU ribosomal protein L29p (L35e)	50S ribosomal protein L29
lrp30	LSU ribosomal protein L30p (L7e)	50S ribosomal protein L30
lrp31	LSU ribosomal protein L31p @ LSU ribosomal protein L31p, zinc-independent	50S ribosomal protein L31

Chapter 4

Assessment of a ROS-targeted bacterial probiotic aimed at improving thermal tolerance in the coral model, the sea anemone *Exaiptasia diaphana*

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4.1 Abstract

Probiotic inoculation is one of several interventions currently being developed with the aim of increasing climate resilience in corals. Inspired by the 'oxidative stress theory of coral bleaching,' we investigated whether a probiotic comprised of bacteria isolated from the coral model, *Exaiptasia diaphana*, and designed to scavenge free radicals, could improve thermal tolerance of this anemone. We tested the high free radical scavenging probiotic alongside a consortium of conspecific low free radical scavengers and a no-inoculum control treatment under ambient (26°C) and elevated temperature conditions (ramped from 26°C to 31.5°C) but found no evidence for improved thermal tolerance. Changes in the relative abundance of anemone-associated *Labrenzia* provided evidence for probiotic integration in the *E. diaphana* microbiome. Uptake of other probiotic members was inconsistent and probiotic members did not persist in the anemone microbiome over time. Consequently, the failure of the probiotic inoculation to confer improved thermal tolerance may have been due to the absence of probiotic bacteria for the full duration of the experiment. Importantly, there were no apparent physiological impacts on the holobiont following inoculation, thus showing that shifting the abundance of native anemone microbiome members was not detrimental to holobiont health. Given the frequency of coral bleaching events, assisted evolution strategies, including probiotics, should be included with action plans to reduce carbon emissions, since without urgent intervention to achieve coral resilience to climate change, coral reefs will not survive. This study will therefore be invaluable for future bacterial bioengineering approaches.

4.2 Importance

Coral reefs are experiencing severe declines due to global climate change. Consequently, strategic interventions are required to ensure their persistence until climate change is addressed through consistent reductions in carbon emissions. Probiotic treatment has been proposed as a novel intervention strategy to improve coral resistance to environmental stress, and initial studies have shown tremendous promise. However, further

research is needed to determine and realize the potential of probiotics for coral. The results presented here will help direct future work that investigates the efficacy of probiotics to mitigate coral bleaching during periods of thermal stress.

4.3 Introduction

The persistence of coral reefs is threatened by environmental conditions caused by climate change, particularly elevated sea surface temperature (SST) (Hughes et al., 2017a). Elevated SST negatively impacts coral, in part, through inhibition of photosynthesis via damage to photosystem II (PSII) of their obligate algal endosymbionts, Symbiodiniaceae (Warner et al., 1999; Tchernov et al., 2004). This impairment leads to increased levels of reactive oxygen species (ROS) in Symbiodiniaceae, which are thought to diffuse into host cells, causing host cell damage (Lesser, 1997; Downs et al., 2002). In a subsequent act of mutual self-preservation, the Symbiodiniaceae and host cells dissociate in a process known as 'bleaching' (Weis, 2008). As corals receive most of their carbon requirements from Symbiodiniaceae (Muscattine et al., 1984), this separation is fatal unless the coral-algae symbiosis is reinstated. With average SST predicted to rise further and associated summer heat waves increasing in frequency and duration (Aral and Guan, 2016), finding effective bleaching mitigation methods beyond controlling carbon emissions has become crucial.

Many innovative approaches have been proposed to mitigate bleaching and enhance coral survival during thermal stress (Damjanovic et al., 2017; Peixoto et al., 2017; van Oppen et al., 2017). However, directly targeting ROS has rarely been investigated despite evidence that exposure to exogenous antioxidants can reduce host ROS levels and bleaching in thermally stressed corals and coral models (Lesser, 1997; Marty-Rivera et al., 2018). In addition, the photosynthetic performance of thermally stressed Symbiodiniaceae has been shown to improve within hours of receiving a single antioxidant dose (Lesser, 1996). Nevertheless, applying antioxidant compounds directly onto corals may not be practical due to the short lifespan of antioxidants when exposed to seawater (King et al., 2016) and ultraviolet radiation (Compton et al., 2019), or desirable due to their potential impact on non-target organisms. Instead, increasing natural antioxidant generation within the coral

holobiont may be more effective and practical.

One suggested way of increasing natural antioxidant generation in the holobiont is through microbiome manipulation (van Oppen et al., 2015). Corals are colonized by a multitude of microorganisms that profoundly influence the animal's health, for example by cycling nutrients (Lesser et al., 2007; Raina et al., 2009; Räddecker et al., 2015) and protecting hosts from pathogens (Shnit-Orland and Kushmaro, 2009; Kuek et al., 2015; Höhener et al., 2016). Research has shown that exposing cnidarian holobionts to beneficial bacteria can reduce the impact of pathogens (Alagely et al., 2011) or environmental stressors such as oil pollution (dos Santos et al., 2015). Inoculation of coral with native bacteria, including catalase positive isolates, has also produced improved bleaching tolerance in corals exposed to elevated temperature (Rosado et al., 2018). Although the results of the latter study were promising, the reason for improved bleaching tolerance was unclear due to the selected bacteria's broad range of traits. For example, the stressed corals may have been supported indirectly through heterotrophic feeding on the introduced bacteria (Ducklow, 1990; Tremblay et al., 2012b; Meunier et al., 2019). Consequently, the influence of antioxidant-producing bacteria on cnidarian bleaching requires further investigation.

To address this, we inoculated the sea anemone *Exaiptasia diaphana* with a probiotic consisting of free radical scavenging (FRS) bacteria (Dungan et al., 2020a) and evaluated its ability to reduce ROS and mitigate bleaching when this coral model was exposed to thermal stress. The holobiont's bleaching response to thermal stress was assessed by measuring Symbiodiniaceae photosynthetic performance and cell densities. Metabarcoding of bacterial 16S rRNA genes was used to track incorporation of the probiotic bacteria into the *E. diaphana* microbiome and changes in bacterial community structure across time and treatments. The influence of host genotype was also explored by comparing the responses of three *E. diaphana* genotypes. This work extends probiotic-based bleaching mitigation research and shows how shifting a native cnidarian microbiome through probiotic inoculation might impact holobiont physiologic performance.

4.4 Results

4.4.1 Probiotic density

The viability and cell density of the bacterial cultures used in each probiotic inoculum were confirmed by counting colony forming units (CFUs) and were generally within one order of magnitude of the target density (10^9 cells mL⁻¹). Exceptions included the Day 7 *Winogradskyella* (negative probiotic) culture, which failed to produce colonies, Day 2 *Micrococcus* and *A. oceani*, and Day 7 *A. oceani* (positive probiotic) cultures, which were of low CFU density (Table 4-1).

Table 4-1 Cell densities calculated from CFU counts of high FRS bacteria cultured for the positive probiotic (left) and low FRS bacteria cultured for the negative probiotic (right), with dosing on Days 0, 2, and 7. Bacterial cultures $< 10^8$ cells mL⁻¹ are in **bold**.

High FRS Strain	Dosing Day	Density (cells mL ⁻¹)	Low FRS Strain	Dosing Day	Density (cells mL ⁻¹)
MMSF00046	0	6.40×10^7	MMSF00910	0	1.56×10^9
<i>Winogradskyella</i>	2	2.34×10^8	<i>Winogradskyella</i>	2	3.27×10^9
	7	1.61×10^9		7	No growth
MMSF00068	0	3.72×10^8	MMSF00107	0	1.33×10^9
<i>Micrococcus</i>	2	3.80×10^7	<i>Micrococcus</i>	2	2.37×10^9
	7	1.29×10^9		7	1.53×10^9
MMSF00132	0	2.23×10^9	MMSF00249	0	6.73×10^9
<i>Labrenzia</i>	2	2.60×10^8	<i>Labrenzia</i>	2	6.00×10^8
	7	1.30×10^8		7	7.73×10^8
MMSF00958	0	2.19×10^8	MMSF00257	0	4.80×10^9
<i>Alteromonas</i>	2	1.43×10^8	<i>Alteromonas</i>	2	2.53×10^9
<i>macleodii</i>	7	4.37×10^8	<i>macleodii</i>	7	2.73×10^9
MMSF01163	0	1.01×10^9	MMSF00404	0	8.67×10^8
<i>Alteromonas</i>	2	2.33×10^7	<i>Alteromonas</i>	2	3.87×10^8
<i>oceani</i>	7	2.53×10^7	<i>oceani</i>	7	4.07×10^9
MMSF01190	0	1.87×10^9	MMSF00964	0	9.27×10^8
<i>Marinobacter</i>	2	2.45×10^8	<i>Marinobacter</i>	2	4.67×10^8
	7	3.39×10^9		7	1.32×10^9

4.4.2 Photosynthetic efficiency of Symbiodiniaceae

Photosynthetic efficiency (F_v/F_m) of Symbiodiniaceae, a commonly-used proxy for cnidarian

holobiont health (Ralph et al., 2016), declined during three weeks of ambient temperature (26°C) acclimation in all anemones (Fig. S4-1). However, F_v/F_m recovered to baseline levels by Day 12, suggesting a true acclimation period of approximately five weeks. All anemones at ambient temperature maintained near-normal F_v/F_m levels after Day 12 but experienced a spike in F_v/F_m at Day 31 compared to Day 28. As there were no changes in F_v/F_m of similar magnitude on adjacent days, the Day 31 values may represent a measurement error.

After the acclimation period, significant changes in F_v/F_m , regardless of anemone genotype, were driven by the interaction of time and temperature ($F_{\text{AIMS2}(13,155)}=5.26$, $p<0.0001$; $F_{\text{AIMS3}(13,153)}=20.17$, $p<0.0001$; $F_{\text{AIMS4}(13,154)}=10.84$, $p<0.0001$) with no impact of probiotic treatment. At Day 43, F_v/F_m was significantly lower in the AIMS2 and AIMS3 anemones at elevated temperature (0.287–0.389) compared to those at ambient temperature (0.433–0.488) for each probiotic treatment (Table S4-1, Table S4-2). For AIMS4, only the negative probiotic treatment had a significantly reduced F_v/F_m from ambient to elevated temperature (Table S4-3). These pairwise comparisons for Day 43 also indicate that there were no significant differences in F_v/F_m among the positive probiotic, negative probiotic, and control anemones at elevated temperature.

4.4.3 Symbiodiniaceae cell density

Symbiodiniaceae cell density declined in most anemones during the acclimation period from Day –21 to Day 0 (Fig. S4-2). During this period, the AIMS3 anemones underwent the greatest decline with a 52% loss of Symbiodiniaceae, on average, whilst AIMS2 and AIMS4 lost 31% and 38%, respectively. The higher reduction in AIMS3 may have been due to their relatively high Symbiodiniaceae density at Day –21, rather than a greater sensitivity to relocation as all anemones had similar Symbiodiniaceae cell densities of $0.5\text{--}1.0 \times 10^7$ cells mg^{-1} host protein at Day 0.

From Day 0 to the conclusion of the experiment, significant changes in Symbiodiniaceae cell density for AIMS2 and AIMS3 anemones were driven by the interaction of time, probiotic treatment, and temperature ($F_{\text{AIMS2}(26,156)}=2.55$, $p=0.0002$; $F_{\text{AIMS3}(26,156)}=1.49$, $p=0.072$). For AIMS4 individuals, Symbiodiniaceae density changed as a result of the

interaction of time and temperature only ($F_{\text{AIMS4}(13,156)}=2.83$, $p=0.0011$). To evaluate the potential effectiveness of probiotic treatment to mitigate the effect of bleaching, we compared Symbiodiniaceae cell densities between probiotic treatments and temperature conditions for Day 43 by anemone genotype. Despite the loss of Symbiodiniaceae cells in response to elevated temperature, at Day 43 there were no significant differences in Symbiodiniaceae cell density based on probiotic treatment in any of the three genotypes. Pairwise comparisons by probiotic treatment showed that at Day 43, Symbiodiniaceae cell density was not significantly different in the AIMS2 anemones at elevated temperature ($3.30\text{--}4.44 \times 10^6$) compared to those at ambient temperature ($5.22\text{--}7.31 \times 10^6$) (Table S4-4). Symbiodiniaceae cell density was not significantly different for the no inoculum control anemones when comparing Day 0 to Day 43 for each temperature condition, indicating that the AIMS2 anemones did not bleach. For AIMS3 anemones in the no inoculum control probiotic treatment, there was a significant decrease in Symbiodiniaceae cell density from Day 0 to 43 under elevated temperature conditions, but not ambient (Table S4-5). On Day 43, Symbiodiniaceae density was significantly lower in AIMS3 anemones exposed to elevated temperature compared to the controls, regardless of probiotic treatment (ambient: $8.41\text{--}9.77 \times 10^6$ vs elevated: $0.43\text{--}2.27 \times 10^6$), demonstrating that probiotic treatment did not mitigate bleaching. Although the AIMS4 comparisons showed a significantly lower cell density at elevated compared to ambient temperature only for the negative probiotic anemones, all heat stressed AIMS4 anemones showed a trend of lower cell densities at elevated temperature (ambient: $3.97\text{--}7.05 \times 10^6$ vs elevated: $0.76\text{--}4.94 \times 10^6$) (Table S4-6).

4.4.4 ROS assay

ROS, the potentially toxic products of thermal stress generated within the holobiont, were quantified using a ROS-activated fluorescent reagent. Levels of ROS varied as a result of the interaction between time and probiotic for AIMS2 ($F_{\text{AIMS2}(24,144)}=3.33$, $p<0.0001$) and the interaction of temperature, time and probiotic in AIMS3 ($F_{\text{AIMS3}(24,144)}=4.91$, $p<0.0001$). There were significant differences in ROS levels for AIMS4 anemones by time only ($F_{\text{AIMS4}(12,144)}=7.30$, $p<0.0001$). Interaction effects related to either probiotic or temperature

treatment are likely influenced by variation in the data (Fig. S4-3) and could be biased by normalization to Symbiodiniaceae cells. Due to sampling error, Day 40 ROS measurements were excluded from the data set.

On Day 43, there were no significant differences in the amount of ROS measured for AIMS2 anemones between ambient and elevated temperature, regardless of probiotic, or between probiotic treatments in elevated temperature (Table S4-7). ROS (measured as fluorescence units) levels for AIMS3 anemones in the no inoculum probiotic treatment at ambient temperature ($1.1-1.9 \times 10^{-3}$) were significantly lower than those in the elevated treatment ($10.7-38.0 \times 10^{-3}$). For AIMS3 anemones subjected to elevated temperature, both positive ($1.4-4.0 \times 10^{-3}$) and negative ($2.2-15.3 \times 10^{-3}$) probiotic treatments had significantly lower ROS than the control (Table S4-8). In AIMS4, ROS levels for anemones in the negative probiotic treatments were significantly lower in ambient temperature ($1.7-2.9 \times 10^{-3}$) compare to elevated temperature ($3.0-21.0 \times 10^{-3}$), with no significant differences between any of the probiotic treatments at Day 43 in elevated temperature (Table S4-9).

4.4.5 Symbiodiniaceae community characterization

Sequencing of the Symbiodiniaceae internal transcribed spacer region 2 (ITS2) region produced 6.56 M reads across 108 *E. diaphana* samples (n=54 for Day 0, n=54 for Day 43, minimum 9 086; mean 60 698, maximum 111 559 reads per sample). After quality control, 2.35 M reads remained (minimum 213, mean 21 725, maximum 67 164 reads per sample). All anemones hosted *Breviolum minutum*, corresponding with previously reported GBR-sourced *E. diaphana* (Tortorelli et al., 2019; Dungan et al., 2020b) and there were no changes in Symbiodiniaceae ITS2 type profiles over the course of the experiment. Four SymPortal ITS2 type profiles were identified: B1-B1o-B1p, B1-B1a-B1b, B1-B1a-B14a-B1n, and B1-B1a-B1b-B1g. All AIMS2 anemones exclusively hosted the B1-B1o-B1p ITS2 type profile Symbiodiniaceae, whereas 89% of AIMS3 anemones hosted B1-B1a-B1b, with 6% each of B1-B1o-B1p and B1-B1a-B1b-B1g. AIMS4 anemones hosted either B1-B1o-B1p (50%), B1-B1a-B1b (44%), or B1-B1a-B14a-B1n (6%).

4.4.6 Bacteria metabarcoding data processing

Sequencing produced 25.7 M reads across 1120 *E. diaphana*, seawater, and control samples (minimum 2; mean 23 003, maximum 112 307 reads per sample). After merging, denoising and chimera filtering, 17.9 M reads remained (minimum 0, mean 15 945, maximum 91 983 reads per sample). Twenty-nine samples were identified with <100 reads per sample and were removed from the dataset. Decontam identified 209 putative contaminant amplicon sequence variants (ASVs), which constituted 1.36% relative abundance of the bacterial communities, and were removed (Table S4-10). After all filtering steps, there were 4 555 ASVs across the remaining samples.

4.4.7 Bacterial community shifts

Principal component analyses (PCoA) based on Jaccard distance showed a general temporal shift in composition of the bacterial communities of each genotype (Fig. 4-1). There was no apparent clustering or separation of datapoints based on probiotic treatment or temperature condition and the primary driver of change in bacterial community composition was time.

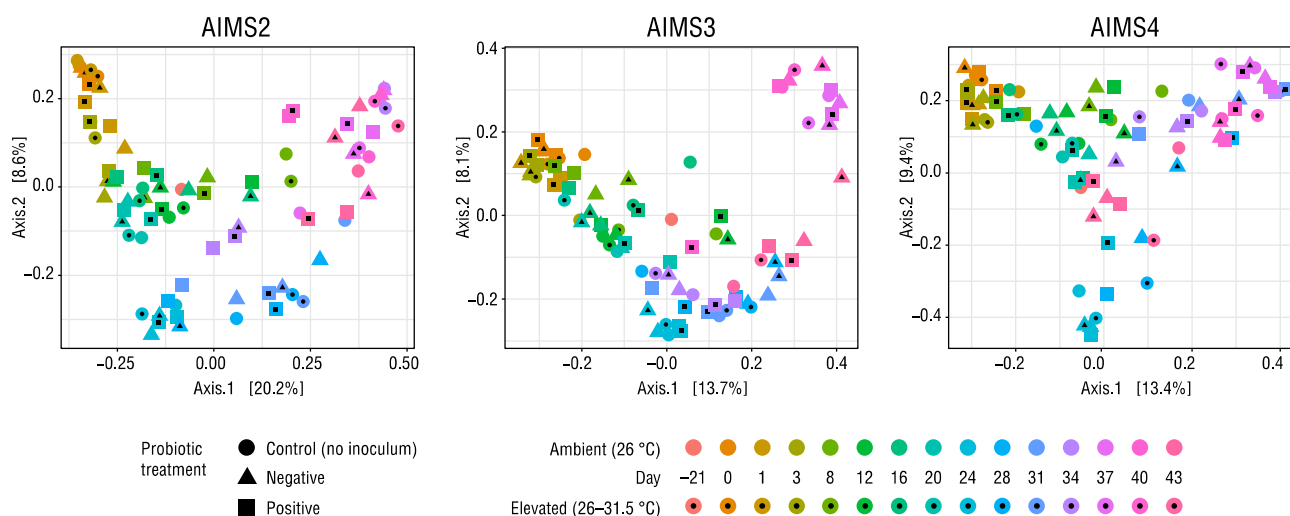


Figure 4-1 PCoA ordinations (Jaccard distance) for each anemone genotype. For each datapoint, n=3.

4.4.8 Incorporation of the probiotic bacteria

ASV-level bubble plots show shifts in relative abundance of the probiotic bacteria within the anemones (Figs. 4-2 – 4-4). High FRS *A. oceani* ASVs showed substantial increases after the first and second inoculations for the positive probiotic treatment, regardless of genotype, and this bacterium remained low in abundance in the no-inoculum controls. In contrast, the relative abundance of *A. macleodii* ASVs increased substantially in all the inoculated anemones and no-inoculum controls. The *Labrenzia* ASV increased in all inoculated anemones of all genotypes compared to the no-inoculum controls, suggesting incorporation of these bacteria from the positive and negative probiotic. An increase of *Marinobacter* in the inoculated anemones also suggested incorporation from the probiotics despite some moderate, albeit unsustained, increases in the control anemones. There were small but notable increases in *Micrococcus* in most inoculated anemones, particularly in the negative probiotic, but also increases in many uninoculated anemones. Increases in *Winogradskyella* in the inoculated anemones were clear but modest. Apart from *A. macleodii* and *Marinobacter* ASVs, none of the probiotic members were maintained at higher than Day 0 levels for the duration of the experiment.

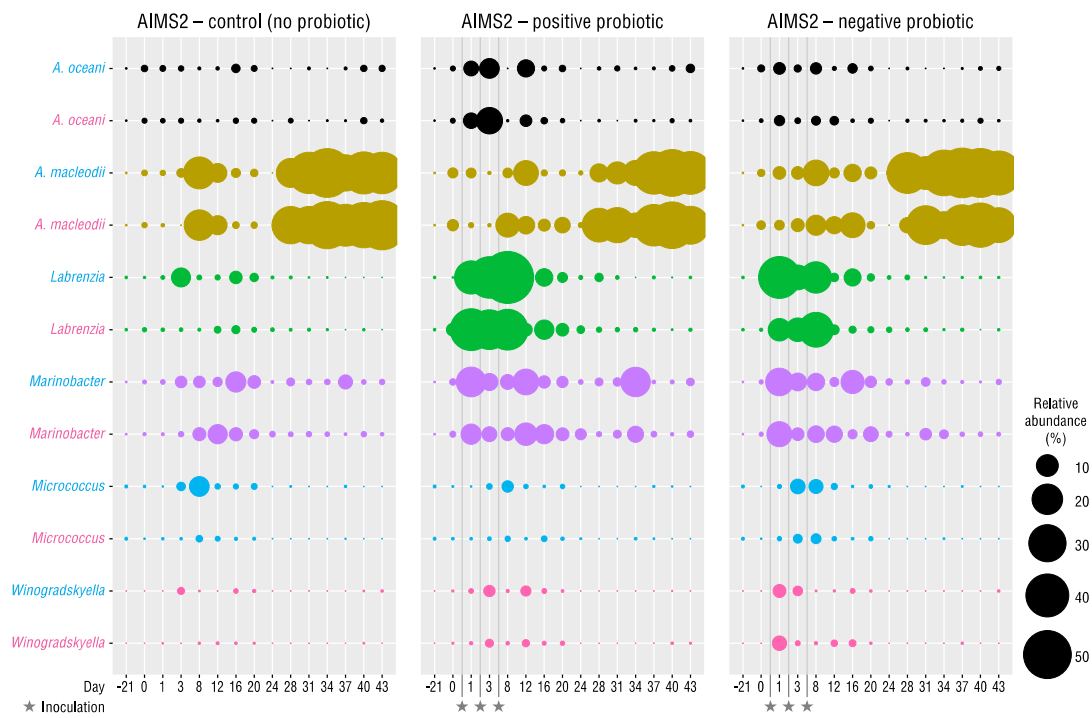


Figure 4-2 Relative abundance of genera of the probiotic bacteria in the AIMS2 anemones. Blue genus names = data for ambient temperature anemones (26°C). Pink genus names = data for elevated temperature anemones (26°C–31°C). Probiotic inoculations were performed at Days 0, 1 and 7 and are indicated by stars. For each bubble, $n=3$.

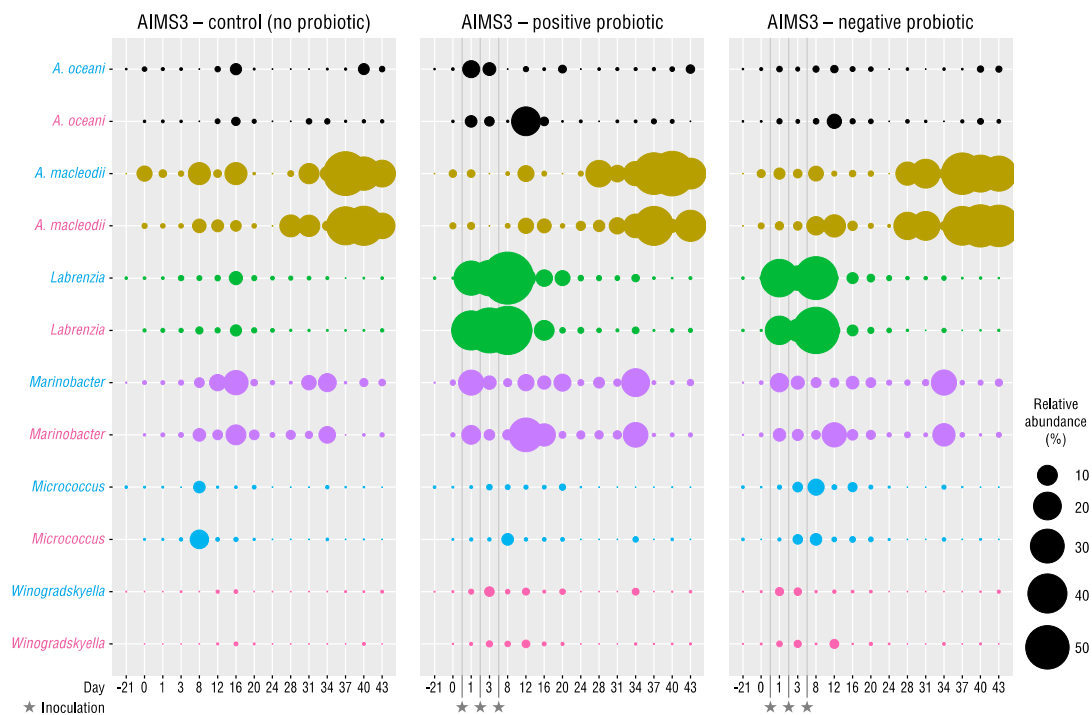


Figure 4-3 Relative abundance of genera of the probiotic bacteria in the AIMS3 anemones. Blue genus names = data for ambient temperature anemones (26°C). Pink genus names = data for elevated temperature anemones (26°C–31°C). Probiotic inoculations were performed at Days 0, 1 and 7 and are indicated by stars. For each bubble, $n=3$.

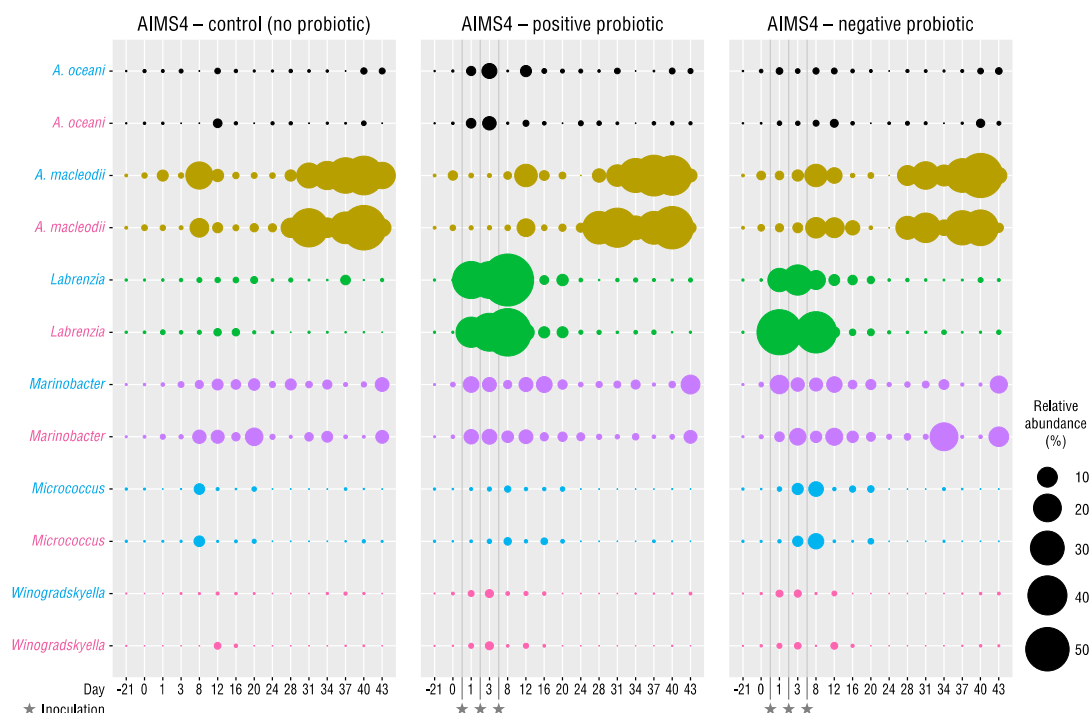


Figure 4-4 Relative abundance of genera of the probiotic bacteria in the AIMS4 anemones. Blue genus names = data for ambient temperature anemones (26°C). Pink genus names = data for elevated temperature anemones (26°C–31°C). Probiotic inoculations were performed at Days 0, 1 and 7 and are indicated by stars. For each bubble, $n=3$.

The bubble plots suggested there was little difference in the relative abundance of probiotic bacteria based on temperature. However, differences appeared to exist between the anemones based on probiotic treatment, particularly in the relative abundance of *Labrenzia* immediately after Day 0. Paired generalized linear model (GLM) analyses confirmed that the bacterial communities that associated with each anemone genotype were significantly different based on treatment (Table 4-2). Importantly though, this was short-lived, with differences generally on Days 1 and 3 only. Therefore, when the anemones were exposed to elevated temperature, there was no significant difference between the bacterial communities of the inoculated and uninoculated anemones.

Table 4-2 p-values from GLM analyses comparing bacterial communities of anemones receiving different probiotic treatments (selected days only). Significant values are in **bold**; $\alpha = 0.05$.

Genotype	Comparison	Day 0	Day 1	Day 3	Day 8	Day 20	Day 31	Day 43
AIMS2	No-inoculum control vs Positive	0.058	0.021	0.028	0.078	0.253	0.286	0.255
	No-inoculum control vs Negative	0.146	0.027	0.020	0.144	0.300	0.205	0.212
AIMS3	No-inoculum control vs Positive	0.086	0.005	0.017	0.009	0.329	0.172	0.484
	No-inoculum control vs Negative	0.190	0.046	0.027	0.058	0.187	0.457	0.315
AIMS4	No-inoculum control vs Positive	0.124	0.020	0.034	0.082	0.175	0.628	0.320
	No-inoculum control vs Negative	0.156	0.006	0.072	0.149	0.194	0.488	0.281

4.5 Discussion

4.5.1 Probiotic inoculation did not improve thermal tolerance

The bleaching response of *E. diaphana* is thought to be initiated by an increased production of ROS by their algal endosymbionts during periods of stress (Lesser, 1997; Weis, 2008). There were significant reductions in F_v/F_m in all anemones at elevated temperature regardless of inoculated or uninoculated treatment, demonstrating that the high FRS probiotic did not mitigate the impact of thermal stress on PSII of the Symbiodiniaceae. Although the AIMS2 anemones resisted bleaching at elevated temperature in all treatments, significant reductions in Symbiodiniaceae cell density of thermally stressed AIMS3 and AIMS4 anemones showed that probiotic treatment did not mitigate bleaching. While the ROS data suggests some neutralization of ROS by probiotic treatment, the overall variation in the ROS data paired with data normalization advises caution. Variation in ROS levels could be a result of stress induced by frequent changes in light and temperature during handling. Fluctuations may also be due to the ephemeral nature of ROS (Diaz et al., 2016), or the low concentrations of ROS in the anemones, which may have been near the CellROX® assay's limit of detection. Bleached AIMS3 and AIMS4 anemones in the elevated temperature regime (Day 43) had ROS values approximately half that of the ambient anemones (prior to normalization to Symbiodiniaceae cells); the

relationship between ROS and Symbiodiniaceae density is not well described and may not be linear. Therefore, no evidence for improved thermal tolerance of *E. diaphana* due to probiotic inoculation was found. However, the probiotic bacteria did not have any apparent negative effect on the anemones.

4.5.2 Uptake of probiotic bacteria by *E. diaphana* was short-lived

Post-inoculation increases in the relative abundance of the probiotic bacteria ASVs and significant differences between the bacterial communities of inoculated and uninoculated anemones on Days 1 and 3 suggest successful probiotic association with the anemones. However, this was short-lived. Consequently, the failure of positive probiotic inoculation to confer improved thermal tolerance could be explained by the absence of probiotic bacteria during temperature ramping and after reaching maximum temperature.

This absence may be due to inadequate dosing. The three-dose strategy employed in the present study was similar to previous cnidarian probiotic experiments. For example, Rosado et al. (2018) inoculated coral samples with a bacterial cocktail twice, five days apart, and Damjanovic et al. (2019b) inoculated coral larvae seven times, at 3-4 day intervals. However, neither study assessed the uptake of inoculated bacteria between doses.

Although previous studies have demonstrated probiotic inoculation can protect *E. diaphana* from pathogenic infection (Alagely et al., 2011; Zaragoza et al., 2014), incorporation of the probiotic bacteria was not assessed. Therefore, to the best of our knowledge, our study is the first time an inoculation-based modification of the *E. diaphana* bacterial microbiome has been described.

4.5.3 Probiotic uptake by *E. diaphana* was uneven

An increase in *Labrenzia* provided the strongest evidence for probiotic association with *E. diaphana*. *Labrenzia* are naturally abundant (~5%) in all the AIMS genotypes (Hartman et al., 2020) and are core members of the Symbiodiniaceae microbiome (Lawson et al., 2017), so may have faced low inhibition from antagonistic interactions with resident bacteria (Rypien et al., 2010). Poor uptake of *Micrococcus* could have been caused by below-target

densities of those bacterial cultures in the probiotics (Table 4-1), emphasizing the need to dose at consistently high cell densities, and possibly higher densities for some bacteria. *Alteromonas* are metabolically-versatile copiotrophs (Pedler et al., 2014), rapidly responding to increases in dissolved organic matter and often dominating mesocosm experiments (McCarren et al., 2010). *A. macleodii* has been found to be highly abundant in *Artemia salina* feedstock used in our *E. diaphana* culture system (Hartman et al., 2020) and increased rapidly throughout the experiment, becoming dominant in inoculated and uninoculated anemones. This suggests that *A. macleodii* likely originated from the *A. salina* feedstock and multiplied due to the culture conditions (Hartman et al., 2019).

4.5.4 Bleaching susceptibility differed by genotype

As the probiotic bacteria were not retained by the anemones, no correlation can be drawn between differences in bleaching tolerance and inoculation. However, the maintenance of near-normal Symbiodiniaceae cell density in the AIMS2 anemones at elevated temperature versus bleaching of the AIMS3 and AIMS4 anemones is noteworthy. Initial Symbiodiniaceae cell densities in the AIMS3 and AIMS4 anemones were higher than AIMS2, which may support a suggestion that higher Symbiodiniaceae cell density is linked to increased bleaching sensitivity (Cunning and Baker, 2012). It is also possible that differences in bleaching tolerance were driven by differences in the *in hospite* Symbiodiniaceae communities harboured by each anemone genotype (Howells et al., 2012; Hawkins et al., 2016b). The AIMS2 anemones, which did not bleach, harboured a distinct Symbiodiniaceae ITS2 type profile, B1-B1o-B1p. Very few AIMS3 individuals had this ITS2 type profile and bleached heavily, whereas 50% of the AIMS4 anemones had this ITS2 type profile and displayed high variation in their bleaching response. These data suggest that Symbiodiniaceae with the B1-B1o-B1p ITS2 type profile could confer thermal resilience to the holobiont. Alternatively, the different anemone genotypes may have different bleaching susceptibilities independent of their Symbiodiniaceae type profile (Gabay et al., 2019), which was not measured in this study. Previous work on corals (Quigley et al., 2018) and GBR-sourced *E. diaphana* (Tortorelli et al., 2019) has shown that the mechanism of recognition and incorporation of Symbiodiniaceae into the holobiont is influenced by both

algal symbiont and host. This phenomenon may translate to the anemone's thermal stress response suggesting that neither the host nor alga independently controls bleaching. Observations from the present study indicate that genotype and algal symbiont type will be critical in future experiments seeking to induce or mitigate bleaching in *E. diaphana*.

4.5.5 Limitations of the study and recommendations for future probiotic work

Although there was evidence for increased relative abundance of some probiotic members, the key limitation of the present study was the inability of probiotic bacteria to persist after inoculation. Therefore, repeated dosing to maintain high levels of probiotic bacteria in the target organisms, as practiced in aquaculture (Hai, 2015), may be necessary. Another method common in aquaculture is probiotic delivery via bioencapsulation in *A. salina* (Hai et al., 2010), which ensures probiotic bacteria are ingested. Although subsequent proliferation is not guaranteed (De et al., 2014), bioencapsulation reduces dilution in open marine environments thus improving practical application, and therefore warrants investigation.

Life stage at inoculation may be an important factor in the uptake of probiotic bacteria by cnidarians. Studies have shown that the microbiomes of coral larvae and juveniles can be altered by inoculation with bacterial consortia (Damjanovic et al., 2017; Damjanovic et al., 2019c), and the manipulation of mature *Pocillopora damicornis* microbiomes by Rosado et al. (2018) suggests this flexibility is maintained as cnidarians age. Nevertheless, the influence of cnidarian life-stage on probiotic uptake has not been fully explored and should be investigated.

The original assessments of FRS ability were performed on bacteria grown in pure culture. However, changes in environment can drive phenotypic plasticity resulting in a loss or decrease of previously observed bacterial traits (KÜMMERLI et al., 2009). Consequently, the persistence of FRS by the bacteria in the positive probiotic may have been impacted by the complex holobiont environment. In addition, the bacterial cultures grown for inoculation were not tested for their FRS phenotype immediately prior to dosing. Given the potential

rapid genetic evolution of bacteria, FRS ability could also have been lost over subsequent generations during sub-culturing to produce each broth culture. Therefore, each culture and cocktail should be checked to ensure FRS ability has been maintained.

Resolving the probiotic bacteria to ASV level allowed us to determine whether they were incorporated by *E. diaphana*. However, future assessments could be enhanced by quantifying probiotic bacteria in the samples by qPCR. Further, qPCR primers based on the genomic region deemed responsible for the FRS phenotype could be used to confirm that the probiotic capability had been retained after culturing.

An element of the present study that we recommend as good practice is the use of a negative probiotic. Unlike comparable probiotic studies, inclusion of a negative probiotic allowed us to account for differences in response to thermal stress due to the introduction of an additional source of nutrition as heterotrophy can reduce the impact of thermal stress on corals (Grottoli et al., 2006b; Aichelman et al., 2016). Increases in probiotic genera in the uninoculated control anemones suggest cross-contamination among culture vessels occurred during the experiment and highlights the importance of sample segregation.

4.5.6 Conclusions

To date, few studies have investigated the ability of probiotic bacteria to increase cnidarian thermal tolerance. In this study we were able show that probiotic inoculation using bacteria with high or low FRS abilities did not harm *E. diaphana*, showing that shifting the abundance of naturally occurring anemone microbiome population did not result in any physiological repercussions. While some members of our probiotic remained with the host after dosing, the observed increases in their relative abundances were short-lived, and the probiotic bacteria did not persist above normal levels. Therefore, whilst probiotic inoculation did not mitigate bleaching in heat-exposed anemones, this cannot be attributed to an inability of the inoculum to confer improved thermal tolerance, which remains unproven. Future studies that maintain elevated levels of probiotic bacteria will provide clearer insights into the potential of the coral bleaching mitigation strategy proposed here.

4.6 Methods

4.6.1 Probiotic preparation

Six *E. diaphana*-sourced bacterial isolates with high free radical scavenging (FRS) ability were selected for inclusion in a 'positive' probiotic (Dungan et al., 2020a). Six closely related strains with low FRS ability were also selected for inclusion in a 'negative control' probiotic. The isolated bacteria were identified from their near-complete length 16S rRNA gene sequences determined by Sanger sequencing (Table 4-3). According to these data, only the *Micrococcus* high and low FRS isolates were different species.

Table 4-3 Bacteria used in the positive and negative probiotic and their 16S rRNA gene GenBank Accession Numbers

Family	Genus	Species	High FRS Strain ID / NCBI Accession Number	Low FRS Strain ID / NCBI Accession Number
Alteromonadaceae	<i>Alteromonas</i>	<i>oceani</i>	MMSF01163/ MN540711	MMSF00404/ MN540719
Alteromonadaceae	<i>Alteromonas</i>	<i>macleodii</i>	MMSF00958/ MN540717	MMSF00257/ MN540713
Alteromonadaceae	<i>Marinobacter</i>	<i>salsuginis</i>	MMSF01190/ MN540716	MMSF00964/ MN540714
Flavobacteriaceae	<i>Winogradskyella</i>	<i>poriferorum</i>	MMSF00046/ MN540715	MMSF00910/ MN540718
Micrococcaceae	<i>Micrococcus</i>	<i>luteus</i> (+), <i>yunnanensis</i> (-)	MMSF00068/ MN540712	MMSF00107/ MN540722
Rhodobacteraceae	<i>Labrenzia</i>	<i>aggregata</i>	MMSF00132/ MN540721	MMSF00249/ MN540720

To prepare the positive and negative probiotics, bacterial isolates were grown from cryo-preserved cells in 50 mL of Reasoner's 2A (R2A) broth supplemented with Red Sea Salt™ seawater (RSS, R11065, Red Sea, USA). The cells were incubated at 37°C for 48 h at 150 rpm in an orbital incubator (OM11, Ratek, Australia). Uninoculated broth was also incubated to confirm medium sterility. Three replicate cultures were grown per isolate. After 48 h, the culture and medium blanks were assessed for cell density by optical density measurement at 600 nm (OD₆₀₀; CLARIOstar PLUS plate-reader, BMG Labtech, Australia), and centrifuged at 3000 × *g* at 4°C for 30 min to pellet the bacteria. The pelleted cells were

washed three times by resuspending in 10 mL 0.2 µm-filtered RSS (fRSS) reconstituted at ~34 parts per thousand (ppt) salinity, and centrifuged at $3000 \times g$ at 4°C for 15 min. After the final centrifugation, the pellet was resuspended in 5 mL fRSS and the three replicate cultures per isolate were combined. OD₆₀₀ measurements were taken of the pooled triplicates at a 1:10 dilution in fRSS, the total number of cells per isolate was calculated based on *Escherichia coli* values (Volkmer and Heinemann, 2011), and the pools were centrifuged and resuspended to a density of 10^9 cells mL⁻¹. Bacteria at 10^9 cells mL⁻¹ were pooled by probiotic treatment; 1 mL of this pool was added to glass jars containing 300 mL RSS and anemones in the select probiotic treatment to a final concentration of 10^6 bacterial cells mL⁻¹. Aliquots (500 µL) of the positive and negative probiotics were flash frozen in liquid nitrogen on inoculation days (Days 0, 2 and 7; see Fig. 4-5 below) and stored at -20°C until DNA extraction. The viability and cell density of each pre-pooled isolate was confirmed by counting CFUs. Three replicate plates of Marine Agar (Difco™ 2216, Thermo Fisher, Australia) were spread inoculated with 50 µL of four serial dilutions (10^{-5} to 10^{-8}) of each isolate. The plates were then incubated at 26°C for seven days before CFUs were counted.

4.6.2 Experimental set-up

Anemones from cultures of three Great Barrier Reef (GBR)-sourced *E. diaphana* genotypes, AIMS2, AIMS3 and AIMS4, were randomly selected from The University of Melbourne culture collection (n=450 per genotype) (Dungan et al., 2020b). Anemones from each genotype were equally distributed among 18 × 300 mL lidded glass culture jars (total jars = 54) and the jars were evenly split between two experimental incubators (Hi-Point 740FHC, Thermo Fisher, Australia) fitted with red, white, and infrared light emitting diode (LED) lights. All anemones were maintained in RSS at ~34 ppt salinity and fed *ad libitum* twice weekly with freshly hatched *A. salina* (Salt Creek, Premium GSL, USA). Jars were cleaned weekly by loosening algal debris with seawater pressure applied through sterile plastic pipettes followed by full RSS changes. All anemones were transferred to clean jars twice during the study period to combat algal growth. Seawater temperatures were monitored using submersible data loggers (Hobo UA-001-08, OneTemp, Australia). The

anemones were acclimated to the experimental incubators for three weeks. Initial light levels were $12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ to match the stock culture conditions, then gradually increased to $28 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ over 72 h on a 12 h:12 h light:dark cycle beginning at 6 a.m. This species requires very low photon levels ($<100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), such as provided in shaded or deep reef habitats. Our experimental light intensity corresponds with previous studies evaluating bleaching via thermal stress on *E. diaphana* (Tollete et al., 2013; Bieri et al., 2016).

4.6.3 Treatment schedule

The experiment began after the three-week acclimation period at a timepoint designated Day 0 (Fig. 4-5). On Day 0, probiotic-treatment anemones were inoculated with the negative or positive probiotics, and no-inoculum control anemones were not inoculated. The probiotic-treated anemones were inoculated again on Days 2 and 7. All anemones were exposed for 43 days to ambient temperature (26°C), or at temperature increasing from ambient to 31.5°C between Day 8 and Day 31 (0.25°C/day), then held at 31.5°C . The temperature ramp rate was designed to approximate GBR summer heatwave conditions (Fig. S4-4 (AIMS, 2019)). The experimental design included three replicate culture jars per genotype for each inoculation-temperature combination. The temperature designation of the incubators was swapped fortnightly and the anemones were randomly rearranged each week to remove incubator and jar position as confounding factors.

4.6.4 Symbiodiniaceae photosynthetic efficiency measurement

Photosynthetic efficiency as measured by the maximum quantum yield (F_v/F_m) of PSII in Symbiodiniaceae, is a commonly-used proxy for general holobiont health as lowered F_v/F_m indicates PSII damage resulting from thermal stress or high irradiance (Ralph et al., 2016). F_v/F_m was measured on the intracellular Symbiodiniaceae by pulse-amplitude-modulated (PAM) fluorometry with an imaging-PAM system (IMAG-MAX/L, Walz Heinz, Germany). On sampling days, measurements were taken from the bodies and proximal tentacles of five anemones per jar 4h after daylight cycle start and after 30 min dark adaptation. PAM settings: saturating pulse intensity 8, measuring light intensity 2 (frequency 1), actinic light

intensity 3, damping 2, and gain 2.

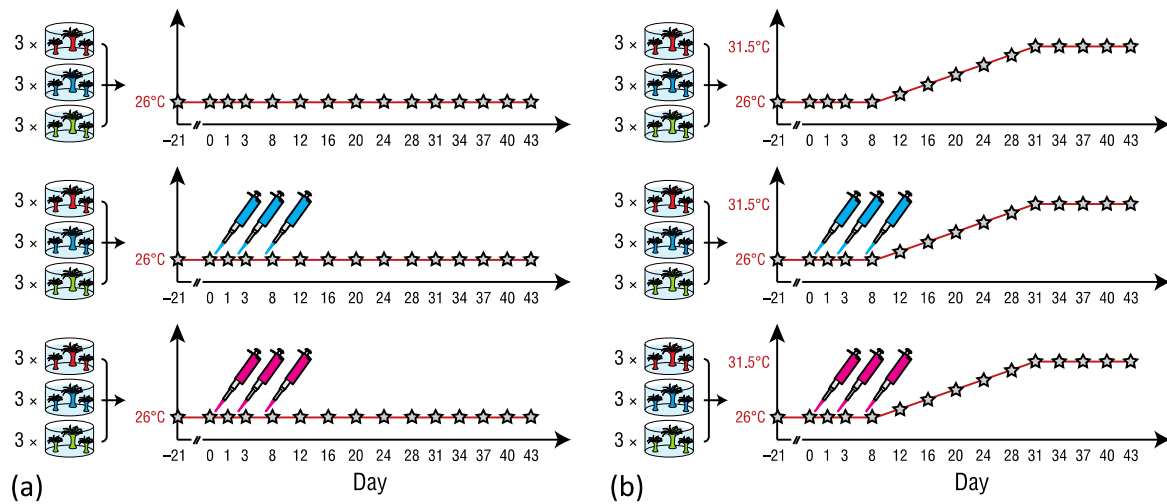


Figure 4-5 Inoculation and sampling schedule for each inoculation-temperature combination. Temperature conditions: (a) ambient (26°C), and (b) elevated (26°C–31.5°C). Positive probiotic inoculations (blue pipettors) and negative probiotic inoculations (pink pipettors) were performed on Days 0, 2 and 7. Stars indicate sample collection timepoints. At Day –21, nine anemones per genotype were collected from stock aquaria for baseline data collection ($n=27$). Thereafter, one anemone from each replicate culture jar ($n=3$) for three *E. diaphana* genotypes (AIMS2, AIMS3, AIMS4) was collected. Total anemone samples, $n=783$.

4.6.5 Anemone tissue processing

On sampling days, one anemone per jar was sacrificed for Symbiodiniaceae cell density, ROS and protein quantification, and DNA extraction for bacterial community analysis. Each anemone was individually homogenized in a sterile glass homogenizer in 1 mL of fRSS and an aliquot of 250 μL was flash frozen in liquid nitrogen for DNA extraction. The remaining homogenate was centrifuged at $5000 \times g$ for 5 min at 4°C to pellet the Symbiodiniaceae, and 50 μL of the supernatant was stored at –20°C for anemone (host) protein measurement, while 600 μL of supernatant was taken for ROS quantification.

4.6.6 Symbiodiniaceae cell density measurement

Pelleted Symbiodiniaceae were washed twice with 700 μL fRSS and centrifuged at $5000 \times g$ for 5 min at 4°C, then resuspended in 700 μL fRSS. Triplicate cell counts (cells mL^{-1}) were performed on 10 μL of sample within 48 hrs of sample collection with an automated cell counter (Countess II FL, Life Technologies, Australia). Cell counts were normalized to host

protein (mg mL^{-1}) determined by Bradford assay (Bradford, 1976). Each protein sample was assayed in triplicate and measured at 595 nm (EnSpire MLD2300 plate-reader, Perkin Elmer, Australia) against bovine serum albumin standards (500-0207, Bio-Rad, Australia).

4.6.7 ROS assay

Anemone ROS levels were quantified with CellROX[®] Orange (C10443, Thermo Fisher, Australia); a ROS-quantifying reagent used in previous cnidarian studies (Levin et al., 2016; Chakravarti et al., 2017; Marty-Rivera et al., 2018; Gegner et al., 2019). For each sample, 594 μL of supernatant, collected as above, was vortexed briefly with 6 μL CellROX[®] Orange (final concentration 5 μM) and incubated in darkness at 37°C for 30 min. Three 200 μL technical replicates of each sample were transferred into a 96-well black, clear-bottom culture plate (Nunc 165305, Thermo Fisher, Australia). Fluorescence readings at excitation 545 nm, emission 565 nm (EnSpire MLD2300, Perkin Elmer, Australia) were then taken and the average reading per sample was normalized to raw Symbiodiniaceae cell counts to give relative ROS values in arbitrary fluorescent units.

4.6.8 Metabarcoding sample preparation

DNA was extracted from the anemone, probiotic and seawater samples according to (Wilson et al., 2002) with modifications described by Hartman et al. (2019). Sample-free negative controls ($n=12$) were also processed to identify contaminants introduced during DNA extractions. Extracted DNA and negative controls were amplified by PCR in triplicate using bacterial primers with Illumina Nextera adapters (underlined) designed at the Walter and Eliza Hall Institute (WEHI) targeting the V5-V6 regions of the 16S rRNA genes: 784F [5' GTGACCTATGAACTCAGGAGTCAGGATTAGATACCCTGGTA 3']; 1061R [5' CTGAGACTTGAC-ATCGCAGCCRRACGAGCTGACGAC 3'] (Andersson et al., 2008). Anemone samples from Days 0 and 43 were also amplified with primers to characterize the Symbiodiniaceae population by targeting the ITS2 region of the rRNA gene using primers Sym_Var_5.8S2 [5' GTGACCTATGAACTCAGGAGTCGAATTGCAGAACTCCGTGAACC 3'] (Hume et al., 2015); Sym_Var_Rev [3' CTGAGACTTGACATCGCAGCCGGGTTCWCTTGTGTGACTTCA-TGC 5'] (Hume et al., 2013). Each PCR contained 12.5 μL MyTaq HS Mix polymerase (Bioline,

Australia), 2 μ L of DNA template, 0.4 μ M of each primer, and nuclease-free water up to 25 μ L. PCR conditions were: 1 cycle $\times$ 95°C for 3 min; 18 cycles $\times$ 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle $\times$ 72°C for 7 min; hold at 4°C. The triplicate PCR products from each sample were then pooled.

To prepare DNA sequencing libraries, 20 μ L of each PCR product pool was purified by size-selection using Ampure XP magnetic beads (Agencourt, Beckman Coulter, Australia). The purified DNA was resuspended in 40 μ L nuclease-free water. Indexing PCRs were created by combining 10 μ L of each DNA suspension with 10 μ L 2 $\times$ Taq master mix (M0270S, New England BioLabs, Australia) and 0.25 μ M of forward and reverse indexing primers. PCR conditions were: 1 cycle $\times$ 95°C for 3 min; 24 cycles $\times$ 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; 1 cycle $\times$ 72°C for 7 min; hold 4°C. Template-free negative controls (n=6) were included to identify contaminants introduced during indexing PCR. Four representative samples were checked for product size and quantity (2200 TapeStation, Agilent Technologies, Australia). Sequencing libraries were created by pooling 5 μ L from each reaction and performing a final bead clean-up on 50 μ L. Each library was checked for quality and quantity to guide pool normalization (2200 TapeStation), then sequenced across three Illumina Mi-Seq runs using v3 (2 $\times$ 300 bp) reagents at WEHI, Melbourne, Australia.

4.6.9 Metabarcoding data processing

Raw 16S rRNA gene sequences were imported into QIIME2 v2019.10.0 (Bolyen et al., 2019) and demultiplexed on a per-sequencing-run basis. Primers were removed with cutadapt v2.6 (Martin, 2011). Filtering, denoising, and chimera checking was performed using DADA2 (Callahan et al., 2016) in the QIIME2 environment to correct sequencing errors, remove low quality bases (mean Qscore <30), and generate bacterial ASVs. The data from each sequencing run were then merged, and taxonomy for each ASV was assigned against a SILVA database (version 132) trained with a naïve Bayes classifier against the same V5-V6 region targeted for sequencing (Bokulich et al., 2018).

4.6.10 Identification of Symbiodiniaceae genetic diversity

Raw sequences for each sample (n=108) were processed using the SymPortal analytical framework (symportal.org) (Hume et al., 2019). Sequencing information was submitted to the SymPortal remote database and underwent quality control including the removal of artefact and non-Symbiodiniaceae sequences. Within-sample informative intragenomic sequences, referred to as 'defining intragenomic variants' (DIVs) were used to identify ITS2-type profiles of putative Symbiodiniaceae taxa (Hume et al., 2019). ITS2 type profiles were designated by SymPortal based on the presence and abundance of the ITS2 sequences in our samples and within the SymPortal database.

4.6.11 Data analysis

All data were analysed in R v3.6.2 (R Core Team, 2018). Statistical tests were considered significant at $\alpha = 0.05$. ASV, taxonomy, metadata and phylogenetic tree files were imported into R and combined into a phyloseq object (McMurdie and Holmes, 2013). Potential contaminant ASVs were identified and removed from the dataset according to their abundance in the extraction (n=12) and PCR (n=12) negative controls relative to the anemone samples using the prevalence method in the R package decontam with $p=0.1$ (Davis et al., 2018). Dark-adapted F_v/F_m , Symbiodiniaceae cell density, and ROS measurements were plotted using the R package ggplot2 (Wickham, 2019) with data separated by genotype. The data were analysed for overall differences using linear mixed effects models against the variables 'probiotic', 'temperature', and 'time', with 'jar' specified as a random effect, in the R package nlme (Pinheiro et al., 2019). *Post hoc* pairwise comparisons were performed using Tukey's honestly significant difference (Tukey, 1949) in the R package emmeans (Searle et al., 1980) with Tukey's adjustment for multiple comparisons. Shifts in the bacterial community compositions of each genotype were visualized in principal coordinate analysis (PCoA) ordinations based on Jaccard distance (Jaccard, 1912). The influence of time (Day 0 to Day 43 only), probiotic and temperature treatments on changes in community composition within each genotype was investigated by comparison of GLMs for each ASV using likelihood ratio tests across 999 iterations in the R package mvabund (Wang et al., 2012). For PCoAs and GLMs the data were collapsed

to genus for clarity and computational efficiency. Incorporation of the probiotic bacteria into the anemone microbiomes was visualized in bubble plots to show changes in relative abundance at the ASV level for the probiotic members in each genotype, temperature, and probiotic condition over time. Further tests with GLMs were performed, as above, to assess whether differences between the bacterial communities for each genotype-temperature-probiotic combination were significant at selected timepoints.

4.7 Acknowledgements

The authors thank Giulia Holland, Louis Dorison, and Emily Derrick for their assistance in anemone maintenance, sample collection and processing, as well as Ben Hume for running SymPortal analysis remotely. This study was supported with funding from the Australian Research Council (grant ID: DP160101468) to MvO and LLB. MvO acknowledges Australian Research Council Laureate Fellowship FL180100036.

4.8 Data availability

Raw Illumina MiSeq data are available under NCBI BioProject ID PRJNA630329 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA630329>).

4.9 Supplementary material

Table S4-1 F_v/F_m comparisons for AIMS2 anemones at Day 43. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Day 43: Ambient-Elevated	Control	12	3.450	0.0048
	Negative	12	3.069	0.0097
	Positive	12	2.655	0.0210
Day 43: Control-Positive	Elevated	12	1.208	0.4709
Day 43: Control-Negative	Elevated	12	1.336	0.4035
Day 43: Positive-Negative	Elevated	12	0.127	0.9911

Table S4-2 F_v/F_m comparisons for AIMS3 anemones at Day 43. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Day 43: Ambient-Elevated	Control	9	3.998	0.0031
	Negative	9	4.664	0.0012
	Positive	9	2.946	0.0163
Day 43: Control-Positive	Elevated	9	0.238	0.9695
Day 43: Control-Negative	Elevated	9	0.160	0.9861
Day 43: Positive-Negative	Elevated	9	0.420	0.9083

Table S4-3 F_v/F_m comparisons for AIMS4 anemones at Day 43. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Day 43: Ambient-Elevated	Control	11	2.021	0.0683
	Negative	11	2.352	0.0384
	Positive	11	1.982	0.0730
Day 43: Control-Positive	Elevated	12	0.468	0.8873
Day 43: Control-Negative	Elevated	12	0.203	0.9776
Day 43: Positive-Negative	Elevated	12	0.750	0.7396

Table S4-4 Symbiodiniaceae cell density comparisons for AIMS2 anemones.

Data: Contrast	Treatment	df	t.ratio	p.value
Ambient: Day 0-Day 43	Control	52	1.890	0.8225
Elevated: Day 0-Day 43	Control	52	1.293	0.9887
Day 43: Ambient-Elevated	Control	12	2.123	0.0552
	Negative	12	0.588	0.5672
	Positive	12	1.723	0.1106
Day 43: Control-Positive	Elevated	12	0.686	0.7757
Day 43: Control-Negative	Elevated	12	0.562	0.8422
Day 43: Positive-Negative	Elevated	12	0.124	0.9916

Table S4-5 Symbiodiniaceae cell density comparisons for AIMS3 anemones. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Ambient: Day 0-Day 43	Control	52	1.714	0.9014
Elevated: Day 0-Day 43	Control	52	6.439	<0.0001
Day 43: Ambient-Elevated	Control	12	7.680	<0.0001
Day 43: Ambient-Elevated	Negative	12	7.795	<0.0001
Day 43: Ambient-Elevated	Positive	12	7.202	<0.0001
Day 43: Control-Positive	Elevated	12	1.774	0.2194
Day 43: Control-Negative	Elevated	12	0.781	0.7213
Day 43: Positive-Negative	Elevated	12	0.993	0.5947

Table S4-6 Symbiodiniaceae cell density comparisons for AIMS4 anemones. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Ambient: Day 0-Day 43	Control	52	0.116	1.000
Elevated: Day 0-Day 43	Control	52	0.955	0.9994
Day 43: Ambient-Elevated	Control	12	0.960	0.3558
Day 43: Ambient-Elevated	Negative	12	2.287	0.0412
Day 43: Ambient-Elevated	Positive	12	1.447	0.1735
Day 43: Control-Positive	Elevated	12	1.602	0.2824
Day 43: Control-Negative	Elevated	12	1.876	0.1879
Day 43: Positive-Negative	Elevated	12	0.274	0.9596

Table S4-7 ROS comparisons for AIMS2 anemones at Day 43.

Data: Contrast	Treatment	df	t.ratio	p.value
Day 43: Ambient-Elevated	Control	12	2.683	0.1502
Day 43: Ambient-Elevated	Negative	12	0.629	0.9865
Day 43: Ambient-Elevated	Positive	12	0.381	0.9987
Day 43: Control-Positive	Elevated	12	2.127	0.3360
Day 43: Control-Negative	Elevated	12	2.170	0.3175
Day 43: Positive-Negative	Elevated	12	0.043	1.000

Table S4-8 ROS comparisons for AIMS3 anemones at Day 43. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Day 43: Ambient-Elevated	Control	12	15.107	<0.0001
Day 43: Ambient-Elevated	Negative	12	3.174	0.0680
Day 43: Ambient-Elevated	Positive	12	0.880	0.9444
Day 43: Control-Positive	Elevated	12	11.981	<0.0001
Day 43: Control-Negative	Elevated	12	14.338	<0.0001
Day 43: Positive-Negative	Elevated	12	2.357	0.2447

Table S4-9 ROS comparisons for AIMS4 anemones at Day 43. Significant values are in **bold**; $\alpha = 0.05$.

Data: Contrast	Treatment	df	t.ratio	p.value
Day 43: Ambient-Elevated	Control	12	1.744	0.5313
Day 43: Ambient-Elevated	Negative	12	3.670	0.0296
Day 43: Ambient-Elevated	Positive	12	0.367	0.9989
Day 43: Control-Positive	Elevated	12	2.215	0.2987
Day 43: Control-Negative	Elevated	12	1.839	0.4787
Day 43: Positive-Negative	Elevated	12	0.376	0.9988

Table S4-10 The 20 most abundant contaminant ASVs identified by decontam across all anemone samples. Some genera have members associated with human skin, suggesting the origin of those putative contaminants e.g. *Cutibacterium*.

	Phylum	Class	Order	Family	Genus	Rel. abundance (%)
1	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	<i>Pelomonas</i>	0.44
2	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	0.15
3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.13
4	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	0.08
5	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	0.06
6	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	<i>Cutibacterium</i>	0.03
7	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcaceae</i>	0.03
8	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	<i>Enhydrobacter</i>	0.03
9	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	<i>Streptococcus</i>	0.02
10	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	<i>Ralstonia</i>	0.02
11	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	0.02
12	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.02
13	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	<i>Cutibacterium</i>	0.02
14	Firmicutes	Clostridia	Clostridiales	Family XI	<i>Finegoldia</i>	0.01
15	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	<i>Cupriavidus</i>	0.01
16	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	0.01
17	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	<i>Corynebacterium</i>	0.01
18	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	<i>Cutibacterium</i>	0.01
19	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	<i>Staphylococcus</i>	0.01
20	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	0.01
21-209	Other					0.24
					Total	1.36

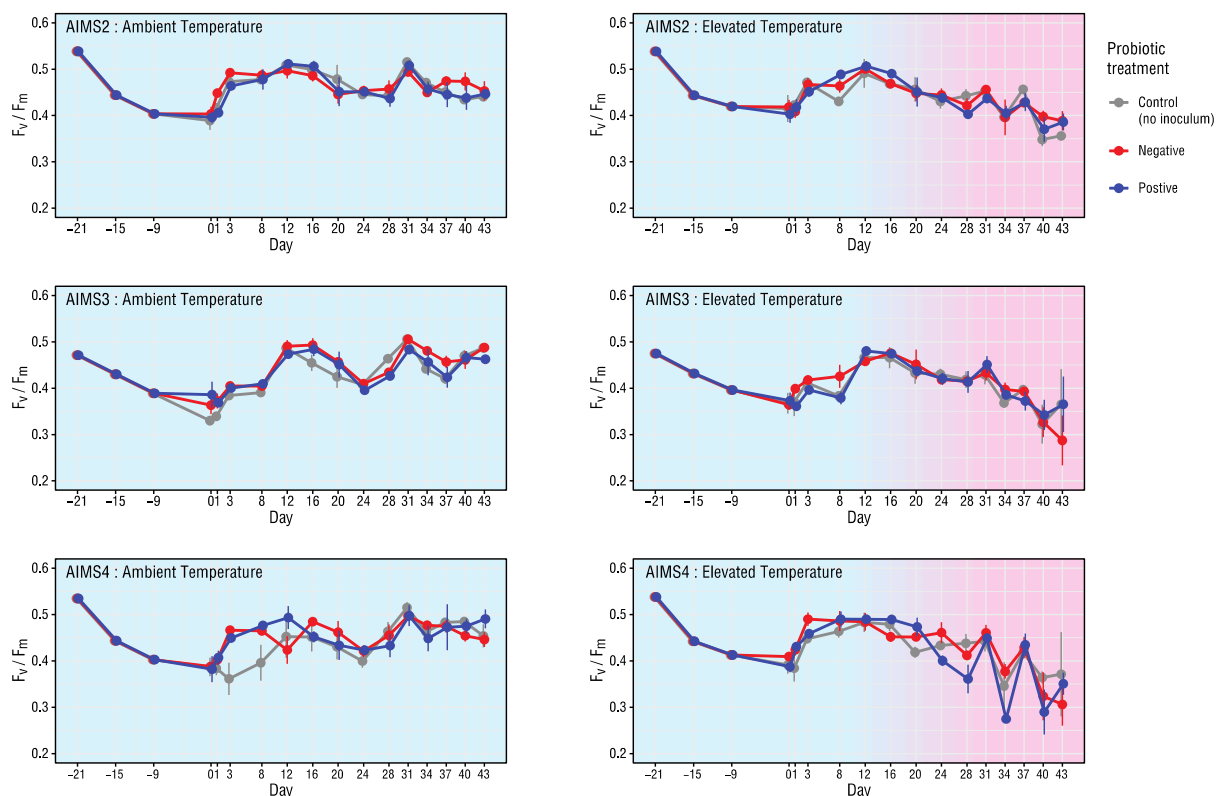


Figure S4-1 F_v/F_m at ambient (26°C) and elevated (26°C–31.5°C) temperature for all treatments for the three genotypes. Probiotic inoculations were performed on Days 0, 1 and 7. For each datapoint, $n=3$. Error bars ± 1 SEM.

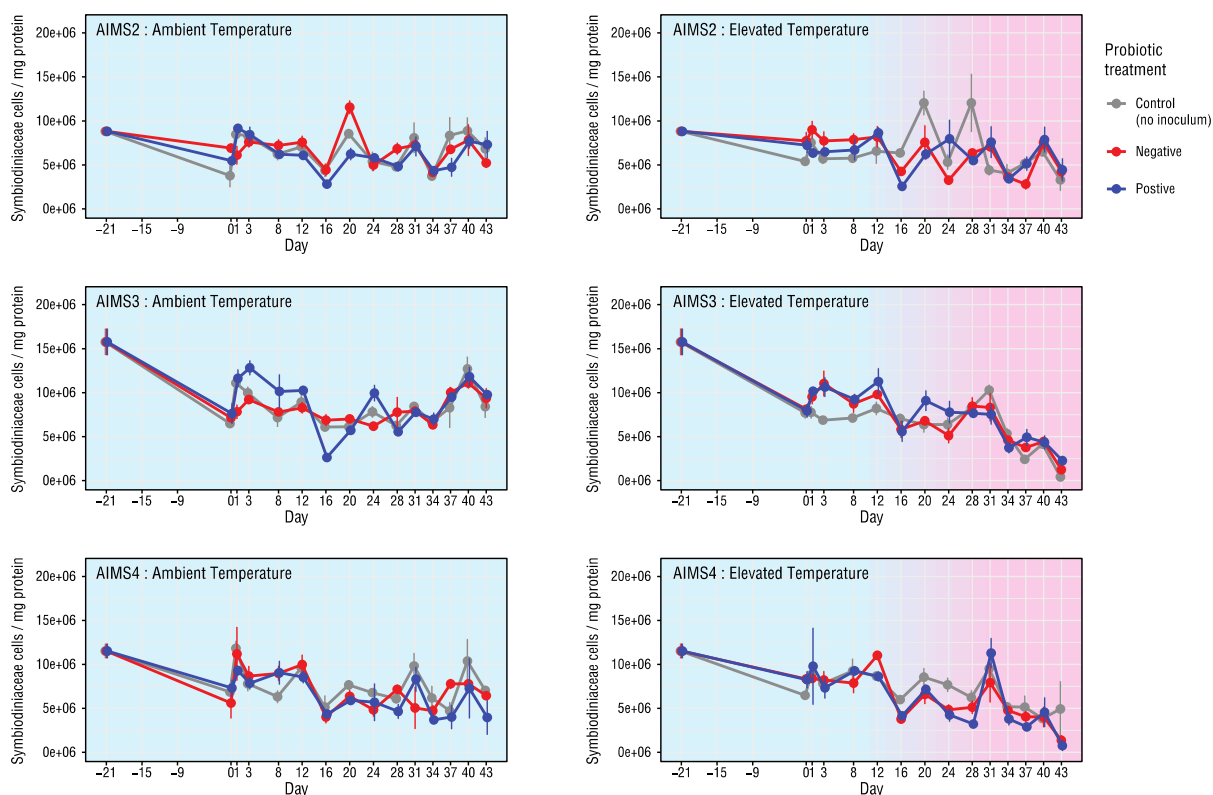


Figure S4-2 Symbiodiniaceae cell density at ambient (26°C) and elevated (26°C–31.5°C) temperature for all experimental treatments and all three genotypes. Probiotic inoculations were performed at Days 0, 1 and 7. For each datapoint, $n=3$. Error bars ± 1 SEM.

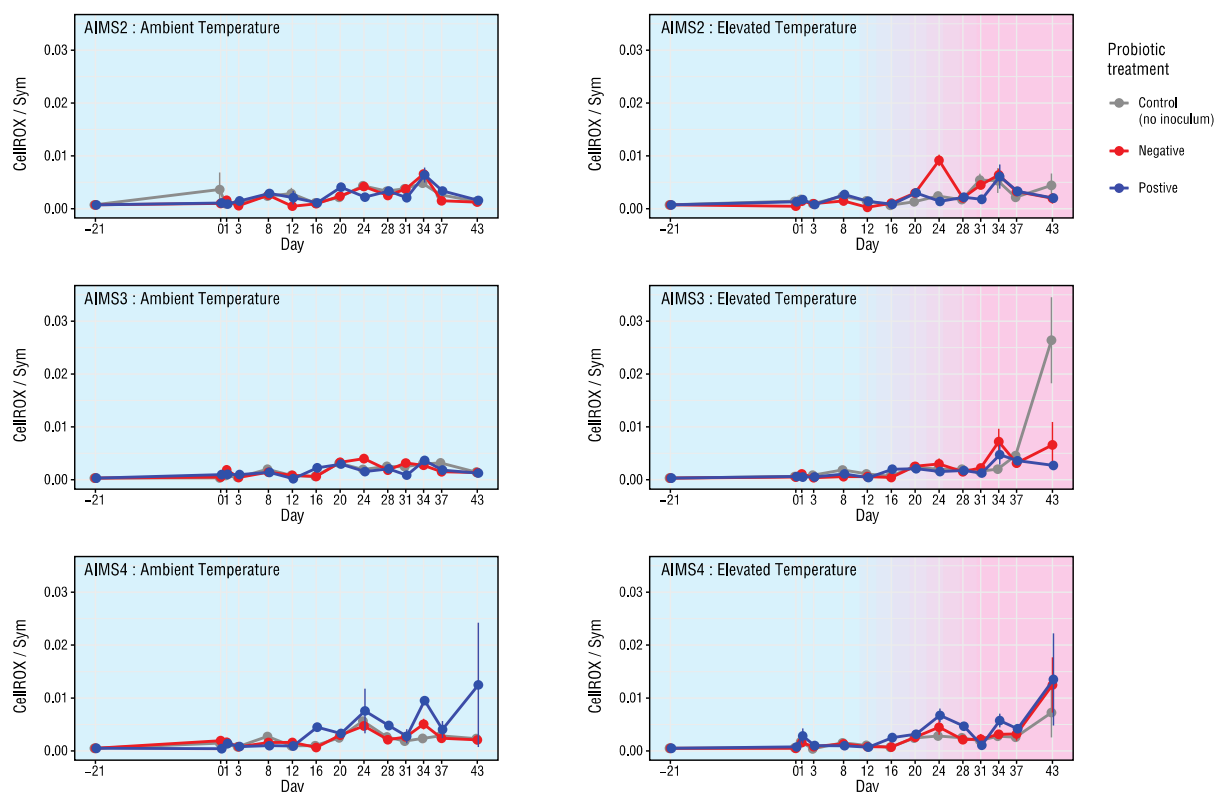


Figure S4-3 ROS levels at ambient (26°C) and elevated (26°C–31.5°C) temperature for all treatments within each genotype. Probiotic inoculations were performed at Days 0, 1 and 7. For each datapoint, $n=3$. Error bars ± 1 SEM.

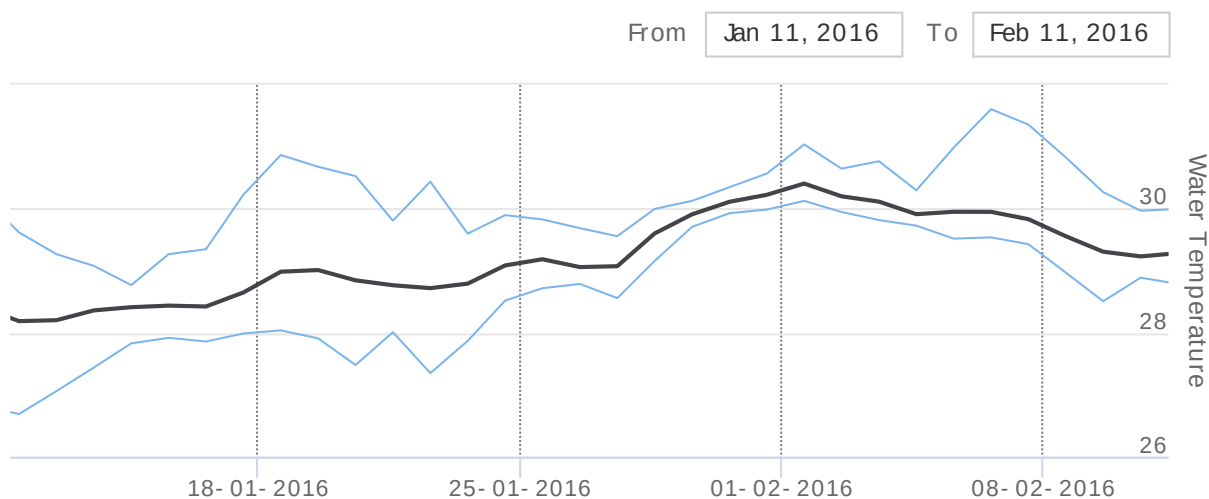


Figure S4-4 Water temperature daily averages (2 m depth) from St Crispin Reef (AIMS 2019). The graph is illustrative for summer heatwave conditions and shows an increase in SST for a northern part of the GBR in summer 2016; a year that saw “unprecedented” mass coral bleaching (Hughes et al., 2017b).

Chapter 5

Lack of evidence for the oxidative stress theory of bleaching in the sea anemone, *Exaiptasia diaphana*

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5.1 Abstract

Nearly half of all living coral has died in the past decade as a result of climate change-driven bleaching. Some of the most compelling evidence in support of the 'oxidative stress theory of coral bleaching' comes from three studies that expose corals, cultures of their algal endosymbionts, or the coral model *Exaiptasia diaphana* to exogenous antioxidants during thermal stress. Here, we replicate these experiments using *E. diaphana* with the addition of mannitol, ascorbate plus catalase, or catechin under ambient and elevated temperatures along with an antioxidant-free control. In the absence of exogenous antioxidants, *E. diaphana* exposed to elevated temperatures for a week experienced bleaching quantified by a significant decrease in Symbiodiniaceae cell density and a reduction in photosynthetic efficiency, but did not show a measurable increase in reactive oxygen species (ROS). Compared to ambient temperature, ROS levels increased for mannitol and ascorbate plus catalase treated anemones under elevated conditions. However, these antioxidant treatments rescued the anemones from bleaching. Further, both mannitol and catechin addition led to significantly increased photosynthetic efficiency under elevated temperatures compared to the no antioxidant controlled. Despite a promising effect of mitigating bleaching, the combination of ascorbate and catalase caused mortality in the anemones with an associated increase in bacteria of the *Vibrio* genus as detected by 16S rRNA gene metabarcoding. This study challenges the oxidative stress theory of bleaching for *E. diaphana* as bleaching was associated with no changes in net ROS, whereas anemones treated with antioxidants and rescued from bleaching, had a significant increase in ROS.

5.2 Introduction

The severe loss of coral cover and decline of coral reefs over the past several decades is primarily driven by climate change-related increases in sea surface temperature (SST)

(Hughes et al., 2017b; Stuart-Smith et al., 2018) . A spike in SST during unusually hot summers can trigger coral bleaching (Hughes et al., 2018a; Hoegh-Guldberg et al., 2019), the dissociation of the coral host and its endosymbiotic, dinoflagellate algae (Symbiodiniaceae). Mass bleaching occurred on the Great Barrier Reef (GBR) in 1998, 2002, 2016, 2017, and 2020, and caused extensive coral mortality, with the 2016 and 2020 events having been the most severe and widespread recorded (Hughes and Pratchett, 2020).

There are several hypotheses detailing the mechanisms driving bleaching (Weis, 2008; Cunning and Baker, 2012; Wiedenmann et al., 2012; Wooldridge, 2013), with a common theme being the overproduction and toxic accumulation of reactive oxygen species (ROS) from the algal symbiont. Experiments and observations have developed and refined the “oxidative stress theory of coral bleaching,” (Downs et al., 2002; Cziesselski et al., 2019), whereby accumulation of ROS lead to signalling cascades and in turn expulsion or xenophagy of Symbiodiniaceae from the coral host (Smith et al., 2005; Weis, 2008; Davy et al., 2012; Suggett and Smith, 2019).

ROS are a normal product of photosynthesis; the light triggered splitting of water molecules in the oxygen evolving complex (OEC) and subsequent transfer of electrons from photosystem II (PSII) to photosystem I (PSI) generates ROS in chloroplasts (Trubitsin et al., 2014; Szabó et al., 2020). Singlet oxygen ($^1\text{O}_2$) can be formed via energy transfer when ground-state molecular oxygen interacts with chlorophyll triplets. This ROS is produced either in the reaction centre of the PSII complex or in the light-harvesting antenna, and reacts with proteins, lipids and pigments to induce photo-oxidative damage to the photosynthetic apparatus (Krieger-Liszkay, 2005). Rehman et al. (2016) documented the light-dependent intracellular production of $^1\text{O}_2$ in Symbiodiniaceae cultures. The major site of superoxide ($\text{O}_2^{\bullet-}$) production is the thylakoid membrane-bound primary electron acceptor of PSI (Szabó et al., 2020). This

$O_2^{\bullet-}$ is further converted to hydrogen peroxide (H_2O_2) within the chloroplast mostly by superoxide dismutase (SOD) (Asada, 1987). Hydroxyl radicals ($OH^{\bullet}$) are produced in the Haber-Weiss or Fenton reactions through the interaction of H_2O_2 and $O_2^{\bullet-}$ or directly from H_2O_2 in the presence of transition metals (Halliwell and Gutteridge, 1990).

Once generated, ROS can trigger the oxidation of essential photosynthetic molecules, such as the D1 protein in PSII (Lesser, 1996; Wang et al., 2011; Mathur et al., 2014), thylakoid membranes (Tchernov et al., 2004; Roberty et al., 2016), and enzymes of the Calvin-Benson cycle (Asada, 1987; Lesser and Farrell, 2004), thereby interfering with the supply of fixed carbon to the holobiont (Lesser, 2004). Increased abundance of ROS in the chloroplast reduces the biosynthesis of chlorophyll (Takahashi et al., 2008; Roberty et al., 2016) and decreases repair rates of the D1 protein (Nishiyama et al., 2006; Schmitt et al., 2014).

If not scavenged by the antioxidant network (e.g. Levin et al., 2016), the damage caused by ROS to the photosynthetic apparatus may trigger the signalling cascade ending with the disruption of the symbiosis (Weis, 2008). Thus, a reduction in photosynthetic capacity (as quantified by chlorophyll *a* content) (Fitt et al., 2001; Takahashi et al., 2004; Venn et al., 2006) or a reduction in photosynthetic efficiency (Takahashi et al., 2004; Bouchard and Yamasaki, 2008), which is most often tracked as the maximum quantum yield (F_v/F_m) (Voolstra et al., 2020), can serve as precursors to bleaching. Because intracellular Symbiodiniaceae translocate photosynthetically fixed carbon to the coral host (Muscatine and Porter, 1977; Tremblay et al., 2013), any one of these measurable changes in the Symbiodiniaceae community may be detrimental to the coral holobiont. Many innovative approaches have been proposed to mitigate bleaching and enhance coral survival during thermal stress (van Oppen et al., 2015; Damjanovic et al., 2017; Peixoto et al., 2017; Dungan et al., 2020a). Directly targeting ROS has been investigated, with promising but limited evidence that exposure to exogenous antioxidants can

reduce host ROS levels and bleaching in thermally stressed corals and coral models (Lesser, 1997; Marty-Rivera et al., 2018). In addition, the photosynthetic performance of thermally stressed Symbiodiniaceae has been shown to improve and net ROS to decrease within hours of receiving a single antioxidant dose (Lesser, 1996; Motone et al., 2020).

In this study, we applied three exogenous antioxidant treatments (ascorbate plus catalase, catechin, and mannitol) to assess their effect on bleaching and net ROS, compared to a no antioxidant control, in the coral model, the sea anemone *Exaiptasia diaphana*. We define net ROS as the difference between ROS produced and ROS scavenged by enzymatic and non-enzymatic antioxidants. Anemones were grown at ambient (26°C) and elevated (ramped from 26°C to 34°C) temperatures with bleaching tracked through Symbiodiniaceae cell densities, photosynthetic efficiency (F_v/F_m), and chlorophyll *a* (chl*a*) content of Symbiodiniaceae cells. Net ROS was quantified using a fluorescent stain that is activated when oxidized and quantified using flow cytometry. Visual health scores were paired with bacterial community composition to track changes in holobiont health associated with antioxidant dosing. This experiment allowed us to ask the following questions:

- 1.) Is thermal stress-induced bleaching, as defined by the loss of Symbiodiniaceae cells, in *E. diaphana* accompanied by elevated ROS levels?
- 2.) Can exogenous antioxidants mitigate thermal stress induced bleaching?
- 3.) If bleaching is mitigated by antioxidant addition, is this associated with a decrease in net ROS?
- 4.) Do exogenous antioxidants cause harm to the holobiont?

5.3 Methods

5.3.1 Experimental set-up

Anemones from Great Barrier Reef (GBR)-sourced *E. diaphana* genotype AIMS3 were randomly selected from The University of Melbourne culture collection (n=256) (Dungan et al., 2020b). Anemones were equally distributed among 12 × 300 mL lidded glass culture jars and placed in an experimental incubator (Model No. LE-509, Thermoline Scientific, Australia) fitted with white light emitting diode (LED) lights (15–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) on a 12 h:12 h light:dark cycle. During acclimation (11 weeks), anemones were maintained in reconstituted Red Sea Salt™ seawater (RSS, R11065, Red Sea, USA) at ~34 parts per thousand (ppt) salinity and fed *ad libitum* twice weekly with freshly hatched *Artemia salina* (Salt Creek, Premium GSL, USA). Jars were cleaned 3–5 h after feeding by loosening algal debris with RSS pressure applied through sterile plastic pipettes followed by full RSS changes. Anemones were considered acclimated once their photosynthetic efficiency (F_v/F_m), as measured by pulse-amplitude-modulated (PAM) fluorometry with an imaging-PAM system (IMAG-MAX/L, Walz Heinz, Germany), stably returned to levels found in the stock culture collection. Seawater temperatures were monitored using submersible data loggers (Hobo UA-001-08) (Fig. S5-1).

Three exogenous antioxidant treatments were assessed for their effect on bleaching and net ROS, compared to a no antioxidant control (Table 5-1), with anemones grown at normal (26°C) and elevated (ramped from 26°C to 34°C) temperatures (Fig. 5-1). Stock antioxidants were made with fRSS at 100x the target concentration and stored at -20°C in several aliquots. On the day of dosing, thawed antioxidant aliquots were diluted in fRSS to the target concentration.

The day prior to beginning the experiment, anemones were transferred from the lidded glass jars to sterile 12 well plates in 0.2 μm -filtered RSS (fRSS); there were eight

anemones per plate (one anemone per well) in a random arrangement for a total of 32 plates (4 plates per antioxidant/temperature combination). Light levels remained at 15-20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ on a 12 h:12 h light:dark cycle. In the elevated temperature treatment, anemones at 26°C were ramped up by 1°C each day, at 11 h into the light cycle. By Day 6, elevated temperature treatments reached 32°C, on Day 10 the temperature was increased to 33°C, then to 34°C on Day 11. Starting on Day 0, antioxidant-treated anemones were immersed in respective antioxidant treatments. fRSS plus antioxidant changes were completed every second day. Anemone plates were randomly rearranged each day inside the incubator to remove plate position as a confounding factor.

Table 5-1 Antioxidants evaluated with anemones at normal (26°C) and elevated (34°C) temperatures. All reagents were purchased from Sigma Aldrich

Antioxidant	Target Concentration	ROS Target	Catalog Number	References
Catechin	5 μM	Singlet oxygen, hydrogen peroxide	43412	(Marty-Rivera et al., 2018)
Ascorbate plus catalase	125 μM 250 U mL^{-1}	Hydroxyl radicals, hydrogen peroxide	A4403, C1345	(Lesser, 1996; 1997)
Mannitol	10 mM	Hydroxyl radicals	M4125	(Lesser, 1997)

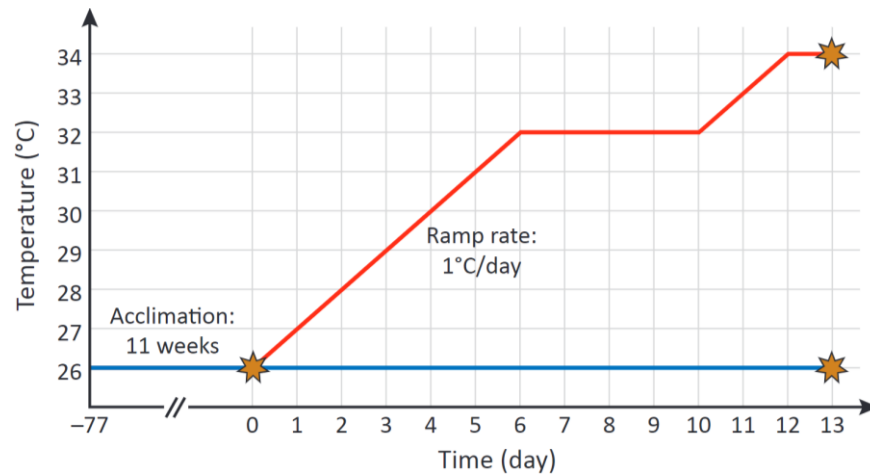


Figure 5-1 Antioxidant dosing and sampling schedule for each antioxidant-temperature combination. Temperature conditions: ambient (26°C, blue), and elevated (26°C–34°C, red). Antioxidant water changes were completed every second day for the duration of the experiment, beginning on Day 0. Stars indicate collection of five anemones from each treatment for Symbiodiniaceae cell counts and ROS quantification and three anemones from each treatment for metabarcoding. An additional eight anemones were collected on Day 13 for Symbiodiniaceae cell density determination. Health scoring and imaging PAM were carried out every day at approximately five h into the light cycle. Total anemone samples, $n=256$.

5.3.2 Symbiodiniaceae photosynthetic efficiency measurement

Photosynthetic efficiency (F_v/F_m) of PSII in Symbiodiniaceae is a commonly used proxy for general holobiont health as lowered F_v/F_m indicates PSII damage (Ralph et al., 2016). F_v/F_m was measured by imaging-PAM fluorometry of the anemone bodies and proximal tentacles at 5 h into the light cycle and after 30 min of dark adaptation. PAM settings were saturating pulse intensity 10, measuring light intensity 3 (frequency 2), actinic light intensity 8, damping 2, and gain 2.

5.3.3 Anemone tissue processing

On Day 0, five anemones were sacrificed for Symbiodiniaceae cell density, host protein quantification, *chl a* determination, and ROS quantification, while three anemones per treatment were flash frozen in liquid nitrogen and stored at -20°C for future DNA

extraction for Symbiodiniaceae and bacterial community composition analysis to document initial control conditions. On Day 13, five anemones per treatment were sacrificed for *chl a* determination and ROS quantification, while 13 anemones in each treatment were used to measure Symbiodiniaceae cell counts and host protein concentrations. An additional three anemones per treatment were flash frozen in liquid nitrogen and stored at -20°C for Symbiodiniaceae and bacterial metabarcoding. Anemones were individually homogenized in a sterile glass homogenizer in 1 mL of treatment fRSS from their well. Homogenate for *chl a* determination and ROS quantification was processed immediately, while aliquots for Symbiodiniaceae cell density and host protein measurement were stored at -20°C until quantification.

5.3.4 Symbiodiniaceae cell density and host protein analysis

The -20°C preserved homogenate was centrifuged at 5000 × *g* for 5 min at 4°C to pellet the Symbiodiniaceae, and 100 µL of the supernatant collected for host protein analysis. Pelleted Symbiodiniaceae were washed twice with 200 µL fRSS and centrifuged at 5000 × *g* for 5 min at 4°C, then resuspended in 200 µL fRSS. Triplicate cell counts (cells mL⁻¹) were calculated with an automated cell counter (Countess II FL, Life Technologies, Australia) from 10 µL of sample. Cell counts were normalized to host protein (mg mL⁻¹) determined by the Bradford assay (Bradford, 1976). Each protein sample was assayed in triplicate and measured at 595 nm (EnSpire MLD2300 plate-reader, Perkin Elmer, Australia) against bovine serum albumin standards (500-0207, Bio-Rad, Australia).

5.3.5 Chlorophyll *a* and ROS quantification

Anemone ROS levels were quantified with CellROX® Orange (C10443, Thermo Fisher, Australia) (Marty-Rivera et al., 2018). A volume of 4 µL CellROX® Orange (stock at 500 µM in dimethyl sulfoxide (DMSO)) was added to 400 µL of the sample and 4 µL of DMSO was added to the remaining 400 µL as a control. Both were incubated at their

experimental temperatures for 60 min. Samples were filtered through a 40 μm mesh and vortexed before processing with a CytoFLEX LX flow cytometer (Beckman Coulter) at a speed of 10 $\mu\text{L min}^{-1}$. Autofluorescent events strongly excited by the 488 nm laser at emissions 525 ± 20 nm and 690 ± 25 nm were used to gate the Symbiodiniaceae cells and separate them from anemone debris. A forward scatter-height/side scatter-height (FSC-H/SSC-H) plot was then used to select homogenous cells, and singlets were gated on a FSC-H/FSC-Area plot. The CellROX® Orange signal was quantified on singlets gated on previous plots with 561 nm excitation (emission 585 ± 42 nm) (Fig. S5-2). At least 5000 Symbiodiniaceae singlets per sample were processed, except in samples where Symbiodiniaceae density was low because of advanced bleaching, where between 700 and 2000 singlets per sample were processed. For each sample, the ratio between the median CellROX® Orange signal of the stained sample and that of the corresponding unstained sample was calculated. This calculation allowed accounting for Symbiodiniaceae's autofluorescence; a higher ratio indicated a higher quantity of ROS in each sample. *Chl a* was also quantified from the gated singlets using 488 nm excitation (emission 690 ± 50 nm) (Bouchard and Yamasaki, 2008). Raw signal intensity, due to *chl a* autofluorescence was recorded in control samples that contained DMSO.

To confirm the ROS quantification results determined by flow cytometry, stained and unstained samples used for flow cytometry were pipetted onto poly-L-lysine (0.1% w v⁻¹ in Milli-Q water) coated coverslip-bottom well slides (Ibidi). Slides were then visualized on an inverted confocal microscope (Nikon A1R) by excitation at 561 nm (emission 575-620 nm).

5.3.6 Symbiodiniaceae community structure

DNA was extracted (38,39) from -20°C stored anemone tissue samples (n=27) to characterize the host-associated Symbiodiniaceae community by targeting the internal

transcribed spacer 2 (ITS2) region. Amplification by PCR was in triplicate using Symbiodiniaceae primers with Illumina Nextera adapters: Sym_Var_5.8S2 [5' GTGACCTATGAACTCAGGAGTCGAATTGCAGAACTCCGTGAACC 3'] (Hume et al., 2015); Sym_Var_Rev [3' CTGAGACTTGACATCGCAGCCGGGTTCTTGTGTGACTTCATGC 5'] (Hume et al., 2013). Each PCR contained 12.5 μ L MyTaq HS Mix polymerase (Bioline, Australia), 2 μ L of DNA template, 0.4 μ M of each primer, and nuclease-free water up to 25 μ L. PCR conditions were: 1 cycle $\times$ 95°C for 3 min; 18 cycles $\times$ 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle $\times$ 72°C for 7 min; hold at 4°C.

To prepare sequencing libraries, 20 μ L of each PCR product pool was purified by size-selection using Ampure XP magnetic beads (Agencourt, Beckman Coulter, Australia). The purified DNA was resuspended in 40 μ L of nuclease-free water. Indexing PCRs were created by combining 10 μ L of DNA suspension with 10 μ L 2x Taq master mix (M0270S, New England BioLabs, Australia) and 0.25 μ M of forward and reverse indexing primers. PCR conditions were: 1 cycle $\times$ 95°C for 3 min; 24 cycles $\times$ 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; 1 cycle $\times$ 72°C for 7 min; hold 4°C. Samples were checked for product size and quantity (2200 TapeStation, Agilent Technologies, Australia). Sequencing libraries were created by pooling 5 μ L from each reaction and performing a final bead clean-up on 50 μ L. Each library was checked for quality and quantity to guide pool normalization, then sequenced on a single Illumina MiSeq run using v3 (2 $\times$ 300 bp) reagents at the Walter and Eliza Hall Institute (WEHI), Melbourne, Australia.

5.3.7 Anemone health

Anemones were assessed for overall health via a qualitative health score (Fig. 5-2) daily, immediately following imaging-PAM fluorometry. Given the connection of bacterial community structure and stability with anemone health, we evaluated the influence of antioxidant treatment on bacterial community composition. DNA was extracted as

described above from *E. diaphana* (n=27), a bacterial mock community (MC) (n=1), and extraction blank controls (n=3), using bacterial primers targeting the V5-V6 regions of the bacterial 16S rRNA genes: 784F [5' GTGACCTATGAACTCAGGAGTCAGGATTAGAT-ACCCTGGTA 3']; 1061R [5' CTGAGACTTGACATCGCAGCCRRACGAGCTGACGAC 3'] (Andersson et al., 2008). Again, sequencing libraries were prepared from pooled PCR products, with the addition of three PCR blank controls. Each library was checked for quality and quantity to guide pool normalization, then sequenced on a single Illumina MiSeq run using v3 (2 × 300 bp) reagents at WEHI.

5.3.8 Metabarcoding data processing

Raw Symbiodiniaceae sequences for each sample (n=27) were processed using the SymPortal analytical framework (symportal.org) (Hume et al., 2019). Sequencing information was submitted to the SymPortal remote database and underwent quality control including the removal of artefact and non-Symbiodiniaceae sequences. Within-sample informative intragenomic sequences, referred to as 'defining intragenomic variants' (DIVs) were used to identify ITS2-type profiles of putative Symbiodiniaceae taxa (Hume et al., 2019). ITS2 type profiles were designated by SymPortal based on the presence and abundance of the ITS2 sequences in our samples and within the SymPortal database.

Raw 16S rRNA gene sequences were imported into QIIME2 v2019.10.0 (Bolyen et al., 2019) and demultiplexed. Primers were removed with cutadapt v2.6 (Martin, 2011). Filtering, denoising, and chimera checking was performed using DADA2 in the QIIME2 environment to correct sequencing errors, remove low quality bases (mean Qscore <30), and generate bacterial amplicon sequence variants (ASVs). Taxonomy for each ASV was assigned against a SILVA database (version 132) trained with a naïve Bayes classifier against the same V5-V6 region targeted for sequencing (Bokulich et al., 2018).

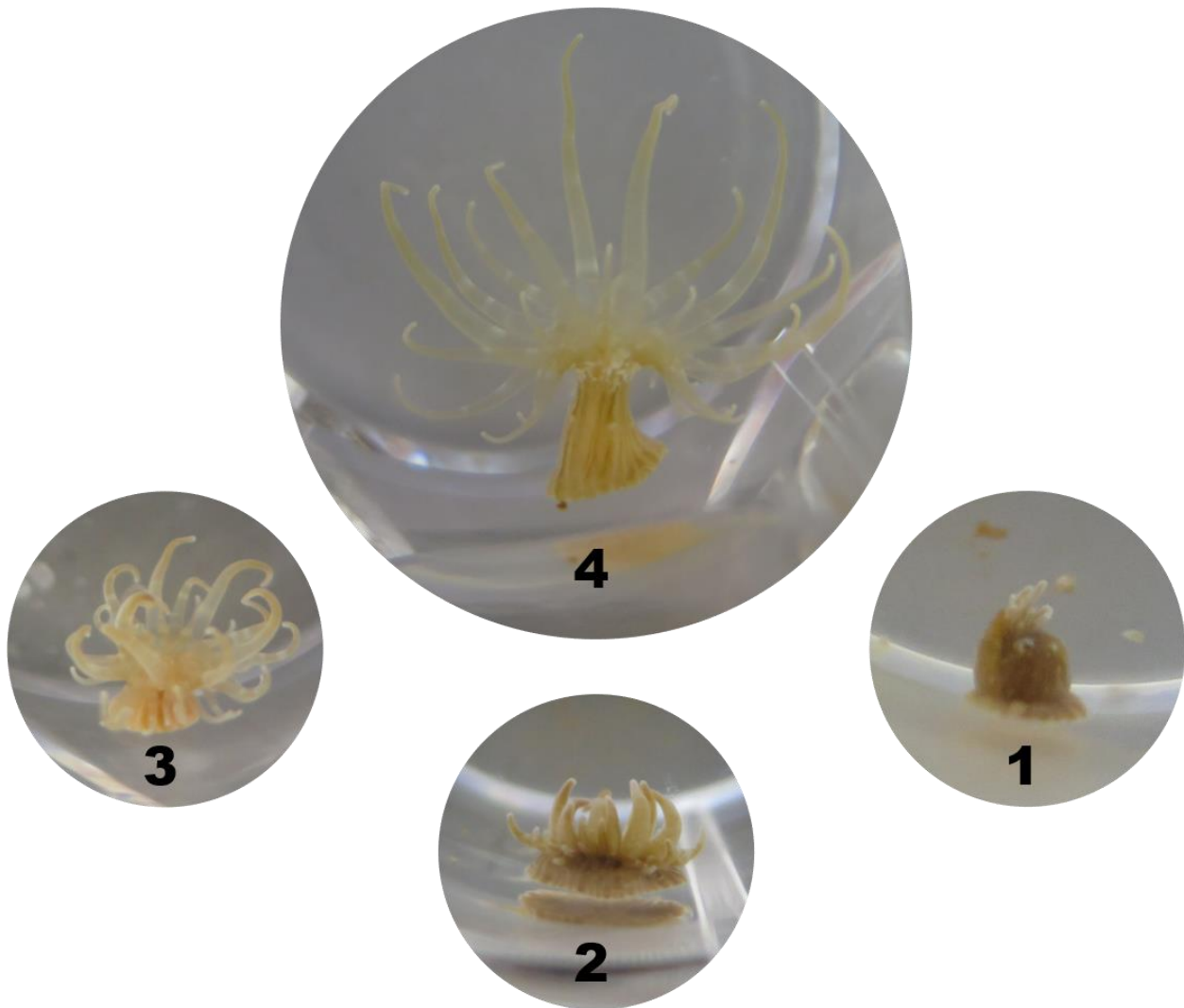


Figure 5-2 Health of anemones was visually scored on a scale from 4 to 0. Anemones with a score 4 (top) had fully extended bodies and tentacles, were actively feeding and responsive to water movement; anemones with a score of 3 (left) had curled tentacles and a partially withdrawn body but were still actively feeding and responding to water movement; anemones with a score of 2 (bottom) had fully retracted bodies with shortened tentacles and showed reduced activity in response to water movement and reduced feeding; anemones that had retracted their tentacles into their bodies and/or were not responding to water movement and feeding, but were not dead, were given a health score of 1 (right); and a score of 0 was a dead anemone. No scale bars.

5.3.9 Data analysis

All data were analysed in R v3.6.2 (R Core Team, 2018). Statistical tests were considered significant at $\alpha = 0.05$. ASV, taxonomy, metadata and phylogenetic tree files were imported into R and combined into a phyloseq object (McMurdie and Holmes, 2013). Potential contaminant ASVs were identified and removed from the dataset according to their abundance in the extraction and PCR negative controls relative to the anemone samples using the prevalence method in the R package decontam with $p=0.1$ (Davis et al., 2018). Dark-adapted F_v/F_m , Symbiodiniaceae cell density, *chl a* fluorescence, and ROS measurements were plotted using the R package ggplot2 with data separated by temperature condition. The data were analysed for overall differences using linear models against the variables 'antioxidant', 'temperature', and 'time' in the R package nlme (Pinheiro et al., 2019). *Post hoc* pairwise comparisons were performed using Tukey's honestly significant difference test in the R package emmeans with Tukey's adjustment for multiple comparisons. Shifts in the bacterial community compositions were visualised in principal coordinate analysis (PCoA) ordinations using a weighted UniFrac distance matrix. The influence of time, antioxidant and temperature treatments on changes in community composition within each genotype was investigated by comparison of generalised linear models (GLM) for each ASV using likelihood ratio tests across 999 iterations in the R package mvabund (Wang et al., 2012). ASVs that were significantly associated with treatment groups were identified using the indicpecies function multipatt and changes in their relative abundance were visualised in bubble plots for each treatment on Day 13.

5.4 Results

5.4.1 Anemone health response to antioxidants

Anemone health was tracked over time (Fig. S5-3) using the qualitative health score system. By Day 7, anemones in the ascorbate+catalase treatment, regardless of temperature condition, showed a drastic decline in health. The health score rating for these two groups continued to decline until the conclusion of the experiment. By Day 11, the elevated temperature anemones displayed a reduced health score compared to the ambient anemones for each antioxidant treatment. This difference was subtle, however, for all but the ascorbate+catalase treatment anemones.

Holobiont health was also assessed with bacterial community analyses. Sequencing of the V5-V6 region of the bacterial 16S rRNA gene produced 3.29 M reads across *E. diaphana* (n=27), MC (n=1), and controls (n=6). After merging, denoising and chimera filtering, 2.37 M reads remained; one control sample had zero reads after quality control and was thus removed. Decontam identified eight putative contaminant ASVs which made up 0.10% of the total reads; these were removed from the data set prior to all statistical analyses (Table S5-1). After all filtering steps, there were 1288 ASVs across the remaining 74 samples. Results from the MC sample (Fig. S5-4) validated the sequencing run.

A two-way analysis of variance (ANOVA) reported a significant interaction of antioxidant and temperature for all alpha diversity measures after thirteen days in experimental conditions ($F_{\text{ObsASVs}(3,16)}=4.01$, $p=0.026$; $F_{\text{Simp}(3,16)}=11.94$, $p=0.0002$; $F_{\text{Shan}(3,16)}=8.98$, $p=0.001$). This interaction was driven largely by anemones in the ascorbate+catalase treatment, whereby this antioxidant dosage led to a substantial decrease in bacterial alpha diversity and this effect was compounded by increased temperature (S5-5, Table S5-2). PCoA showed a shift in bacterial composition, particularly for anemones treated

with ascorbate+catalase, which was also the only antioxidant treatment to show separation by temperature condition (Figure A). GLM results confirm that the bacterial community structure (Fig. 5-3B) varied significantly with the interaction of antioxidant and temperature treatments (LRT=1468, $p=0.002$). Pairwise comparisons between ambient and elevated temperature anemones for each antioxidant treatment revealed a significant change in bacterial community composition for ascorbate+catalase (LRT=590, $p=0.044$), with no significant differences for the other antioxidant treatments or control group. The indicator species analysis provided 56 ASVs that were characteristic of the microbiomes of the different treatment conditions (Fig. S5-6, Table S5-3), Anemones in the ascorbate+catalase treatment had an increase in the abundance of Halomonadaceae and Vibrionaceae while losing Rhodobacteraceae and Crocinitomicaceae, with ASV017, a *Vibrio* sp., identified as an indicator member for both ambient and elevated ascorbate+catalase treated anemones.

5.4.2 Symbiodiniaceae communities

Sequencing of the Symbiodiniaceae ITS2 region produced 318,936 reads across 27 *E. diaphana* samples ($n=3$ for Day 0, $n=24$ for Day 13, minimum 4,077; mean 11,812, maximum 14,993 reads per sample). After quality control, 196,113 reads remained (minimum 948, mean 7,263, maximum 9,970 reads per sample). All anemones hosted *Breviolum minutum*, corresponding with previously reported GBR-sourced *E. diaphana* (Tortorelli et al., 2019; Dungan et al., 2020b). Four ITS2 profiles were present in the dataset: B1-B1a-B1b ($n=16$), B1-B1a-B1b-B1ap ($n=5$), B1-B1a-B1b-B1g ($n=3$), and B1-B1o-B1p ($n=3$).

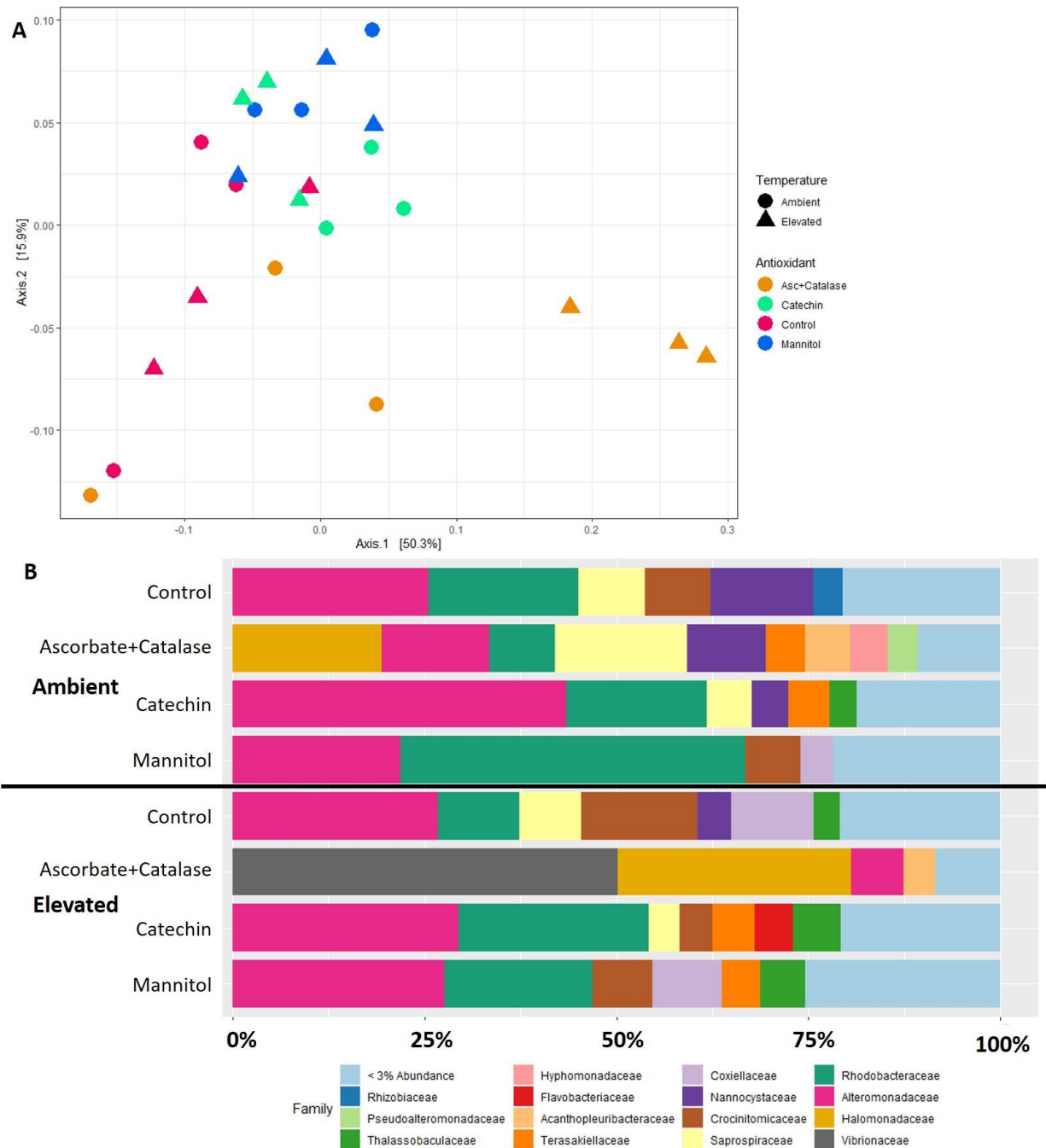


Figure 5-3 Principle coordinate analysis (A) and relative abundance of bacterial families (B) for AIMS3 anemones after two weeks in experimental conditions. In the PCoA, ambient (circles) or elevated (triangles) temperature regimes are visualized across control (purple), ascorbate+catalase (orange), catechin (green), and mannitol (blue) antioxidant treatments. The ordination was plotted based on a weighted UniFrac distance matrix. In the bar plot, ambient temperature anemones are on the top and elevated temperature anemones are on the bottom. Families whose relative abundance was less than 3% across the whole data set were pooled into a single category. $n=3$ for each treatment.

5.4.3 Bleaching and photosynthetic health

On Day 13, an analysis of variance revealed a significant effect of temperature ($F_{(1,86)}=25.78$, $p<0.0001$) on Symbiodiniaceae density. Pairwise comparisons revealed a significant reduction in Symbiodiniaceae cell density between ambient and elevated temperature for the control anemones ($t_{(86)}=4.27$, $p=0.0001$), thus confirming that elevated temperature induced bleaching. Catechin treated anemones also experienced a significant decline in Symbiodiniaceae cell density from ambient to elevated temperature ($t_{(86)}=2.53$, $p=0.0134$), with no significant changes for ascorbate+catalase or mannitol treated anemones between ambient and elevated temperatures (Fig. 5-4A).

Changes in photosynthetic efficiency were tracked each day for the duration of the experiment (Fig. S5-7). A two-way analysis of variance revealed a significant interaction of antioxidant dose and temperature regime on F_v/F_m on Day 13 ($F_{(3,142)}=5.46$, $p=0.0012$). Pairwise comparisons show that, for each antioxidant treatment, anemones in the ambient temperature condition had a significantly higher F_v/F_m compared to those experiencing elevated temperature, with no significant difference between antioxidant treatments under ambient temperature conditions (Table S5-4, Table S5-5). Under elevated conditions, however, mannitol and catechin treatments resulted in a significantly higher F_v/F_m on Day 13 compared to the no antioxidant controls (Fig. 5-4B).

Chl a fluorescence was quantified at Day 13 by the median emission at 690 nm of Symbiodiniaceae cells passing through the flow cytometer when excited with a 488 nm laser. There was a significant interaction of antioxidant treatment and temperature on chl a fluorescence ($F_{(3,32)}=4.42$, $p=0.0104$) (Figure 5-4C). Only ascorbate+catalase treated anemones had a significant response to temperature; in this antioxidant treatment, anemones in the ambient treatment had a significantly higher chl a fluorescence compared to the elevated temperature condition ($t_{(32)}=4.066$, $p=0.0003$). In both

ambient and elevated conditions, anemones in the ascorbate+catalase treatment had significantly higher *chl a* fluorescence compared to all other antioxidant treatments ($p < 0.0001$).

5.4.4 Net ROS

Prior to running samples through the flow cytometer, samples were visualized using confocal microscopy, confirming that the presence of ROS would increase the CellROX® Orange signal (Fig. S5-8). On Day 13, net ROS varied significantly with the interaction of antioxidant treatment and temperature ($F_{(3,32)} = 9.52$, $p = 0.0001$) (Fig. 5-4D). At elevated temperature, ascorbate+catalase, catechin, and mannitol treated anemones had higher CellROX® Orange staining compared to ambient ($t_{\text{asc+cat}(32)} = 7.75$, $p < 0.0001$; $t_{\text{cat}(32)} = 2.43$, $p = 0.0211$; $t_{\text{man}(32)} = 6.43$, $p < 0.0001$). There were no significant differences, however, in net ROS between ambient and elevated temperature for the control antioxidant treatment. Under elevated temperature conditions both ascorbate+catalase and mannitol treated anemones had significantly increased CellROX® Orange signal compared to the no antioxidant controls ($t_{\text{asc+cat}(32)} = 5.23$, $p = 0.0001$; $t_{\text{man}(32)} = 5.44$, $p < 0.0001$), with no differences in net ROS at ambient temperature between antioxidant treatments.

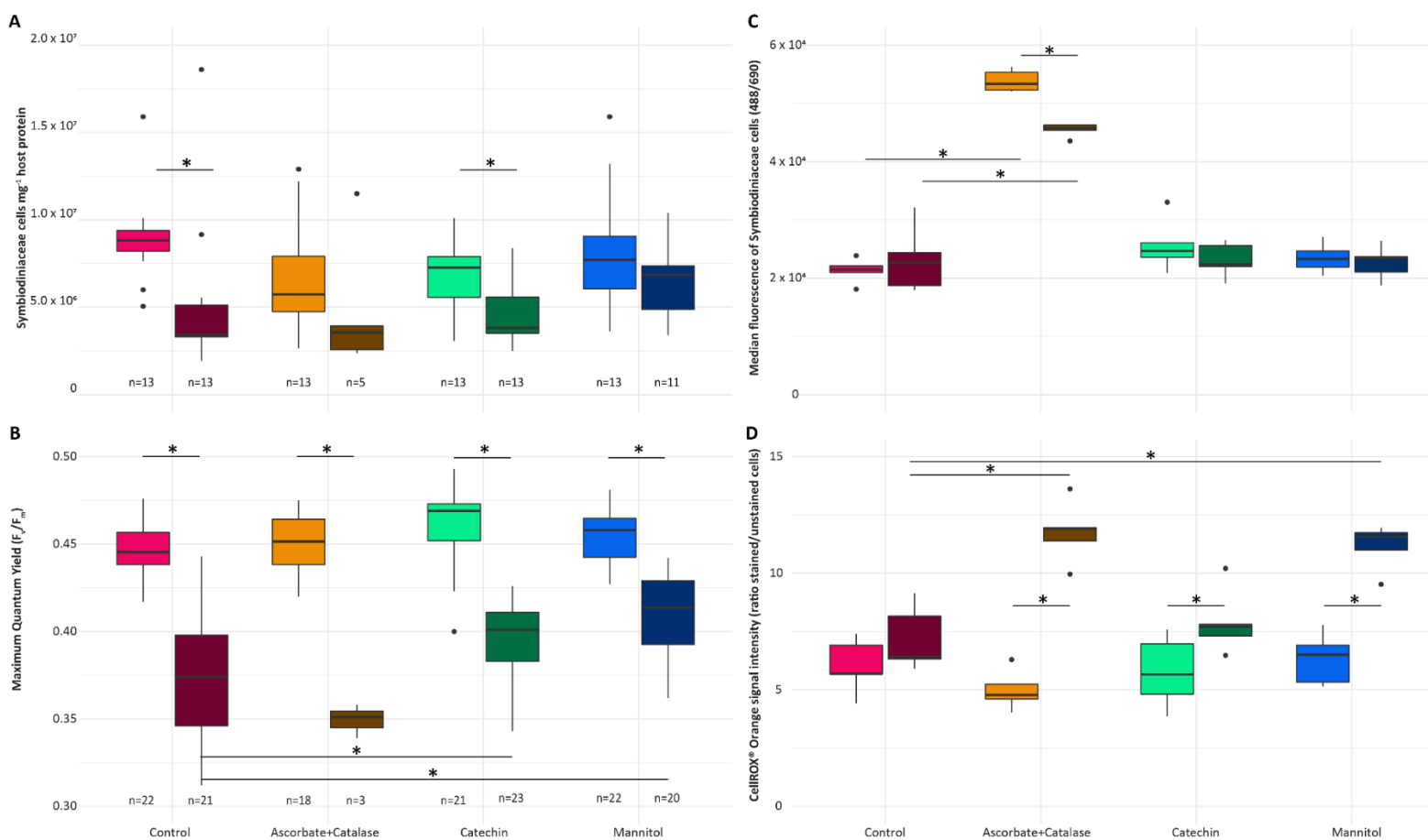


Figure 5-4 Bleaching metrics of (A) Symbiodiniaceae cell density normalized to host protein, (B) photosynthetic efficiency (F_v/F_m), (C) Chl *a* content, and (D) net ROS (D) measured on Day 13 from anemones reared at ambient (light colours) or elevated (dark colours) temperature for the no antioxidant control (purple), ascorbate+catalase (orange), catechin (green), and mannitol (blue) antioxidant treatments. Chl *a* content of Symbiodiniaceae cells was measured as median emitted fluorescent signal (488/690 (excitation/emission)), with $n=5$ per treatment. Net ROS as measured as signal intensity of CellROX® Orange stained Symbiodiniaceae cells separated from anemones, where signal intensity was quantified as the median signal ratio of stained/unstained cells. $n=5$ per treatment for net ROS. Asterisks indicate significant pairwise differences.

5.5 Discussion

5.5.1 Thermal stress-induced bleaching was not accompanied by elevated ROS

In the absence of exogenous antioxidants, *E. diaphana* exposed to elevated temperatures had a significant reduction in Symbiodinaceae cell densities (i.e., bleaching) compared to the ambient control. There was no evidence of chl*a* pigment bleaching, but a decline in photosynthetic health (F_v/F_m) was apparent, with no significant change in net ROS. We confirmed that all anemones harboured *B. minutum* as their homologous symbiont. Our data shows that bleaching, caused by exposure of elevated temperature, does not increase ROS levels in the anemone holobiont.

Marty-Rivera et al. (2018) measured net ROS in a similar manner, using confocal images to quantify fluorescence signal of CellROX® Orange. They found that thermal stress of *E. diaphana* resulted in significantly higher ROS signal and that the application of catechin at 5 μ M (same concentration as used in this experiment) lead to significantly reduced ROS under both ambient and elevated temperatures, with no significant differences between catechin treated anemones between temperature regimes. Despite this seemingly promising result, all *E. diaphana* treatments in that study, regardless of antioxidant dosage, experienced a significant loss of Symbiodiniaceae cells after four days at 33°C (Marty-Rivera et al., 2018). Taken together, these studies suggest that increased ROS may not always be the driving factor in bleaching, particularly for *E. diaphana*, which does not have an obligate symbiosis with Symbiodiniaceae.

The Oxidative Theory of Coral Bleaching suggests that hydrogen peroxide (H_2O_2) derived from photo-oxidative stress in the symbiont might leak into the host tissue when symbiont antioxidant defences are overwhelmed in bleaching corals (Downs et al., 2002). However, changes in Symbiodiniaceae net ROS as a result of exposure to elevated temperature is highly variable by species (Lesser, 1996; Suggett et al., 2008; McGinty et al., 2012; Rehman et al., 2016; Wietheger et al., 2018; Lesser, 2019). Under elevated temperatures, some Symbiodiniaceae species, such as *B. psygmophilum* (formerly Symbiodinium clade B2,

Lajeunesse et al., 2018), experience a decrease in net ROS with a corresponding increase in cellular antioxidant activities, such as catalase (McGinty et al., 2012). Additionally, thermally stressed *B. minutum* shows increased activity of the H₂O₂ scavenging gene ascorbate peroxidase (Krueger et al., 2015a).

Despite the variability in net ROS levels from Symbiodiniaceae exposed to elevated temperatures, there is support for the production of H₂O₂ in the thylakoid lumen and stroma during photosynthesis (Szabó et al., 2020). While H₂O₂ can easily pass through chloroplast membranes and accumulate in the Symbiodiniaceae cytosol (Mubarakshina et al., 2010; Borisova et al., 2012), the theory that H₂O₂ passes from the Symbiodiniaceae cytosol into the coral host cell is speculative as no studies have tracked the production and movement of H₂O₂ in this system. Our observations show that, when temperature is the dominant stressor, coral bleaching does not necessarily involve a connection of ROS fluxes between host and symbiont as there were no significant increases in ROS for anemones that bleached. Our bleaching response in the absence of elevated ROS corresponds with other studies that show that bleaching can occur without photosynthetically produced ROS (Tollete et al., 2013) and with discrepancies in enzymatic antioxidant activity between host and symbiont tissue portions (Krueger et al., 2015b), raising questions about the importance of symbiont-derived ROS in initiating cnidarian bleaching. Nielsen et al. (2018) found that bleaching independent of ROS can also occur under light. Although ROS were produced in the symbiont, they were not released to the host tissues and no attributable physiological effects were detected in either host or symbiont (Nielsen et al., 2018), corroborating field observations that coral superoxide production is unrelated to bleaching status (Diaz et al., 2016).

5.5.2 Exogenous antioxidants prevented bleaching in *E. diaphana*

Each antioxidant treatment reduced the impact of thermal stress on bleaching, but each treatment saw improvements in different bleaching parameters. Treatment with catechin lead to a significant increase in F_v/F_m under elevated temperature compared to the no antioxidant controls. This rescue of PSII was also apparent for mannitol treated anemones,

which did not have a significant loss of Symbiodiniaceae cells with exposure to elevated temperatures. The addition of antioxidants ascorbate+catalase also prevented bleaching, but at the expense of holobiont health.

Catechin and mannitol treated anemones had a significantly greater F_v/F_m compared to the no antioxidant controls under elevated conditions. This is a promising finding as F_v/F_m has been described as a proxy for thermal stress response in corals (Voolstra et al., 2020) and Symbiodiniaceae cultures (Ragni et al., 2010; Goyen et al., 2017; Dang et al., 2019a), with higher or retained F_v/F_m values indicating increased bleaching resistance during stress events. The only other study to administer exogenous catechin to *E. diaphana* also showed a significantly higher F_v/F_m in catechin treated anemones compared to no antioxidant controls in the elevated temperature (33-34°C) treatment with a significant loss of Symbiodiniaceae cell density under elevated temperature, regardless of antioxidant treatment (Marty-Rivera et al., 2018).

Catechin is a carotenoid; carotenoids play a pivotal role in quenching singlet oxygen radicals produced at the reaction centres of PSII (Schmitt et al., 2014). Because F_v/F_m is a measure of photosynthetic efficiency of PSII, the oxidation of catechin may explain why anemones in this treatment had significantly higher F_v/F_m values. However, other points in the light reaction of photosynthesis, such as the electron transport chain between PSII and PSI, could have been damaged by thermal stress (Szabó et al., 2017), which was not captured in our measurement. Supplementation with other carotenoids, such as zeaxanthin produced by Symbiodiniaceae-associated *Muricauda* spp., have been shown to improve F_v/F_m and neutralize ROS in thermally stressed Symbiodiniaceae cultures (Motone et al., 2020). The application of catechin in this experiment did not cause a change in health score compared to the antioxidant controls (Fig. S2). This is likely due to the fact that carotenoids are a natural component of phototrophic organisms (Takaichi, 2011).

Like catechin, dosing anemones with mannitol did not cause any adverse changes in the health scores compared to the no-antioxidant controls. Mannitol has multiple functions in plants and algae, including osmoregulation, storage, regeneration of reducing power, and scavenging of ROS (Iwamoto and Shiraiwa, 2005). The presence of mannitol in chloroplasts

can protect plants against oxidative damage by hydroxyl radicals (Shen et al., 1997b). During photosynthesis, hydroxyl radicals are produced in the Haber-Weiss or Fenton reactions through the interaction of H_2O_2 and superoxide or directly from H_2O_2 in the presence of transition metals (Halliwell and Gutteridge, 1990). Targeting mannitol biosynthesis in tobacco chloroplasts has been shown to enhance the hydroxyl radical-scavenging capacity of the plants and increased their resistance to oxidative stress (Shen et al., 1997a). If the exogenous addition of mannitol leads to the quenching of hydroxyl radicals for *in hospite* Symbiodiniaceae, we would expect increased resistance to oxidative stress, potentially explaining why mannitol rescued PSII performance under elevated temperature and mitigated bleaching.

The phenomenon of PSII rescue has also been observed with Symbiodiniaceae cultures when exposed to ascorbate+catalase for one hour at elevated temperatures, with the caveat that the improvement in photosynthetic capacity did not completely mitigate the effects of thermal stress (Lesser, 1996). In our experiment, treatment of anemones with ascorbate+catalase did not result in a change of F_v/F_m under elevated temperature conditions. However, the ascorbate+catalase treatment had significantly increased chl *a* fluorescence, regardless of temperature. Exogenous addition of ascorbate to plants (i.e. maize, wheat, barley) has resulted in increased chlorophyll content (Akram et al., 2017), suggesting that ascorbate is driving the change we see here in Symbiodiniaceae chl *a* content. An increase in chl *a* per Symbiodiniaceae cell can be used to cover higher metabolic demands under lower photosynthetic efficiencies (Iglesias-Prieto et al., 1992). This chl *a* boost in ascorbate+catalase treated anemones with the accompanying capacity to maintain higher metabolic rates may explain why these anemones did not bleach under elevated temperatures.

During bleaching, some Symbiodiniaceae species experience a loss of chl *a* pigment due to photo-oxidative damage (Venn et al., 2006). The results from our control, mannitol, and catechin treatment support previous findings that chl *a* content in *B. minutum* does not always change as a result of thermal stress (Venn et al., 2006; Krueger et al., 2015a).

5.5.3 Mitigation of bleaching was not associated with lower ROS

Compared to ambient temperature, ROS levels (as quantified by the ratio of CellROX® Orange stained/unstained Symbiodiniaceae cells) increased for ascorbate+catalase, catechin, and mannitol treated anemones under elevated temperature conditions. Two of these treatments (ascorbate+catalase and mannitol) did not bleach, i.e., showed no significant loss of symbiont cells, under elevated temperatures. Our data suggests that adding an exogenous antioxidant exacerbated the accumulation of net ROS in the holobiont. Our exogenous addition of antioxidants could have been interfering with the coral/Symbiodiniaceae's own cellular response to photoinhibition, potentially explaining why net ROS levels increased for all antioxidant treated anemones from ambient to elevated temperature.

5.5.4 Ascorbate+catalase treatment was lethal to anemones

Vitamin C (ascorbic acid) is an antioxidant molecule and a key substrate for the detoxification of ROS derived from photosynthesis. Its primary role is photoprotection by either acting as a reducing agent capable of transferring an electron to a light-excited chlorophyll molecule, or detoxifying H_2O_2 produced in the thylakoid lumen via Haber-Weiss reactions (Ivanov, 2014; Akram et al., 2017). The term "ascorbate" is often used to describe this compound because it mainly takes the anionic form at physiological pH values. Ascorbate was used in this experiment in combination with catalase to mimic previous experiments (Lesser, 1996; 1997). When exogenously applied on its own, ascorbate has been shown to improve activity of endogenous catalase (Athar et al., 2008; 2009) and increase endogenous ascorbate levels (Athar et al., 2008). Where ascorbate typically has highest concentrations inside chloroplasts, catalase is most abundant in the cytosol (Ivanov, 2014), potentially driving the original decision to use these antioxidants together as one treatment.

Hydrogen peroxide accumulation in the stroma can inhibit up to 90% of CO_2 fixation in isolated intact chloroplasts, with complete reversal after the addition of catalase (Kaiser, 1979). Previous work found that, compared to ambient temperature controls, there were

no significant differences in photosynthesis and respiration rates when thermally stressed corals (*Agaricia tenuifolia*) were dosed with a combination of catalase (250 U mL⁻¹) and ascorbate (125 µM) (Lesser, 1997). Additionally, there were significantly more Symbiodiniaceae cells lost from thermally stressed corals without antioxidants compared to those exposed to thermal stress plus antioxidants (either ascorbate+catalase or mannitol (10 mM)). Despite those promising results, the health of anemones that were dosed with ascorbate+catalase declined after Day 7, regardless of temperature. Additionally, several anemones in this treatment group, particularly under elevated temperature, died prior to sampling because of the antioxidant dose. For those anemones that survived, this group had the lowest F_v/F_m values on Day 13. The dosage of ascorbate+catalase also shifted the bacterial communities of *E. diaphana*. Bacterial communities had strong evidence of disease with increased levels of taxa associated with known coral pathogens, Vibrionaceae (ASV017, *Vibrio* sp.) (Sunagawa et al., 2009a; Gignoux-Wolfsohn and Vollmer, 2015; Tout et al., 2015) and reduced levels of other common coral-associated bacteria taxa Rhodobacteraceae (Huggett and Apprill, 2019) (ASV007, *Pelagibaca* sp.; ASV009, *Ruegeria* sp. (Miura et al., 2019); ASV043, *Rosei* sp.) and Crocinitomicaceae (Weber et al., 2020) (ASV002, ASV036, *Crocinitomix* spp.).

Jointly, ascorbate+catalase mitigated bleaching, but caused extensive mortality. High concentrations of antioxidants can be detrimental in biological systems (Villanueva and Kross, 2012). Future experiments should explore their application independently with toxicity tests to determine how each antioxidant might contribute to bleaching rescue and which concentration would be most suited.

5.5.5 Conclusions

The global decline of coral reefs heightens the need to understand how corals may persist under changing environmental conditions. Here we have shown that the exogenous application of antioxidants can mitigate bleaching in *E. diaphana* exposed to thermal stress despite an increase in net ROS. We speculate that there is an alternative pathway to coral bleaching, whereby, reductions in photosynthate transfer, as a result of photoinhibition,

challenge the symbiotic relationship status quo.

In other systems, when the symbiont is defective and not productively contributing to the relationship, symbiosis breaks down. In the squid-*Vibrio fischeri* system, *V. fischeri* bacteria unable to induce normal luminescence levels in the host were unable to form prolonged symbiotic association with their squid host (Visick et al., 2000). In a similar fashion, soybean plants decrease resource allocation to rhizobia that fail to fix nitrogen inside their root nodules (Kiers et al., 2003). In fact, plants can detect, discriminate, and reward the best fungal partners with more carbohydrates (Kiers et al., 2011). In turn, their fungal partners enforce cooperation and stabilize symbiosis by increasing nutrient transfer only to those roots providing more carbohydrates (Selosse and Rousset, 2011). Thus, there is a penalty or sanction to the symbiont and/or host for disobeying the “rules” of the established symbiosis.

For the coral-Symbiodiniaceae symbiosis, one critical “rule” is the sharing of resources between partners, namely the translocation of photosynthate from the algae to the coral. During thermal stress and bleaching, there is evidence for a reduction in the translocation of photoautotrophically acquired carbon from Symbiodiniaceae to their coral host (Hughes et al., 2010; Baumann et al., 2014; Tremblay et al., 2016; Krueger et al., 2018), with thermally resilient *Durisdinium* spp. able to fix and translocate more carbon compared to thermally susceptible *Cladocopium* spp. (Baker et al., 2013). These changes in symbiont productivity may shift interactions from mutualistic to antagonistic (Lesser et al., 2013; Baker et al., 2018), leading to bleaching. Thus, the extent of reciprocal nutrient/photosynthate supply and not an accumulation of ROS (Blackstone and Golladay, 2018; Morris et al., 2019), may be the driving force behind bleaching in the ‘sanction theory of coral bleaching.’

This theory is supported by the consistent findings that alternative mechanisms of acquiring fixed carbon, such as by heterotrophic feeding, are triggered by thermal bleaching (Grottoli et al., 2006a; Levas, 2012; Baumann et al., 2014) and that heterotrophy decreases the need for autotrophic carbon translocation (Tremblay et al., 2012a). Additionally, heterotrophic input of carbon is an important determinant of colony survival after bleaching (Anthony et al., 2009; Hughes and Grottoli, 2013; Levas et al., 2013; Bessell-

Browne et al., 2014). Nitrate (NO_3^-) enrichment (skewing N:P ratios) is routinely shown to reduce thermal tolerance of corals (Wiedenmann et al., 2012; Shantz and Burkepile, 2014; Vega Thurber et al., 2014; Zaneveld et al., 2016; Rosset et al., 2017; Burkepile et al., 2019; Donovan et al., 2020; Fernandes de Barros Marangoni et al., 2020) and concomitantly induces a significant decrease in carbon fixation and translocation for *in hospite* Symbiodiniaceae (Ezzat et al., 2015). Seawater enrichment with ammonium (NH_4^+), however, increases carbon fixation and translocation (Ezzat et al., 2015), presents with a less severe bleaching response compared to NO_3^- (Burkepile et al., 2019), and may possibly protect corals during thermal stress (Fernandes de Barros Marangoni et al., 2020). For heat-evolved *Cladocopium goreau* that increased thermal tolerance in *Acropora tenuis* larvae, there was a consistent increase in expression of algal carbon fixation genes (Buerger et al., 2020). Thus, bleaching is more reliably attributed to changes in autotrophic carbon metabolism, which depend on nutrient form and ratio (Morris et al., 2019), compared to photo-oxidative stress, as shown in this study.

5.6 Data availability

Raw Illumina MiSeq data are available under NCBI BioProject ID PRJNA630325.

5.7 Acknowledgments

The authors thank Leon Hartman, Sarah Jane Tsang Min Ching, and Xavier Smith for their assistance in anemone maintenance, sample collection and processing, as well as Ben Hume for running SymPortal analysis remotely. We thank the BioSciences Microscopy Unit (The University of Melbourne) and the Biological Optical Microscopy Platform for the use of their confocal microscope, and Dr. Gabriela Segal for training. Leon Hartman reviewed the manuscript and designed Fig. 5-1. This study was supported with funding from the Australian Research Council (grant ID: DP160101468) to MvO and LLB and Australian Research Council Laureate Fellowship FL180100036 to MvO.

5.8 Supplementary material

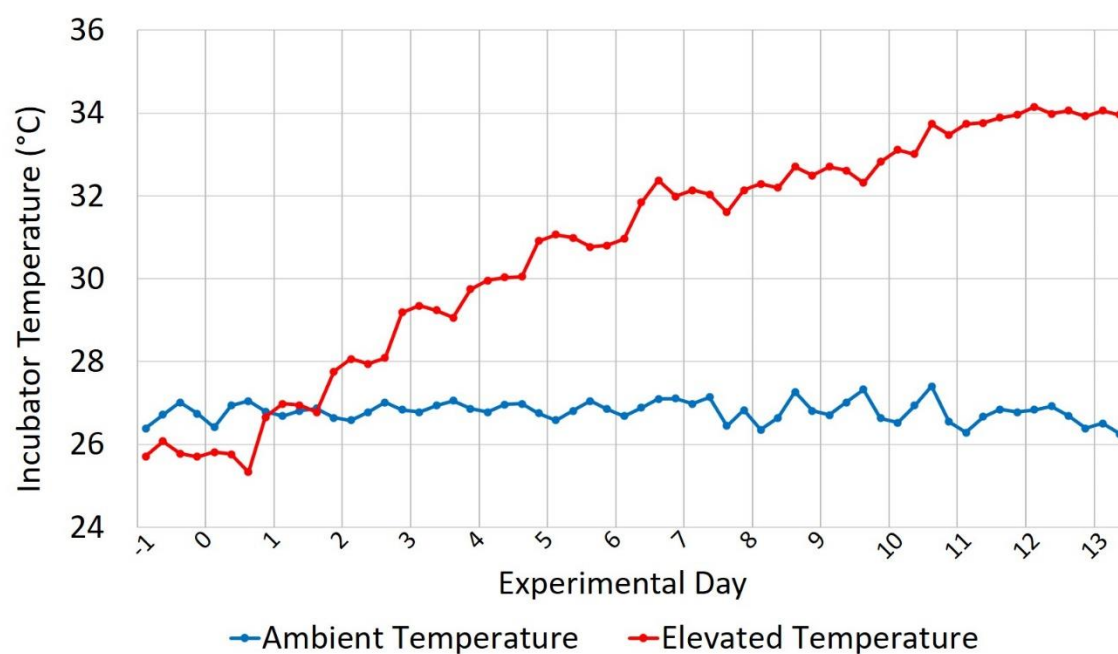


Figure S5-1 Average temperature for every six hours during the experimental period beginning the day prior to initial antioxidant dosing (Day -1) to Day 13 for the ambient (blue) and elevated (red) conditions. The ambient treatment averaged ($\pm$ SE) $26.80 \pm 0.03^\circ\text{C}$ during this two-week period. Data was collected on submersible HOBO loggers.

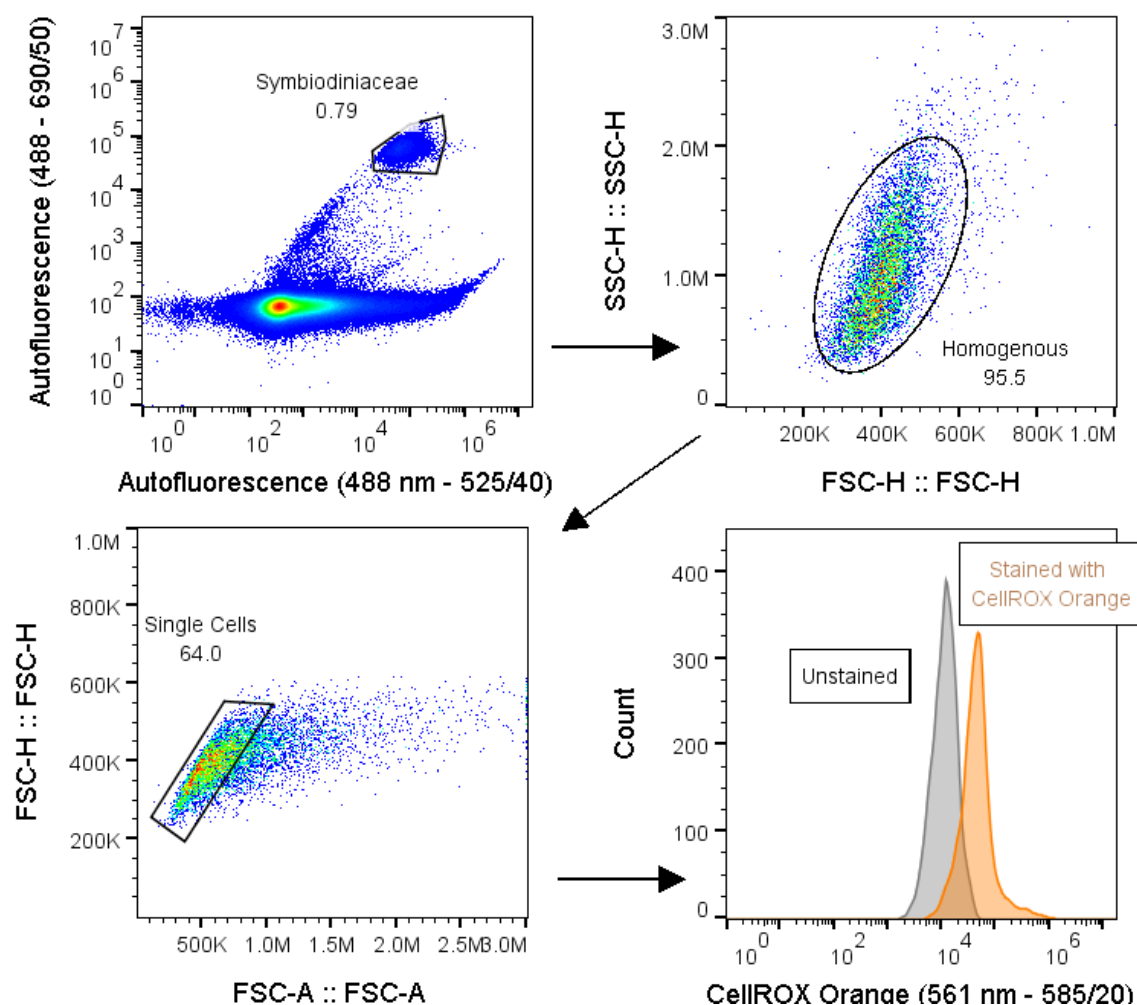


Figure S5-2: To isolate and quantify chlorophyll a and CellROX® Orange fluorescence in singlet Symbiodiniaceae cells, we selected events strongly excited by the 488 nm laser at emissions 525 ± 40 nm and 690 ± 25 nm (A). An FSC-H/SSC-H plot was then used to select homogenous cells (B), and singlets were gated on an FSC-H/FSC-A plot (C). CellROX® Orange signal was quantified as the median signal ratio of stained versus unstained cells on singlets gated on previous plots with the 561 nm laser (emission 585 ± 42 nm) (D). At least 5000 Symbiodiniaceae singlets per sample were processed, except in samples where Symbiodiniaceae density was low because of advanced bleaching (between 700 and 2000 singlets per sample).

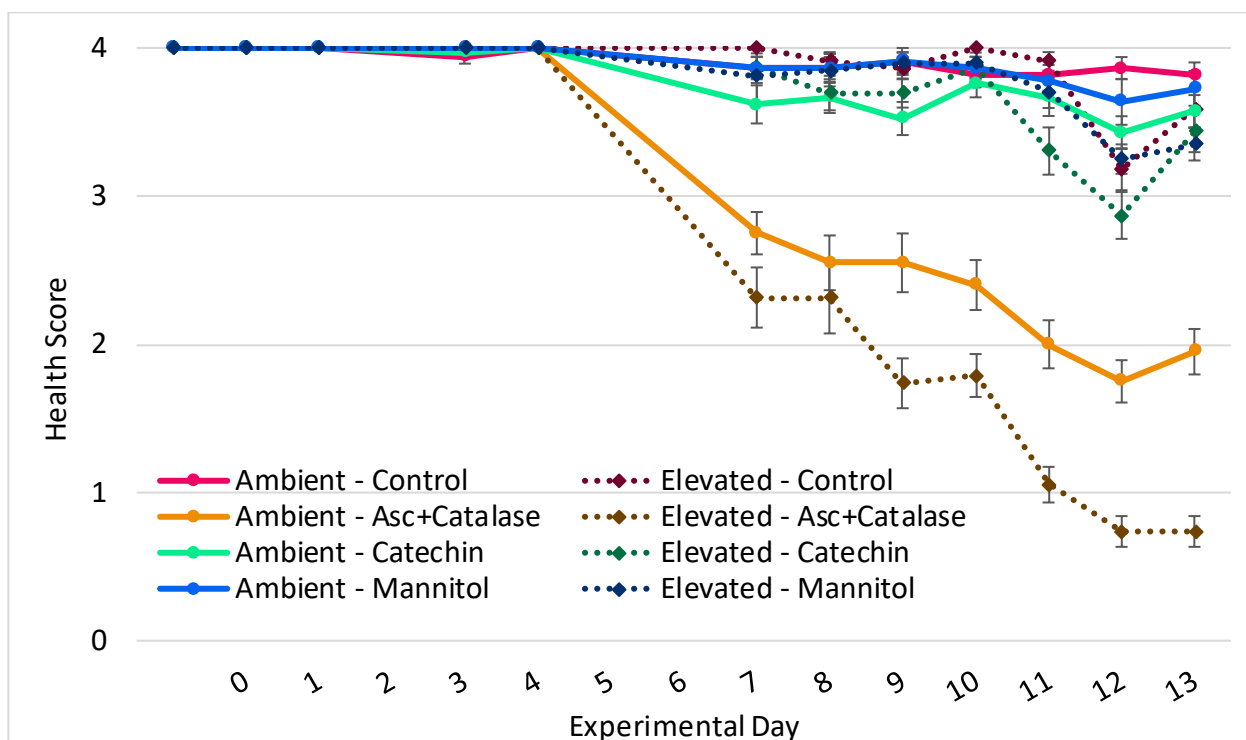


Figure S5-3 Anemone health scores over time for anemones reared at ambient (light colours, solid line) or elevated (dark colours, dashed line) temperature for the no antioxidant control (purple), ascorbate+catalase (orange), catechin (green), and mannitol (blue) antioxidant treatments. The anemones were moved to fRSS on Day -1, the experiment began on Day 0 with anemones sampled on Day 13.

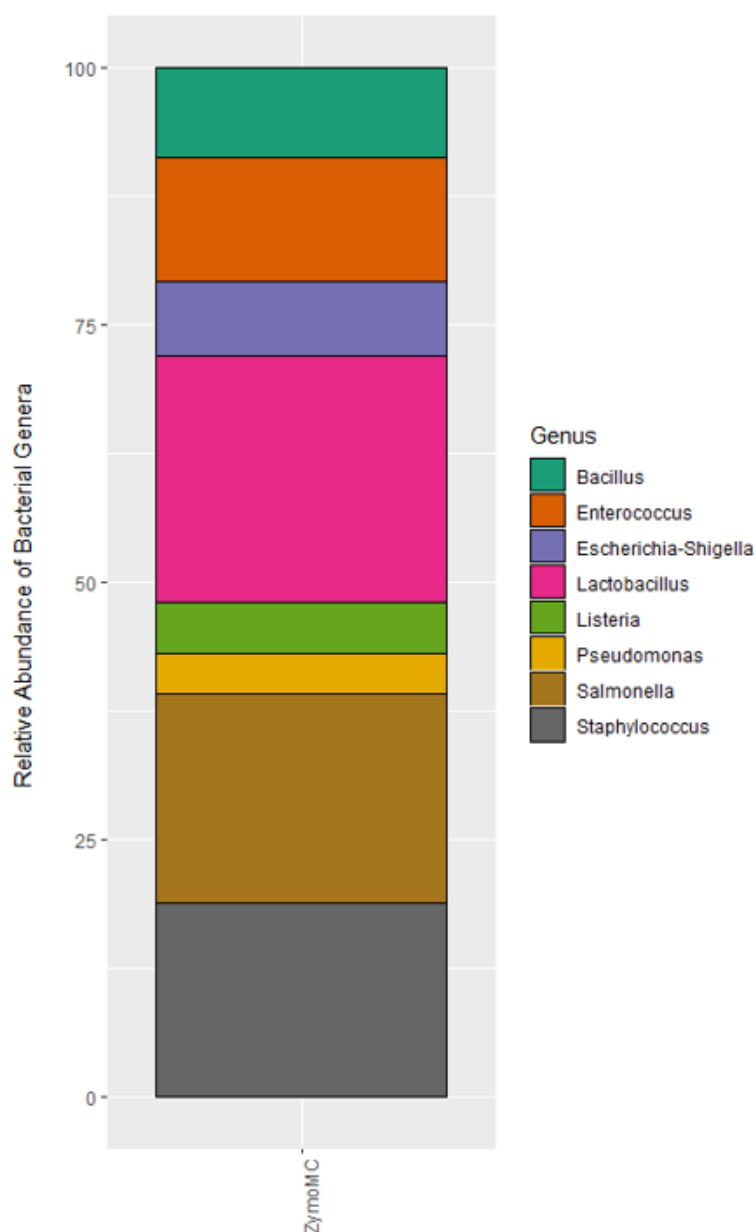


Figure S5-4 Relative abundance of genera found after sequencing of a ZymoBIOMICS™ Microbial Community DNA Standard. Expected contributions for each genus are: *Bacillus*– 17.4%, *Enterococcus*– 9.9%, *Escherichia* – 10.1%, *Lactobacillus* – 18.4%, *Listeria* – 14.1%, *Pseudomonas* – 4.2%, *Salmonella* – 10.4%, and *Staphylococcus*– 15.5%.

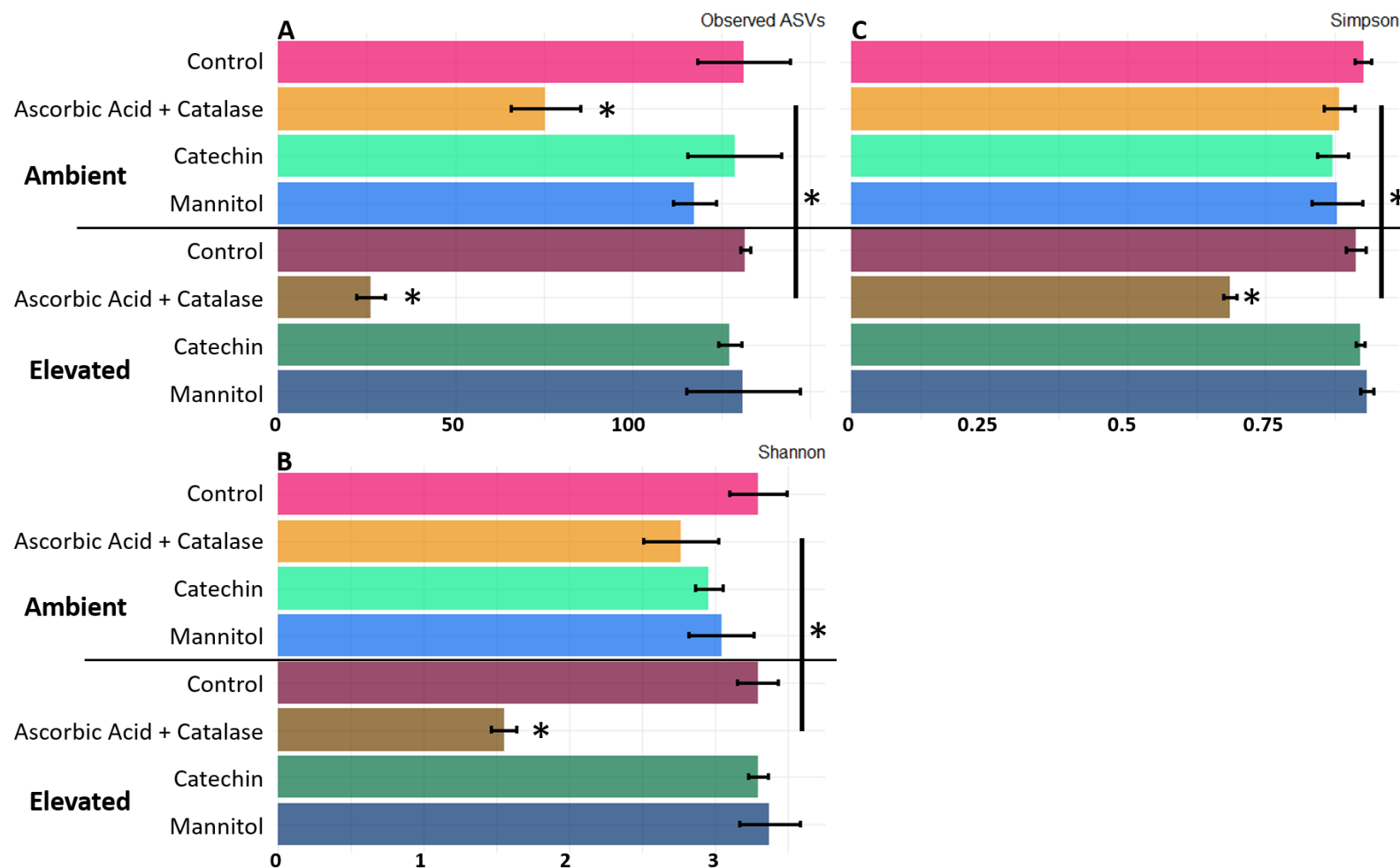


Figure S5-5 Observed ASVs (A), Simpson's Index (B), and Shannon's Index (C) for AIMS3 anemones after two weeks under ambient (light colours) or elevated (dark colours) temperature across control (purple), ascorbate+catalase (orange), catechin (green), and mannitol (blue) antioxidant treatments. The data set was rarefied to 22,509 reads per sample. Asterisks indicate significant pairwise differences between antioxidant treatments by temperature or between temperature conditions for a given antioxidant treatment. Statistical output for pairwise comparisons is in Table S5-2.

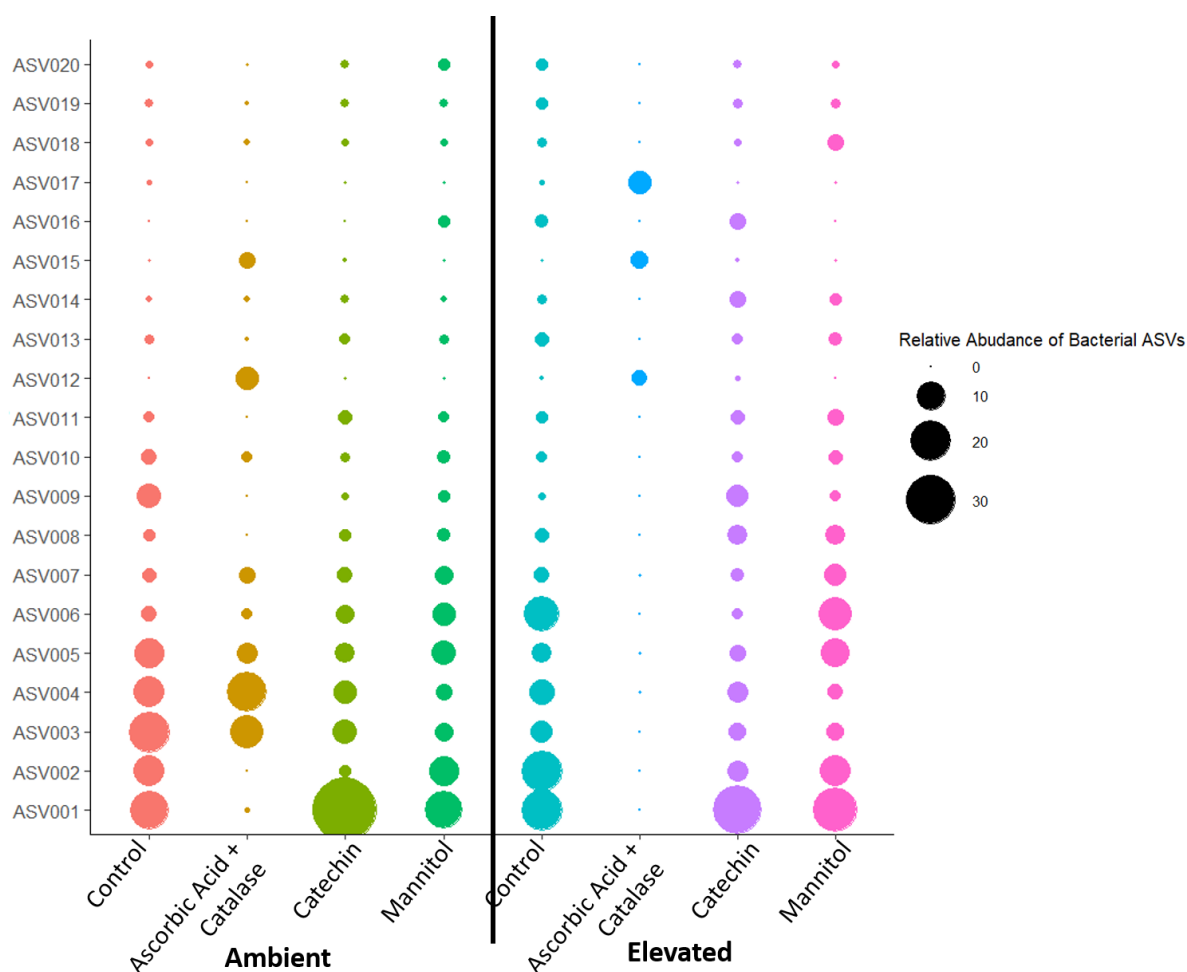


Figure S5-6 Bubble plot showing the relative abundance of bacterial ASVs for each replicate of the four treatment groups for AIMS3 anemones. The size of the bubble corresponds with the relative abundance. ASVs were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity. The identities of each ASV as well as statistics can be found in Table S5-3.

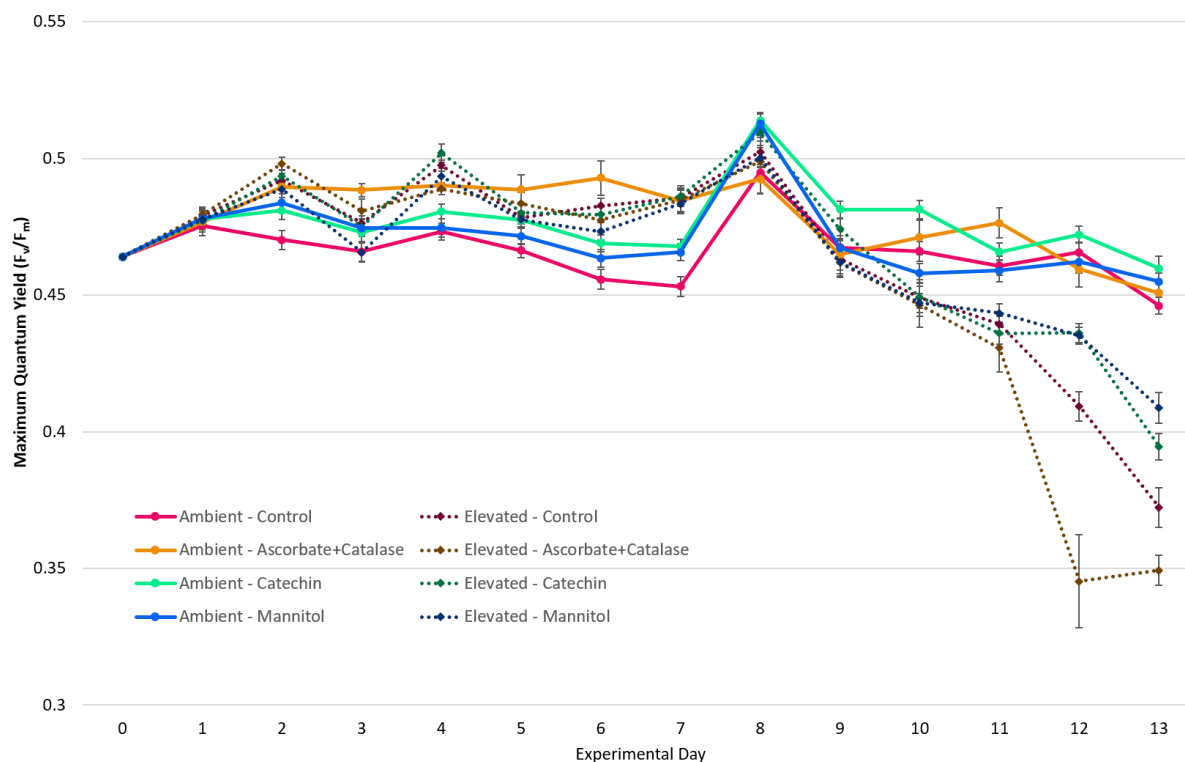


Figure S5-7 Maximum quantum yield (F_v/F_m) over time for anemones reared at ambient (light colours, solid line) or elevated (dark colours, dashed line) temperature for the no antioxidant control (purple), ascorbate+catalase (orange), catechin (green), and mannitol (blue) antioxidant treatments.

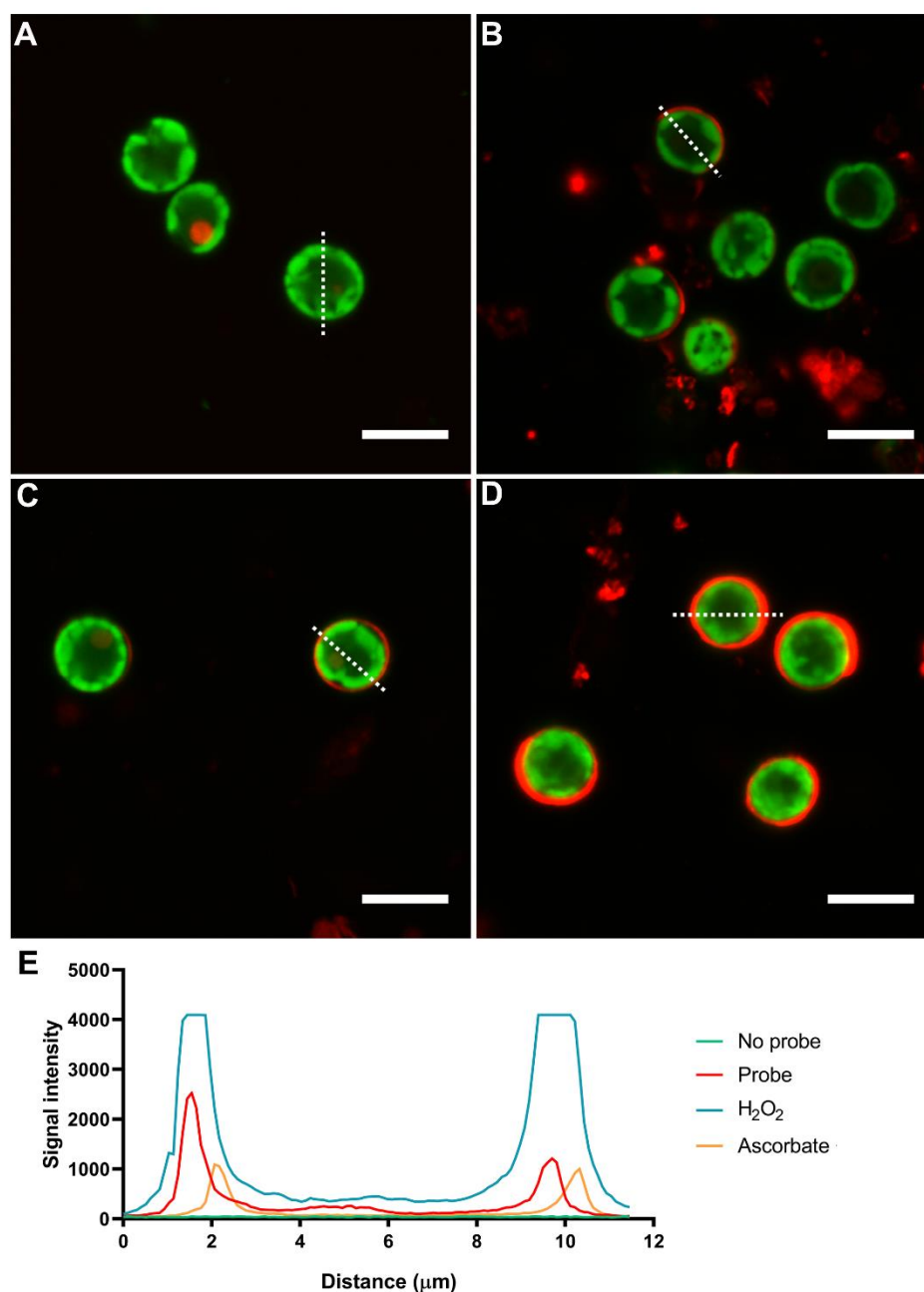


Figure S5-8 Observations of ROS production from *in hospite Breviolum minutum* cells in confocal microscopy. (A) *B. minutum* not loaded with CellROX® Orange; (B-D) pictures of CellROX® Orange-loaded cells. Anemones were kept overnight at 27°C and 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in (A,B) RSS at 34 ppt salinity, (C) RSS + ascorbate 0.5% (w/v), or (D) RSS + 20 mM H₂O₂. Cells were excited with the 488 nm laser (emission collected in the 650-720 nm range) to visualize the autofluorescence of the cells (green) or with the 561 nm laser (emission collected in the 571-621 nm range) to visualize the CellROX® Orange fluorescence when ROS is produced (red). (E) Signal intensities were measured on ImageJ across the white dashed lines indicated on the respective images. Each scale bar is 10 μm .

Table S5-1 Potential contaminant ASVs

ASV	Relative Abundance (%)	Phylum	Family	Genus
Contaminant01	0.0003	Proteobacteria	Pasteurellaceae	<i>Actinobacillus</i>
Contaminant02	0.0003	Proteobacteria	Hyphomicrobiaceae	<i>Hyphomicrobium</i>
Contaminant03	0.0006	Epsilonbacteraeota	Arcobacteraceae	<i>Arcobacter</i>
Contaminant04	0.0011	Proteobacteria	Rhizobiaceae	<i>Phyllo</i>
Contaminant05	0.0025	Proteobacteria	Pseudomonadaceae	<i>Pseudomonas</i>
Contaminant06	0.0138	Proteobacteria	Enterobacteriaceae	<i>Serratia</i>
Contaminant07	0.0379	Proteobacteria	Sphingomonadaceae	<i>Sphingomonas</i>
Contaminant08	0.0432	Proteobacteria	Burkholderiaceae	<i>Cupriavidus</i>
Total Relative Abundance (%)	0.0997			

Table S5-2 Significant pairwise comparisons of alpha diversity for Day 13.

Alpha Diversity Measure	Temperature	Antioxidant	df	t.ratio	p.value
Observed ASVs	Ambient > Elevated	Asc+Catalase	16	3.580	0.0025
Observed ASVs	Ambient	Asc+Catalase <Control	16	4.064	0.0045
Observed ASVs	Ambient	Asc+Catalase <Catechin	16	3.870	0.0067
Observed ASVs	Ambient	Asc+Catalase <Mannitol	16	3.048	0.0348
Observed ASVs	Elevated	Asc+Catalase <Control	16	7.668	< 0.0001
Observed ASVs	Elevated	Asc+Catalase <Catechin	16	7.353	< 0.0001
Observed ASVs	Elevated	Asc+Catalase <Mannitol	16	7.619	< 0.0001
Simpson's Index	Ambient > Elevated	Asc+Catalase	16	5.786	< 0.0001
Simpson's Index	Elevated	Asc+Catalase <Control	16	6.681	< 0.0001
Simpson's Index	Elevated	Asc+Catalase <Catechin	16	6.922	< 0.0001
Simpson's Index	Elevated	Asc+Catalase <Mannitol	16	7.272	< 0.0001
Shannon's Index	Ambient > Elevated	Asc+Catalase	16	4.952	0.0001
Shannon's Index	Elevated	Asc+Catalase <Control	16	7.115	< 0.0001
Shannon's Index	Elevated	Asc+Catalase <Catechin	16	7.118	< 0.0001
Shannon's Index	Elevated	Asc+Catalase <Mannitol	16	7.443	< 0.0001

Table S5-3 ASVs were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus. The treatment column shows which experimental treatments the corresponding ASV is strongly and significantly associated with. The first character indicates the temperature treatment (A=Ambient or E=Elevated) and the second character indicates the antioxidant treatment (0=control, A=ascorbate+catalase, M = mannitol, and C = catechin). The final eight columns indicate the relative abundance (%) of that specific ASV compared to all other ASVs in each treatment.

ASV	Stat	p	Treatment	Class	Family	Genus	A0	AA	AC	AM	E0	EA	EC	EM
ASV001	1	0.003	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Alteromonadaceae	NA	11.49	0.13	31.64	10.49	12.92	0.01	18.20	14.74
ASV002	1	0.001	A0+AC+AM+E0 +EC+EM	Bacteroidia	Crocinitomicaceae	<i>Crocinitomix</i>	7.07	0.00	0.88	6.60	12.70	0.00	3.27	7.12
ASV003	1	0.003	A0+AA+AC+A M+E0+EC+EM	Deltaproteobacteria	Nannocystaceae	NA	12.31	7.98	4.41	2.37	3.66	0.02	2.21	2.29
ASV004	1	0.003	A0+AA+AC+A M+E0+EC+EM	Bacteroidia	Saprospiraceae	<i>Lewinella</i>	6.87	12.09	4.01	1.80	4.57	0.03	3.01	1.62
ASV005	1	0.003	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Alteromonadaceae	<i>Alteromonas</i>	6.74	3.04	2.68	4.16	2.74	0.02	1.96	6.19
ASV006	0.984	0.014	A0+AC+AM+E0 +EC+EM	Gammaproteobacteria	Coxiellaceae	<i>Coxiella</i>	1.72	0.71	2.39	3.90	9.38	0.00	0.77	8.29
ASV007	0.999	0.003	A0+AA+AC+A M+E0+EC+EM	Alphaproteobacteria	Rhodobacteraceae	<i>Pelagibaca</i>	1.45	1.82	1.71	2.49	1.66	0.03	1.09	3.34
ASV008	1	0.001	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	NA	NA	1.02	0.00	0.96	1.16	1.36	0.00	2.80	2.72
ASV009	0.972	0.01	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Rhodobacteraceae	<i>Ruegeria</i>	4.08	0.00	0.34	0.87	0.29	0.00	3.44	0.78
ASV010	1	0.003	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Alteromonadaceae	<i>Alteromonas</i>	1.71	0.76	0.63	1.15	0.79	0.00	0.75	1.48
ASV011	1	0.001	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Terasakiellaceae	NA	0.76	0.00	1.33	0.69	0.87	0.00	1.31	1.93
ASV012	0.988	0.003	AA+EA+EC	Alphaproteobacteria	Hyphomonadaceae	<i>Oceanicaulis</i>	0.00	3.81	0.03	0.03	0.07	1.69	0.14	0.01
ASV013	0.989	0.001	A0+AC+AM+E0 +EC+EM	Gammaproteobacteria	Alteromonadaceae	<i>Alteromonas</i>	0.59	0.10	0.79	0.57	1.39	0.00	0.73	1.11
ASV014	0.976	0.03	A0+AA+AC+A M+E0+EC+EM	Bacteroidia	Cyclobacteriaceae	NA	0.19	0.20	0.44	0.21	0.56	0.00	1.89	1.00
ASV015	0.972	0.027	AA+EA+EC	Alphaproteobacteria	Terasakiellaceae	<i>Terasakiella</i>	0.03	1.87	0.09	0.04	0.05	2.22	0.07	0.03
ASV016	1	0.001	AM+E0+EC	Gammaproteobacteria	Alteromonadaceae	NA	0.00	0.00	0.00	1.01	1.25	0.00	1.86	0.00
ASV017	0.969	0.022	E0+EA	Gammaproteobacteria	Vibrionaceae	<i>Vibrio</i>	0.13	0.00	0.04	0.04	0.11	3.67	0.02	0.02
ASV018	1	0.003	A0+AA+AC+A M+E0+EC+EM	Actinobacteria	Propionibacteriaceae	<i>Cuti</i>	0.35	0.19	0.32	0.32	0.64	0.00	0.36	1.80
ASV019	0.961	0.001	A0+AC+AM+E0 +EC+EM	Gammaproteobacteria	Alteromonadaceae	<i>Alteromonas</i>	0.51	0.07	0.51	0.48	0.99	0.00	0.53	0.56

ASV020	0.97	0.006	A0+AC+AM+E0 +EC+EM	Rhodothermia	Balneolaceae	<i>Balneola</i>	0.30	0.02	0.44	0.87	0.87	0.00	0.45	0.33
ASV021	0.999	0.001	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Stappiaceae	<i>Stappia</i>	0.07	0.00	0.61	1.01	0.55	0.00	0.62	0.30
ASV022	1	0.001	A0+AC+AM+E0 +EC+EM	Bacteroidia	Saprospiraceae	NA	0.20	0.00	0.66	0.24	1.09	0.00	0.36	0.34
ASV023	0.943	0.008	A0+AC+AM+E0 +EC+EM	Deltaproteobacteria	Bdellovibrionaceae	OM27 clade	0.20	0.00	0.31	0.26	0.69	0.00	0.78	0.65
ASV024	0.921	0.01	AA	Alphaproteobacteria	NA	NA	0.00	2.27	0.47	0.00	0.00	0.00	0.00	0.00
ASV025	1	0.001	A0+AC+E0+EC	Bacteroidia	NA	NA	0.33	0.00	0.14	0.00	1.00	0.00	1.19	0.00
ASV026	0.957	0.002	A0+AC+E0	Alphaproteobacteria	Rhodobacteraceae	NA	1.38	0.15	0.72	0.02	0.32	0.02	0.00	0.00
ASV027	1	0.003	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Burkholderiaceae	<i>Pelomonas</i>	0.36	0.56	0.14	0.17	0.07	0.00	0.16	0.56
ASV028	0.927	0.006	AC+E0+EC	Deltaproteobacteria	Bdellovibrionaceae	OM27 clade	0.06	0.00	0.07	0.00	1.23	0.00	0.59	0.00
ASV029	0.919	0.014	E0+EM	Alphaproteobacteria	Terasakiellaceae	NA	0.03	0.00	0.00	0.06	0.11	0.00	0.16	1.34
ASV030	0.976	0.025	A0+AA+AC+A M+E0+EC+EM	Alphaproteobacteria	Sphingomonadaceae	<i>Sphingomonas</i>	0.20	0.80	0.17	0.13	0.05	0.00	0.08	0.18
ASV031	0.943	0.012	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Rhizobiaceae	NA	0.42	0.00	0.07	0.26	0.14	0.00	0.22	0.39
ASV032	1	0.001	A0+AC+AM+E0 +EC+EM	Deltaproteobacteria	Bdellovibrionaceae	OM27 clade	0.14	0.00	0.13	0.17	0.32	0.00	0.34	0.32
ASV033	0.967	0.004	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Rhizobiaceae	NA	0.29	0.01	0.33	0.28	0.11	0.00	0.16	0.10
ASV034	0.976	0.019	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Moraxellaceae	<i>Enhydrobacter</i>	0.21	0.27	0.15	0.15	0.05	0.00	0.10	0.25
ASV035	0.913	0.025	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Kiloniellaceae	NA	0.29	0.00	0.08	0.11	0.12	0.00	0.32	0.23
ASV036	0.894	0.013	E0+EC	Bacteroidia	Crocinitomicaceae	<i>Crocinitomix</i>	0.04	0.00	0.00	0.00	0.54	0.00	0.51	0.00
ASV037	0.913	0.021	A0+AC+AM+E0 +EC+EM	Alphaproteobacteria	Hyphomonadaceae	<i>Hyphomonas</i>	0.19	0.00	0.11	0.10	0.15	0.00	0.42	0.10
ASV038	0.951	0.028	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Alteromonadaceae	<i>Alteromonas</i>	0.07	0.06	0.23	0.11	0.23	0.00	0.14	0.15
ASV039	0.943	0.016	A0+AC+AM+E0 +EC+EM	Deltaproteobacteria	P3OB-42	NA	0.06	0.00	0.06	0.31	0.14	0.00	0.17	0.15
ASV040	0.951	0.042	A0+AA+AC+A M+E0+EC+EM	Gammaproteobacteria	Solimonadaceae	<i>Oceanococcus</i>	0.09	0.18	0.11	0.05	0.08	0.00	0.15	0.23
ASV041	0.876	0.027	A0+AA+AM	Spirochaetia	Spirochaetaceae	<i>Spirochaeta 2</i>	0.43	0.20	0.00	0.21	0.01	0.00	0.00	0.00
ASV042	0.972	0.003	A0+AC+AM+E0 +EC+EM	Gammaproteobacteria	Alteromonadaceae	<i>Alteromonas</i>	0.19	0.00	0.03	0.14	0.07	0.00	0.06	0.23
ASV043	0.911	0.003	E0+EC+EM	Alphaproteobacteria	Rhodobacteraceae	<i>Rosei</i>	0.07	0.00	0.03	0.00	0.14	0.00	0.25	0.13

ASV044	0.943	0.01	A0+AC+AM+E0+EC+EM	Alphaproteobacteria	Terasakiellaceae	NA	0.05	0.00	0.06	0.17	0.10	0.00	0.12	0.08
ASV045	0.898	0.007	A0+AC+E0+EC	Bacteroidia	Flavobacteriaceae	<i>Winogradskyella</i>	0.11	0.00	0.24	0.02	0.06	0.00	0.12	0.00
ASV046	0.976	0.006	AA	Alphaproteobacteria	Rhodobacteraceae	<i>Litorimicrobium</i>	0.02	0.44	0.00	0.00	0.00	0.00	0.00	0.00
ASV047	0.896	0.012	AC+AM+EC+EM	Alphaproteobacteria	AB1	NA	0.00	0.00	0.09	0.08	0.02	0.00	0.10	0.17
ASV048	0.924	0.011	E0+EC+EM	Alphaproteobacteria	NA	NA	0.01	0.02	0.01	0.00	0.04	0.00	0.13	0.10
ASV049	1	0.003	EA	Holophagae	Acanthopleuribacteraceae	<i>Acanthopleuribacter</i>	0.00	0.00	0.00	0.00	0.00	0.30	0.00	0.00
ASV050	0.906	0.015	AA	Alphaproteobacteria	Rhodobacteraceae	<i>Marinovum</i>	0.00	0.18	0.00	0.01	0.01	0.00	0.00	0.02
ASV051	0.972	0.008	AA	Bacteroidia	Saprospiraceae	<i>Membranicola</i>	0.00	0.18	0.00	0.00	0.00	0.00	0.01	0.00
ASV052	0.845	0.018	AA	Deltaproteobacteria	NA	NA	0.00	0.12	0.01	0.00	0.06	0.00	0.00	0.00
ASV053	0.86	0.012	A0+AC+AM	Alphaproteobacteria	NA	NA	0.06	0.00	0.05	0.03	0.01	0.00	0.00	0.00
ASV054	0.867	0.02	A0+AA	Gammaproteobacteria	Algiphilaceae	<i>Algiphilus</i>	0.05	0.06	0.01	0.01	0.01	0.00	0.01	0.00
ASV055	0.828	0.015	A0+AM+E0	Alphaproteobacteria	Rickettsiaceae	<i>Candidatus Megaira</i>	0.02	0.00	0.00	0.05	0.04	0.00	0.02	0.00
ASV056	1	0.006	AA	Alphaproteobacteria	Devosiaceae	<i>Maritalea</i>	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00

Table S5-4 Pairwise comparisons between temperature conditions by antioxidant treatment of F_v/F_m for Day 13. Values in **bold** indicate significant differences; $\alpha = 0.05$.

Temperature	Antioxidant	estimate	SE	df	t.ratio	p.value
Ambient > Elevated	Control	0.0738	0.00673	142	10.959	< 0.0001
Ambient > Elevated	Ascorbate+Catalase	0.1014	0.01377	142	7.369	< 0.0001
Ambient > Elevated	Catechin	0.0651	0.00666	142	9.771	< 0.0001
Ambient > Elevated	Mannitol	0.0461	0.00682	142	6.761	< 0.0001

Table S5-5 Pairwise comparisons between antioxidant treatments by temperature condition of F_v/F_m for Day 13. Values in **bold** indicate significant differences; $\alpha = 0.05$.

Temperature	Antioxidant	estimate	SE	df	t.ratio	p.value
Ambient	Control < Ascorbate+Catalase	-0.00469	0.00702	142	-0.668	0.909
Ambient	Control < Catechin	-0.01362	0.00673	142	-2.023	0.1845
Ambient	Control < Mannitol	-0.00877	0.00666	142	-1.318	0.553
Ambient	Ascorbate+Catalase < Catechin	-0.00894	0.00709	142	-1.26	0.5897
Ambient	Ascorbate+Catalase < Mannitol	-0.00409	0.00702	142	-0.582	0.9373
Ambient	Catechin < Mannitol	0.00485	0.00673	142	0.72	0.8889
Elevated	Control > Ascorbate+Catalase	0.02295	0.01363	142	1.685	0.3356
Elevated	Control < Catechin	-0.02232	0.00666	142	-3.35	0.0056
Elevated	Control < Mannitol	-0.03646	0.0069	142	-5.287	< 0.0001
Elevated	Ascorbate+Catalase < Catechin	-0.04528	0.01355	142	-3.341	0.0058
Elevated	Ascorbate+Catalase < Mannitol	-0.05942	0.01367	142	-4.347	0.0002
Elevated	Catechin < Mannitol	-0.01414	0.00675	142	-2.095	0.1597

Chapter 6

Short-term exposure to sterile seawater reduces bacterial community diversity in the sea anemone, *Exaiptasia diaphana*

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6.1 Abstract

The global decline of coral reefs heightens the need to understand how corals may persist under changing environmental conditions. Restructuring of the coral-associated bacterial community, either through natural or assisted strategies, has been suggested as a means of adaptation to climate change. A low complexity microbial system would facilitate testing the efficacy of microbial restructuring strategies. We used the model organism for corals, *Exaiptasia diaphana*, and determined that short-term (three weeks) exposure to filter-sterilised seawater conditions alone reduced the complexity of the microbiome. Metabarcoding of the V5-V6 region of the bacterial 16S rRNA gene revealed that alpha diversity was approximately halved in anemones reared in filter-sterilized seawater compared to controls reared in unfiltered seawater and that the composition (beta diversity) differed significantly between the two. By reducing the complexity of the *E. diaphana* microbiome, the development of a system for testing assisted strategies such as probiotics, is more feasible.

6.2 Introduction

Corals are colonized by microbes, including bacteria, eukaryotes, archaea, and viruses (Blackall et al., 2015; Ainsworth et al., 2017; Huggett and Apprill, 2019), that contribute to the overall health of this complex host-microbe association, or holobiont (Rohwer et al., 2002). A noted eukaryote in the coral holobiont is the algal endosymbiont, a dinoflagellate of the family Symbiodiniaceae, which actively interact with other coral-associated microbes (Camp et al., 2020; Matthews et al., 2020). Coral-associated bacteria, hereafter referred to as the “microbiome,” likely consist of a combination of commensals, transients, and long-term stable members, and combined with the host, form a mutually beneficial, stable symbiosis. Evolution of the coral microbiome to confer benefits to the holobiont may occur naturally (Ziegler et al., 2017) or be directed through assisted evolution strategies, in which the microbiome of cnidarians is manipulated (van Oppen et al., 2015; van Oppen et al., 2018; Epstein et al., 2019a). Manipulation of the coral microbiome could be achieved through the addition of a bacterial consortium with specific purposes such as degradation

of oil (dos Santos et al., 2015), nutrient cycling, disease prevention (Alagely et al., 2011), or mitigation of bleaching (Rosado et al., 2018). These consortia, also known as probiotics, are developed to target specific aspects of holobiont health and functioning and should be based on the management of the already complex ménage à trois of host-bacteria-algae interactions.

One strategy to unravel the functions of and interactions between host-associated bacteria is to identify and synthetically recreate the minimal microbiome (MM). The MM is defined as the fewest microbes and/or microbial functions needed to maintain health and functioning of the holobiont (de Vos, 2013). The concept of a MM has been explored in plant science (Qin et al., 2016) and the human gut microbiome (de Vos, 2013; Clavel et al., 2017), with suggestions for its application in coral science (van Oppen and Blackall, 2019). While the ability to achieve the MM is important for functional microbiome studies, realizing this with antibiotics is difficult for many marine invertebrates (D'Agostino, 1975), including the model organism for corals, the sea anemone *Exaiptasia diaphana* (Hartman, 2020).

As an alternative approach to creating the MM, we have explored a community reduction strategy of the *E. diaphana* microbiome by manipulating the external environment (Clavel et al., 2017), instead of the application of antibiotics. Studies on the microbiome of *E. diaphana* indicate a similar phylum level diversity to corals (Blackall et al., 2015) for anemones sourced from the Great Barrier Reef (GBR) (Hartman et al., 2019; Dungan et al., 2020a; Hartman et al., 2020), Hawaii (Herrera et al., 2017), Pacific and Caribbean (Brown et al., 2017), Atlantic (Röthig et al., 2016) and Red Sea (Ahmed et al., 2019). We hypothesize that selective forces acting on *E. diaphana* and its microbiome, when placed in sterile seawater, will reduce the complexity of the microbiome with the loss of some (e.g., transient) members. This hypothesis is driven by existing evidence that environment has a significant influence on the cnidarian microbiome (Zhang et al., 2015; Webster and Reusch, 2017; Hartman, 2020). If their microbiomes were successfully reduced, we sought to identify bacterial taxa that persisted in anemones reared in sterile seawater as these taxa may be critical to holobiont health.

6.3 Methods

6.3.1 Sample collection

Anemones and the surrounding seawater from each of four GBR-sourced genotypes (Dungan et al., 2020b) were sampled once from each of two conditions:

- 1) control anemones - maintained in Red Sea Salt™ (R11065, Red Sea, USA; RSS) seawater prepared with reverse osmosis water at ~34 parts per thousand (ppt) salinity, or
- 2) 'sterile' anemones – defined as anemones removed from stock conditions (see 1), maintained in sterilised glass jars with 300 mL of 0.2 µm filtered RSS (fRSS), and sampled after three weeks.

Before fRSS was added to the anemones, the water was plated on Marine Agar (Difco™ Marine Agar 2216; MA); the absence of any bacterial growth on agar plates after seven days confirmed sterility. In both conditions, anemones were fed *ad libitum* with freshly hatched *Artemia salina* (brine shrimp, Salt Creek, UT, USA) nauplii twice weekly. *A. salina* feedstock was not sterile prior to feeding (see Hartman et al., 2020 for details on feedstock bacterial community composition). Control and 'sterile' anemone tanks were cleaned each week after feeding with complete RSS or fRSS water changes, respectively. Each anemone (n=6 for control anemones and n=15 for 'sterile' anemones from each genotype) was individually homogenized in a sterile glass homogenizer in 1 mL fRSS, flash frozen in liquid nitrogen, and stored at –20°C for later DNA extraction. Immediately following anemone acquisition, RSS and fRSS was collected from each anemone culture container (n=3 for RSS and n=5 for fRSS for each genotype container). The collected RSS and fRSS had hosted the anemones for 7 days since the last water change. Briefly, 250 mL of RSS or fRSS was filtered through a 0.2 µm Supor hydrophilic polyether sulfone membrane (Pall, New York) with a Sentino peristaltic pump (Pall). The membrane was flash frozen then stored at –20°C until genomic DNA extraction.

6.3.2 Anemone health metrics

Health metrics were measured in control and 'sterile' anemones. Photosynthetic efficiency, according to maximum quantum yield (F_v/F_m) was measured on the intracellular algae by pulse-amplitude-modulated (PAM) fluorometry with an imaging-PAM system (IMAG-MAX/L, Walz Heinz, Germany) weekly, and immediately prior to sampling for both control and 'sterile' anemones. Additionally, a subset (n=5 per genotype for both control and 'sterile' anemones) were sacrificed for host protein and Symbiodiniaceae cell density determinations as described in (Dungan et al., 2020b). Additionally, feeding behaviours, anemone body and tentacle position, and asexual propagation were qualitatively documented throughout the experiment.

6.3.3 DNA extraction and MiSeq library preparation

DNA was extracted (Wilson et al., 2002; Hartman et al., 2019) from the anemones and RSS/fRSS samples for bacterial community analysis by metabarcoding of the 16S rRNA genes. Extraction blanks (n=7) were processed simultaneously to identify contaminants introduced during DNA extractions. Extracted DNA along with no template negative PCR controls (n=2) were amplified by PCR in triplicate using bacterial primers with Illumina Nextera adapters (underlined) targeting the V5-V6 regions of the 16S rRNA genes: 784F [5' GTGACCTATGAACTCAGGAGTCAGGATTAGATACCCTGGTA 3']; 1061R [5' CTGAGACTTGCACTATCGCAGCCRRRCACGAGCTGACGAC 3'] (Andersson et al., 2008). Each PCR contained 12.5 μ L MyTaq HS Mix polymerase (Bioline, Australia), 2 μ L of DNA template, 0.4 μ M of each primer, and nuclease-free water up to 25 μ L. PCR conditions were: 1 cycle $\times$ 95°C for 3 min; 18 cycles $\times$ 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle $\times$ 72°C for 7 min; hold at 4°C. Triplicates were not pooled and instead treated as technical replicates in library preparation and sequencing.

To prepare DNA sequencing libraries, 20 μ L of each PCR product was purified by size-selection using Ampure XP magnetic beads (Agencourt, Beckman Coulter, Australia). Indexing PCRs were created by combining 10 μ L of purified DNA with 10 μ L 2x Taq master mix (M0270S, New England BioLabs, Australia) and 0.25 μ M of forward and reverse

indexing primers. PCR conditions were: 1 cycle $\times$ 95°C for 3 min; 24 cycles $\times$ 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; 1 cycle $\times$ 72°C for 7 min; hold 4°C. A subset of samples, chosen at random, were checked for product size and quantity (2200 TapeStation, Agilent Technologies, Australia); sequencing libraries were created by pooling 5 μ L from each reaction and performing a final bead clean-up on 50 μ L. Each library was checked for quality and quantity to guide pool normalization (2200 TapeStation), then sequenced on a single Illumina MiSeq run using v3 (2 $\times$ 300 bp) reagents at the Walter and Eliza Hall Institute, Melbourne, Australia.

6.3.4 Metabarcoding data processing and analysis

Raw 16S rRNA gene sequences were imported into QIIME2 v2019.10.0 (Bolyen et al., 2019) and demultiplexed. Primers were removed with cutadapt v2.6 (Martin, 2011). Filtering, denoising, and chimera checking was performed using DADA2 (Callahan et al., 2016) in the QIIME2 environment to correct sequencing errors, remove low quality bases (mean Qscore <30), and generate bacterial amplicon sequence variants (ASVs). Taxonomy for each ASV was assigned against a SILVA database (version 132) trained with a naïve Bayes classifier against the same V5-V6 region targeted for sequencing (Bokulich et al., 2018).

All data were analysed in R (v3.6.2; R Core Team, 2019). Statistical tests were considered significant at $\alpha = 0.05$. ASV, taxonomy, metadata and phylogenetic tree files were imported into R and combined into a phyloseq object (McMurdie and Holmes, 2013). Potential contaminant ASVs were identified and removed sequentially from the dataset according to their abundance in the extraction and PCR negative controls relative to the anemone and seawater samples using the prevalence method in the R package decontam with $p=0.1$ (Davis et al., 2018).

Alpha diversity of the bacteria was investigated to assess the impact of RSS type on the bacterial communities. Alpha diversity metrics were calculated in the R package vegan (Oksanen et al., 2018) after rarefying the samples to 16 993 reads per sample. Alpha diversity data were then analysed for overall differences using linear mixed effects models (LME) against the variables genotype, maintenance in RSS or fRSS, and sample type

(anemone or seawater) with culture container specified as a random effect, in the R package nlme (Pinheiro et al., 2019). *Post hoc* pairwise comparisons were performed by genotype using Tukey's honestly significant difference test (Tukey, 1949) in the R package emmeans (Searle et al., 1980) with Tukey's adjustment for multiple comparisons. Standard deviations from the LME model were used to determine the within and between sample variation for triplicate sequencing data. Bar charts were created by agglomerating taxa at the family level based on relative abundances using the R package phyloseq.

Differences in the bacterial community compositions of each genotype were visualised in principal coordinates analysis (PCoA) ordinations based on a weighted UniFrac distance matrix. To compare bacterial community compositions, we applied a nonparametric permutational multivariate analysis of variance (PERMANOVA) using the weighted UniFrac distance matrix as implemented in the vegan function `adonis` (permutation=999) (Oksanen et al., 2018). Data were centre log ratio transformed (Morton et al., 2017) prior to PERMANOVA and all data were evaluated for homogeneity of dispersion among groups as an assumption for `adonis`. Genera that were significantly associated with treatment groups were identified using the `indicspecies` function `multipatt` (Cáceres and Legendre, 2009) and changes in their relative abundances were visualised in bubble plots for each genotype.

6.4 Results

6.4.1 Anemone health

Throughout the three-week period, all *E. diaphana* maintained good health based on the density and photophysiological performance of their *in hospite* Symbiodiniaceae, tentacle extension, active feeding on *A. salina* nauplii, and asexual propagation. Average Symbiodiniaceae cell densities (normalized to host protein) were not significantly different between control and 'sterile' anemones by genotype ($p > 0.05$). After an initial drop in F_v/F_m following the transfer of anemones to fRSS and in a different incubator, F_v/F_m values returned to control levels.

6.4.2 Metabarcoding data processing

Sequencing produced 8.8 M reads across *E. diaphana* (n=84), RSS/fRSS (n=32), extraction blanks (n=7), and negative control samples (n=2) sequenced as triplicate technical replicates for a total of 375 samples. After merging, denoising and chimera filtering, 6.2 M reads remained; three replicates were removed as they had <20 reads. Decontam identified 138 putative contaminant ASVs constituting 4.25% relative abundance of the bacterial communities. Upon review of these putative contaminants, 20 were found to be common marine bacteria. To be conservative, these 20 were removed from the decontam output and thus included in the subsequent data analyses. The remaining 118 ASVs (3.91% relative abundance) were removed from the data set prior to all statistical analyses (Table S6-1). After all filtering steps, there were 1171 ASVs across the remaining samples.

6.4.3 Triplicate technical replicates

For amplicon sequencing of the bacterial 16S rRNA gene V5-V6 region, the standard error within the three replicates of a single sample was greater than the error between biological replicates for the observed number of ASVs (13.12 and 9.35, respectively). For both Shannon's and Simpson's diversity metrics, the error between biological replicates and within a biological replicate was approximately the same. Given that these technical replicates all stem from one biological sample, the triplicates were merged for visualization and exploration of the bacterial microbial communities.

6.4.4 Bacterial community shifts

For all alpha diversity metrics, a one-way analysis of variance (ANOVA) reported a significant interaction of sample type (anemone versus seawater) and environment (RSS versus fRSS) ($F_{\text{ObsASVs}(1,81)}=62.35$, $p<0.0001$; $F_{\text{Simp}(1,81)}=12.26$, $p=0.0008$; $F_{\text{Shan}(1,81)}=51.67$, $p<0.0001$). Substantial decreases in bacterial alpha diversity were observed in all 'sterile' anemone genotypes (Fig. 6-1, Table S6-2) with the average number of observed ASVs dropping 55% from (mean $\pm$ SE) 134 ± 6 in control anemones to 74 ± 3 ($t(18)=8.85$, $p<0.0001$) in 'sterile' anemones. Simpson's and Shannon's bacterial diversity also decreased in all anemones, regardless of genotype, with significant reductions in AIMS2 (Shannon's only),

AIMS3 (both Shannon's and Simpson's), and AIMS4 (Shannon's only) (Fig. 6-1, Table S6-2). A total of 13 bacterial families were found in at least three genotypes of control anemones and absent in at least three genotypes of 'sterile' anemones (Table S6-3). These families were all part of the rare bacterial biosphere (Sogin et al., 2006) with the most abundant, Microtrichaceae, having an average of 0.12% relative abundance across all control genotypes. Conversely, there were five families that were present in the 'sterile' anemones, yet absent in control anemones (Table S6-3). The most abundant of these was Nisaeaceae at 0.18% relative abundance in 'sterile' anemones.

Principal coordinate analyses (PCoA) showed a change in composition of the bacterial communities within each genotype (Figure). There was clear clustering of data points by sample type (anemone versus fRSS/RSS) and environmental condition. PERMANOVA results by genotype confirmed that the bacterial community structure (Fig. 6-2B) varied significantly with the interaction of sample type and environment ($F_{\text{AIMS1}(1,86)}=6.59$, $p=0.001$; $F_{\text{AIMS2}(1,83)}=5.82$, $p=0.001$; $F_{\text{AIMS3}(1,85)}=6.18$, $p=0.001$; $F_{\text{AIMS4}(1,86)}=5.64$, $p=0.001$). The indicator species analysis highlighted genera that are characteristic of the microbiomes in these distinct groups (Fig. 6-3, Figs. S6-1 - S6-4). *Erythrobacter*, *Winogradskyella*, *Phaeodactylibacter*, *Muricauda*, and unresolved Rhodobacteraceae genera were significantly more abundant in control anemones compared to 'sterile' anemones (Table S6-4). Few genera were significantly more abundant in 'sterile' anemones (only *Nisaea*, *Thalassobaculum*, and *Alteromonas* in AIMS3 and *Tropicibacter* in AIMS4). Groups that were significantly more abundant in the anemones, regardless of environment or genotype, compared to seawater were Alteromonadaceae, Terasakiellaceae, *Coxiella*, *Labrenzia*, Nannocystaceae, Sphingobacteriales, and *Fulvivirga*. In the RSS *Roseibacterium* and *Pseudohongiella* were consistently more abundant while *Algiphilus* was in greater abundance in fRSS. Saprospiraceae was the only group that was significantly more abundant in all seawater samples, regardless of genotype or environment (Table S6-4).

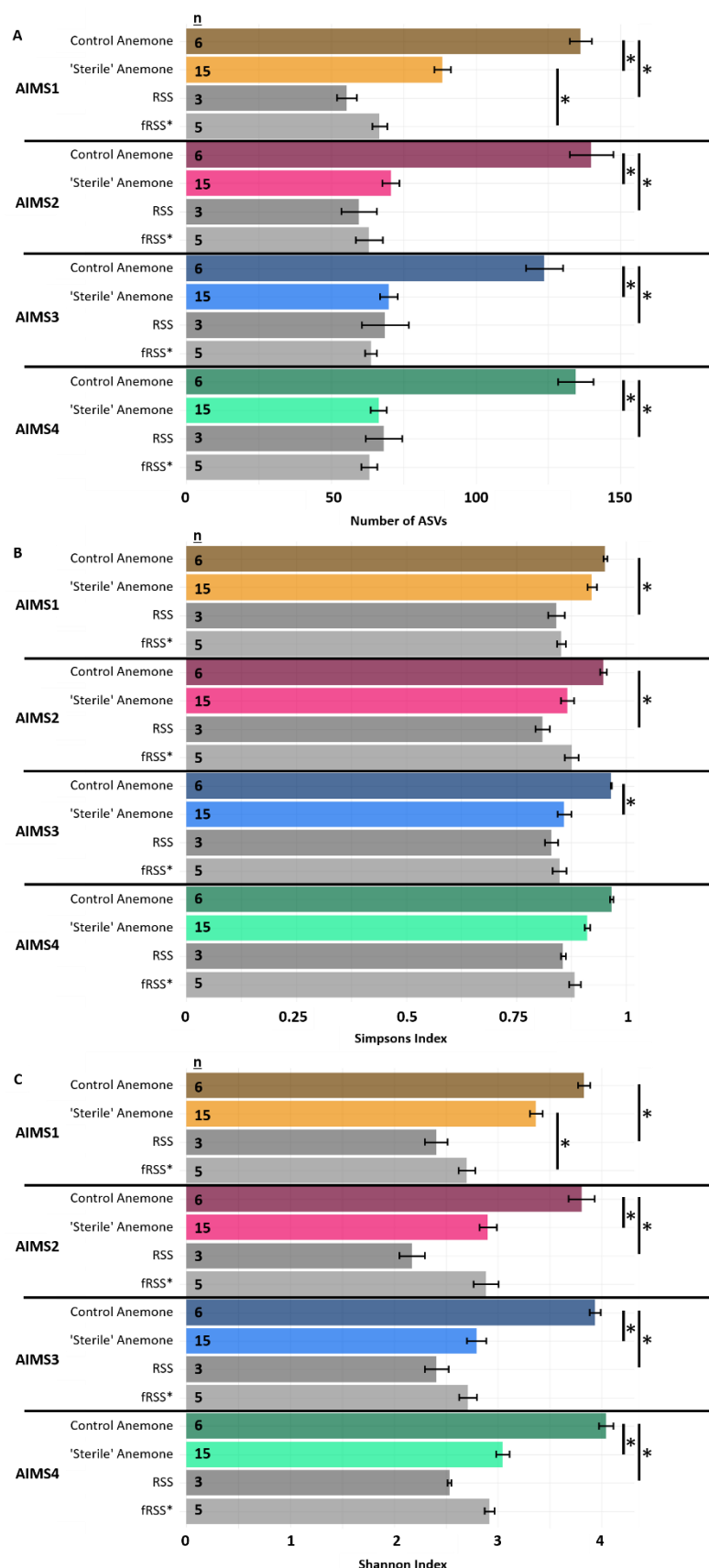
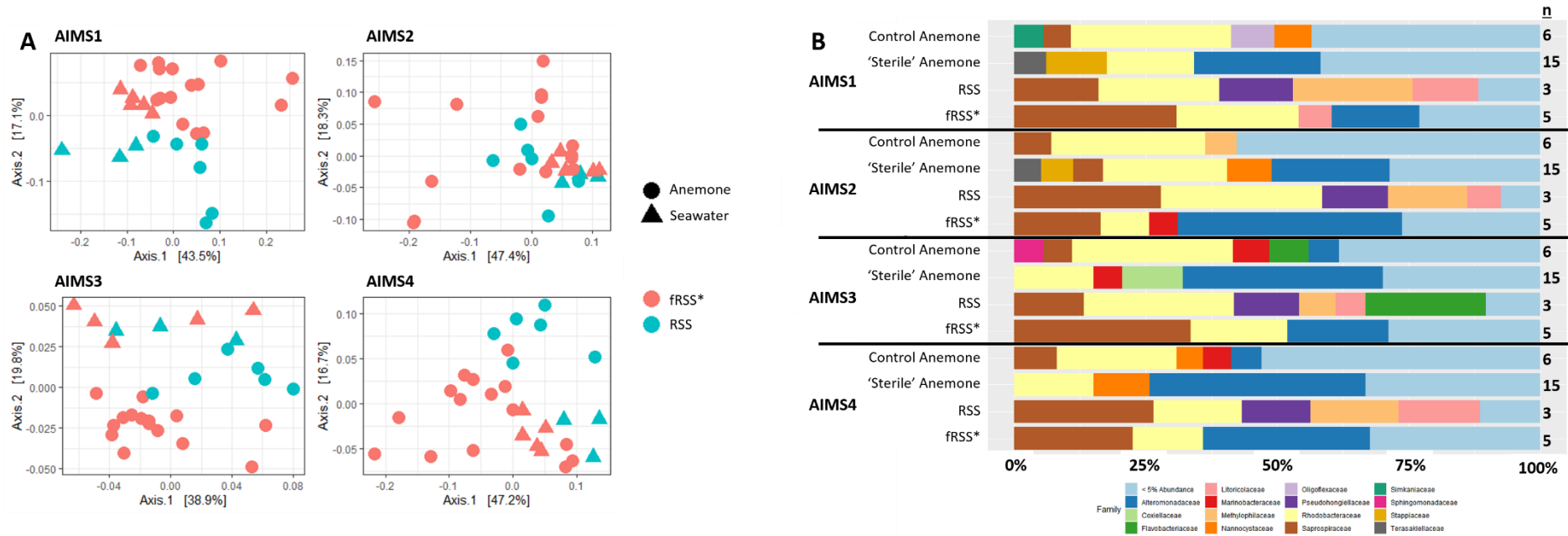


Figure 6-1 Observed ASVs (A), Simpson's Index (B), and Shannon's Index (C) for AIMS1–4 anemones (orange, purple, blue, green) and seawater (grey) samples in control (unfiltered seawater) or sterilized seawater (SW). The data set was rarefied to 16,993 reads per sample with technical triplicates pooled. Asterisks indicate significant pairwise differences between sample types by genotype. Averages $\pm$ SE are shown. Statistical output for pairwise comparisons is in SI Table 6-2. *fRSS was collected after hosting anemones for 7 days.



1
2 **Figure 6-2** Principle coordinate analysis (PCoA) (A) for AIMS1-4 using a weighted UniFrac distance matrix for the anemone (circles) and seawater
3 (triangles) samples in the control (blue symbols) and sterile (red symbols) treatments. Each point is an individual sample with technical triplicates merged.
4 Relative abundance of bacterial families (B) for anemone and seawater samples in RSS and fRSS treatments between GBR-sourced anemone genotypes
5 AIMS1-4. Families whose relative abundance was less than 5% across the whole data set were pooled into a single category.

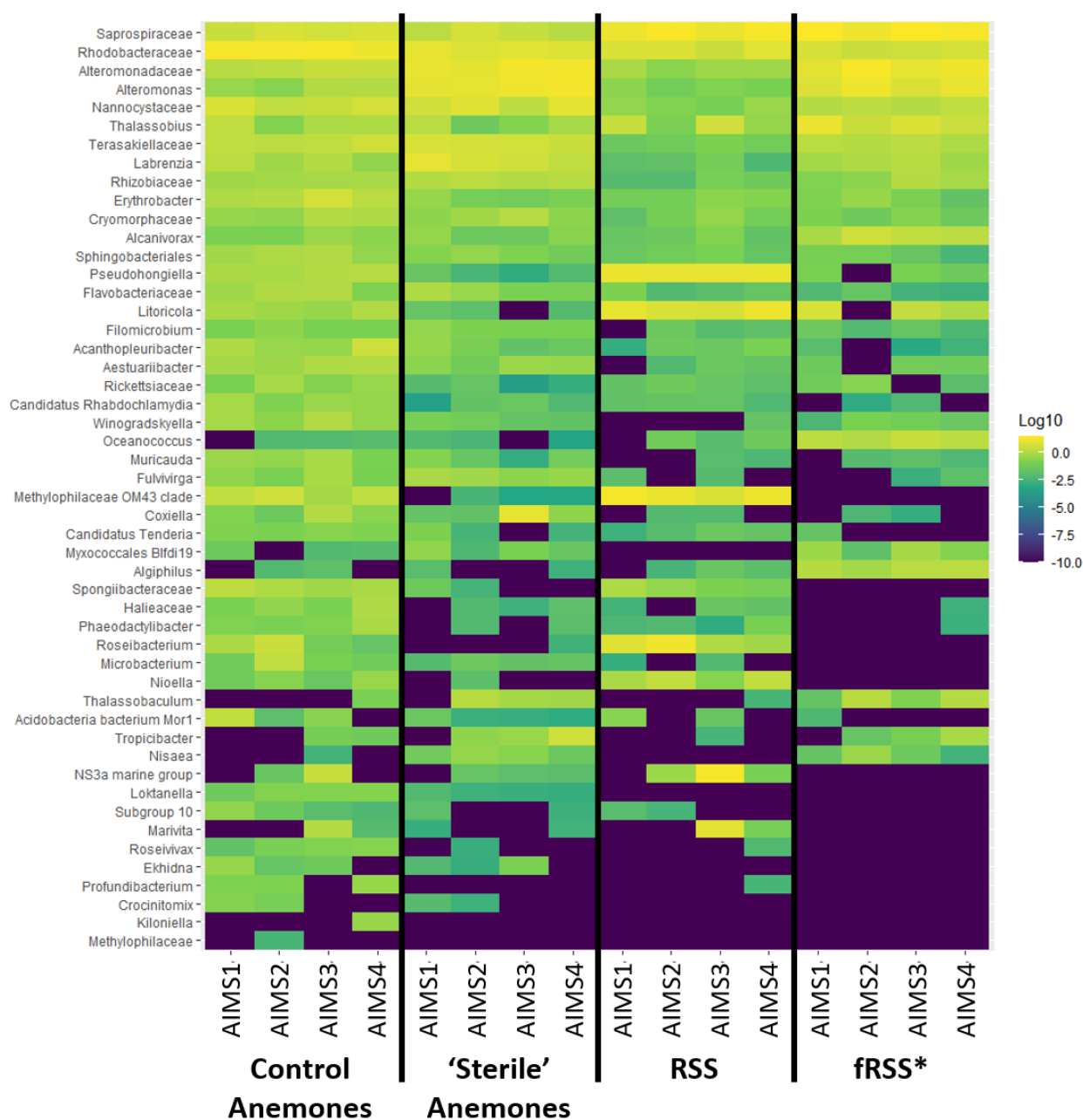


Figure 6-3 Heat map showing the relative abundance of bacterial taxa for each treatment group (control and 'sterile' anemones, RSS and fRSS) for AIMS1-4 anemones. The colour of each square corresponds with the relative abundance, which was log10 normalized. Taxa were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity. *fRSS was collected after hosting anemones for 7 days.

6.5 Discussion

The coral holobiont has the capacity to restructure its bacterial component, either through natural (Ziegler et al., 2017) or assisted strategies (Damjanovic et al., 2017), which has been suggested as a means of holobiont adaptation to climate change (Ziegler et al., 2019). We found that the bacterial diversity and community structure for each of four anemone genotypes was significantly different with a reduction in alpha diversity when anemones were moved from RSS to fRSS and maintained in fRSS for three weeks. Several taxa, including Alteromonadaceae, Terasakiellaceae, *Coxiella*, *Labrenzia*, Nannocystaceae, Sphingobacteriales, and *Fulvivirga*, maintained high abundances in 'sterile' anemones despite changes in other aspects of community structure. We suggest that these groups have a key role in holobiont health. Our results have implications for future manipulation of the *E. diaphana* holobiont, as these anemones can be manipulated to reduce the diversity of their microbiome while remaining healthy and thus are good models. The loss of bacterial diversity in the 'sterile' anemones and likely passage of these microbes into the seawater hints at the interaction of *E. diaphana* with their seawater environment.

6.5.1 *Exaiptasia diaphana* microbiome can be reduced with sterile seawater

From this study, the average number of ASVs from GBR-sourced *E. diaphana* was 134, which is substantially lower than >750 ASVs, which was previously reported for these anemones (Hartman et al., 2020). After anemones were maintained for three weeks in fRSS, the number of observed bacterial ASVs was significantly reduced in all genotypes while maintaining holobiont health. It has been previously reported that *E. diaphana* has a reduction of bacterial ASVs when cultured in fRSS, regardless of temperature (Hartman et al., 2019). A common approach to reduce the microbiome of a complex system is the application of antibiotics, which has been successful in marine invertebrates, including corals (Sweet et al., 2011), but often at the expense of holobiont health (D'Agostino, 1975; Gilbert et al., 2012; Hartman, 2020). Even when there are no visible adverse effects of antibiotic treatment, the artificial shift in or depletion of the holobiont microbiome may

have unintended consequences such as perturbations of native microbial communities and proliferation of pathogens (Raymann et al., 2017), or the development of antibiotic resistance. A disruption to the bacterial community of coral via antibiotic treatment has resulted in diminished resilience to thermal stress (Gilbert et al., 2012), bleaching and necrosis (Glasl et al., 2016). We have demonstrated that the microbial diversity of *E. diaphana* is rapidly (in three weeks) reduced in sterile seawater with an average of 55% of ASVs lost, critically, with no changes in Symbiodiniaceae photophysiology or anemone health.

6.5.2 Potential key microbiome members of *E. diaphana*

Bacterial genera were identified as indicators that reflect the treatment (RSS or fRSS) of the anemones and seawater (Fig. 6-3). *Erythrobacter*, *Winogradskyella*, *Phaeodactylibacter*, *Muricauda*, and unresolved Rhodobacteraceae genera were significantly more abundant in control anemones compared to 'sterile' anemones. At least three of these groups (*Erythrobacter*, *Winogradskyella*, and *Muricauda*) are known carotenoid producers (Prabhu et al., 2014; Zhang et al., 2016; Setiyono et al., 2019), which can serve as potent antioxidants protecting the algal symbiont during periods of oxidative stress (Schmitt et al., 2014). Given their absence in healthy 'sterile' anemones, they might be considered opportunistic colonizers. The only bacterial genera that were significantly more abundant in 'sterile' anemones were *Nisaea*, *Thalassobaculum*, and *Alteromonas* (AIMS 3) and *Tropicibacter* (AIMS4). These four genera are all Alphaproteobacteria, with the former two being members of the order Thalassobaculales and the latter two being Rhodobacterales members.

Several taxa were significantly more represented in the anemones versus seawater, regardless of genotype or seawater filtration status. These included Alteromonadaceae, *Labrenzia*, Terasakiellaceae, *Coxiella*, Nannocystaceae, Sphingobacteriales, and *Fulvivirga*. These potentially obligate anemone-associated taxa likely contribute to an important anemone feature like health or metabolism. The taxa we identified as stably associated with *E. diaphana* can be divided into those that are frequently found in cnidarians or their

algal symbionts, including Alteromonadaceae (Rothig et al., 2017; Ahmed et al., 2019; Damjanovic et al., 2019a; Epstein et al., 2019b), *Labrenzia* (Lawson et al., 2017), *Fulvivirga* (Glasl et al., 2016; Ziegler et al., 2017; Epstein et al., 2019b; Pootakham et al., 2019; Damjanovic et al., 2020) and Sphingobacteriales (Meron et al., 2012; Kellogg et al., 2013; Li et al., 2014; van de Water et al., 2017; Bonthond et al., 2018), and those that occur much more infrequently, including Terasakiellaceae, *Coxiella*, and Nannocystaceae. *Labrenzia* is part of the core microbiome of the anemones' Symbiodiniaceae (Lawson et al., 2017) and may have a role in the production of antioxidants such as dimethyl sulfoniopropionate (DMSP) and its breakdown products (Sunda et al., 2002). The DMSP biosynthesis (Curson et al., 2017) and degradation genes, were found in the whole genome sequences of *E. diaphana*-sourced *Labrenzia* isolates (Dungan et al., 2020a). Sphingobacteriales in soil systems are believed to be copiotrophic, referring to their ability to metabolize a wide array of carbon sources (Hester et al., 2018).

Terasakiellaceae (Rhodospirallales) were reported in the cold water coral *Paragorgia arborea* (Weiler et al., 2018) where they were implicated in nitrogen cycling, and they could have a similar role in the anemone holobiont. *Coxiella* are members of the order Legionellales, which are described as facultative or obligate intracellular parasites known to infect invertebrate and vertebrate species (Garritty and Brenner, 2005), including marine animals; their role in anemones should be determined. While there are few references to Nannocystaceae in the literature (i.e. Gignoux-Wolfsohn et al., 2020), the order Myxococcales has been reported in coral metabarcoding studies (Godoy-Vitorino et al., 2017; Ziegler et al., 2017; Pollock et al., 2018; Rosales et al., 2019). Myxococcales has been associated with disease resistant corals (Rosales et al., 2019) and could have some importance in pathogen control.

6.5.3 Mitigating metabarcoding biases

Recent evidence suggests that pooling triplicate PCRs prior to sequencing is redundant and that a single replicate is sufficient as modern amplicon data analysis is robust enough to reduce the influence of artefacts introduced during the PCR (Marotz et al., 2019). We

compared triplicate technical replicates from single reactions for amplicon sequencing of the bacterial 16S rRNA gene V5-V6 region and found that the standard error within was greater than the error between biological samples from a single sampling replicate for the observed number of ASVs. Because each sample was sequenced in the same MiSeq run, we would expect less variation between the technical replicates than the biological samples. However, in the case of observed numbers of ASVs, there was a strong, consistent effect of technical replicate. The variation between technical replicates is likely an artefact of errors that arise during the early stages of PCR, which are then amplified exponentially, and chimera formation (Polz and Cavanaugh, 1998), thus these results support the continued practice of pooling triplicate PCRs prior to the indexing step as outlined in the Earth's Microbiome Project protocol (Gilbert et al., 2014). Like the Earth's Microbiome Project, having standard procedures for cnidarian sampling and processing, as well as the establishment of a dynamic mock community comprised of coral-associated taxa, could mitigate some of these potential biases.

6.5.4 Conclusions

The results from this study have implications for future manipulation of the *E. diaphana* microbiome and perhaps that of corals. Recent studies have shown that cnidarians can acquire bacteria introduced into their external environment (Damjanovic et al., 2017; Damjanovic et al., 2019c; Damjanovic et al., 2020) and, with the addition of probiotic bacteria, may have the ability to mitigate the effects of climate change (Rosado et al., 2018). Future studies should test the ability of *E. diaphana* to acquire bacteria from their environment with a focus on microbes that have potentially beneficial traits for the holobiont.

The use of probiotics or synthetic bacterial communities has been effective in treating disease in humans (de Vos, 2013; Shetty et al., 2019) and aquaculture (Verschuere et al., 2000; Ringø, 2020) as well as promoting plant growth (Qin et al., 2016). Following these systems, modifying or engineering the microbiome of corals with probiotics may be an option to enhance coral recovery from bleaching or disease (Peixoto et al., 2017; Rosado et

al., 2018). These types of interventions will likely require extensive experimentation, thus, the ability to work with a reduced microbiome in a healthy anemone holobiont is important. We found that culturing *E. diaphana* in fRSS versus RSS was able to halve the microbiome diversity while still maintaining holobiont health and function. This finding will be invaluable for bacterial bioengineering approaches to increase the climate resilience of the coral holobiont.

6.6 Data availability

The raw Illumina MiSeq dataset for this study can be found under NCBI BioProject ID PRJNA576556.

6.7 Acknowledgments

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6.8 Supplementary material

Table S6-1 Potential contaminants identified from extraction blanks and negative controls. A total of 118 potential contaminants were identified from extraction blanks (n=113) and negative controls (n=5). Contaminants 013-118 each had > 0.1% relative abundance and were pooled in the last row for brevity.

ASV	Group	Relative Abundance (%)	Phylum	Family	Genus
Contaminant001	Extraction Blanks	1.59	Proteobacteria	Burkholderiaceae	<i>Cupriavidus</i>
Contaminant002	Extraction Blanks	0.89	Firmicutes	Staphylococcaceae	<i>Staphylococcus</i>
Contaminant003	Extraction Blanks	0.67	Proteobacteria	Sphingomonadaceae	<i>Novosphingobium</i>
Contaminant004	Extraction Blanks	0.31	Proteobacteria	Burkholderiaceae	<i>Pelomonas</i>
Contaminant005	Extraction Blanks	0.20	Actinobacteria	Propionibacteriaceae	<i>Cutibacterium</i>
Contaminant006	Extraction Blanks	0.13	Bacteroidetes	Chitinophagaceae	<i>Hydrothalea</i>
Contaminant007	Extraction Blanks	0.11	Firmicutes	Staphylococcaceae	<i>Staphylococcus</i>
Contaminant008	Negative	0.04	Firmicutes	Staphylococcaceae	<i>Staphylococcus</i>
Contaminant009	Extraction Blanks	0.03	Actinobacteria	Corynebacteriaceae	<i>Lawsonella</i>
Contaminant010	Extraction Blanks	0.03	Proteobacteria	Sphingomonadaceae	<i>Sphingomonas</i>
Contaminant011	Extraction Blanks	0.02	Proteobacteria	Pseudomonadaceae	<i>Pseudomonas</i>
Contaminant012	Extraction Blanks	0.02	Actinobacteria	Nocardiaceae	<i>Rhodococcus</i>
Contaminant013-118	All	0.20			

Table S6-2 Pairwise comparisons of alpha diversity metrics by genotype. Significant differences are in **bold**; $\alpha = 0.05$.

			Observed ASVs			Simpson's Index			Shannon's Index		
Genotype	Pairwise Comparison		df	t.ratio	p.value	df	t.ratio	p.value	df	t.ratio	p.value
AIMS1	Control Anemone	RSS	81	6.735	<0.0001	81	2.8	0.0318	81	5.119	<0.0001
AIMS1	Control Anemone	'Sterile' Anemone	18	4.228	0.0026	18	0.748	0.8765	18	1.71	0.3474
AIMS1	RSS	fRSS	18	-0.407	0.9766	18	-0.733	0.8825	18	-1.135	0.6736
AIMS1	'Sterile' Anemone	fRSS	81	3.157	0.0118	81	2.015	0.1909	81	3.285	0.0081
AIMS2	Control Anemone	RSS	81	6.995	<0.0001	81	3.477	0.0045	81	5.915	<0.0001
AIMS2	Control Anemone	'Sterile' Anemone	18	6.813	<0.0001	18	2.174	0.1684	18	3.794	0.0066
AIMS2	RSS	fRSS	18	0.345	0.9854	18	-1.823	0.2952	18	-2.108	0.1883
AIMS2	'Sterile' Anemone	fRSS	81	1.346	0.5368	81	-0.181	0.9979	81	0.304	0.9902
AIMS3	Control Anemone	RSS	81	5.006	<0.0001	81	2.393	0.0866	81	5.045	<0.0001
AIMS3	Control Anemone	'Sterile' Anemone	18	5.157	0.0004	18	2.942	0.0397	18	4.715	0.0009
AIMS3	RSS	fRSS	18	0.5	0.958	18	0.001	1	18	-0.685	0.9015
AIMS3	'Sterile' Anemone	fRSS	81	1.108	0.6857	81	0.33	0.9875	81	0.449	0.9697
AIMS4	Control Anemone	RSS	81	5.922	<0.0001	81	2.311	0.1041	81	5.386	<0.0001
AIMS4	Control Anemone	'Sterile' Anemone	18	7.024	<0.0001	18	1.586	0.411	18	4.26	0.0024
AIMS4	RSS	fRSS	18	1.093	0.6979	18	-0.551	0.9451	18	-1.215	0.6255
AIMS4	'Sterile' Anemone	fRSS	81	0.966	0.7693	81	0.771	0.8673	81	0.609	0.929

Table S6-3 Relative abundance (%) of bacterial families that were present in at least three genotypes of the 'sterile' anemones but absent in the control anemones (dark grey) and families that were absent in the 'sterile' anemones but present under control conditions (white).

Class	Order	Family	Contro l AIMS1	Contro l AIMS2	Contro l AIMS3	Contro l AIMS4	Sterile SW AIMS1	Sterile SW AIMS2	Sterile SW AIMS3	Sterile SW AIMS4
Alphaproteobacteria	Thalassobaculales	Nisaeaceae	0.000	0.000	0.002	0.000	0.059	0.430	0.203	0.033
Alphaproteobacteria	Caulobacterales	Caulobacteraceae	0.000	0.000	0.000	0.000	0.395	0.129	0.024	0.097
Bacilli	Bacillales	Paenibacillaceae	0.000	0.000	0.000	0.000	0.171	0.042	0.011	0.005
Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	0.000	0.000	0.000	0.001	0.001	0.001	0.005	0.003
Clostridia	Clostridiales	Family XVII	0.000	0.000	0.000	0.000	0.004	0.001	0.000	0.001
Deltaproteobacteria	Myxococcales	Haliangiaceae	0.053	0.043	0.023	0.051	0.000	0.000	0.000	0.001
Gammaproteobacteria	Ga0077536	Unknown	0.037	0.144	0.006	0.025	0.001	0.000	0.000	0.000
Gammaproteobacteria	Legionellales	Legionellaceae	0.006	0.057	0.009	0.040	0.001	0.000	0.000	0.000
Deltaproteobacteria	Myxococcales	Unknown	0.007	0.016	0.002	0.006	0.000	0.000	0.000	0.000
Gracilibacteria	Candidatus Peregrinibacteria	Unknown	0.000	0.003	0.001	0.062	0.000	0.000	0.000	0.000
Acidimicrobiia	Microtrichales	Microtrichaceae	0.441	0.169	0.033	0.246	0.000	0.000	0.000	0.000
Alphaproteobacteria	NRL2	Rhizobiales bacterium NRL2	0.011	0.027	0.010	0.027	0.000	0.000	0.000	0.000
Alphaproteobacteria	NRL2	Unknown	0.017	0.000	0.001	0.114	0.000	0.000	0.000	0.000
Alphaproteobacteria	Puniceispirillales	Unknown	0.105	0.100	0.001	0.020	0.000	0.000	0.000	0.000
Bacteroidia	Cytophagales	Microscillaceae	0.009	0.015	0.000	0.008	0.000	0.000	0.000	0.000
Chlamydiae	Chlamydiales	Criblamydiaceae	0.012	0.003	0.003	0.133	0.000	0.000	0.000	0.000
OM190	Unknown	Unknown	0.001	0.005	0.000	0.009	0.000	0.000	0.000	0.000
Rhodothermia	Balneolales	Balneolaceae	0.005	0.002	0.021	0.026	0.000	0.000	0.000	0.000

Table S6-4 Indicator bacterial genera that characterize environmental treatments by genotype. Genera were selected from an indicator species analysis with parameters $At=0.8$ and $Bt=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity.

Treatment	Genotype	Class	Order	Family	Genus	stat	p.value
Control Anemones	AIMS1	Subgroup 22	Acidobacteria bacterium Mor1	Acidobacteria bacterium Mor1	<i>Acidobacteria bacterium Mor1</i>	0.95	0.001
frSS	AIMS1	Gammaproteobacteria	Salinisphaerales	Algiphilaceae	<i>Algiphilus</i>	0.927	0.001
Control Anemones	AIMS1	Gammaproteobacteria	Coxiellales	Coxiellaceae	<i>Coxiella</i>	0.954	0.001
Control Anemones	AIMS1	Bacteroidia	Flavobacteriales	Crocinitomicaceae	<i>Crocinitomix</i>	0.896	0.001
Control Anemones	AIMS1	Bacteroidia	Cytophagales	Cyclobacteriaceae	<i>Ekhidna</i>	0.963	0.001
Anemones	AIMS1	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Erythrobacter</i>	0.878	0.001
Anemones	AIMS1	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Filomicrobium</i>	0.893	0.001
Anemones	AIMS1	Bacteroidia	Cytophagales	Cyclobacteriaceae	<i>Fulvivirga</i>	0.921	0.001
Anemones	AIMS1	Alphaproteobacteria	Rhizobiales	Stappiaceae	<i>Labrenzia</i>	0.974	0.001
Anemones	AIMS1	Bacteroidia	Flavobacteriales	Flavobacteriaceae	<i>Muricauda</i>	0.9	0.001
Control Anemones	AIMS1	Gammaproteobacteria	Betaproteobacteriales	Methylophilaceae	OM43 clade	0.95	0.001
Control Anemones	AIMS1	Bacteroidia	Chitinophagales	Saprospiraceae	<i>Phaeodactylibacter</i>	0.902	0.001
Control Anemones	AIMS1	Gammaproteobacteria	Oceanospirillales	Pseudohongiellaceae	<i>Pseudohongiella</i>	0.961	0.001
Control Anemones	AIMS1	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Roseibacterium</i>	0.951	0.001
Control Anemones	AIMS1	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	Subgroup 10	0.911	0.001

Seawater	AIMS1	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Thalassobius</i>	0.91	0.001
Control Anemones	AIMS1	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.951	0.001
Anemones	AIMS1	Gammaproteobacteria	Alteromonadales	Alteromonadaceae		0.998	0.001
Anemones	AIMS1	Bacteroidia	Flavobacteriales	Cryomorphaceae		0.827	0.001
Anemones	AIMS1	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae		0.95	0.001
Anemones	AIMS1	Deltaproteobacteria	Myxococcales	Nannocystaceae		0.923	0.001
Anemones	AIMS1	Alphaproteobacteria	Rhodospirillales	Terasakiellaceae		0.909	0.001
Control Anemones	AIMS1	Gammaproteobacteria	KI89A clade			0.934	0.001
Control Anemones	AIMS1	Bacteroidia	Flavobacteriales	Flavobacteriaceae	<i>Winogradskyella</i>	0.931	0.001
Control Anemones	AIMS1	Gammaproteobacteria	Cellvibrionales	Halieaceae		0.936	0.001
Anemones	AIMS1	Bacteroidia	Flavobacteriales	Flavobacteriaceae		0.908	0.001
Anemones	AIMS1	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.899	0.001
Anemones	AIMS1	Alphaproteobacteria				0.896	0.001
Seawater	AIMS1	Bacteroidia	Chitinophagales	Saprospiraceae		0.954	0.001
Control Anemones	AIMS2	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	<i>Aestuariibacter</i>	0.847	0.001
fRSS	AIMS2	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	<i>Alcanivorax</i>	0.97	0.001
Control Anemones	AIMS2	Gammaproteobacteria	Tenderiales	Tenderiaceae	<i>Candidatus Tenderia</i>	0.935	0.001
Anemones	AIMS2	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Filomicrobium</i>	0.873	0.001
Anemones	AIMS2	Bacteroidia	Cytophagales	Cyclobacteriaceae	<i>Fulvivirga</i>	0.98	0.001

						4	
Anemones	AIMS2	Alphaproteobacteria	Rhizobiales	Stappiaceae	<i>Labrenzia</i>	0.90 6	0.001
Control Anemones	AIMS2	Gammaproteobacteria	Oceanospirillales	Litoricolaceae	<i>Litoricola</i>	0.95	0.001
Control Anemones	AIMS2	Bacteroidia	Flavobacteriales	Flavobacteriaceae	<i>Muricauda</i>	0.90 8	0.001
Control Anemones	AIMS2	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Nioella</i>	0.94 7	0.001
Control Anemones	AIMS2	Bacteroidia	Chitinophagales	Saprospiraceae	<i>Phaeodactylibacter</i>	0.86 9	0.001
Control Anemones	AIMS2	Gammaproteobacteria	Oceanospirillales	Pseudohongiellaceae	<i>Pseudohongiella</i>	0.96 4	0.001
Control Anemones	AIMS2	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Roseibacterium</i>	0.92 1	0.001
Control Anemones	AIMS2	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.97 2	0.001
Anemones	AIMS2	Gammaproteobacteria	Alteromonadales	Alteromonadaceae		0.99 1	0.001
Anemones	AIMS2	Deltaproteobacteria	Myxococcales	Nannocystaceae		0.89 2	0.001
Anemones	AIMS2	Alphaproteobacteria	Rhodospirillales	Terasakiellaceae		0.90 2	0.001
Control Anemones	AIMS2	Deltaproteobacteria	Myxococcales	Nannocystaceae		0.88 5	0.001
Control Anemones	AIMS2	Gammaproteobacteria	KI89A clade			0.88 4	0.001
Control Anemones	AIMS2	Bacteroidia	Chitinophagales			0.91 2	0.001
Anemones	AIMS2	Bacteroidia	Chitinophagales	uncultured Sphingobacteriales bacterium		0.92 8	0.001
Control Anemones	AIMS2	Gammaproteobacteria	Cellvibrionales	Halieaceae		0.90 2	0.001
Anemones	AIMS2	Bacteroidia	Flavobacteriales	Flavobacteriaceae		0.90 6	0.001

Anemones	AIMS2	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.91 4	0.001
Anemones	AIMS2	Bacteroidia	Chitinophagales			0.83 5	0.001
fRSS	AIMS2	Deltaproteobacteria				0.88 6	0.001
Seawater	AIMS2	Bacteroidia	Chitinophagales	Saprospiraceae		0.89 9	0.001
fRSS	AIMS3	Gammaproteobacteria	Salinisphaerales	Algiphilaceae	<i>Algiphilus</i>	0.98 4	0.001
'Sterile' Anemones	AIMS3	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	<i>Alteromonas</i>	0.88 6	0.001
Control Anemones	AIMS3	Chlamydiae	Chlamydiales	Simkaniaceae	<i>Candidatus Rhabdochlamydia</i>	0.86 4	0.001
Anemones	AIMS3	Gammaproteobacteria	Coxiellales	Coxiellaceae	<i>Coxiella</i>	0.99 2	0.001
Control Anemones	AIMS3	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Erythrobacter</i>	0.96 9	0.001
Anemones	AIMS3	Bacteroidia	Cytophagales	Cyclobacteriaceae	<i>Fulvivirga</i>	0.92	0.001
Control Anemones	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Loktanella</i>	0.93 9	0.001
Control Anemones	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Marivita</i>	0.93 4	0.001
Control Anemones	AIMS3	Actinobacteria	Micrococcales	Microbacteriaceae	<i>Microbacterium</i>	0.87 1	0.001
Control Anemones	AIMS3	Bacteroidia	Flavobacteriales	Flavobacteriaceae	<i>Muricauda</i>	0.98 3	0.001
'Sterile' Anemones	AIMS3	Alphaproteobacteria	Thalassobaculales	Nisaeaceae	<i>Nisaea</i>	0.82 6	0.001
Control Anemones	AIMS3	Bacteroidia	Flavobacteriales	Flavobacteriaceae	NS3a marine group	0.94 2	0.001
fRSS	AIMS3	Gammaproteobacteria	Salinisphaerales	Solimonadaceae	<i>Oceanococcus</i>	0.89 3	0.001
Control Anemones	AIMS3	Gammaproteobacteria	Betaproteobacteriales	Methylophilaceae	OM43 clade	0.95 1	0.001

Control Anemones	AIMS3	Gammaproteobacteria	Oceanospirillales	Pseudohongiellaceae	<i>Pseudohongiella</i>	0.94 9	0.001
Control Anemones	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Roseibacterium</i>	0.91	0.001
Control Anemones	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Roseivivax</i>	0.94 3	0.001
'Sterile' Anemones	AIMS3	Alphaproteobacteria	Thalassobaculales	Thalassobaculaceae	<i>Thalassobaculum</i>	0.85 5	0.001
Seawater	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Thalassobius</i>	0.88 6	0.001
Control Anemones	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.92 5	0.001
Anemones	AIMS3	Gammaproteobacteria	Alteromonadales	Alteromonadaceae		0.96 7	0.001
Anemones	AIMS3	Bacteroidia	Flavobacteriales	Cryomorphaceae		0.81 4	0.002
Anemones	AIMS3	Alphaproteobacteria	Rhodospirillales	Terasakiellaceae		0.95 9	0.001
fRSS	AIMS3	Deltaproteobacteria	Myxococcales	Blfdi19		0.80 8	0.001
Anemones	AIMS3	Bacteroidia	Chitinophagales	uncultured Sphingobacteriales bacterium		0.89 7	0.001
Control Anemones	AIMS3	Bacteroidia	Flavobacteriales	Flavobacteriaceae	<i>Winogradskyella</i>	0.95 4	0.001
Anemones	AIMS3	Gammaproteobacteria	Alteromonadales	Alteromonadaceae		0.91 5	0.001
Anemones	AIMS3	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.9	0.001
fRSS	AIMS3	Deltaproteobacteria				0.89 6	0.001
Seawater	AIMS3	Bacteroidia	Chitinophagales	Saprospiraceae		0.92 9	0.001
Control Anemones	AIMS4	Holophagae	Acanthopleuribacterales	Acanthopleuribacteraceae	<i>Acanthopleuribacter</i>	0.96 1	0.001
fRSS	AIMS4	Gammaproteobacteria	Salinisphaerales	Algiphilaceae	<i>Algiphilus</i>	0.99 6	0.001

Control Anemones	AIMS4	Gammaproteobacteria	Tenderiales	Tenderiaceae	<i>Candidatus Tenderia</i>	0.88 6	0.001
Anemones	AIMS4	Gammaproteobacteria	Coxiellales	Coxiellaceae	<i>Coxiella</i>	0.9	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Erythrobacter</i>	0.90 9	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Rhodovibrionales	Kiloniellaceae	<i>Kiloniella</i>	0.91 3	0.001
Anemones	AIMS4	Alphaproteobacteria	Rhizobiales	Stappiaceae	<i>Labrenzia</i>	0.9	0.001
Control Anemones	AIMS4	Gammaproteobacteria	Oceanospirillales	Litoricolaceae	<i>Litoricola</i>	0.91 8	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Loktanelia</i>	0.94	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Nioella</i>	0.91 4	0.001
fRSS	AIMS4	Gammaproteobacteria	Salinisphaerales	Solimonadaceae	<i>Oceanococcus</i>	0.96 6	0.001
Control Anemones	AIMS4	Gammaproteobacteria	Betaproteobacteriales	Methylophilaceae	OM43 clade	0.94 4	0.001
Control Anemones	AIMS4	Bacteroidia	Chitinophagales	Saprospiraceae	<i>Phaeodactylibacter</i>	0.92 7	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Profundibacterium</i>	0.96 8	0.001
Control Anemones	AIMS4	Gammaproteobacteria	Oceanospirillales	Pseudohongiellaceae	<i>Pseudohongiella</i>	0.95 4	0.001
'Sterile' Anemones	AIMS4	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	<i>Tropicibacter</i>	0.85 8	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.97 1	0.001
Control Anemones	AIMS4	Alphaproteobacteria	Rickettsiales	Rickettsiaceae		0.97	0.001
Anemones	AIMS4	Gammaproteobacteria	Alteromonadales	Alteromonadaceae		0.91 7	0.001
Anemones	AIMS4	Deltaproteobacteria	Myxococcales	Nannocystaceae		0.86 4	0.001

Anemones	AIMS4	Alphaproteobacteria	Rhodospirillales	Terasakiellaceae		0.88 6	0.001
Control Anemones	AIMS4	Deltaproteobacteria	Myxococcales	Nannocystaceae		0.98 3	0.001
Control Anemones	AIMS4	Gammaproteobacteria	Cellvibrionales	Spongiibacteraceae		0.90 4	0.001
Control Anemones	AIMS4	Alphaproteobacteria	NRL2			0.92 4	0.001
Control Anemones	AIMS4	Bacteroidia	Chitinophagales	uncultured Sphingobacteriales bacterium		0.84 6	0.001
Control Anemones	AIMS4	Bacteroidia	Flavobacteriales	Flavobacteriaceae	<i>Winogradskyella</i>	0.84 3	0.001
Seawater	AIMS4	Bacteroidia	Chitinophagales	Saprospiraceae		0.92 5	0.001

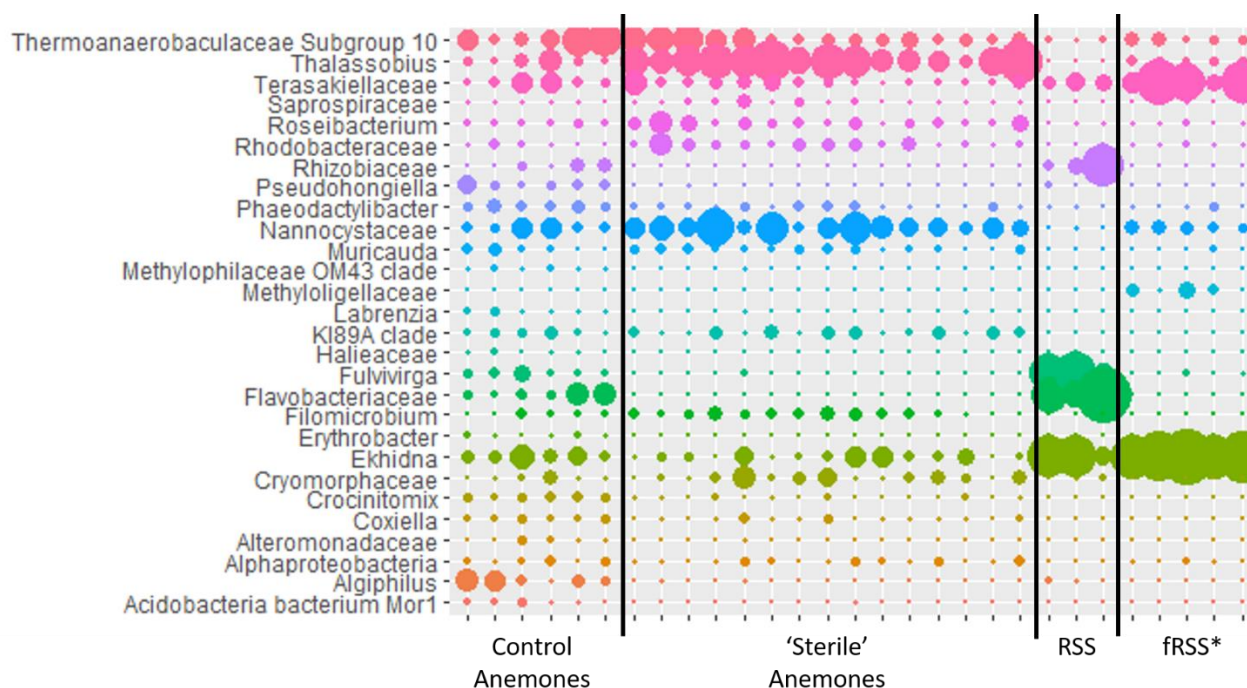


Figure S6-1 Bubble plot showing the relative abundance of bacterial taxa for each replicate of the four treatment groups for AIMS1 anemones. The size of the bubble corresponds with the relative abundance. Taxa were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity. *fRSS was collected after hosting anemones for 7 days.

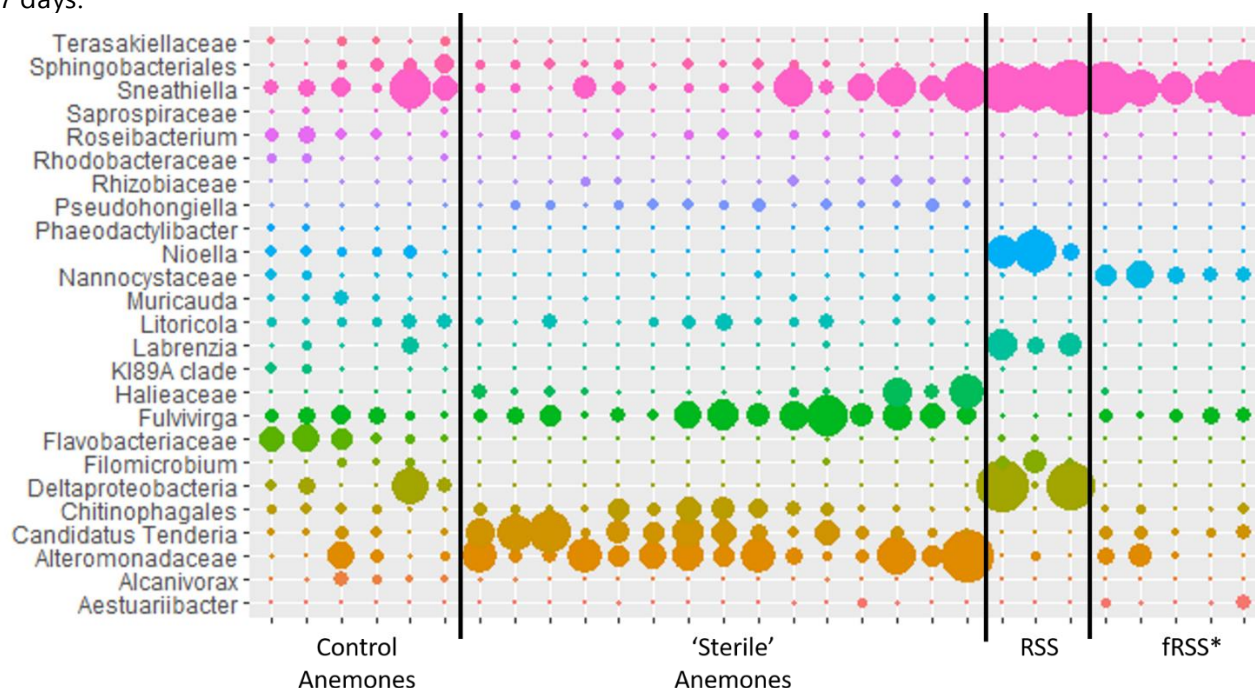


Figure S6-2 Bubble plot showing the relative abundance of bacterial taxa for each replicate of the four treatment groups for AIMS2 anemones. The size of the bubble corresponds with the relative abundance. Taxa were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity. *fRSS was collected after hosting anemones for 7 days.

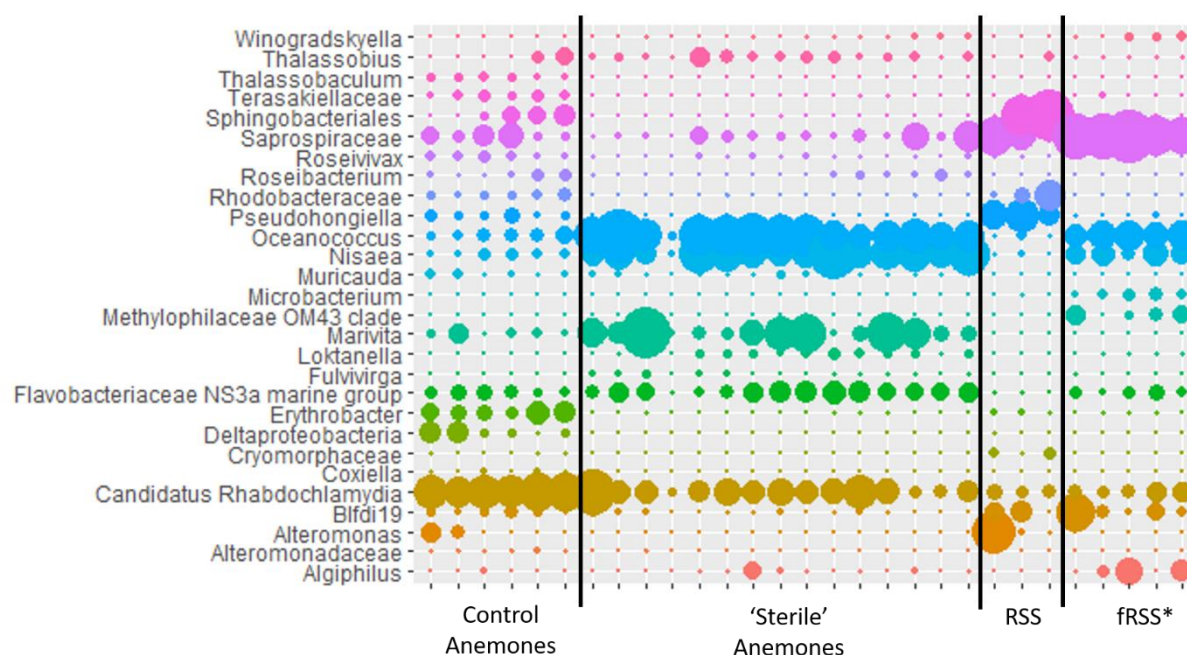


Figure S6-3 Bubble plot showing the relative abundance of bacterial taxa for each replicate of the four treatment groups for AIMS3 anemones. The size of the bubble corresponds with the relative abundance. Taxa were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity. * fRSS was collected after hosting anemones for 7 days.

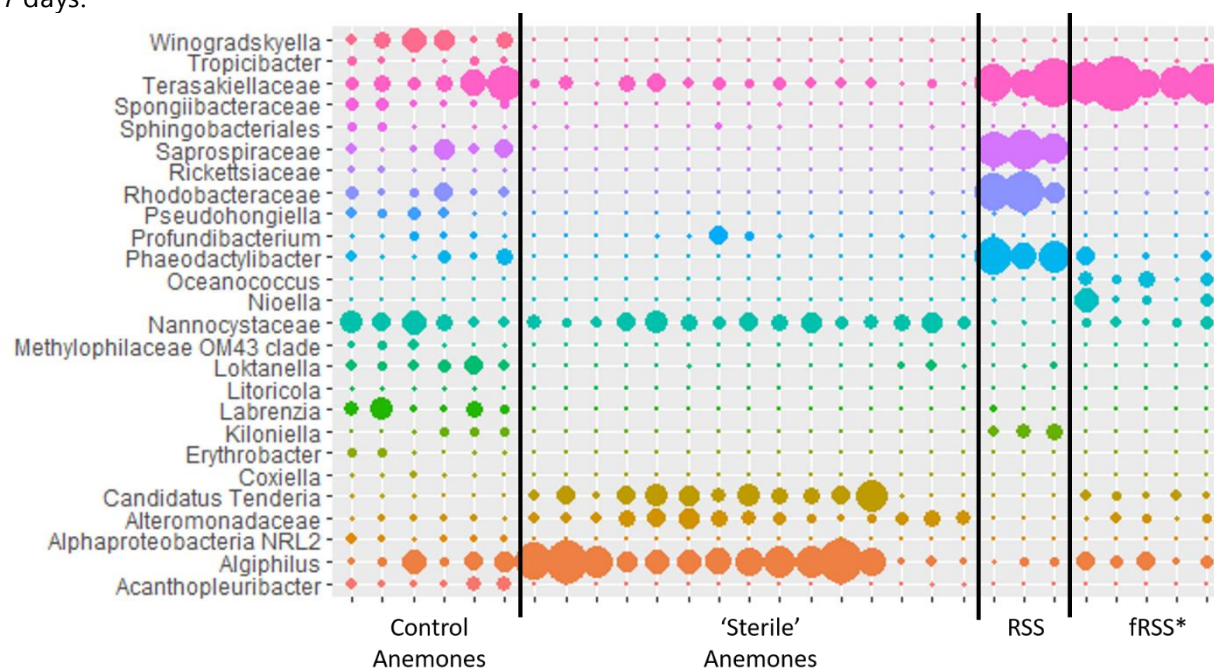


Figure S6-4 Bubble plot showing the relative abundance of bacterial taxa for each replicate of the four treatment groups for AIMS4 anemones. The size of the bubble corresponds with the relative abundance. Taxa were selected from an indicator species analysis with parameters $A_t=0.8$ and $B_t=0.8$, where A is the probability that a sample belongs to its target group or specificity and B is the probability of finding the genus in samples belonging to the sample group or fidelity. * fRSS was collected after hosting anemones for 7 days.

Chapter 7

General discussion

Coral scientists have long been thinking about the manipulation of coral-associated bacteria to manage coral health (Reshef et al., 2006; Teplitski and Ritchie, 2009; Alagely et al., 2011; Ritchie, 2012; Krediet et al., 2013). In this thesis, I explored the exploitation of bacterial oxidative stress responses as an avenue for probiotics to mitigate bleaching. Using Great Barrier Reef-sourced *Exaiptasia diaphana* (Chapter 2), I identified several diverse bacterial isolates that have a high capacity to scavenge free radicals, such as reactive oxygen species (ROS), along with a conspecific/congeneric low free radical scavenging (FRS) strains (Chapter 3). Application of these selected high and low FRS isolates did not mitigate thermal bleaching of *E. diaphana* (Chapter 4); this may be explained by the loss of the probiotic members from the anemone microbiome shortly after inoculation.

The ROS assay did not incontrovertibly show a ROS increase with heat exposure and bleaching in *E. diaphana*, which led me to explore the oxidative stress theory of coral bleaching by applying exogenous antioxidants to *E. diaphana* during thermal stress (Chapter 5). The administration of ascorbate plus catalase and mannitol were effective in rescuing *E. diaphana* from bleaching but, contrary to expectations, ROS levels under elevated temperatures increased in these treatments compared to the ambient temperature controls. Further, ROS levels in the no antioxidant controls did not increase in the thermally bleached anemones. Combined these observations challenge the oxidative stress theory of coral bleaching in *E. diaphana* and whether it is an appropriate model for studying coral bleaching. Taken together, the outcomes from this thesis will influence future coral probiotic work (Table 7-1). Below, I will discuss several of the implications that

this thesis has for probiotic research and propose additional approaches for future probiotic studies to mitigate coral bleaching.

Table 7-1 Summary of the outcomes of this thesis and their relevance to probiotics research as well as future research directions.

Outcome	Implications for probiotics research	Further research needed
Probiotic inoculation led to uneven and short-lived uptake of bacteria (Chapter 4)	The ability of a probiotic to associate with the host and maintain that association over time is crucial to the success of that probiotic.	Future research needs to evaluate alternative dosing strategies, considering the addition of chemical cues, and track how probiotic members persist over time.
A genetically and phenotypically diverse set of a bacterial isolates can be cultured from <i>E. diaphana</i> (Chapter 3)	A diverse culture collection allows for exploration of alternative strategies for mitigating bleaching during thermal stress.	Functions beyond ROS scavenging, such as nitrogen metabolism, sulfur metabolism, and disease mitigation should be explored in cnidarian-sourced bacteria for future probiotics.
Host-associated bacteria with the capacity to scavenge free radicals can be identified and prepared as a probiotic inoculum (Chapters 3 and 4)	Consistent evidence identifies ROS as a key player in the biological mechanism of bleaching. Bacteria with the capacity to neutralize ROS <i>in hospite</i> are promising for future probiotic applications.	Using natural products chemistry, the antioxidants that high FRS bacteria synthesize to neutralize free radicals can be identified. These compounds could then be applied during thermal stress exposure to explore their capacity to mitigate thermal stress in <i>E. diaphana</i> .
Despite the lack of correlation between ROS accumulation and bleaching, the application of some exogenous antioxidants mitigated bleaching in <i>E. diaphana</i> (Chapter 5).	For the first time, I comprehensively show that exogenous factors can mitigate bleaching in a cnidarian under thermal stress.	Host-associated bacteria can be metabolically engineered to produce antioxidants. Future research should consider this application with a focus on coral-associated bacteria that also symbiotically associate with <i>in hospite</i> Symbiodiniaceae.
An increase in ROS does not accompany bleaching for <i>E. diaphana</i> in symbiosis with its homologous symbiont, <i>Breviolum minutum</i> (Chapters 4 and 5).	To use probiotics as an intervention strategy to mitigate bleaching, we must first have a comprehensive understanding of the biological process that occurs from the onset of temperature stress to the dysbiosis of the coral host and Symbiodiniaceae.	In situ visualization of ROS could be completed using live cell imaging techniques paired with the exogenous addition of ROS-sensitive fluorescent dyes or the application of genetically encoded fluorescence proteins.
The microbiome of <i>E. diaphana</i> can be experimentally reduced (Chapter 6)	Working with a reduced microbiome would simplify probiotics research. Additionally, the reduced or minimal microbiome (MM) members likely have a key function in the holobiont and may be ideal probiotic candidates.	By selectively removing bacteria and tracking holobiont physiology future studies may identify members of the MM. Probiotics could then be developed as the synthetic recreation of the MM.

7.1 Effective incorporation of bacterial probiotics

In Chapter 4, I showed that repeated dosing of bacterial probiotics into the external seawater of *E. diaphana* at 10^6 cell mL⁻¹ was not sufficient to establish a long-term association of probiotic members in the microbiome. Bioencapsulation, the enrichment of live feed with probiotics, is a common inoculation strategy for bacterial probiotics in aquaculture (Hai et al., 2010; Hai, 2015) and has been suggested as a means of coral probiotic administration (Peixoto et al., 2017; Damjanovic, 2019; Hartman, 2020). However, this dosing strategy does not support effective incorporation of the probiotic members into the microbiome of the host. In aquaculture, probiotics are routinely supplemented in feed daily to provide short-term benefits but are often not detected in the gut of dosed organisms beyond a few weeks (Kim and Austin, 2006; Balcázar et al., 2007). Probiotic colonization is often transient as shown from aquaculture and with *E. diaphana* (Chapter 4), and the continuous addition of probiotics would be required for long-term maintenance. Ultimately, this is not a feasible approach for the persistence of probiotic communities in corals due to the scale of coral reefs that are impacted by thermal bleaching.

To effectively incorporate into the host-associated microbiota, probiotic bacteria must be recognized by the host or attracted to chemical cues and resistant to active antimicrobial compounds (Krediet et al., 2013). Because our initial repository of bacteria is host-sourced, we can be confident that they are resistant to antimicrobial compounds that may be secreted by the host or other associated microorganisms. The challenge is coercing the cnidarian host to accept the probiotic members and create enduring shifts in the structure of the microbial community. Modifications of quorum sensing (QS) signals in the cnidarian, *Hydra vulgaris*, promoted the colonization of bacterial symbionts in host tissue (Pietschke et al., 2017). QS has also been hypothesized to initiate bacteria-coral (Apprill et al., 2012), *Vibrio fischeri*-bob tail squid (Visick et al., 2000), and *Sodalis praecaptivus*-tsetse fly (Medina Munoz et al., 2020) symbiosis. Correspondingly, approaches aimed at promoting bacterial QS signals may adjust the cnidarian hosts ability to recognize these microorganisms and accept them as symbionts (Dobretsov et al., 2009). Of the few studies that have investigated the onset of bacterial symbiosis with the coral host, the focus has

been on bacterial community structure over time (Aprill et al., 2009; Damjanovic et al., 2019a) with some bacterial symbionts taken up more easily than others (Aprill et al., 2012). Future studies should examine the establishment of coral-bacterial relationships to understand the changes in chemical cues by the host or proteins involved in bacterial recognition (Kvennefors et al., 2008).

7.2 Potential beneficial traits of bacterial probiotics to mitigate thermal stress

From the work completed in this thesis, there is now a repository of over 1000 phenotypically diverse, *E. diaphana*-sourced, bacterial isolates available, 168 of which have been whole genome sequenced. Based on extensive support for the oxidative stress theory of coral bleaching (Chapter 1), I applied a phenotypic test to identify individuals with a high capacity to scavenge free radicals, such as ROS (Chapter 3). While a valid hypothesis, there are alternative strategies beyond ROS scavenging for mitigating bleaching during thermal stress (Peixoto et al., 2017). This diverse culture collection allows for exploration of genetic and phenotypic markers for other bacterial functions, including nitrogen metabolism and disease mitigation in cnidarian-sourced bacteria. These functions should be evaluated in future studies developing coral probiotics for their potential roles in mitigating bleaching.

Mass coral bleaching represents one of the greatest threats to coral reefs and has mainly been attributed to elevated sea surface temperature (SST). However, reduced water quality can also interact with warming to increase coral bleaching. Nitrate (NO_3^-) enrichment is routinely shown to reduce thermal tolerance of corals (Wiedenmann et al., 2012; Shantz and Burkepile, 2014; Vega Thurber et al., 2014; Zaneveld et al., 2016; Burkepile et al., 2019; Donovan et al., 2020; Fernandes de Barros Marangoni et al., 2020). Seawater enrichment with ammonium (NH_4^+), however, presents with a less severe bleaching response compared to NO_3^- (Burkepile et al., 2019) and may possibly protect corals during thermal stress (Fernandes de Barros Marangoni et al., 2020). Bacteria can reduce NO_3^- in anaerobic environments via denitrification and assimilatory or dissimilatory NO_3^- reduction to NH_4^+ ,

with the former producing nitric oxide (NO) as an intermediate (Tiedje, 1988). Denitrifying microorganisms are present in coral microbiomes (Siboni et al., 2008; Kimes et al., 2010; Yang et al., 2013; Tilstra et al., 2019), with a coupling of nitrification and denitrification suggested as a means for the biological removal of inorganic nitrogen from the coral holobiont by bacterial and/or archaeal communities (Siboni et al., 2008; Rådecker et al., 2015). Genes that can serve as functional markers for nitrate reduction and denitrification in host-associated bacteria include nitrate reductases (*narBGHI*, *napAB*, *nasAB*), nitrite reductases (*nirABDKS*, *nrfAH*), nitric oxide reductases (*norBCV*) and nitrous oxide reductases (*nosZ*) (Fig. 7-1).

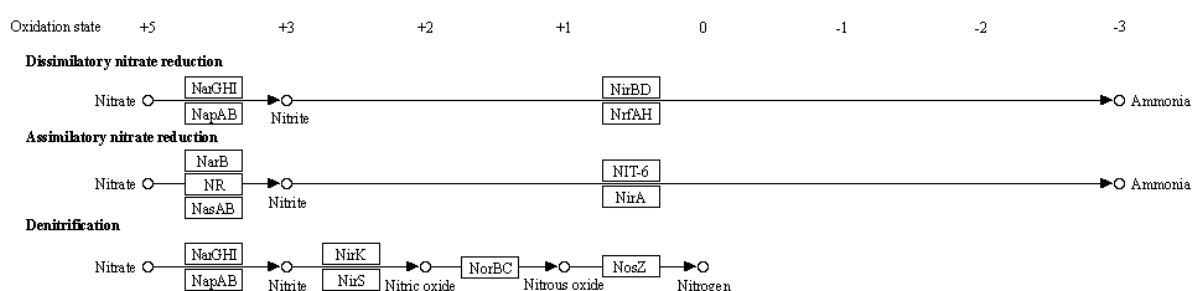


Figure 7-1 Bacterial nitrogen metabolism pathways for dissimilatory and assimilatory nitrate reduction and denitrification. Image adapted from https://www.genome.jp/kegg-bin/show_pathway?map00910

In addition to ROS, other free radicals such as reactive nitrogen species (RNS), specifically NO, have been proposed as messengers of programmed cell death (PCD), promoting coral bleaching (Perez and Weis, 2006; Bouchard and Yamasaki, 2008; Weis, 2008; Hawkins and Davy, 2012; Hawkins et al., 2013; Hawkins and Davy, 2013; Hawkins et al., 2014). Bacteria can enzymatically detoxify NO in aerobic conditions, identified with the flavohemoglobin protein encoded by the *hmp* gene (Cramm et al., 1994; Gardner et al., 2000). This nitric oxide dioxygenase plays a central role in the response of bacteria to nitrosative stress (Cramm et al., 1994). Additionally, the anaerobic nitric oxide reductase protein uses NADH to detoxify NO, protecting several NO-sensitive enzymes (Silaghi-Dumitrescu et al., 2005). Bacteria that have the genes for nitric oxide reductases (*norBCV*, Fig. 7-1) or nitric oxide dioxygenases (*hmp*), may be ideal candidates for future probiotic work.

Nitrogen (N₂) fixation is performed by prokaryotes called diazotrophs. Diazotrophically-derived nitrogen in dissolved and particulate organic form can play an important role for the nutrition of corals (Benavides et al., 2017). This additional source of nitrogen is likely to

be important to sustain net growth of corals particularly when the coral host nutritional status is impaired such as during coral bleaching events (Bednarz et al., 2017). Nitrogenase reductase gene (*nifHDK*) sequences in corals (Lesser et al., 2004; Fiore et al., 2010), revealed a high diversity of both N₂-fixing heterotrophic bacteria and cyanobacteria (Olson et al., 2009; Lema et al., 2014). Coral-associated diazotrophs may play an important role in determining the response of coral holobionts to elevated SST (Cardini et al., 2016), suggesting that probiotics should include host-associated bacteria capable of N₂ fixation.

Microbes have evolved sophisticated strategies to gauge their own population densities and accordingly adapt their phenotypes to the biotic environment. Such population density-dependent cell-to-cell signalling and gene regulation is often termed 'quorum sensing' (QS) (Bassler, 2002). Acyl-homoserine lactones (AHLs) act as signals in QS; when AHLs build up to a certain threshold concentration within a diffusion-limited environment, the 'quorum' is sensed by the AHL receptor on the bacterium surface (Teplitski and Ritchie, 2009). The receptor–AHL complex then binds within promoters of regulated genes to control their expression. QS has important roles in the interactions between mutualistic (Visick et al., 2000), commensal, and pathogenic (Ng and Bassler, 2009) bacterial communities and their eukaryotic hosts (Rasmussen and Givskov, 2006; Dobretsov et al., 2009). Key genes involved in QS, including the homoserine lactone synthase (*ypeI*) and response regulators (*yenR* and *ypeR*) are integral for promoting bacteria-host symbiosis (Medina Munoz et al., 2020). The presence of these genes in selected probiotic members may lead to their long-term colonization in the cnidarian host.

Coral-bleaching events are often followed by disease outbreaks (Selig et al., 2006; Bruno et al., 2007; Muller et al., 2008), such as white pox (Patterson et al., 2002). Thus, probiotic treatment to mitigate disease should also be considered to protect coral reefs during periods of thermal stress. QS can be inhibited by other bacteria via the enzymatic degradation of AHL signals – this biological phenomenon is termed quorum quenching (QQ) (Romero et al., 2008). Microbial communities isolated from white band disease-infected corals and exposed to a QQ compound lost the ability to establish disease (Certner and Vollmer, 2018). In probiotics, QQ activity has been a selection criterion (Ringø,

2020) with coral-sourced probiotics capable of QQ and preventing progression of the coral pathogen *Serratia marcescens* in *E. diaphana* (Alagely et al., 2011). AHL lactonases (Dong et al., 2000; Leadbetter and Greenberg, 2000) and acylases (Romero et al., 2008) produced by bacteria can degrade AHLs and thus 'quench' AHL-mediated gene regulation in pathogenic bacteria. The presence of these genes that regulate AHL lactonases and acylases (*aiiACD*, *ahIM*, *pvdQ*, *quiP*, *qsdA*) should be used as selection criteria for future probiotic candidates. Because QQ can not only reduce the virulence of a pathogen, but could also block the production of antibiotics and attachment by beneficial bacteria (Teplitski and Ritchie, 2009), QQ of probiotic isolates should be tested against known pathogenic isolates as well as established commensal strains.

In Chapter 3, I reviewed the whole genome sequences of the selected probiotic members for the presence or absence of genes for potential functions that could be useful when it comes to neutralizing ROS and mitigating coral bleaching. These functions include dimethyl sulfoniopropionate (DMSP) metabolism, biosynthesis of DMSP and vitamin B₁₂, and catalase production. While presence of these genes in probiotic bacteria is a valid selection criterion, future work should functionally characterize these potential traits in selected strains. DMSP metabolism from coral-sourced bacteria has been validated by confirming that strains can use DMSP as a carbon source and tracking dimethyl sulfide production via gas chromatography (GC) (Tandon et al., 2020). Likewise, DMSP biosynthesis can be phenotypically confirmed with growth assays that detect DMSP production with GC from media that lacks any methylated sulfur DMSP pathway intermediates (Curson et al., 2017). Vitamin B₁₂ can be extracted from bacteria cultures and quantitatively determined using a microbial assay, the VitaFast® Vitamin B12 assay (Piwowarek et al., 2018). While I did phenotypically characterize catalase production with the addition of hydrogen peroxide to freshly grown cells, a more quantitative assay can be completed across the bacterial growth curve to assess catalase activity (Hadwan, 2018).

7.3 Metabolic engineering of host-associated bacteria

While there is evidence for antioxidant dosage to provide relief against oxidative stress in cnidarians or their algal symbiont (Lesser, 1996; 1997; Marty-Rivera et al., 2018; Chapter 5), applying antioxidant compounds directly onto corals may not be practical due to the short lifespan of antioxidants when exposed to seawater (King et al., 2016) and ultraviolet radiation (Compton et al., 2019), or desirable due to their potential impact on non-target organisms. Additionally, antioxidants can be lethal when administered directly to *E. diaphana* (Chapter 5). Increasing natural antioxidant generation within the cnidarian holobiont may be more effective and practical. One suggested way of increasing natural antioxidant generation in the holobiont is through microbiome manipulation (van Oppen et al., 2015; Dungan et al., 2020a). As corals are complex symbiotic organisms formed by the polyp, endosymbiotic dinoflagellates, other protists, bacteria, fungi, archaea and viruses (Rosenberg et al., 2007; Rosenberg et al., 2009), the bioactive compounds, specifically antioxidants, secreted by symbiotic bacteria could contribute to the health of the holobiont. Host-associated bacteria can be metabolically engineered to produce antioxidants, such as carotenoids (Misawa and Shimada, 1998; Sanchez et al., 2013). The *crt* genes derived from *Pantoea* spp. have been successfully used for the *de novo* biosynthesis of the carotenoid zeaxanthin in formerly non-carotenogenic microbes (Ruther et al., 1997; Beuttler et al., 2011). Zeaxanthin is of special interest to coral bleaching as its supplementation to Symbiodiniaceae cultures has rescued photosynthetic efficiency (F_v/F_m) during a thermal stress event (Motone et al., 2020), similar to the results from Chapter 5. Additionally, Symbiodiniaceae-associated bacteria of the genus *Muricauda* are known producers of zeaxanthin (Prabhu et al., 2014; Motone et al., 2020), but they failed to successfully grow in probiotic culture (Chapter 3). Thus, genetic engineering of other probiotic candidates to synthesize zeaxanthin may be an option to reduce oxidative stress in symbiotic cnidarians during periods of increased SST.

7.4 Minimal microbiome and bacteria functions *in vivo*

No microorganisms exist in isolation, but all are part of complex communities comprised of many different individuals with different functions that form an active, dynamically changing community. While individual bacterial species were isolated from *E. diaphana* and studied (Chapter 3), often the function of the microbe in the environment is more than the sum of individuals who inhabit the area. Cells within a mixed community interact, communicate, influence, and alter the biochemical and physiological processes among the members. In complex microbial systems, such as the coral holobiont, these interactions are notoriously difficult to characterize. These limitations can be lessened when working with a minimal microbiome. The fewest microbes and/or microbial functions needed to maintain health and functioning of the holobiont has been called the minimal microbiome (MM) (de Vos, 2013; van Oppen and Blackall, 2019). The concept of a MM has been explored in plant science (Qin et al., 2016) and the human gut microbiome (de Vos, 2013), with suggestions for its application in coral science (van Oppen and Blackall, 2019). Functions of the coral MM may include the ideal probiotic functions as described above including roles in holobiont nitrogen metabolism, nutrient cycling, antibacterial mechanisms, and defence against disease (Ritchie, 2012; Krediet et al., 2013; Bourne et al., 2016).

Future research to identify bacterial functions should involve the characterization of the coral MM, which will likely be species-specific and require metagenomic, metatranscriptomic, and metabolomic analyses of healthy and bleached corals (van Oppen and Blackall, 2019). From these results, a synthetic microbiome functionally resembling this MM can subsequently be reconstructed from the well-characterized culturable microbiome (Chapter 3; Sivasubramaniam and Franks, 2016) and tested in the host. As an alternative strategy to identify bacterial functions *in vivo* or determine if select strains form an obligate symbiosis with the coral holobiont, programmed inhibitor cells (PICs) can be used to target stains of interest and deplete them from the microbiota (Ting et al., 2020). This method uses antigen-specific cell-cell adhesion of the PICs and target species, which allows for controlled removal of that species with minimal impact on other members of the complex community. The resultant holobiont phenotype following variations in the

structure of synthetic microbiomes or species targeted by PICs will be predictive of whether selected strains or functional roles are critical for holobiont health (van Oppen and Blackall, 2019). Given the challenges in establishing the MM, a suitable alternative may be working in a reduced microbial system (Chapter 6).

7.5 Evaluating the oxidative stress theory of coral bleaching

"It is proposed that algal-generated [hydrogen peroxide] H_2O_2 can diffuse from the algal symbiont into the coral cytoplasm." (Downs et al., 2002). Despite the frequency of which this founding principle for the 'oxidative stress theory of coral bleaching' is repeated in the literature, this principle has never been fully validated. To do so would require the visualization of H_2O_2 produced in Symbiodiniaceae during photo-oxidative stress, followed by the tracking of this (and other ROS) molecule over space and time.

Biochemically, it is plausible for H_2O_2 to be produced intracellularly and move from the Symbiodiniaceae to the host cell. H_2O_2 is neutral and is less toxic compared to other ROS, with a longer lifetime of about 1 ms. Comparatively, the lifetime of singlet oxygen ($^1O_2$) in aqueous solution is about 3.5 μs diffusing over a range of approximately 10 nm (see review by Schmitt et al., 2014). Literature suggests very short lifetimes for superoxide radicals ($O_2^{\cdot-}$) in the range of 1-4 μs , largely determined by the presence of super oxide dismutase (SOD) (Schmitt et al., 2014). With the permeability coefficient for $O_2^{\cdot-}$ estimated a 2.1 nm s^{-1} at pH 7.3 and 25°C, chloroplast thylakoids show little permeability to $O_2^{\cdot-}$ (Takahashi and Asada, 1983). The hydroxyl radical ($HO^{\cdot}$) is the most reactive species; in aqueous solutions its mean lifetime is 0.1 μs (Sutherland, 1991), and its mean diffusion distance estimated at a few molecular diameters from the site or origin (Mattila et al., 2015).

Principally, H_2O_2 can diffuse across great distances and eventually penetrate through membranes, possibly through water channels like aquaporins. It is assumed that H_2O_2 can travel at a few μm while interacting with H_2O_2 -metabolizing enzymes and, therefore, can play an important role in signalling (Schmitt et al., 2014). H_2O_2 can diffuse through the chloroplast envelope aquaporins (Mubarakshina et al., 2010; Borisova et al., 2012), moving from the stroma to the cytosol of a Symbiodiniaceae cell. The distance from the

Symbiodiniaceae cytosol to the host cell cytosol would vary but might average 200 nm (Wakefield et al., 2000) and include several membranes plus the Symbiodiniaceae cell wall.

The most well described approach to visualize ROS *in vivo*, involve fluorophores that are activated once oxidized. In the case of fluorophores, two different approaches exist: i) the addition of exogenous fluorescent probes, which penetrate into the cell and change their fluorescence properties due to reaction with ROS or RNS (Table 7-2), and ii) the expression of ROS-sensitive fluorescent proteins, mostly variants of the green fluorescent protein (GFP), which act as real-time redox reporters. These strategies to visualize ROS and RNS produced in Symbiodiniaceae during photo-oxidative stress are rarely applied to corals or other cnidarians. Exogenous application of the ROS probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) using *in vivo* microscopy techniques has shown an increase in ROS production in the algal endosymbionts of symbiotic *Fungia granulosa* (Gardner et al., 2017) and *Pocillopora damicornis* (Nielsen et al., 2018), with no significant differences in ROS for three species of octocoral after one hour at elevated temperature (Parrin et al., 2017). Exposure to elevated temperature resulted in an increase of RNS synthesis, as visualized *in vivo* using the fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) for and *E. diaphana* (Hawkins et al., 2013). In addition to the challenges of detecting and quantifying fluorescent signal in an organism that has extensive autofluorescence, there are almost as many ways to measure ROS/RNS as there are studies that have done so in cnidarians. Scientists should consider standardizing this approach as to streamline future research and allow for comparison between coral species and research groups.

Table 7-2 Compilation of RNS and ROS-sensitive exogenous fluorescence probes. Adapted from Schmitt et al. (2014)

Compound	Specificity/localizability	References
2',7'-dichlorodihydro-fluorescein diacetate (H ₂ DCFDA)	Unspecific ROS, can penetrate algal cell walls	(Franklin et al., 2004; Sandeman, 2006; McGinty et al., 2012; Krueger et al., 2015b; Roberty et al., 2016; Gardner et al., 2017; Parrin et al., 2017; Nielsen et al., 2018; Wietheger et al., 2018; Lesser, 2019; Fernandes de Barros Marangoni et al., 2020; Motone et al., 2020)
Singlet oxygen sensor green (SOSG)	¹ O ₂ ; unequal penetration, and photosensitization	(Rehman et al., 2013; Rehman et al., 2016; Prasad et al., 2018; Wietheger et al., 2018; Lesser, 2019)
3,3'-diaminobenzidine (DAB)	Generates a dark brown precipitate which reports the presence and distribution of H ₂ O ₂ in presence of peroxidase. DAB can penetrate cell walls and is not light sensitive. Not yet trialed in cnidarians or dinoflagellates	(Šnrychová et al., 2009; Mattila et al., 2015)
3'-(p-Hydroxyphenyl) fluorescein (HPF) and 3'-(p-aminophenyl) fluorescein (APF)	HPF and APF are cell permeable and resistant to autooxidation. They detect HO [•] , peroxyxynitrite (ONOO ⁻) and hypochlorite (OCl ⁻) production in cells.	(Hawkins and Davy, 2013; Mattila et al., 2015)
nitroblue tetrazolium (NBT)	Specific to superoxide and with slightly reduced reactivity to H ₂ O ₂ . Used most frequently in cnidarian or Symbiodiniaceae literature to detect the presence of SOD	(Mattila et al., 2015) Used to detect SOD (Liñán-Cabello et al., 2010; Hawkins et al., 2015; Krueger et al., 2015b)
Hydroethidine (dihydroethidium)	Predominately oxidized by superoxide, can penetrate algal cell walls	(Perez and Weis, 2008; Rosental et al., 2017; Wietheger et al., 2018; Lesser, 2019)
diphenyl-1-pyrenylphosphine (DPPP)	Unspecific ROS; DPPP can be used as a fluorescent probe to monitor lipid peroxidation in cell membranes.	(Mattila et al., 2015; Rosental et al., 2017)
CellROX® Green	Unspecific ROS	(Rosental et al., 2017; Cziesielski et al., 2018)
CellROX® Orange	Unspecific ROS	(Chapters 4 and 5; Levin et al., 2016; Marty-Rivera et al., 2018; Buerger et al., 2020; Hartman, 2020)
4-amino-5-methyl-amino-2',7'-difluorofluorescein diacetate (DAF-FM-DA)	NO	(Perez and Weis, 2006; 2008; Hawkins and Davy, 2012; Hawkins et al., 2013; Fernandes de Barros Marangoni et al., 2020)
Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	The Amplex® Red reagent, in combination with horseradish peroxidase (HRP), has been used to detect H ₂ O ₂ released from biological samples	(Suggett et al., 2008; Šnrychová et al., 2009; Mattila et al., 2015; Roberty et al., 2015; Goyen et al., 2017)
Methyl Cypridina Luciferin Analogue (MCLA)	O ₂ ^{-•}	(Saragosti et al., 2010; Mattila et al., 2015; Diaz et al., 2016)

One approach to overcome problems regarding the specificity and localization of the applied dyes (Table 7-2) is the application of genetically-encoded fluorescence proteins. These proteins can be selectively expressed as fluorescence markers, fused to specific target proteins or to organelle specific targeting sequences, thus enabling a specific and localized monitoring of ROS at a molecular level (Meyer and Dick, 2010; Schmitt et al., 2014). One such marker is the roGFP2-Orp1 sensor, in which proximity between a peroxidase (Orp1) and an artificial target protein (roGFP2) is established by genetic fusion. Oxidation of Orp1 by H_2O_2 is passed to the roGFP2 protein, permitting the tracking of H_2O_2 through space and time (Schwarzländer et al., 2016). In the HyPer family of H_2O_2 sensing probes, the OxyR protein from *E. coli* forms an intramolecular disulfide upon oxidation by H_2O_2 , modifying a fused fluorescent protein, which can then be visualized under confocal microscopy (Bilan and Belousov, 2016). Fluorescent probes have been established for the malaria-causing parasite, *Plasmodium falciparum*, allowing *in vivo* redox imaging of the intracellular pathogen within their host cells (Kasozzi et al., 2013; Schuh et al., 2018). The close relationship between *Plasmodium* and dinoflagellates, such as the coral endosymbiont (McFadden, 2000), provides precedent for application of genetically-encoded fluorescence proteins to the cnidarian-Symbiodiniaceae system.

7.6 Conclusions

Coral bleaching events can be predicted using remote sensing and *in situ* SST data, however no effective mitigation strategies currently exist to lessen the impacts of bleaching. Given the increasing frequency of climate change driven coral mass bleaching and mass mortality events, intervention strategies aimed at enhancing coral thermal tolerance (assisted evolution) are urgently needed in addition to strong action to reduce carbon emissions (Peixoto et al., 2019). This thesis contributes to the biological knowledge on the potential beneficial roles of host-associated bacteria and the influence of ROS in cnidarian bleaching, but more importantly, the outcomes of this thesis provide useful information that can be applied to future assisted evolution strategies, particularly with the continuation of probiotics research. Corals may adapt to changing environmental conditions on their own by changing their associated microbiota (Reshef et al., 2006). The

application of probiotics speeds up the process of natural selection to promote the proliferation of beneficial microorganisms.

There is an urgent need to move away from simply documenting the demise of coral reefs and explore interventions that can assist corals and build their resilience to the increasing number of threats they face. Without such interventions, such as probiotics, coral reefs will not survive.

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