The volatilome of the cnidarian-dinoflagellate symbiosis

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A thesis submitted to the
Victoria University of Wellington
in fulfilment of the requirements for the degree of
Doctor of Philosophy in Science

2023

General Abstract

With a rapidly changing environment, understanding the endosymbiotic relationship between cnidarians and dinoflagellates is crucial to elucidate the ways in which corals may respond to future conditions. At the basis of this symbiosis is the exchange of metabolites and signalling molecules between partners, all of which contribute to the establishment, maintenance, and ultimately dissociation of this important relationship. A subset of metabolites, biogenic volatile organic compounds (BVOCs) are low molecular weight, weakly lipophilic molecules that diffuse quickly through water and air, making them potential candidates for signalling molecules in inter-species interactions. This thesis sought to characterise patterns of BVOC generation in the cnidarian-dinoflagellate symbiosis across symbiotic states and thermal dysfunction in the model cnidarian Aiptasia (*Exaiptasia diaphana*).

In Chapter 2, I characterised the suite of BVOCs (collectively, the 'volatilome') emitted by the Aiptasia model system in symbiosis, and by each partner in isolation. Relative to symbiotic anemones, the volatilome of cultured symbionts (*Breviolum minutum*) was more distinct than it was to aposymbiotic (symbiont-free) anemones, suggesting that symbiosis alters the physiological state of the dinoflagellate more dramatically than that of Aiptasia. For example, cultured *B. minutum* produced dimethyl sulphide (DMS) in highest abundance, while anemones produced halogenated methanes like bromochloromethane, bromodichloromethane, tribromomethane and trichloromethane, regardless of symbiotic state. Alternatively, the relative lack of BVOC alteration in the cnidarian host, regardless of symbiotic state, may suggest a high degree of metabolic integration between the symbiotic partners.

In Chapter 3, I examined the role of symbiont identity on the microbiome and volatilome of Aiptasia. Microbiome analysis revealed distinct populations of bacteria in each symbiotic state, with bacteria in the family Vibrionaceae being the most abundant in aposymbiotic anemones. As prominent members of bacterial pathogens, the higher proportion of bacteria in this family could indicate disease susceptibility in the aposymbiotic state. Relative to the volatilomes of aposymbiotic anemones and those symbiotic with native *B. minutum*, symbiosis with the non-native dinoflagellate *Durusdinium trenchii* emitted a volatilome indistinct from either aposymbiosis or symbiosis with the native *B. minutum*. This suggests that the presence of a symbiont that is known to form a sub-optimal and potentially stressful symbiosis with Aiptasia impacts the metabolome. Indeed, anemones with native symbionts produced the BVOC isoprene in highest abundance, while aposymbiotic anemones and those containing the non-native symbiont produced a higher abundance of the aldehydes octanal, nonanal and dodecanal, suggesting a potentially future role for these molecules as biomarkers.

In Chapter 4, I investigated the impact of thermal stress on the microbiome and volatilome of Aiptasia in symbiosis with its native symbiont *B. minutum*, and in the aposymbiotic state. Aposymbiotic and symbiotic anemones were exposed to control (25 °C), sub-bleaching (30 °C) and bleaching (33.5 °C) temperatures. In both aposymbiotic and symbiotic anemones, I observed a restructuring of the microbiome between 25 °C and 33.5 °C, with anemones at 30 °C exhibiting an intermediate state. This is consistent with previous experiments showing that cnidarian microbiota can shift in response to changing environmental conditions. Anemones at 30 °C produced the highest number of significantly different BVOCs, including acetone and naphthalene. In contrast, symbiotic anemones at the highest temperature (33.5 °C) produced a distinct volatilome relative to the lower temperature treatments, a shift largely driven by higher quantities of dimethyl sulphide, eucalyptol and 1-iodododecane. Overall, aposymbiotic anemones exhibited a decline in BVOC richness at progressively higher temperatures, perhaps revealing the onset of metabolic collapse; this decrease was not observed for symbiotic anemones at higher temperature, suggesting a stabilising effect of the dinoflagellate endosymbionts.

In Chapter 5, I describe a method with which to assess chemotactic responses in Symbiodiniaceae, and defined tryptone as a positive control in *B. minutum*, *Cladocopium* spp., and *D. trenchii*. I assessed the chemotactic response of *B. minutum* and *Cladocopium* spp. to a pervasive marine metabolite dimethylsulphoniopropionate (DMSP), and volatiles bromodichloromethane ($BrCl_2CH$) and diiodomethane (I_2CH_2). Despite their production by aposymbiotic anemones in Chapter 2, neither $BrCl_2CH$ nor I_2CH_2 elicited a chemotactic response in *B. minutum* or *Cladocopium* sp. The precursor to BVOC dimethyl sulphide (DMS), multifunctional and widespread DMSP has functions in osmoregulation, antioxidant defence and acts as a chemoattractant for multiple marine organisms. I found that, while *B. minutum* was repelled by DMSP, *Cladocopium* spp. did not respond chemotactically to this molecule. These differing responses by distinct species of Symbiodiniaceae may reflect differing chemical cues used by Symbiodiniaceae to locate and establish a symbiosis with new cnidarian hosts, and adds to the literature describing the functional diversity of these endosymbionts.

Collectively, my thesis elucidates the synthesis and release of BVOCs by the cnidarian-dinoflagellate symbiosis, both in response to symbiotic state and thermal stress. This foundational study provides a platform from which to explore the functional roles of identified BVOCs and bacterial associates. Additionally, the non-invasive technology of volatilomics applied here may serve to identify biomarkers for ecosystem health in natural habitats. Ultimately, this work contributes to our understanding of the ways in which cnidarian-dinoflagellate symbiosis is altered in response to stress at a time when coral reefs are threatened with extinction.

Contributions, publications, and a pandemic

Over the course of this thesis, there was a global pandemic that caused a disruption to laboratory access locally, and to intended travel plans. As a result, some research questions shifted, travel plans cancelled, and laboratory work restarted. Despite these setbacks, four manuscripts were prepared, the first of which was already published at the time of thesis writeup. The following three chapters are prepared for submission soon. As a result of this format, there is some repetition between chapters, particularly in the 'Introduction' and 'Methods' sections. Tables too large for individual chapters are available in Supplementary Materials. This thesis represents the intellectual and analytical work of the author, with assistance as described below.

Chapter 2: This chapter is formatted as an independent manuscript and has been published at the time of thesis completion in the Journal of Experimental Biology:

Wuerz, M., Lawson, C., Ueland, M., Oakley, C. A., Grossman, A. R., Suggett, D. J. & Davy, S. K. (2022). Symbiosis induces unique volatile profiles in the model cnidarian Aiptasia. *Journal of Experimental Biology, 225(19)*, jeb244600. http://doi.org/10.1242/jeb.244600.

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Visualization: M.W., C.A.O.
Supervision: S.K.D., D.J.S., C.A.O.
Funding acquisition: M.W., A.R.G., V.M.W., D.J.S., S.K.D., C.A.O.

Chapter 3: This chapter is formatted as an independent manuscript and will be submitted to Biology:

Wuerz, M., Lawson, C., Oakley, C. A., Possell, M., Wilkinson, S. P., Grossman, A. R., Weis, V. M., Suggett, D. J. & Davy, S. K. Symbiont identity impacts the microbiome and volatilome of the symbiotic cnidarian Aiptasia.

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Conceptualization: M.W., C.A.L., C.A.O., A.R.G., V.M.W., D.J.S., S.K.D.
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Validation: M.W., C.A.L., C.A.O., D.J.S., S.K.D.
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Visualization: M.W., C.A.O.
Supervision: C.A.O., D.J.S., S.K.D.
Funding acquisition: M.W., A.R.G., V.M.W., D.J.S., S.K.D., C.A.O.

Chapter 4: This chapter is formatted as an independent manuscript and will be submitted to ISME J:

Wuerz, M., Lawson, C. A., Oakley, C. A., Possell, M., Wilkinson, S. P., Grossman, A. R., Weis, V. M., Suggett, D. J & Davy, S. K. Thermal stress induces restructuring of volatile gas emissions by the model symbiotic cnidarian Aiptasia.

Author contributions:

Conceptualization: M.W., C.A.L., C.A.O., A.R.G., V.M.W., D.J.S., S.K.D.
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Visualization: M.W., C.A.O.
Supervision: C.A.O., D.J.S., S.K.D.
Funding acquisition: M.W., A.R.G., V.M.W., D.J.S., S.K.D., C.A.O.

Chapter 5: This chapter is formatted as an independent manuscript and will be submitted as a note to Coral Reefs:

Wuerz, M., Raina, J. B., Nitschke, M. R., Suggett, D. J & Davy, S. K. Chemotactic responses to DMSP, but not halogenated methane BVOCs, vary among Symbiodiniaceae species.

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Visualization: M.W.
Supervision: C.A.O., D.J.S., S.K.D.
Funding acquisition: M.W., D.J.S., S.K.D., J.B.R.

Acknowledgements

Thank you to the scientists who guided and mentored me throughout these past four years. First and foremost, I am grateful to my PhD supervisor Prof. Simon Davy for his continuous encouragement, advice and patience throughout this degree. His wealth of knowledge and positive attitude were inspiring and a pleasure to work with. I am grateful to my co-supervisors Prof. David Suggett for his motivation and to Dr. Clint Oakley for bringing realism into the lab. Thank you to Dr. Matt Nitschke for teaching me to code, and to Dr. Caitlin Lawson for volatilomics analysis support. Thank you to the Davy lab for keeping things lively and interesting. A big thank you to my friends, many of whom I made during this degree - thank you to Randy Lacey for the cribbage, to Evan Heit for the crosswords, to Rose McGruddy for the banter and to Bobby Lust for the laughs. Thank you to my family for encouraging me at every turn, and to my partner Brad for being a stabilizing force. I am grateful for you all.

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Chapter 1 General Introduction

1.1 Symbiosis

Anton de Bary defined symbiosis in 1878 as "the living together of differently named organisms" (Oulhen et al., 2016). De Bary further described symbiosis as existing in the natural world on a continuum, encompassing a broad range of interactions from parasitism, in which one organism benefits to the other's detriment, through commensalism, in which one organism benefits while the other is unaffected, to mutualism, in which both organisms benefit from the interaction. Though the original etymological meaning of symbiosis was clearly stated to encompass a broad variety of associations, a lack of consensus over its definition has permeated the symbiosis field since its onset. A common misconception includes the confounding of symbiosis with mutualism, a confusion that restricts the meaning of symbiosis to include only mutually beneficial interactions (Martin & Schwab, 2012). A modern definition of symbiosis is one that describes an intimate association between individuals of different species and recognizes that interactions between individuals are dynamic in time and space (Goff, 1982). Although there is a tendency among scientists to categorize associations and behaviours into discrete groups, it cannot be overstated that associations between organisms exist on a spectrum, and the degree to which one organism benefits or is harmed, can change over time and with varying environmental conditions (Bronstein, 1994; Baker et al., 2018). In this thesis, the term symbiosis will be applied to all associations between organisms that involve prolonged physical contact in which at least one organism benefits, regardless of whether the other organism benefits (e.g., clownfish living within anemone tentacles; Litsios et al., 2012), is unaffected (e.g., barnacles on the teeth of cetaceans; Carrillo et al., 2015) or is harmed (e.g., tapeworms infecting human intestines; Tsai et al., 2013). Mutualism is the most widely studied type of symbiosis and is an association so common that it is now thought that all microorganisms rely on microbial symbionts for optimal health (McFall-Ngai et al., 2013).

A symbiosis can be further classified as either an ectosymbiotic relationship, in which the bodies of both partners remain separate (though in physical contact), or endosymbiotic, in which the smaller partner resides within the larger partner; the association of both partners is referred to as the 'holobiont' (Queller & Strassmann, 2016). In endosymbiotic relationships, the 'host' is typically the larger partner, while the smaller partner is termed the 'symbiont'. Endosymbiosis has played a key role in the evolution of life on Earth; mitochondria and plastids, which include chloroplasts, began as endosymbionts through engulfment by another microorganism. These symbionts evaded digestion by their host cell because they provided a benefit to that host and have subsequently undergone over a

billion years of evolution, giving rise to the mitochondria and plastids we are familiar with today (Sagan, 1967; Keeling, 2010).

The evolution of mutualistic symbioses can offer competitive advantages to both partners through the utilization of biological processes that they have not themselves evolved and can be found in diverse ecosystems across Earth and between distantly related organisms (Uchiumi & Sasaki, 2020). In the soil, plant roots often form associations with microorganisms, which allow the plant to sequester and assimilate inorganic nutrients into their metabolism. A common association in the rhizosphere is that of arbuscular mycorrhizae, an endosymbiosis between arbuscular mycorrhizal (AM) fungi and the roots of vascular flowering plants. In this symbiosis, AM fungi benefit from the receipt of photosynthetically-derived carbon from their host, while the host benefits from the acquisition of inorganic nutrients, particularly phosphorus, acquired by the symbiont from the soil and transferred to the plant's cortical cells (Harrison, 2005). The legume-rhizobia symbiosis is crucial to global atmospheric nitrogen cycling, in which bacteria (rhizobia, rhizobacteria) colonise the roots of legumes in the form of root nodules. Rhizobia in root nodules fix atmospheric nitrogen (N_2) into ammonium (NH_4^+), which is then released to the plant through its roots. The plant will then fix ammonium into amino acids and release these back to the rhizobia, thus eliminating the need for the bacteria to assimilate NH_4^+ into amino acids on their own (Lodwig et al., 2003). In the marine environment, sponges form complex symbioses with microbial consortia that can reach densities of 10^9 microbial cells/cm^3 of sponge and exhibit a surprising degree of complexity given the phylogenetic position of sponges as basal animals (Pita et al., 2016). This symbiosis is of interest in the biotechnology field due to the production of antimicrobial compounds by the bacterial symbionts and can provide insights into the mechanics of symbiosis in a basal animal model (Indraningrat et al., 2016). The Hawaiian bobtail squid, *Euprymna scolopes* forms an association with the luminescent bacterium, *Vibrio fischeri*, whose light provides an anti-predatory camouflage called counterillumination. In this strategy, *V. fischeri* provides luminescence to the animal's ventral surface which acts to mimic natural moonlight, thus camouflaging itself against the night sky (McFall-Ngai, 2014). While these examples highlight the diversity and complex nature of the evolution of symbiosis, the focus of this thesis is the symbiosis between cnidarians (the phylum to which corals and sea anemones belong) and their dinoflagellate symbionts, which is of ecological importance due to its central role in the growth and maintenance of coral reefs (Weis, et al., 2008; Nitschke et al., 2022).

1.2 Coral Reefs

Among the most diverse ecosystems on Earth are tropical coral reefs, which are critical for ecosystem goods and the services that they provide to economies worldwide. Although coral reefs cover just ~0.1% of the ocean floor (Hoegh-Guldberg et al., 2017), they support one quarter of marine species, indicating their importance as biodiversity hotspots (Connell, 1978). Due to their diverse nature (Fig. 1.1), coral reefs can support a robust fishing and tourism industry. Communities of fishes supported by coral reef ecosystems provide a significant food source for over a billion people worldwide, including individuals living far from reefs, and a variety of other economically valuable goods and services, collectively sustaining an estimated one billion people worldwide, often in developing countries (Moberg & Folke, 1999; Sing Wong et al., 2022). Composed of individual polyps, corals are colonial organisms with soft bodies, which secrete a calcium carbonate skeleton, providing a rigid substrate and structural framework to the reef (Buddemeier & Kinzie III, 1976). This rigid structure produced by corals provides protection to vulnerable coastlines by providing a place for waves, tsunamis and hurricanes to break, thereby dissipating energy before it reaches the shore (Kunkel et al., 2006).

Coral reefs are at a critical time in history, and the ecosystem services they provide are at risk (Hoegh-Guldberg et al, 2007). With increasing global temperatures and environmental extremes, coral reefs are threatened with extinction by the end of the century if global temperatures rise by more than 1.5°C (Hoegh-Guldberg & Ormond, 2018; Goreau & Hayes, 2021). Given the multitude of ways in which coral reefs are important for global biodiversity, local economies, sustenance, and coastal protection, and the host of stressors they face, the preservation of these ecosystems is a priority for scientists and citizens around the globe. Central to the maintenance of coral reef growth is the symbiotic relationship between the cnidarian and their dinoflagellate symbiont (Rosset et al., 2021). Though this symbiosis is critical for the flourishing of coral reefs, this relationship is sensitive to environmentally induced dysbiosis. If stressors are prolonged or extreme, dysbiosis can lead to bleaching in which symbionts are lost from host tissue, which can ultimately trigger the collapse of this ecosystem (Suggett & Smith, 2020). Given the integral nature of this symbiosis to reef growth at a time when corals are threatened with extinction, understanding the nature between cnidarians and their endosymbionts is more crucial now than ever.

One piece of optimism with respect to coral bleaching is the idea that after symbionts have been lost from host tissue, corals can acquire novel symbionts that are better suited to the current environment than previous symbionts, a phenomenon termed symbiont switching (Boulotte et al., 2016).

Alternatively, corals that were previously co-colonized with multiple, lower abundance Symbiodiniaceae symbionts can alter their symbiont composition to comprise a different assemblage of symbionts, a phenomenon termed symbiont shuffling (Baker, 2003). In both cases, this shift in the physiological attributes of the symbiont population would confer increased thermal tolerance to the holobiont, though the potential for switching especially seems limited (Gabay et al., 2019) as more thermally tolerant symbionts are not necessarily nutritionally beneficial to their host (Matthews et al., 2017, 2018).

Figure 1.1: Coral reefs form the basis of a rich variety of marine life. A) Cultured Symbiodiniaceae cells (*Cladocopium* sp.), which provide the energetic basis for reef building corals; B) Coral reef diversity provides a habitat for an abundance of marine life. Photo credit: Matthew Nitschke.

1.3 Cnidarian-dinoflagellate symbiosis

1.3.1 Dinoflagellate associates

One of the best studied, and the most abundant marine symbioses is that between members of the phylum Cnidaria and dinoflagellate algae of the family Symbiodiniaceae. These algae live inside a host-derived membrane (the 'symbiosome') within the animal host's gastrodermal cells (Wakefield & Kempf, 2001). Because they are enclosed within this symbiosome, their immediate environment is much different than it otherwise would be in the free-living state in terms of access to ions, nitrogen and carbon (Allemand & Furla, 2018). Under the environmental conditions in which this symbiosis evolved, it is considered mutually beneficial to both partners: the algae receive a stable and high-light

position in the water column in which to photosynthesize, safety from predators and access to nitrogenous waste products from the host, while the host receives photosynthetic products which can support most or all its metabolic needs (Muscatine et al., 1984). This association thus provides a tight cycling of metabolites in an otherwise prohibitively nutrient poor habitat, ultimately allowing for the ecological success of coral reefs (Muscatine & Porter, 1977; Roth, 2014; Iwasaki et al., 2016). However, this symbiosis can shift along the mutualism-parasitism continuum should environmental conditions change. For example, when ocean waters become warmer, symbionts sequester more nutrients for their own reproduction and withhold resources from their host, potentially causing a shift towards parasitism (Lesser et al., 2013; Baker et al., 2018).

Originally thought to be a single pandemic species in the genus *Symbiodinium*, *S. microadriaticum* (Freudenthal, 1962), phylogenetic reassessment has now identified nine clades within the family Symbiodiniaceae and up to 15 genus-level lineages (LaJeunesse et al., 2018; Nitschke et al., 2022), with an unknown, but increasingly defined (e.g., Nitschke et al., 2020) number of species. Symbionts in the family Symbiodiniaceae are diverse, occupying divergent habitats, including free-living and endosymbiotic niches with a variety of different hosts including sponges, foraminiferans, ciliates and cnidarians (Baker, 2003; LaJeunesse et al., 2018). Symbiotic dinoflagellates were previously categorized into clades (e.g., A, B, C, etc.) based on DNA analysis of the second internally transcribed spacer (ITS2) of the rRNA gene, and remains a popular method with which to assess Symbiodiniaceae diversity (Fujise et al., 2018; van der Windt et al., 2020). Classification with this region alone has proven insufficient however, due to the multi-copy nature of this gene and high degree of intragenomic variability within this region (Hume et al., 2019). Consequently, taxonomic classification has moved to incorporate additional genetic markers including the ribosomal large sub-unit (28S; LaJeunesse et al., 2018; Sikorskaya et al., 2022), the chloroplast *psbA* noncoding region (*psbA*^{ncr}) (Hume et al., 2019; Wee et al., 2020), and the *cob* region of mitochondrial DNA (Brian et al., 2019; LaJeunesse, Casado-Amezúa, et al., 2022). Incorporation of the integrated elements of genetic content and host-specificity have identified 11 genus-equivalent lineages: *Symbiodinium*, *Breviolum*, *Cladocopium*, *Durusdinium*, *Effrenium*, *Freudenthalidium*, *Fugacium*, *Gerakladium*, *Halluxium*, *Miliolidium* and *Philozoon* (Nitschke et al., 2022), with an additional five lineages yet to be designated to the genus-level (Fr2, Fr4, Foraminifera-specific Clade G, Clade I, and Clade J; LaJeunesse et al., 2018; Nitschke et al., 2022). With next generation sequencing (NGS) technology, contemporary analyses are employing these approaches to afford greater sequencing depth (Cunning et al., 2017), but this is an evolving field and the use of additional markers will undoubetably be uncovered in the years to come (Davies et al., 2022).

Symbiosis specificity refers to the taxonomic range within which an organism associates (Silverstein et al., 2012). Both cnidarians and dinoflagellates in a symbiosis can be either specialists or a generalists (Saad et al., 2022). A specialist host will associate with only one or very few species of symbionts, and a specialist symbiont will only associate with one species of host, even if that host contains multiple symbiont species (Baker, 2003). For example, *Cladocopium goreaui* are considered generalists, as they are widely distributed across different hosts and habitats, while other species of *Cladocopium* (C17 type) are specific to coral *Montipora* spp. and thus considered specialists (LaJeunesse et al., 2003; Saad et al., 2022). The ability of a host to associate with a variety of symbiont species thus gives the host access to a diversity of physiological traits and has been proposed as a mechanism by which cnidarians can withstand environmental change (Suggett et al., 2017; Davies et al., 2018). Geographic location can also influence the type(s) of symbiont with which a host associates. For example, colonies the coral of *Plesiastrea versipora* in southern (temperate) Australia associate with *Breviolum* sp., while *P. versipora* colonies further north (subtropical or tropical) associate with *Cladocopium* sp. (Rodriguez-Lanetty et al., 2001). Likewise, *Pocillopora acuta* is found to associate with *Cladocopium* spp. in a reef habitat, and with *Durusdinium* spp. in a nearby mangrove (Ros et al., 2021), demonstrating the utility of associating with different symbionts, depending on environmental conditions.

Physiological diversity within Symbiodiniaceae is extensive (Nitschke et al., 2022), with differences found in: fixation of inorganic carbon (Brading et al. 2013; Ros et al., 2020), photosynthetic and respiration rate (Starzak et al., 2014), production of antioxidants (Yost & Mitchelmore, 2009; Goyen et al., 2017), translocation of metabolic products to the host (Matthews et al., 2017) and thermal tolerance (Nitschke et al., 2015; Swain et al., 2017; Xiao et al., 2022). Symbiodiniaceae diversity is important ecologically to the success of coral reefs, particularly considering environmental change. Due to the physiological diversity in the family Symbiodiniaceae, a given host can associate with different species of Symbiodiniaceae, thus providing a broader geographic range for that host. For example, a study assessing the physiological differences of Symbiodiniaceae associated with *Montastraea cavernosa* at different depths (10 and 16 m *vs*. 25 and 35 m) revealed differences in Symbiodiniaceae communities (Eckert et al., 2020). Additionally, it has been suggested that corals may adapt to environmental stressors without changing their physical location by altering their Symbiodiniaceae landscape, an idea termed the ‘Adaptive Bleaching Hypothesis’ (Buddemeier & Fautin, 1993). The ability of a coral to withstand such a change in symbiont dominance depends on the physiological performance of that species, which in turn necessitates our understanding of Symbiodiniaceae physiology if we want to predict the ability of a coral to withstand, and recover from, stress events.

Dinoflagellate symbionts are usually acquired during early life-stages, either through maternal transfer (vertical transmission) or from the environment (horizontal transmission) (Quigley et al., 2017). While coral offspring from vertically transmitted lines exhibit more stable intergenerational patterns of Symbiodiniaceae populations, corals acquiring symbionts from the environment can potentially better adapt to changing conditions between generations by selecting for symbionts better adapted to their current environment (Fabina et al., 2012; Breusing et al., 2022). Symbiodiniaceae densities in the water column are quite low (Littman et al., 2008), though they are higher in the sediment (Nitschke et al., 2020), and coral larvae are poor swimmers (Hata et al., 2017). Nonetheless, corals are able to establish symbiont populations early in their life history and typically in a matter of weeks following settlement (Takeuchi et al., 2017).

1.3.2 Bacteria in the coral holobiont

The importance of the cnidarian-dinoflagellate symbiosis has long been recognised, but only more recently has the potentially crucial role of other microorganisms in the coral holobiont been acknowledged (Reshef et al., 2006). In addition to dinoflagellate symbionts, corals associate with a variety of other microorganisms such as archaea, viruses, fungi and even filamentous green algae in the genus *Ostreobium* (Oppen & Blackall, 2019). *Ostreobium* sp. have been found underneath coral tissue, or in the coral skeleton (Shashar et al., 1997), while fungi can also inhabit the coral skeleton and may attack *Ostreobium* spp. (Bentis et al., 2000). Coral-associated viruses likely infect all members of the coral holobiont, including archaeal, bacterial and eukaryotic associates, the most common being bacteriophages (Wood-Charlson et al., 2015), thus may play an important role in controlling bacterial community composition. Bacteria represent the majority of prokaryotic diversity associated with corals, the most common belonging to the phyla Gammaproteobacteria, Alphaproteobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Tenericutes (Huggett & Apprill, 2019), and can inhabit coral tissue, mucus, the gastrovascular cavity and skeleton (Oppen & Blackall, 2019). The composition of cnidarian-associated microbiota is dependent on a variety of abiotic factors including geography (Rohwer et al., 2002), water flow rates (Lee et al., 2017a), environmental nutrient conditions (Wang et al., 2018) and thermal stress (Lee et al., 2015). Additionally, there is increasing evidence that, as with the cnidarian-dinoflagellate symbiosis, host-microbial specificity is important to the stability of prokaryotic-cnidarian associations (Kvennefors et al., 2010; Carlos et al., 2013).

Although the roles of most bacterial species associated with the coral holobiont remain unknown, multiple functional roles are understood to be beneficial (Shnit-Orland & Kushmaro, 2009; Krediet et al., 2013; Peixoto et al., 2017). Nitrogen-fixing bacteria make inorganic nitrogen available for

symbiodiniacean use in photosynthesis (Moynihan et al., 2022), as do the cyanobacterial symbionts of some corals (Lesser et al., 2004). Antimicrobial compounds have been shown to be produced by species of beneficial bacteria, thus aiding the holobiont in disease prevention (Nissimov et al., 2009). Finally, some bacteria can degrade polysaccharides and thus provide the holobiont with partially digested nutrients (Kimes et al., 2010). Recently, it has been shown that population with *Endozoicomonas* sp. provides a trade-off to corals, allowing them to grow more quickly at the cost of disease vulnerability (Epstein et al., 2023). This interesting trade-off shows that the presence of potentially harmful microbes may be beneficial in the short-term. These beneficial roles all contribute to the healthy functioning of the coral reef ecosystem; understanding the ways in which bacterial populations change with altered environments is crucial to understand how coral reef habitats will continue to respond to the changing climate on Earth.

1.4 Metabolic exchange in cnidarian-dinoflagellate symbiosis

The exchange of metabolites is crucial for the health and ecological success of the cnidarian-dinoflagellate holobiont. In its free-living state, dinoflagellates assimilate inorganic nutrients including nitrogen, carbon and phosphorus directly from the seawater. In symbiosis however, the alga does not have direct access to seawater and the cnidarian host supplies these nutrients to the symbionts (Fig. 1.2).

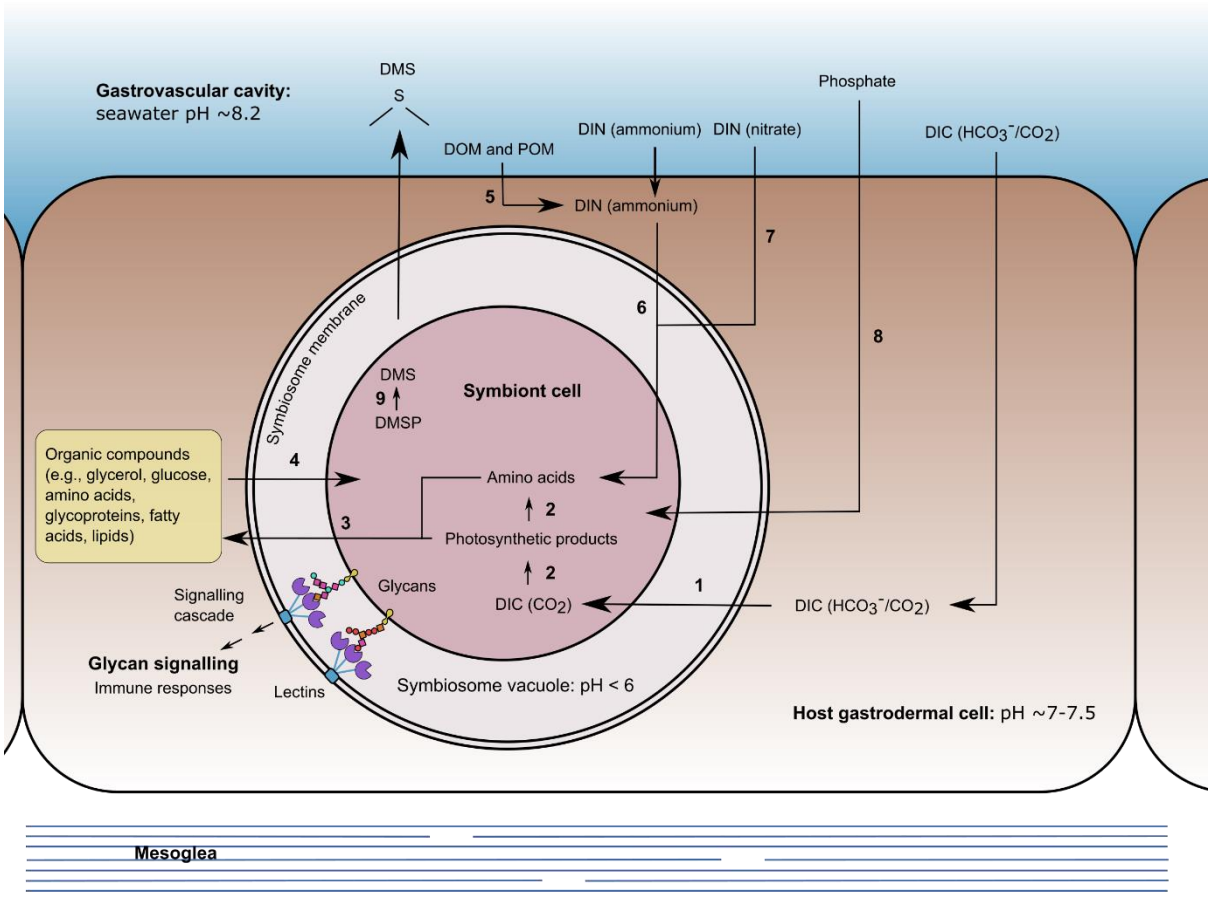

Figure 1.2: Schematic diagram summarising the bilateral exchange of nutrients between host and symbiont in the cnidarian-dinoflagellate symbiosis. 1) Uptake of dissolved organic carbon (DIC) either as bicarbonate (HCO_3^-) from surrounding seawater or as CO_2 from surrounding seawater or host metabolism. 2) CO_2 is fixed photosynthetically, producing a range of organic compounds including amino acids. 3) Translocation of photosynthetic products to host tissue. 4) Reverse translocation of organic compounds from the host to symbiont. 5) Dissolved organic matter (DOM) and particulate organic matter (POM) are used to support host metabolism. 6) Ammonium assimilation into amino acids by symbiont. 7) Nitrate assimilation into amino acids by the symbiont. 8) Phosphate assimilation by the symbiont from the seawater. 9) Dimethylsulphoniopropionate (DMSP) degradation into dimethyl sulphate (DMS) by DMSP lyase in symbiont.

1.4.1 Carbon

Required for photosynthesis, inorganic carbon can come from: host or symbiont respiration in the form of carbon dioxide (CO_2; Harland & Davies, 1995; Rädecker et al., 2017), the host as a biproduct of coral calcification in the form of CO_2 (Ware et al., 1992; Comeau et al., 2017), or from the surrounding seawater in the form of CO_2 or bicarbonate (HCO_3^-) (Furla et al., 2000). For use in photosynthesis (see below), carbon must be in the form of CO_2, thus necessitating the conversion of HCO_3^- to CO_2 by the enzyme carbonic anhydrase (CA) which has been identified in algae and symbiotic cnidarians (Weis, 1991).

1.4.2 Nitrogen, Phosphorus, and Iron

Nitrogen is often considered to be a limiting nutrient since it is in short supply relative to other nutrients (Zehr & Kudela, 2011), so the transport of nitrogen between partners is one of the most significant nutrient cycles in the cnidarian-dinoflagellate symbiosis (Davy et al., 2012). Dissolved inorganic nitrogen (DIN) in the ambient seawater exists predominantly as ammonium (NH_4^+) or nitrate (NO_3^-) ions (Badgley et al., 2006), or it can be obtained from host waste products as NH_4^+ (Rahav et al., 1989). Both symbionts and symbiotic anemones are able to assimilate ammonium and nitrate, however the dinoflagellate is the primary site of ammonium assimilation (Grover et al., 2003). Non-symbiotic anemones are not able to biologically incorporate inorganic nitrogen into their metabolism and must obtain organic nitrogen through heterotrophic feeding (Furla et al., 2005). Symbiotic cnidarians and dinoflagellates are involved in a bidirectional exchange of nitrogenous products (Wang & Douglas, 1998). When anemones feed heterotrophically, ammonia is generated as a waste product and is excreted from the animal, which is released to the symbiont where it is assimilated into glutamate using the NADPH-linked glutamate dehydrogenase (GDH) enzyme (Yellowlees et al., 1994), which can then be incorporated as amino acids and released back to the host. In this way, nitrogen is recycled from the animal host to the algae and back to the host, allowing the tight coupling of this environmentally limited nutrient (Wang & Douglas, 1998). Further to this, the receipt of photosynthetically fixed carbon from their symbiotic partners leads to a decrease in cnidarian amino acid degradation and thus reduced ammonium production (Rees & Ellard, 1989), thus resulting in nitrogen conservation by the animal (Wang & Douglas, 1998).

Dissolved inorganic phosphorus (DIP) in the surrounding seawater mainly exists as phosphate (PO_4^{3-}) and needs to be actively transported against a concentration gradient through the host and into symbiont cells, where it is assimilated (Godinot et al., 2009; Ferrier-Pagès et al., 2016). Corals can also use dissolved organic phosphorus by first transforming DOP into DIP with phosphatase enzymes

(Blanckaert et al., 2022), which is then assimilated by the dinoflagellate symbionts. A healthy symbiotic relationships between corals and their dinoflagellate symbionts relies on the appropriate relative abundance of DIN to DIP; with increasing DIN inputs into reef habitats through anthropogenic disturbances (Brodie et al., 2012; D'Angelo & Wiedenmann, 2014; Houk et al., 2020), corals are left in a relatively phosphorus deficient state. This P deficit can lead to coral bleaching and thus ultimately coral starvation (Rosset et al., 2017).

Iron is an essential trace metal needed for the healthy functioning of multiple biological pathways (Ilbert & Bonnefoy, 2013), and is the most widely studied of all trace elements. It is a cofactor bound to proteins needed in photosystem I, photosystem II, the electron transport chain and ferredoxin activity (Raven et al., 1999; Crichton & Pierre, 2001). Additionally, it is a ROS scavenger (Wolfe-Simon et al., 2005) and important for nitrate assimilation (Rueter et al., 1990), making it crucial for photosynthetic organisms. Iron availability is increasingly recognised in the physiology of coral reefs (Biscéré et al., 2018), as low iron can increase coral sensitivity to thermal stress, by decreasing the reefs' antioxidative protection, potentially leading to bleaching (Song et al., 2015). It is recognized that trace elements including iron are essential to the biochemistry of host-symbiont compatibility in cnidarians (Reich et al., 2022), a balance which is crucial for shaping the biochemical and ecological niches of corals (Grima et al., 2022), which may become increasingly altered with climate change.

1.4.3 Symbiont – host translocation

Once carbon and other nutrients are acquired by the symbiont, it is photosynthetically fixed through the Calvin-Benson (C_3) cycle to produce a variety of organic molecules that can be either metabolized or stored by the algae, or translocated to the host (Muscatine & Hand, 1958; Ros et al., 2021), where photosynthetically derived sugars can support most of the host's daily carbon requirements for respiration, maintenance and growth (Muscatine & Porter, 1977; Muscatine et al., 1981). The translocation of photosynthetically fixed carbon from symbiont to host is an important step in the cnidarian-dinoflagellate symbiosis and is one of the most-cited features of this symbiosis. So-called 'mobile-compounds' transferred between symbiont and host include low-molecular weight compounds like glycerol, glucose, amino acids, and organic acids (e.g., malate; Muscatine & Cernichiari, 1969; Whitehead & Douglas, 2003), as well as larger more complex compounds like free fatty acids (Papina et al., 2003) and glycoconjugates (Markell & Trench, 1993). Originally, it was thought that glycerol was the primary photosynthetic product translocated from symbiont to host (Muscatine, 1967), but it is now known that glucose is the photosynthate transferred in highest abundance (Whitehead & Douglas, 2003; Burriesci et al., 2012). Very little is known about the 'reverse translocation' of metabolites from host to symbiont, but there is some evidence for the

phenomenon, through the transfer of ^{35}S labelled material to Symbiodiniaceae by food ingested by Aiptasia (Cook, 1971).

1.5 Molecular signalling in symbiosis

Inter-partner signalling in the cnidarian-dinoflagellate symbiosis is essential to the establishment and maintenance of this dynamic relationship. Nutrient exchange (Matthews et al., 2017), cell cycle regulation (Gorman et al., 2022) and host immune system modulation (Mansfield et al., 2017) are all processes affected by symbiotic state, with a variety of organic compounds having been shown to act as agents of intracellular signalling, and communication across biological systems, including lipids, glycans, proteins, nucleotides and gases (Rosset et al., 2021); of these, glycans, lipids and reactive species are thought to play a crucial role in cnidarian-dinoflagellate symbiosis establishment and maintenance (Rodriguez-Lanetty et al., 2006; Matthews et al., 2018; Blackstone, 2022; Tortorelli et al., 2022). These will be introduced in further detail here.

1.5.1 Glycan-lectin signalling

Glycans are carbohydrates often associated with cell surfaces, playing a variety of biological signalling roles and are of particular importance in the establishment of the cnidarian-dinoflagellate symbiosis (Tivey et al., 2020a; Tortorelli et al., 2022). Microbe-associated molecular patterns (MAMPs) are an encompassing term, including glycans, to describe a suite of extracellular moieties on Symbiodiniaceae cell surfaces. Lectins on the host cell surface are carbohydrate-binding proteins, called pattern recognition receptors (PRRs), and function in recognition of MAMPs (Weis, 2019). While invertebrates lack an advanced immune system homologous to that of vertebrates (Arala-Chaves & Sequeira, 2000), their sophisticated innate immune system (Rowley & Powell, 2007) allows for the discernment between a pathogenic microbe and a potentially beneficial symbiont (Nyholm & Graf, 2013). The species-specific glycan-lectin binding that occurs at the onset of a successful symbiosis triggers phagocytosis of the prospective symbiont, stimulates inter-partner signalling and begins host immune system modulation to prevent digestion of the dinoflagellate (Jacobovitz et al., 2021).

1.5.2 Lipid signalling

Lipids constitute a group of non-polar molecules, within which are subgroups of lipids, both implicated in cnidarian-dinoflagellate symbiosis: oxylipins, and sphingolipids. Oxylipins are created through the oxidation of polyunsaturated fatty acids, and sphingolipids, which are components of

biological membranes. Oxylipins are membrane-diffusible and have roles in inflammation regulation and cellular homeostasis, as well as stress and defence responses (Wasternack & Feussner, 2018). In corals, oxylipins are also produced in response to heat stress and infection, suggesting a crucial role in the stress response (Lõhelaid & Samel, 2018). Additionally, oxylipin synthesis genes are downregulated in anemones symbiotic with their native symbiont, relative to when aposymbiotic or symbiotic with a non-native symbiont (Matthews et al., 2017). This suggests that an optimal symbiotic pairing downregulates the synthesis of metabolic pathways associated with stress, leading to a quiescent immune state.

As essential components of biological membranes, sphingolipids activate G-protein coupled receptors that regulate diverse processes such as apoptosis, cell survival, inflammation, and autophagy (Hannun & Obeid, 2018). With roles in both cell survival (sphingosine-1-phosphate: S1P) and apoptosis (sphingosine and ceramide), this 'sphingosine rheostat' provides a crucial balance of sphingolipids needed for cellular homeostasis (Rosset et al., 2021). Successfully invading pathogens have been shown to manipulate host immunity by inhibiting autophagy through the sphingosine rheostat (Heaver et al., 2018). Similarly, in the establishment of the cnidarian-dinoflagellate symbiosis, the sphingosine rheostat is shifted in favour of pro-survival signalling (Detournay & Weis, 2011). Conversely, during symbiosis dysfunction, the rheostat is shifted towards pro-apoptosis (Kitchen & Weis, 2017).

1.5.3 Reactive species signalling

Once simply considered by-products of oxidative metabolism, reactive forms of oxygen, nitrogen and sulphur are now recognised as important signalling molecules (D'Autréaux & Toledano, 2007). 'Free radicals' are molecules produced as a by-product of many biochemical reactions, including respiration and photosynthesis in mitochondria and chloroplasts, respectively (Murphy, 2009; Khorobrykh et al., 2020), and are highly biologically reactive (Zuo et al., 2015). Reactive oxygen species include singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^\cdot$) (Tripathy & Oelmüller, 2012).

When the ground state of molecular oxygen ($^3O_2$) absorbs sufficient energy, the spin of its two unpaired electrons is altered, resulting in a highly reactive singlet oxygen molecule (Tripathy & Oelmüller, 2012). Usually, detoxification of ROS is achieved by the excess energy of $^1O_2$ absorption by carotenoids, which dissipate excess energy as heat (Edge et al., 1997). Under periods of extreme environmental stress, ROS generation can increase and accumulate beyond the organism's ability to quench these species (Muchlin & Bendich, 1987), leading to tissue damage, which can occur through

the oxidation of biological macromolecules, such as lipids, proteins, and DNA (Zuo et al., 2015; Juan et al., 2021).

1.5.4 Reactive nitrogen species

Nitric oxide (NO) is a reactive species and signalling molecule involved in physiological regulation (Paul & Roychoudhury, 2020), and in the immune response against pathogen invasion (Fang, 2004). NO has been reported in symbiotic cnidarians (Hawkins & Davy, 2012) and dinoflagellates (Wang & Ruby, 2011), which increases during periods of environmental stress, alongside bleaching (Perez & Weis, 2006). Additionally, interaction of NO with ROS generates the highly reactive peroxynitrate ($ONOO^-$), further contributing to oxidative stress and inhibiting the mitochondrial electron transport chain. Bacteria capable of neutralizing ROS have been demonstrated as successful pathogens to corals (Munn et al., 2008). ROS and RNS have been shown to accumulate in parallel with stress-associated metabolic dysregulation (Weis, 2008; Hawkins et al., 2014; Gardner et al., 2017). Collectively, these data suggest that reactive species could indicate the onset of symbiosis breakdown (Hawkins et al., 2013; Weis, 2008).

1.5.5 Cnidarian bleaching

Carbon fixation in corals relies on the efficient transfer of energy through the photosynthetic electron transport chain (ETC). Under periods of increased heat, electron flow can increase to a point that surpasses the Symbiodiniaceae ETC capacity (Warner and Suggett, 2016), leading to an increase in intracellular oxygen concentration, which in turn increases the production of ROS (Imlay, 2013). ROS generation due to ETC saturation occurs most commonly through the Mehler reaction (Roberty et al., 2014; Roberty et al., 2015), in which PSI reduces O_2 to superoxide (O_2^-), which can be quenched by antioxidants such as superoxide dismutase (SOD). Produced by both cnidarian and dinoflagellate, SOD converts O_2^- to hydrogen peroxide (H_2O_2; Fig. 1.3) (Roberty et al., 2015), which, if not quickly reduced to H_2O and O_2, itself is also damaging to host tissue (Lesser, 2006), and can react with ferrous iron to produce a reactive hydroxyl radical ($^{\cdot}OH$; Lesser, 2006). As the rate of ROS production increases with increasing temperatures, enzymes such as SOD become overwhelmed, ROS leak out of the symbionts into host tissue and ultimately, it is proposed, the bleaching cascade is initiated (Fig. 1.3; Nielsen et al., 2018). It should be acknowledged that the precise cellular mechanism of bleaching remains unresolved (Oakley and Davy, 2018), however the molecular signalling cascades such as this are crucial for the interactions between symbiotic partners. Additionally, a diversity of signals also operates between organisms outside of symbiosis as well, with chemicals acting as agents of communication among organisms at the ecosystem level more broadly.

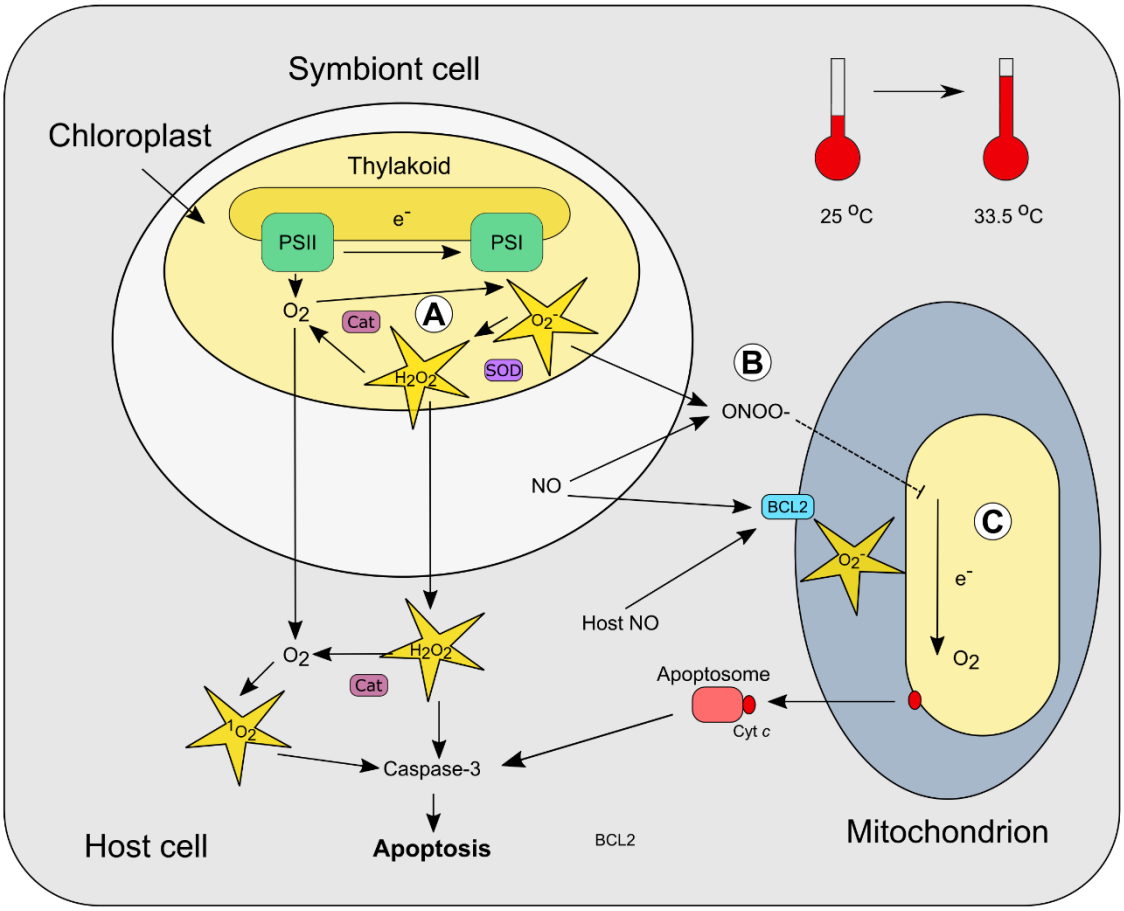

Figure 1.3: Reactive oxygen species generation that may initiate cnidarian bleaching. A) Photosynthetic electron transport between photosystem II (PSII) and photosystem I (PSI) produces superoxide (O_2^-) via the Mehler reaction. O_2^- is converted to H_2O_2 via superoxide dismutase (SOD) and catalase (Cat). B) Nitric oxide reacts with O_2^- to produce peroxynitrite ($ONOO^-$), which in turn inhibits the mitochondrial electron transport chain. C) Thermal stress increases the mitochondrial electron transport chain generation of ROS due to high respiration or electron transport inhibition. Figure modified from Oakley and Davy, 2018.

1.6 Chemical communication

Wilson (1970) defined biological communication as 'action on the part of one organism (or cell) that alters the probability pattern of behaviour in another organism (or cell) in an adaptive fashion. The chemical signals of olfaction and taste are fundamentally different from those of vision, sound, or touch, which rely on energy through light, vibration, or pressure; chemical cues rely on the physical movement of odour molecules from the signaller to the receiver. Whereas sight and sound can convey a signal at the time they are made, chemical messages can stay long after the emitter has gone. Arguably the oldest and most widespread form of information transfer, chemical communication occurs widely among bacteria, fungi, plants, and animals (Wyatt, 2003). Due to the long evolutionary

history of chemical communication, it is perhaps unsurprising that the chemicals with which communication is achieved is particularly diverse.

Signalling compounds can be transmitted in a variety of different ways, including through physical contact (e.g., pheromone-binding proteins in insects; Tegoni et al., 2004), or release into the environment to affect its action through air or water. For example, the volatile class terpenoids are a group of emitted 5-carbon chemicals containing over 40,000 compounds, many of which have defined signalling roles in predator defence and pollinator attraction (Theis & Lerdau, 2003). While the structure of signalling molecules is indeed diverse, the rest of this thesis will focus on volatile compounds.

1.6.1 Volatiles

Volatile organic compounds (VOCs) are low molecular weight chemicals with low boiling points, high vapour pressure and thus tend to transform between liquid and gas phases readily at atmospheric pressure and temperature (Mansurova et al., 2018). Many VOCs originate from anthropogenic sources such as vehicle emissions, and lead to the formation of pollutants such as secondary organic aerosols, forming toxic and carcinogenic human health concerns (He et al., 2019). On a global scale, however, VOCs originating from biological organisms account for most VOC emissions into the atmosphere (Sahu, 2012), out-emitting those produced by anthropogenic sources by an order of magnitude (Messina et al., 2016), and are termed biogenic volatile organic compounds (BVOCs). Interestingly, BVOCs are produced by organisms from all kingdoms of life (Vlot & Rosenkranz, 2022), including bacteria (Murata et al., 2022), fungi (Wang et al., 2020), algae (Zuo, 2019), terrestrial plants (Peñuelas & Llusià, 2003), insects (Gomez-Diaz & Benton, 2013) and mammals (Kücklich et al., 2017) including humans (Gallagher et al., 2008; Landini et al., 2022).

A subset of larger metabolites, which typically have molecular weights of less than 2000 Da (Dittrich & Ibáñez, 2015), BVOCs are smaller, with 5-20 carbon atoms and molecular weights below 500 Da. Their lipophilic nature, low water solubility, and small size allow BVOCs to cross cell membranes freely and pass into the surrounding environment (Pichersky et al., 2006), making them excellent candidates for intercellular or interspecies communication. Compared to primary metabolites, BVOCs are released into the environment and can be measured in the gas phase, thus allowing non-invasive analysis of organismal state (Sinha et al., 2017; Mansurova et al., 2018).

BVOCs can be metabolic by-products, end-products in themselves (e.g., serving as attractants to pollinators) or precursors to other molecules (e.g., hormones in plants). Altering BVOC emission in response to herbivore damage can be adaptive, to deter other herbivores (Shiojiri et al., 2006), attract enemies of herbivores (Turlings & Wäckers, 2009) or transmit information about tissue damage to other parts of the same plant (Luna et al., 2012). Neighbouring plants can respond to these cues by mounting their own defence against upcoming herbivory (Schnake et al., 2020). The evolution of benefit to the emitter of such 'warning signals' has hypotheses in kin selection, in which only genetically similar individuals will discern and respond to BVOC messages, thereby selectively enhancing the fitness of their own genes (Kalske et al., 2019). Alternatively, unrelated neighbouring individuals may mount herbivore defences and thus reduce the probability of herbivores entering the shared patch of increasingly resistant plants (Kalske et al., 2019).

It has previously been assumed that volatiles diffuse passively out of a cell, however BVOC production in flowers and roots has been localized to the epidermal cells, allowing their release directly into the atmosphere (Kolosova et al., 2001) or rhizosphere (Chen et al., 2004), respectively. BVOCs produced by vegetative organs are synthesized in trichomes (Schilmiller et al., 2008) and diffuse into the atmosphere through mechanical disruption as seen in peppermint plants (Gershenzon et al., 2000). Alternatively, BVOCs in other plants are made in leaf mesophyll, and exit through the stomata (Fall & Monson, 1992) or cuticle (Liao et al., 2021).

Classification of BVOCs is a matter of current review; the Model of Emissions of Gases and Aerosols from Nature version 2.1 (MEGAN2.1; Guenther et al., 2012) developed an estimate of 147 BVOC emissions deemed to be significant in atmospheric interactions (Guenther et al., 2012). These BVOCs have been classified into ten groups based on a combination of their structure and biological functions: 1) terpenoid compounds; 2) methanol and acetone; 3) short-chained oxygenated VOCs; 4) stress compounds; 5) leaf surface volatiles; 6) organic halides; 7) organic sulphur; 8) alkanes; 9) benzenoid compounds; 10) alkenes (Guenther et al., 2012; Guenther, 2013).

1.6.2 History of BVOC research

Research into organic compounds in the Earth's atmosphere began to gain attention in 1952, when Haagen-Smit showed that VOCs and nitric oxides (NO_x) combined to produce ozone (Haagen-Smit, 1952). Subsequently, research into urban atmospheric emission, including from vehicle combustion, was spurred (Grohse & Saline, 1958; Monkhouse, 1957). Research into organically produced volatiles lagged behind that of combustion by-products but took root when Went (1960) theorized that

organically-produced VOCs created the blue haze above the Blue Mountains in Australia (Went, 1960), a phenomenon now known to be caused by isoprene (Sharkey et al., 2008). Since then, quantification and classification of individual BVOCs have increasingly gained attention. By 1978, 606 BVOCs had been identified in the atmosphere; by 1986, 2857 had been identified (Graedel, 1978, 1986). By 2007, Goldstein and Galbally estimated that tens of thousands of BVOCs had been measured in the atmosphere, with an estimated hundreds of thousands yet to be identified (Guenther et al., 2012).

1.6.3 Evolution of volatile synthesis

To investigate the evolution of volatile synthesis, evolutionary biologists have researched loss-of-function of BVOCs in flowering plants. Biologists often investigate vestigial organs to discern evolutionary history of an organism, however scent is not a fossilized trait, so studies of related species that bracket pollinator shifts have been used in the investigation of BVOC evolution. It is thought that BVOC production is the ancestral state in angiosperms, with loss of scent evolving independently in the transition to pollinator types in which scent is unnecessary (e.g., bird, wind, or self-pollination; Raguso, 2016).

Evolutionary biologists often frame loss of function as trade-offs of energetic benefits in loss of metabolically expensive organs such as eyes, wings, or flight muscles (Helsen et al., 2020). With respect to volatile biosynthesis, the cost of producing BVOCs may be trivial compared to the ecological cost of 'eavesdropping' by herbivores and seed predators. Under this paradigm, pollinator-mediated selection must be sufficiently strong to select for scent to counterbalance the negative consequences of natural enemy attraction to floral scent. Benzaldehyde production by different species of *Petunia* flowers provides an example for the selection of BVOC production among closely related species. Bee-pollinated *P. integrifolia* emits benzaldehyde to attract insect pollinators, while hummingbird pollinated *P. exserta* produces red flowers lacking scent. Since *P. exserta* evolved a pollination strategy without chemical cues, loss of BVOC production may prevent eavesdroppers from locating their flowers (Raguso, 2016). Despite this, vestigial scent in self-pollinating or hummingbird-pollinated flowers may still confer a competitive advantage over non-emitting flowers – null mutants for (E)- β-caryophyllene in self-pollinating *Arabidopsis thaliana* flowers are more susceptible to bacterial infection, suggesting an advantage of low-level production of this BVOC at levels below florivore detection levels (Raguso, 2016).

1.6.4 Volatiles in terrestrial ecosystems

Among terrestrial ecosystems, forests produce BVOCs in highest abundance (Šimpraga et al., 2019). Of the organisms living there, living trees are the biggest BVOC producer, with emissions also originating from leaf and wood litter, microorganisms and the rhizosphere (Šimpraga et al., 2019). As major components of the global carbon cycle, forests provide a sink for atmospheric CO_2, and store ~45% of terrestrial carbon in their biomass (Bonan, 2008). The most abundantly produced terrestrial volatiles are isoprenoids, also called terpenoids; formed from the 5-carbon precursors dimethylallyl diphosphate (DMAPP) or isopentenyl diphosphate (IPP), tens of thousands of isoprenoid compounds have been identified (Tetali, 2019). Produced as a product from both primary and secondary metabolism, isoprenoids provide essential cellular functions. As a product of primary metabolism, isoprenoids are components of photosynthetic pigments (Bohlmann & Keeling, 2008) and growth hormones (Moses et al., 2013). Secondary metabolite isoprenoids provide diverse ecological functions such as pollinator attraction, antioxidants, thermotolerance, herbivore deterrence and antibacterial compounds (Gershenzon & Dudareva, 2007; Tholl, 2015; Croteau et al., 2020).

Isoprene, a terpenoid volatile compound released from plants is perhaps the best studied terrestrial BVOC. Produced in abundance, isoprene emissions equal approximately 5×10^8 metric tons carbon *per* year globally (Pollastri et al., 2021). Isoprene is costly to produce: 14 NADPH and 21 ATP are used to make one molecule of isoprene (Pollastri et al., 2021; Sharkey & Yeh, 2001). There are multiple known roles for isoprene at the cellular and ecosystem level, including protection from abiotic stresses. For example, isoprene-emitting plants are better protected against heat stress (Sharkey et al., 2001) and drought (Tattini et al., 2015), and can act as a ROS scavenger, quenching ozone (Loreto et al., 2001), hydrogen peroxide (Loreto & Velikova, 2001), singlet oxygen (Velikova et al., 2004) and nitric oxide (Velikova et al., 2005).

1.6.5 Volatiles in aquatic ecosystems

Though less well studied than that of terrestrial ecosystems, aquatic ecosystems are increasingly becoming recognised as hotspots for BVOC emissions (Exton et al., 2015; Lawson et al., 2021; Saha & Fink, 2022). As with terrestrial organisms, aquatic organisms produce a diversity of BVOCs, with over 35,000 algal species found to produce these compounds (Watson, 2004), including terpenoids, carotenoids, fatty acid derivatives and sulphur compounds (Watson, 2004). The evaluation of cyanobacteria-originated BVOCs has also been applied to the evaluation of drinking water quality (Lee et al., 2017b), as cyanobacteria are a causative agent of offensive taste in water and are prolific producers of toxins often lethal to humans (Carmichael et al., 2001). Cellular functions and ecological

roles of aquatic BVOCs are less well documented, than those of terrestrial origin, though some evidence of inter-species BVOC signalling in aquatic environments does exist (Saha & Fink, 2022). As with terrestrial invertebrates responding to herbivore-damaged plants, aquatic invertebrates have been shown to respond behaviourally to wounded algae. For example, β-cylocitral release upon rupture of toxic blue-green alga *Microcystis* sp. causes negative chemotaxis of the zooplankton *Daphnia magna* (Jüttner et al., 2010).

While trees are well recognised as prolific producers of BVOCs with described functions in inter-species interactions (Šimpraga et al., 2019), seaweeds are likewise beginning to gain recognition as producers of BVOCs involved in associated microbial communities, and oxidative stress response in the aquatic realm (Saha et al., 2021). The roles of seaweed-produced BVOCs may be equally diverse as their above-water counterparts. The seaweeds *Corallina pilulifera*, *Lithophyllum yessoense* and *Asparagopsis armata* all produce bromoform, which have been shown to inhibit growth of epiphytic microalgae and bacteria (Ohsawa et al., 2001; Paul et al., 2006). Seaweed volatiles have also been shown to influence the microbial community associated with the seaweeds *Bonnemaisonia hamifera* (Persson et al., 2011) and *Taonia atomaria* (Othmani et al., 2016). Additionally, oxidative stress in the brown kelp *Laminaria digitata* induces the production of volatile aldehydes (Goulitquer et al., 2009) and halocarbons (Palmer et al., 2005).

Most research on marine volatilomics has focused on one abundantly produced BVOC, dimethyl sulphide (DMS; Deschaseaux et al., 2016; Exton et al., 2015). DMS is a breakdown product of dimethylsulphoniopropionate (DMSP; Zhang et al., 2019), which is produced by cnidarians (Raina et al., 2013), coccolithophores (Franklin et al., 2010), bacteria (Zheng et al., 2020) and dinoflagellates (Hatton et al., 2012), including Symbiodiniaceae (Keller et al., 1989). DMSP is cleaved by an enzyme called DMSP lyase, which converts DMSP to the volatile DMS. First identified in bacteria (Yoch et al., 1997), DMSP lyase has now been identified in eukaryotes, including a coccolithophore (Alcolombri et al., 2015), Symbiodiniaceae (Alcolombri et al., 2015) and the coral *Acropora digitifera* (Chiu & Shinzato, 2022). It is a multifunctional molecule with osmoregulatory (Sunda et al., 2002) and antioxidant (Jones & King, 2015) roles. Furthermore, since the oxidation products of DMS act as cloud condensation nuclei, there is a proposed role for DMS in cloud formation and local climate regulation (Charlson et al., 1987; Ayers & Cainey, 2007). While these functional roles of DMS are indeed important to the physiology of reef systems, the role of many other BVOCs produced by marine ecosystems remains unknown. It is becoming increasingly recognised that corals produce an enormous diversity of BVOCs, many of which have proposed functions in signalling, stress response and antimicrobial activity (Lawson et al., 2021). This chemical richness necessitates the

transition from the measurement of targeted BVOCs like DMS, towards an untargeted shotgun approach with which to explore the chemical diversity of these remarkable ecosystems (Lawson et al., 2022). Given the abundance of BVOCs in aquatic ecosystems, and their important role in communication in other inter partner relationships, I hypothesise a widespread role for BVOCs as agents of inter-partner signalling in the cnidarian-dinoflagellate symbiosis

1.7 Aiptasia as a model organism for coral research

There are many technical difficulties associated with working with corals that have impeded cnidarian-dinoflagellate symbiosis research; slow growth, infrequent reproduction and high sensitivity to environmental conditions make coral research difficult (Baumgarten et al., 2015). Their calcareous skeletons create barriers to cell biological studies as well. Like corals, *Exaiptasia diaphana* (hereafter, 'Aiptasia') is an anthozoan that forms an intracellular symbiosis with Symbiodiniaceae. Relative to corals, Aiptasia is hardier, easier to maintain in the laboratory, readily reproduces asexually and can live symbiont-free (aposymbiotic; Weis et al., 2008; Lehnert et al., 2012). Given that aposymbiotic anemones continue to reproduce asexually through pedal laceration, the health of symbiont-free anemones is considered sufficient for experimental purposes (Puntin et al., 2022). Tolerant to a variety of non-native symbionts, various Symbiodiniaceae species can populate and persist inside host tissues, allowing the study of comparative symbiosis repopulation dynamics, mechanisms of symbiosis establishment and specificity and differential susceptibility to environmental stressors (Puntin et al., 2022). This ability of Aiptasia to form steady symbioses with a variety of symbiont species was exploited in this thesis to examine the metabolic interactions in holobionts containing symbionts not typically found in association with the host (heterologous). Several cell biological processes have been first described in Aiptasia, and then later validated in corals (Weis, 2019). For these reasons, Aiptasia has become an accepted model organism for coral reef research (Weis et al., 2008; Baumgarten et al., 2015; Van Treuren et al., 2019). While there are many reasons to use Aiptasia as a tool for studying metabolic interactions between cnidarians and dinoflagellates, there are also limitations to its use. Aiptasia does not synthesise a calcium carbonate skeleton, and thus cannot be used to study calcification in corals. Additionally, the uniformity of using a genetically identical population in a lab is not representative to the genetic diversity found in corals nature, thus necessitating conclusions found in Aiptasia to be repeated on corals to determine the ecological relevance of the results.

1.8 Aims and Objectives

The primary aim of this thesis was to elucidate the impact of symbiotic state on the holobiont volatilome, and the potential role of volatiles in host-symbiont communication. The emerging field of volatilomics was applied to characterise patterns of BVOC generation in the cnidarian-dinoflagellate symbiosis, and to elucidate the potential roles of BVOCs in inter-partner signalling and thermal dysfunction. While recent studies have explored BVOC emissions from corals and cultured Symbiodiniaceae, never before have BVOCs been investigated in the context of symbiotic state. To achieve this, the project consisted of the following specific objectives and hypotheses:

1) To define the volatilome of the model symbiotic cnidarian Aiptasia and its dinoflagellate symbiont, both in isolation and in symbiosis. This work provides a baseline to explore BVOC production by each partner in isolation, and in symbiotic association to determine the conservation of BVOCs with previously described volatilomes.

   H_1: The volatilome of each partner in isolation will differ from that of the holobiont, reflecting symbiosis-specific changes in partner physiology.

2) To determine the influence of symbiont identity on the volatilome of Aiptasia. This was performed to explore the ways in which metabolic functioning is altered in response to symbiosis with a non-native, sub-optimal symbiont.

   H_1: The volatilome produced by anemones harbouring a non-native (heterologous) symbiont will differ from the volatilome produced by anemones containing the native (homologous) symbiont.

3) To measure changes in the volatilome of Aiptasia in response to thermal stress in both the aposymbiotic and symbiotic states. This next step was performed to explore the ways in which BVOCs are altered from their baseline production to further elucidate the ways in which thermal stress alters symbiosis stability.

   H_1: The volatilome of anemones exposed to thermal stress will shift under increased temperature, with the abundance and diversity of BVOCs reflecting increased cellular stress and symbiotic dysfunction.

4) To determine whether different species of free-living Symbiodiniaceae respond *via* chemotaxis to BVOCs, with particular emphasis on the volatile precursor DMSP which is known to be produced in abundant quantities by reef corals. This work adds functional

information about an abundantly produced BVOC, DMS, identified in previous chapters. Two halogenated methane BVOCs identified in my work were also tested, bromodichloromethane and diiodomethane.

H_1: Symbiodiniaceae species will exhibit different chemotactic responses to DMSP, bromodichloromethane, and diiodomethane, and thus reflect differences in host-symbiont specificity.

Collectively, these objectives all contribute to our understanding of the ways in which BVOC emission will change in response to symbiotic state and thermal stress, and potentially identify chemical biomarkers with which to apply this powerful and non-invasive technique to ecosystem management.

Chapter 2 Symbiosis induces unique volatile profiles in the model cnidarian Aiptasia

2.1 Abstract

The establishment and maintenance of the symbiosis between a cnidarian host and its dinoflagellate symbionts is central to the success of coral reefs. To explore the metabolite production underlying this symbiosis, I focused on a group of low weight secondary metabolites, biogenic volatile organic compounds (BVOCs). BVOCs are released from an organism or environment, and can be collected in the gas phase, allowing non-invasive analysis of an organism's metabolism (i.e., 'volatilomics'). I characterised volatile profiles of the sea anemone *Exaiptasia diaphana* ('Aiptasia'), a model system for cnidarian–dinoflagellate symbiosis, using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry. I compared volatile profiles between: 1) symbiotic anemones containing their native symbiont, *Breviolum minutum*; 2) aposymbiotic anemones; and 3) cultured isolates of *B. minutum*. Overall, 152 BVOCs were detected, and classified into 14 groups based on their chemical structure, the most numerous groups being alkanes and aromatic compounds. A total of 53 BVOCs were differentially abundant between aposymbiotic anemones and *B. minutum* cultures; 13 between aposymbiotic and symbiotic anemones; and 60 between symbiotic anemones and cultures of *B. minutum*. More BVOCs were differentially abundant between cultured and symbiotic dinoflagellates than between aposymbiotic and symbiotic anemones, suggesting that symbiosis may modify symbiont physiology more than host physiology. This is the first volatilome analysis of the Aiptasia model system and provides a foundation from which to explore how BVOC production is perturbed under environmental stress, and ultimately the role they play in this important symbiosis.

2.2 Introduction

All organisms synthesise a unique chemical composition of metabolites which reflects their underlying metabolic processes and relationship to the environment. A subset of these metabolites are termed biogenic volatile organic compounds (BVOCs) that are often synthesised as a by-product of central metabolism and emitted directly into the environment (Sharkey et al., 2008; Schmidt et al., 2016). BVOCs are a functionally diverse group of chemicals characterised by their low molecular weight (< 200 Da), low boiling point and high vapour pressure (Mansurova et al., 2018). In contrast to other metabolites, which must be extracted from tissues and fluids, BVOCs diffuse into the external environment, thereby allowing their detection without disruption of the producing organism and hence non-invasive analysis of metabolic state (Mansurova et al., 2018).

Terrestrial ecosystems are known to be prolific producers of BVOCs (Guenther, 2013), many of which play important physiological and ecological roles, such as pollinator attraction (Schiestl, 2015), pathogen defence (Huang et al., 2012), plant growth promotion (Bailly et al., 2014) and antioxidant production (Stashenko et al., 2004). BVOCs also function as agents of rapid communication in intra- and inter-species communication (Briard et al., 2016; Mansurova et al., 2018). The BVOC produced in highest quantities by terrestrial ecosystems is isoprene, which functions in thermal tolerance in plant cell walls (Behnke et al., 2007), predator defence (Laothawornkitkul et al., 2008) and as an antioxidant (Loreto and Velikova, 2001). The dominant BVOC produced by marine ecosystems is dimethyl sulphide (DMS) (Kettle et al., 1999; Jackson et al., 2021), which functions as an antioxidant (Sunda et al., 2002), attractant for macrofauna to a productive marine environment (Nevitt et al., 1995; Wright et al., 2011), and local climate regulator (Park et al., 2021). Notably, coral reefs have been identified as marine 'hotspots' of BVOC emissions (Exton et al., 2015), with corals producing highly diverse BVOC cocktails – characterised as "volatilomes" (Steinke et al., 2018) – that vary by species and environmental conditions (Lawson et al., 2019; Lawson et al., 2021). Despite the diversity and abundance of BVOCs in the marine environment, resolving BVOC form and function in marine ecosystems is a comparatively new area of focus (Saha et al., 2021).

Metabolite exchange is critical for sustaining a stable and healthy symbiosis between reef-building coral hosts and their microalgal dinoflagellate partners (family: Symbiodiniaceae) (Matthews et al., 2017; Rosset et al., 2021). Dinoflagellate symbionts reside within membrane-bound vesicles in the host gastrodermis (Wakefield and Kempf, 2001), meaning that inter-partner signalling across this host-symbiont interface, including the exchange of metabolites, is crucial for maintaining the symbiosis (Rosset et al., 2021). Diverse molecules such as glycans (Markell and Wood-Charlson, 2010), lipids (Kitchen et al., 2017) and noncoding RNA (Baumgarten et al., 2017) have been identified as signalling molecules in the cnidarian–dinoflagellate symbiosis. BVOCs have likewise been identified as agents of inter-kingdom signalling molecules in other systems (Schmidt et al., 2016; Netzker et al., 2020). For example, p-anisaldehyde and phenylacetaldehyde production attracts pollinators to flowers (Theis, 2006), herbivore-damaged *Nicotiana attenuate* produce linalool to stimulate herbivore predation (Kessler and Baldwin, 2001), and *Laccaria bicolor* stimulate root formation in *Arabidopsis* and *Populus* through sesquiterpene production (Ditengou et al., 2015). Given their importance as infochemicals in other systems, BVOCs may therefore also be important for regulating the cnidarian–dinoflagellate symbiosis.

The metabolism of both cnidarian and dinoflagellate partners is altered in response to symbiotic state (Rodriguez-Lanetty et al., 2006; Yuyama et al., 2021), and these changes may also be detectable in

the volatilome. These metabolic shifts function to optimise this relationship and allow the growth of both organisms in symbiosis, allowing each partner to benefit from their close association with the other. Alterations can be reflected through gene expression (Rodriguez-Lanetty et al., 2006), nutrient exchange (Xiang et al., 2020) and assimilation (Wang and Douglas, 1998), cell cycle regulation (Tivey et al., 2020b), and the modulation of the immune response (Neubauer et al., 2016). Since other, non-BVOC molecules are known to change in response to symbiotic state (Rosset et al., 2021), I hypothesise that BVOC profiles will also differ among symbiotic states in this system, thus reflecting differing metabolic processes.

The symbiotic sea anemone *Exaiptasia diaphana* ('Aiptasia') is a model system for the study of the cnidarian–dinoflagellate symbiosis that can extend to other symbiotic cnidarians, including ecologically-important reef corals. Like corals, Aiptasia is a known producer of BVOCs such as DMS when in symbiosis (Franchini and Steinke, 2017). The Aiptasia model system has been adopted for its ease of laboratory culture and ability to separate symbiont from host to study each organism in isolation, providing insight into the roles that the cnidarian and dinoflagellate play in the symbiosis (Weis et al., 2008; Baumgarten et al., 2015), including metabolite exchange (Matthews et al., 2017, 2018). The aim of this study was to therefore profile the BVOCs of the Aiptasia model system. I describe the BVOCs that are produced by the intact symbiosis, as well as the host anemone and homologous (i.e., native) symbiotic dinoflagellate (*Breviolum minutum*) when in isolation from one another, which may inform how symbiotic state alters the volatilome. The unique volatile signatures characterised here provide a baseline for future non-invasive analysis of how symbiotic state and environmental change influence symbiosis physiology, as well as studies of inter-partner communication and functionality of BVOCs in the cnidarian–dinoflagellate symbiosis.

2.3 Methods

2.3.1 Experimental Organisms

The homologous symbiont of Aiptasia, *Breviolum minutum* (ITS2 type B1, culture ID 'FLAp2'), was grown in 35 ppt 0.22 µm filtered seawater (FSW) enriched with f/2-medium (AusAqua Pty Ltd., SA, Australia) and maintained at 25 °C in a climate-controlled incubator. Cultures were grown under light provided by fluorescent lamps (Osram Dulux 36/W890 fluorescent bulbs) at approximately 70 µmol photons m^{-2} s^{-1} on a 12:12 h light dark cycle. One week prior to experimentation, cultures for BVOC analysis were diluted with fresh medium to ensure that they were in exponential growth. On the day of sampling, maximum photochemical efficiency, F_v/F_m (dimensionless), was used as an indicator of culture health (Table S1; Suggett et al., 2009); cultures (n = 5) were dark acclimated in 125 mL serum

glass bottles (Wheaton, Millville, NJ, USA) for 15 min before measurement with an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3 and damping = 3).

A long-term (15+ years) clonal culture of the sea anemone Aiptasia (culture ID: NZ1) of unknown Pacific origin (Matthews et al., 2017) was maintained in the laboratory in FSW at 25 °C and approximately 70 µmol photons m^{-2} s^{-1} on a 12:12 h light dark cycle. Anemones (n = 100) were rendered aposymbiotic (i.e., symbiont-free) using menthol-induced bleaching, which was achieved by exposure to menthol (20% w/v in ethanol; Sigma-Aldrich, Auckland, NZ) at a final concentration of 0.19 mmol L^{-1} in 0.22 µm FSW (Matthews et al., 2016). Anemones were incubated in menthol for 8 h during the 12-h light period, after which photosynthesis was inhibited by replacing menthol/FSW with FSW containing 5 µmol L^{-1} 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 100mmol L^{-1} dissolved in EtOH, Sigma-Aldrich), for 16 h to prevent recolonisation by inhibiting photosynthesis of the remaining symbionts. This 24-h cycle was repeated for four consecutive days, after which anemones were maintained in 0.22 µm FSW for the three days between menthol and DCMU treatments. Anemones were fed once weekly with *Artemia* sp. nauplii, with fresh FSW changes 8 hours post-feeding. This protocol was continued for 6 weeks, and aposymbiotic anemones were maintained in the dark at 25°C for 6 months prior to BVOC sampling.

Given that the culture of *B. minutum* did not originate from our NZ1 lab stock of Aiptasia, symbiotic anemones were generated by recombining aposymbiotic anemones with the cultured symbionts. To achieve this, aposymbiosis was confirmed through fluorescence microscopy (Olympus IX53 inverted microscope; 100× magnification), and a sub-set of aposymbiotic anemones (n = 25) were starved for seven days prior to inoculation with cultured *B. minutum*. An aliquot (~20 µL) of *B. minutum* culture, concentrated by centrifugation to a density of 3×10^6 cells mL^{-1}, was pipetted directly onto the oral disc of individual aposymbiotic anemones. *Artemia* sp. nauplii were mixed into this suspension to encourage phagocytosis of algal cells (Davy et al., 1997). Inoculated anemones were fed twice weekly with *Artemia* sp. nauplii and maintained under the same ambient temperature and light conditions as described above until a fully symbiotic state was reached after three months. Anemones were fully symbiotic for six months prior to BVOC sampling. The presence of intracellular symbionts was confirmed by fluorescence microscopy three months prior to sampling as described above.

2.3.2 BVOC sampling and volatilome characterisation

Volatile signatures were collected and analysed from three states: vials of aposymbiotic anemones (n = 5; ~15 anemones/vial); symbiotic anemones colonised with *B. minutum* (n = 4; ~15 anemones/vial); and free-living cultures of *B. minutum* (n = 5). The experimental setup (Fig. S1) and BVOC retrieval were performed using a modified method of Lawson et al. (2019, 2021). The night before BVOC sampling, experimental organisms were transferred into sterile 150 mL glass crimp cap vials containing 75 mL FSW, where they were retained for a minimum of 12 h under conditions identical to those used for growth, and vials were refreshed with FSW and sealed immediately prior to sampling using 20 mm PTFE/Si crimp caps (Agilent, USA). BVOCs were collected by passing instrument grade air (100 mL min^{-1}; BOC Gases, Wellington, NZ) into sampling vials for 20 min, during which time air was bubbled through the FSW, facilitating the release of BVOCs into the headspace. The outgoing air then passed through open-ended thermal desorption tubes (TDTs; Markes International Ltd, Llantrisant, UK) containing the sorbent Tenax TA, onto which BVOCs adhered (Fig. S1). After the 20 min sampling time, tubes were immediately sealed with brass storage caps and stored at 4 °C until processing. All TDTs were analysed within two weeks of sampling to minimise sample degradation using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-ToFMS) as *per* Olander et al. (2021).

Prior to desorption, TDTs were injected with 0.2 µL of 150 ppm bromobenzene (GC grade, Sigma-Aldrich, Castle Hill, NSW, Australia) in methanol (HPLC grade, Sigma-Aldrich) using an automated eVol® XR analytical pipette (SGE Analytical Science, Wetherill Park BC, NSW, Australia) as an internal standard to ensure injection repeatability. Samples were desorbed using a Markes UNITY 2 series thermal desorber and ULTRA multi-tube autosampler (Markes International Ltd. Llantrisant, UK) for 2.5 min at 300 °C and concentrated on a Markes General Purpose Carbon C4/5-C30/32 cold trap at -30 °C. Desorbed compounds were then injected into a Pegasus 4D GC×GC-ToFMS platform (LECO Corp., St Joseph, MI, USA) *via* a linked 1 m uncoated silica transfer line at 150 °C. High purity helium with a flow rate of 1 mL min^{-1} was used as the carrier gas. The GC×GC-ToFMS was equipped with a cryogenic quad jet modulator, using two liquid-nitrogen cold jets in conjunction with two pulsed hot-air jets to trap and refocus compounds eluting between the primary (Rxi-624Sil MS column (30 m×0.25 mm inner diameter (ID), 1.40 µm film thickness); Restek Corp.) and secondary (Stabilwax column (2 m×0.25 mm ID, 0.50 µm film thickness); Restek Corp) columns. The primary and secondary columns were bridged by a SilTite µ-Union connector (SGE Analytical Science Pty., Ltd, Ringwood, VIC, Australia). The temperature program was as follows: initial temperature of 35 °C with a 5 min hold followed by an increase to 240 °C at a rate of 5 °C min^{-1} with a final hold of 5 min. Temperature offsets of + 5 °C and + 15 °C for the modulator and second dimension column,

respectively, were used. The modulation period was 5 s and included a 1 s hot pulse. The ToFMS collected at a rate of 100 spectra s^{-1} with a range of 29–450 amu.

Data processing was performed using ChromaTOF® (version 4.51.6.0; LECO). A signal-to-noise ratio of 150 was used with a baseline offset of 0.8. The peak widths for the first and second dimensions were 30 s and 0.15 s, respectively. Compounds were tentatively identified by comparing mass spectra against a commercial library (NIST08 library in NIST MS Search v.2.2f; NIST, Gaithersburg, MD) where a spectral match of 80% was required. Unclassified compounds were denoted as UC, with the numbers following indicating the retention times of the compound in the first and second dimension, respectively. All samples were normalised to the internal standard. BVOCs were also collected from filtered (0.22 µm) seawater blanks (n = 6) using identical methods at the time of sample collection. All samples were aligned using the Statistical Compare feature in ChromaTOF®. For alignment, the samples were classified according to treatment method, as aposymbiotic anemones (n = 5), symbiotic anemones (n = 4), cultures of *B. minutum* (n = 5) and seawater blanks (n = 6). A requirement of the BVOCs to be present in a minimum of two samples within one treatment was used to eliminate BVOCs that were not deemed relevant. After this, average values for BVOCs present in the blanks were subtracted from all samples. Finally, peak abundance was normalised to the protein content of each replicate. Protein was released from samples by sonication (VCX500; Sonics & Materials Inc., Newtown, CT, USA) with a detergent (5% sodium deoxycholate) to lyse cells. Detergent was subsequently precipitated by acidification and removed by centrifugation (Eppendorf 5424 Centrifuge; Thermo Fisher Scientific, Waltham, MA, USA). Protein content was measured using the fluorometric Qubit Protein Assay Kit (Vergauwen et al., 2017). Compounds identified as likely methodological artifacts e.g. silicon-containing compounds, were also removed from the dataset as they are suspected contamination from dimethylpolysiloxane (a GC column component) hydrolysis (Cella and Carpenter, 1994).

2.3.3 Statistical Analysis

Differential abundance of BVOCs was estimated using the limma R package (Ritchie et al., 2015); the *voom* function was used to convert counts to $\log_2$-counts-*per*-million and to assign weights to each observation based on the mean-variance trend. The counts assigned to each observation roughly corresponds to abundance of the BVOC, which can then be used to compare BVOC production between treatments. Functions *lmFit*, *eBayes*, and *topTable* were used to fit weighted linear regression models, calculate empirical Bayes moderated t-statistic, and calculate FDR-corrected p-values (Phipson et al., 2016). Bubble plots, bar graphs and pie charts were created with ggplot2 (Wickham, 2016) in RStudio.

Pairs of biological replicates, standardised using the *decostand* function in the vegan package (Oksanen et al., 2020) in R (version 1.2.5033), were compared using the Bray-Curtis similarity measure, and this output was subjected to non-metric multidimensional scaling (NMDS) to visualise differences among groups based on BVOCs.

2.4 Results

2.4.1 BVOC emissions are affected by symbiotic state

A total of 152 BVOCs were detected across the dataset of aposymbiotic anemones, symbiotic anemones and symbiont cultures (Fig. 2.1a). Of these, 7 BVOCs were produced solely by aposymbiotic anemones, 11 by symbiotic anemones, and 29 by cultures of *B. minutum;* 42 BVOCs were detected in aposymbiotic and symbiotic anemones but were absent in cultures of *B. minutum*; 5 were detected in symbiotic anemones and cultures of *B. minutum* but were absent from aposymbiotic anemones; and 23 were detected in aposymbiotic anemones and cultures of *B. minutum* but were absent in symbiotic anemones (Fig. 2.1a). Only BVOCs detected in a minimum of two biological replicates in at least one symbiotic state were classed as 'present' in a state.

35 BVOCs were present across all samples, and so were designated as 'core compounds' (Fig. 2.1a). All BVOCs were grouped according to their chemical class (Fig. 2.1b). Unknown compounds represented the majority of BVOCs in all states, followed by aromatic compounds in aposymbiotic anemones, and alkanes in symbiotic anemones and cultures of *B. minutum*. BVOCs across the three states were distinct, with clear NMDS clustering of BVOCs by aposymbiotic anemones, symbiotic anemones and cultures of *B. minutum* (Fig. 2.2), indicating that each state was characterised by a unique volatilome. The numbers of differentially abundant BVOCs relative to the holobiont were contrasted (Fig. 2.3), revealing that cultured *B. minutum* produces a more distinct volatilome than aposymbiotic anemones.

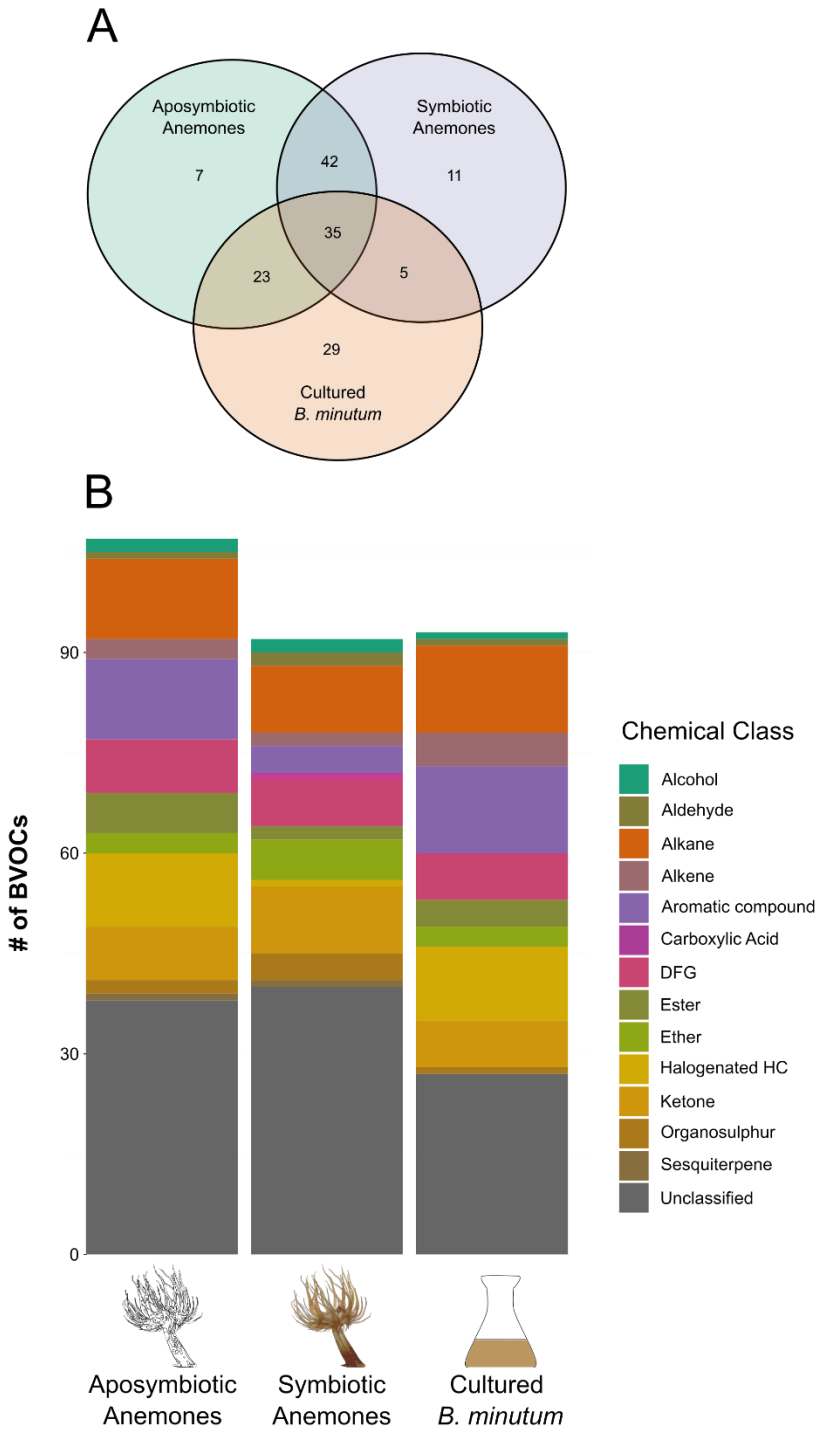

Figure 2.1: Comparison of BVOC diversity among three symbiotic states. A) BVOCs detected in each symbiotic state; B) Number of BVOCs present in each state, grouped based on their chemical class. BVOCs had to be present in at least two biological replicates in at least one of three states (aposymbiotic anemones (n = 5), symbiotic anemones (n = 4), cultures of *B. minutum* (n = 5)) to be included. For differentially abundant BVOCs, see Figure 3.

2.4.2 Contrasting volatilomes between symbiotic states

A total of 71 BVOCs were differentially abundant between the three symbiotic states, as determined with pairwise differential abundance testing (Table S2):

(i) Aposymbiotic anemones *versus B. minutum* cultures:

53 differentially abundant BVOCs were recorded between aposymbiotic anemones and cultures of *B. minutum*. Of these, 25 were detected in higher quantities in the volatilome of aposymbiotic anemones, including 5 halogenated hydrocarbons (bromochloromethane, bromodichloromethane, dibromochloromethane, dibromomethane, and trichloromethane), plus 1,3-octadiene and styrene. By comparison, 28 BVOCs were detected in higher quantities in the volatilome of *B. minutum*, including DMS, anisole, methylal and amylene hydrate.

(ii) Aposymbiotic anemones *versus* symbiotic anemones:

When anemones were compared between the aposymbiotic and symbiotic states (colonised with *B. minutum*), their volatilomes were very similar, with only 13 compounds being differentially abundant. Only 7 compounds were detected in higher quantities in the volatilome of aposymbiotic anemones: dimethoxy-1,3-benzene, 1,3-octadiene, amylene hydrate, 3,4-dimethyl-2-pentanone, and 2 unknown compounds. By comparison, 6 compounds were detected in higher quantities in the volatilome of symbiotic anemones: DMS, ethylidenecyclopropane and 4 unknowns.

(iii) *B. minutum* culture *versus* symbiotic anemones:

Comparison between the cultured *B. minutum* and symbiotic anemones revealed the greatest divergence in the volatilome, with 60 BVOCs being differentially abundant. Of these, 33 were more abundant in *B. minutum* cultures, including DMS, amylene hydrate, 2-pentanone, 2-butanone and isopropylsulphonyl chloride. In comparison, 27 were more abundant in symbiotic anemones, including 6 halogenated methane BVOCs (bromochloromethane, bromodichloromethane, dibromochloromethane, dibromomethane, trichloromethane and tribromomethane), butanal, styrene and propylbenzene.

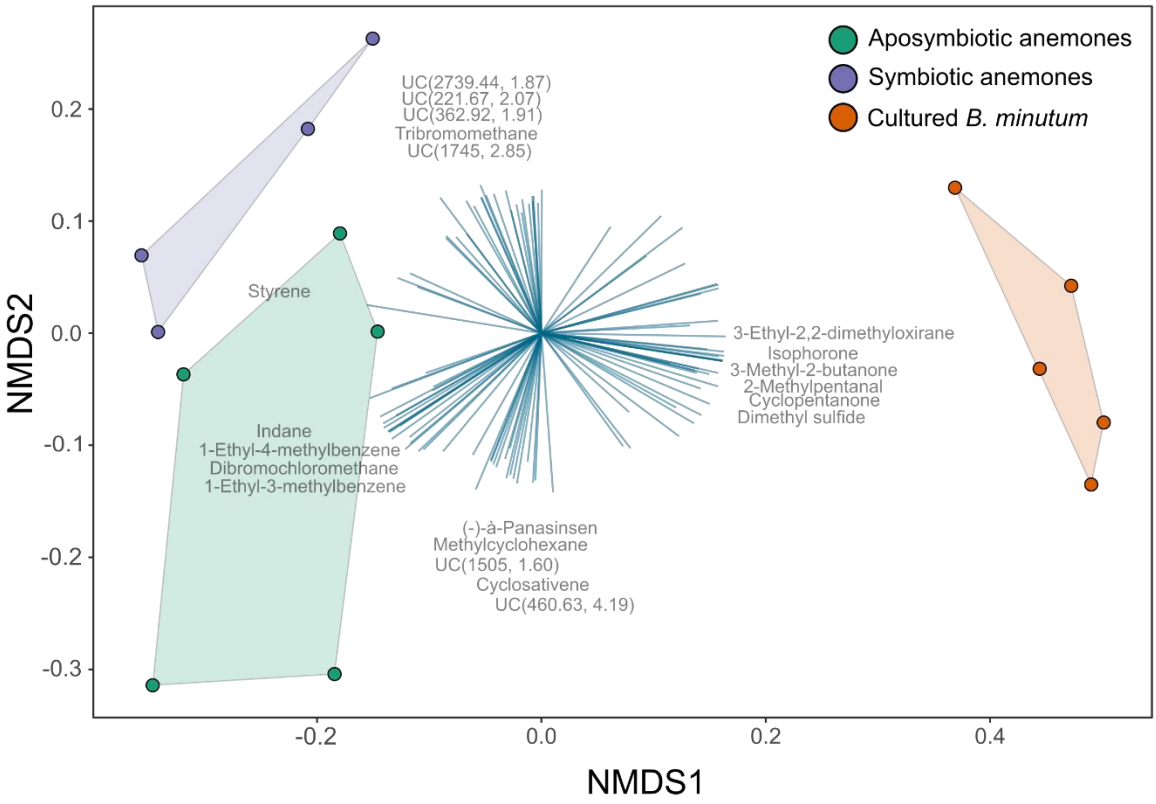

Figure 2.2: Non-metric multidimensional scaling (stress = 0.058) plot of BVOCs produced by the Aiptasia model system. Plotted using the *vegan* package in R. BVOCs had to be present in at least two replicates in at least one of three states: (aposymbiotic anemones (n = 5), symbiotic anemones (n = 4) or cultures of *B. minutum* (n = 5)) to be included. Displayed BVOCs were chosen based on the top 5 loading scores for each NMDS dimension.

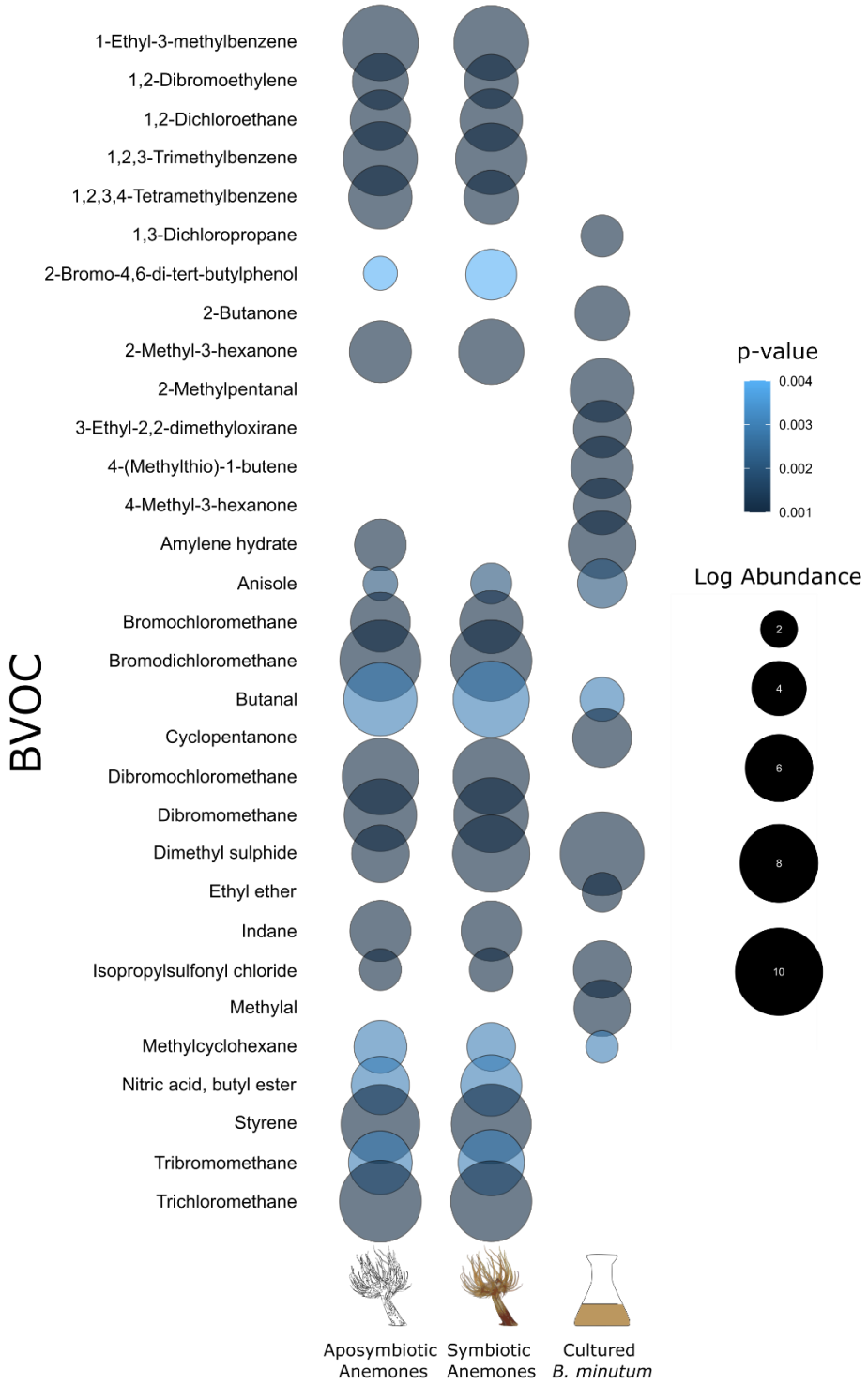

Figure 2.3: Differentially abundant BVOCs detected from aposymbiotic anemones, symbiotic anemones and cultures of *B. minutum*. Bubble size is based on the log of the normalised BVOC peak area. Bubble colour is based on p-value (differential abundance testing). Only BVOCs that were tentatively identified and changed significantly among treatments are shown; unidentified BVOCs were not included in this plot. BVOCs had to be detected in at least two biological replicates in at least one of three states (aposymbiotic anemones (n = 5), symbiotic anemones (n = 4) and cultures of *B. minutum* (n = 5) to be included.

2.5 Discussion

BVOC emissions by organisms provide important insight into underlying metabolic regulation *via* external environmental and biotic factors (Niinemets, 2010; Mansurova et al., 2018). While recent research has shown that – as with terrestrial organisms (Hrebien et al., 2020; Peñuelas and Llusià, 2003) – reef building corals emit highly diverse BVOC mixtures (volatilomes) (Lawson et al., 2021), how such volatilomes are influenced by symbiotic state is unexplored. Since coral metabolism is altered in response to symbiosis (Matthews et al., 2017; Sproles et al., 2019; Rosset et al., 2021), BVOC analysis can provide a non-invasive technique with which to assess the intracellular mechanisms and interactions involved in symbiosis. To date, the volatilomes of corals have only been retrieved from a single heat-stress experiment, where symbiotic state between the host coral and its endosymbiotic dinoflagellate community was largely retained, but physiological competency compromised (Lawson et al., 2021). The purpose of this study was therefore to characterise BVOC production by the Aiptasia model system and its individual constituents (aposymbiotic anemones and cultures of *B. minutum*), using non-invasive analysis. In doing so, I identified distinct volatilomes between symbiotic states. My results show that cultured *B. minutum* produce a more distinct volatilome than that of aposymbiotic anemones or anemones symbiotic with their homologous symbiont, *B. minutum*. Aposymbiotic *versus* symbiotic anemones had fewer statistically differentially abundant BVOCs (13) and a more similar composition of BVOC diversity than cultured *B. minutum* *versus* symbiotic anemones (60), indicating that the volatilome of the host cnidarian may be less impacted by symbiotic state than that of the symbiont.

2.5.1 Core Compounds

Of the 152 BVOCs detected in this study, 35 were present in all three symbiotic states, and thus were classified as ‘core compounds’. Of the 35 core compounds, the most common group was unclassified BVOCs (12), followed by ketones (5). Notably, dimethyl sulphide (DMS) is a core compound; to my knowledge the production of DMS in aposymbiotic anemones has not been reported, though previous studies show that cnidarians may be a cryptic source of DMS not produced by their endosymbiotic partner (Raina et al., 2013). Alternatively, it is possible that DMS was produced by members of the associated bacterial community, as coral-associated bacteria have been shown to produce the precursor to DMS, dimethylsulphoniopropionate (DMSP; Kuek et al., 2022). Regardless of its source, this information serves to highlight the ubiquitous nature of DMS in marine systems, where it serves osmoregulatory (Wittek et al., 2020), antioxidant (Sunda et al., 2002) and chemoattractant roles (DeBose et al., 2008).

2.5.2 Volatilomes of Aiptasia and B. minutum in the absence of symbiosis

Comparing the volatilome of aposymbiotic anemones *versus* cultured symbionts is important for identifying those BVOCs that are synthesised by the algae or cnidarian (or their microbial associates) in the absence of symbiosis. For example, halogenated methane compounds (chloroform, bromodichloromethane, chlorodibromomethane, bromochloromethane and dibromomethane) were detected in significantly higher quantities in aposymbiotic anemones relative to cultured *B. minutum*. This outcome is consistent with previous research showing that halogenated hydrocarbons, including bromoform and chlorodibromomethane are likely core components of coral (*Acropora intermedia* and *Pocillopora damicornis*) volatilomes under ambient and elevated temperatures (Lawson et al., 2021), but are not detectable in the volatilomes of bacteria and dinoflagellate symbionts isolated from these corals (Lawson et al., 2019, 2020). This suggests that the cnidarian host may be the primary source of these molecules in Aiptasia, and that the production of halogenated hydrocarbons is conserved among symbiotic cnidarians.

In contrast, ethylidenecyclopropane was increased in cultures of *B. minutum* as well as symbiotic anemones relative to aposymbiotic anemones, suggesting that it originated from *B. minutum*. Previously identified as a by-product of thermal stress *via in vitro* experiments simulating thermal and light stress in artificial membranes, ethylidenecyclopropane may serve a protective role in the holobiont to stabilise symbiont lipid membranes (Halahan, 2013). It is therefore plausible that the symbionts are responsible for production of this molecule and potentially upregulate its production during periods of stress.

A well-studied BVOC in marine systems is DMS, which was likewise detected in significantly higher quantities in both cultures of *B. minutum* and symbiotic anemones relative to aposymbiotic anemones (495× and 31× greater, respectively), suggesting that *B. minutum* produces DMS in both its symbiotic and cultured states. DMS is a breakdown product of dimethylsulphoniopropionate (DMSP), and marine phytoplankton – including Symbiodiniaceae – produce it prolifically (Keller et al., 1989; Yoch, 2002; Lawson et al., 2019), as do corals in response to stress (Hopkins et al., 2016a). DMS is an antioxidant molecule that can penetrate thylakoid membranes and scavenge free-radicals produced by harmful lipid peroxidation reactions (Sunda et al., 2002), and conceivably may be passed from symbiont to host to provide antioxidative protection. While the symbiont is a source of free radical production during photosynthesis (Khorobrykh et al., 2020), and especially so during periods of thermal and/or light stress (Nielsen et al., 2018), the significantly higher levels of DMS produced in the symbiotic *vs.* aposymbiotic state may indicate that the symbiont is providing a molecule important for the mitigation of oxidative damage to the host (Oakley and Davy, 2018).

BVOCs that are common between aposymbiotic anemones and cultured *B. minutum* but seemingly absent from symbiotic anemones may indicate that these BVOCs, or their upstream precursors, are consumed in symbiosis, or by bacteria specific to symbiotic anemones prior to experimental detection. Alternatively, these BVOCs could be of bacterial origin, with the holobiont providing a less ideal environment to support bacterial growth and therefore bacterial BVOC synthesis. Due to the tight nutritional/metabolite exchange between cnidarians and dinoflagellates (Davy et al. 2012) there could be fewer metabolites, which support bacterial growth, leaking out of the holobiont (Morris, 2015). For example, amylene hydrate and 3,4-dimethyl-2-pentanone were detected in higher quantities in aposymbiotic anemones and cultures of Symbiodiniaceae than in the intact symbiosis, suggesting that one of these processes affected the detection of these molecules. These BVOCs are prime candidates for future functional studies, to better understand how metabolism changes with symbiotic state.

2.5.3 Symbiosis affects the cnidarian volatilome

Differentially abundant BVOCs between symbiotic states could represent a true alteration in the volatilome of one or both partners in response to association with the other, underlining the foundational ways in which symbiotic partners can influence one another. It could also be the case that products are consumed by the dinoflagellate symbiont or associated bacteria, resulting in a failure of detection in symbiotic anemones. Finally, BVOCs could change configuration prior to detection through reaction with other biologically produced airborne molecules (Kai et al., 2018), that may not be present in the absence of symbiosis. With the identification of key BVOCs that differ among symbiotic states, future functional studies should focus on unravelling these complex relationships.

Comparing the BVOCs of symbiotic *versus* aposymbiotic anemones allows us to infer the influence of symbiosis on the hosts volatilome. BVOCs produced by the holobiont could be produced by either partner independent of symbiotic state. Symbiosis has previously been shown to alter the host transcriptome in the symbiotic anemone *Anthopleura elegantissima* (Rodriguez-Lanetty et al., 2006), and proteome of Aiptasia (Oakley et al., 2016). Relative to aposymbiotic anemones, symbiotic anemones upregulate genes controlling lipid degradation, preventing apoptosis, and downregulate genes involved in antioxidant response and prevention of cell proliferation (Rodriguez-Lanetty et al., 2006). Likewise, symbiotic anemones show higher expression of proteins involved in lipid, nitrogen and carbon transport, intracellular trafficking and endocytosis, processes that reflect changes in host metabolism due to nutritional exchange with algal symbionts (Oakley et al., 2016). BVOCs detected in higher abundance in symbiotic anemones could be downstream products of these processes, or be end-products in themselves, serving to aid in the exchange of nutrients between host and symbiont.

Aposymbiotic anemones have been shown to produce increased proteases and chitinases, reflective of a shift towards heterotrophy in the aposymbiotic state, in addition to proteins involved in mediating reactive oxygen stress (Oakley et al., 2016). BVOCs observed in the aposymbiotic state could be breakdown products of these proteins, function in symbiont attraction, have antibacterial properties, or be a by-product of a more active immune system not suppressed by colonisation with symbionts (Detournay et al., 2012). For example, 1,3-dimethoxybenzene, used as an alarm substance produced by the terrestrial hexapod *Neanura muscorum* (Porco & Deharveng, 2007) was detected in higher quantities in aposymbiotic anemones relative to symbiotic anemones and could similarly be a product of stress in anemones that have failed to establish symbiosis.

The relatively minimal change in the cnidarian volatilome between symbiotic states is particularly notable, perhaps reflecting host-symbiont compatibility during symbiosis establishment. Indeed, during establishment of a successful symbiosis, it has been shown that the host immune system is modulated and there are few signs of cellular stress in the host (Zamioudis and Pieterse, 2012, Matthews et al., 2017). The mechanisms underlying successful inter-partner integration and communication in the cnidarian-dinoflagellate symbiosis are still unclear (Davy et al., 2012; Poole et al., 2016; Neubauer et al., 2017, Rosset et al., 2021), but my data are consistent with the symbiont successfully evading host detection and/or a high degree of host-symbiont cellular integration. In future, it will be interesting to compare my observations with the volatilome that arises during colonisation with less compatible symbionts, which can induce cellular stress and even host mortality (Starzak et al., 2014; Matthews et al., 2017; Tortorelli et al., 2020).

2.5.4 Symbiosis affects the B. minutum volatilome

Comparing the volatilome of cultured symbionts *versus* symbiotic anemones is important for identifying the ways in which symbiosis alters metabolism of the symbiont and/or how the host metabolises compounds synthesised by the symbiont. Specifically, BVOCs in higher quantities in cultured *B. minutum* relative to the holobiont may be suppressed in symbiosis, consumed by the host, or modified prior to release into the environment. BVOCs detected in symbiotic anemones but not in cultured *B. minutum* could represent compounds produced by the host, or compounds produced by the symbiont in response to being in symbiosis. When making these comparisons, however, it is important to recognise the limitations of working with cultured dinoflagellate symbionts (Maruyama and Weis, 2021). For example, bacteria associated with algal culture medium could differ in terms of both abundance and composition relative to bacterial populations associated with the intact symbiosis, once again impacting the volatilome, given that bacteria are also prolific producers of BVOCs (Netzker et al., 2020; Ping and Boland, 2004; Lawson et al., 2020). Furthermore, previous studies

have highlighted important physiological differences that arise due to nutrient availability, independent of symbiotic state in Symbiodiniaceae. For example, C/N ratios of Symbiodiniaceae *in hospite* (11.5) are more like Symbiodiniaceae cultured in N-deprived medium (13.5) compared to those cultured in N-replete medium (3.3) (Xiang et al., 2020). Likewise, transcripts associated with N-acquisition are upregulated in *in hospite* Symbiodiniaceae and N-deprived cultured Symbiodiniaceae, but not in Symbiodiniaceae cultured in N-replete medium (Xiang et al., 2020). Considering this, the observed differences in volatilomes between Symbiodiniaceae and Aiptasia cultures could be a product of physiological differences due to nutrient availability, rather than differences in symbiotic state alone. Nevertheless, this approach still has the potential to inform our functional understanding of the cnidarian–dinoflagellate symbiosis. For example, cyclopentanone levels increased in cultured *B. minutum* relative to symbiotic anemones. Cyclopentanone is a precursor to jasmonic acid, which plays a role in the response to environmental stresses in plants (León and Sánchez-Serrano, 1998). Should this BVOC be transferred from dinoflagellate to cnidarian host, a protective benefit could be provided to the host through symbiotic association.

My observation that the volatilome of *B. minutum* may potentially be altered more dramatically in response to symbiosis than that of the host Aiptasia, could reflect a shift in Symbiodiniaceae metabolism between cultured and symbiotic states. As free-living cells, Symbiodiniaceae are motile with two flagella and a characteristic gymnodinioid shape (Trench and Blank, 1987), while in symbiosis, their cells are coccoid, lack flagella, and are larger, with smaller plastids and thinner cell walls (Trench and Blank, 1987; Pasaribu et al., 2015). Moreover, it is known from physiological and 'omics' studies, that symbiosis induces an increase in symbiont metabolism, and metabolite transport and release (Trench, 1971; Maor-Landaw et al., 2020), with some of these released metabolites being linked to host-symbiont recognition (Wood-Charlson et al., 2006). Recent molecular studies have highlighted how genes associated with various other processes are down-regulated in the symbiotic state, including those involved in stress responses and immune regulation (Mohamed et al., 2020; Yuyama et al., 2021); these latter observations are consistent with the more stable environment provided by the intracellular habitat. When in symbiosis, there is more tissue separating the symbiont cells from the external environment, and thus more tissue through which algal-derived BVOCs must pass before release into the environment. This may provide more opportunity for BVOC modification by other metabolites as has been observed in bacteria, where BVOCs can be synthesised through the non-enzymatic combination of volatile precursors to produce a novel BVOC (Kai et al., 2018).

2.6 Conclusion

The marine environment is known to be an important source of BVOCs, however the diversity of BVOC production, and its relationship to symbiosis, has only begun to be understood. Here I have demonstrated not only that the Aiptasia model system produces a diverse array of BVOCs, but that these volatilomes vary with symbiotic state, providing insight into the ways in which metabolism changes in response to symbiotic state. Relative to the intact symbiosis, the volatilome of the cultured symbiont *B. minutum* was more distinct than that of the anemone host in isolation. While this may be indicative of a more dramatic adjustment of algal physiology *versus* host physiology in response to symbiosis, it is important to resolve the possible conflating role of different nutrient and bacterial loads in algal cultures relative to Aiptasia stocks. Ultimately, however, the extension of volatilomics to this model system will facilitate more powerful studies of the cnidarian–dinoflagellate mutualism using a non-invasive technique and provide a platform for studying the role of BVOCs in reef-building corals.

Chapter 3 Symbiont identity impacts the microbiome and volatilome of the Aiptasia holobiont

3.1 Abstract

The establishment and maintenance of a symbiosis between cnidarians and dinoflagellates underpins the success of reef building corals in otherwise nutrient-poor habitats. This study characterised the suite of biogenic volatile organic compounds (BVOCs) that comprise the volatilome of the sea anemone *Exaiptasia diaphana* ('Aiptasia'), when aposymbiotic and in symbiosis with either its native dinoflagellate symbiont *Breviolum minutum* or the non-native *Durusdinium trenchii*. The microbiome in these different symbiotic states was characterised to more fully define the experimental holobiont: 147 unique amplicon sequence variants (ASVs) were observed across symbiotic states. The microbiomes were distinct among groups: bacteria in the family Vibrionaceae were most abundant in aposymbiotic anemones, bacteria in the family Crocinitomicaceae were most abundant in anemones symbiotic with *D. trenchii*, and anemones symbiotic with *B. minutum* had the highest proportion of low abundance ASVs. Overall, 142 BVOCs were detected and classified into 17 groups based on their chemical structure. Aposymbiotic anemones produced a volatilome of 107 BVOCs, *D. trenchii*-populated anemones 97 BVOCs, and *B. minutum*-populated anemones 70 BVOCs. A total of six BVOCs were differentially abundant between aposymbiotic anemones and anemones symbiotic with *B. minutum*; three between aposymbiotic anemones and anemones symbiotic with *D. trenchii* and one between anemones symbiotic with *B. minutum vs.* those with *D. trenchii*. Isoprene was detected in higher abundance when anemones hosted their native symbiont, and dimethyl sulphide was detected in higher abundance in the volatilome of both Aiptasia-Symbiodiniaceae combinations relative to aposymbiotic anemones. The volatilomes of aposymbiotic anemones and anemones symbiotic with *B. minutum* were distinct, while the volatilome of anemones symbiotic with *D. trenchii* overlapped both. Collectively, the distinct microbial communities and Volatilome that is indistinct between aposymbiosis and symbiosis with a non-native symbiont is consistent with the idea that *D. trenchii* produces a metabolically sub-optimal symbiosis with Aiptasia. This may potentially reflect how symbiotic cnidarians, including corals, may respond to climate change should they acquire novel dinoflagellate partners.

3.2 Introduction

Coral reefs are highly diverse marine ecosystems and provide a habitat and food supply for a wealth of marine life (Bouchet, 2006). At the core of these diverse ecosystems is the symbiotic relationship between dinoflagellate algae (family: Symbiodiniaceae) and their cnidarian hosts (Davy et al., 2012; Rosset et al., 2021). The dinoflagellate symbionts are housed intracellularly within cnidarian gastrodermal cells, where photosynthetic products are translocated from the symbiont to the cnidarian host, thereby sustaining host metabolism, growth, reproduction and ultimately survival in a resource poor environment (Roth, 2014). In return, the dinoflagellate is supplied with inorganic nutrients such as nitrogen and phosphorus (Davy et al., 2012), and a stable habitat in which to photosynthesise in the absence of predators. The nature of this symbiosis has been intensively researched for decades, not only because of its importance for maintaining the foundation of reef ecosystems, but also because of the vulnerability of the symbiosis to anthropogenic stressors (Suggett & Smith, 2020; Hughes et al., 2021), particularly increased ocean temperatures as a consequence of climate change (Glynn, 1993; Hoegh-Guldberg et al., 2017).

Comprised of taxonomically distinct genera and species (LaJeunesse et al., 2018, 2022; Nitschke et al., 2020), the Symbiodiniaceae family is also physiologically diverse, for example with respect to photo- and nutritional physiology, cellular growth and stress tolerance (Suggett et al., 2015; Nitschke et al., 2022). While many cnidarian hosts exhibit specificity for the species of Symbiodiniaceae with which they naturally associate (Thornhill et al., 2013), others can associate with a variety of algal symbionts (Baker & Romanski, 2007). The species of Symbiodiniaceae with which a cnidarian naturally associates is called the native, or homologous symbiont. Following bleaching, hosts can acquire novel symbiont taxa (Boulotte et al., 2016), or more commonly be repopulated by species that were previously less abundant within their tissues (Cunning et al., 2015); these changes in the symbiont population are termed “switching” and “shuffling”, respectively. While repopulation or reorganisation with thermally tolerant symbionts can help corals withstand changing environmental conditions, it can also result in an association with altered, suboptimal nutritional exchange and differences in the activities of specific metabolic pathways (Matthews et al., 2017, 2018). Such changes in the metabolic characteristics of cnidarian associations with non-native (heterologous) symbionts, relative to associations with homologous symbionts, have been documented through differences in the transcriptome (Matthews et al., 2017), proteome (Medrano et al., 2019; Sproles et al., 2019) and metabolome (Matthews et al., 2017; Tsang Min Ching et al., 2022).

In addition to algal symbionts, cnidarians also associate with an array of other microorganisms, including bacteria, fungi, viruses, and archaea (Bosch & Mcfall-Ngai, 2011; van Oppen & Blackall, 2019). It is now recognised that the association with these microorganisms is crucial to the optimal functioning of coral reefs; collectively, along with algal associates, these microorganisms all contribute to a metabolically intertwined metaorganism, referred to as a 'holobiont'. This aggregation of prokaryotes and eukaryotes has a dynamic relationship, and the community composition will reflect the current environmental conditions (Osman et al., 2020). The importance of bacteria in the coral holobiont led to the proposal of the 'coral probiotic hypothesis' (Reshef et al., 2006), postulating that the microbiome composition is optimised to support coral biology given current environmental conditions; consequently, the cnidarian-associated microbial community is an outcome of the ability of the microbes to compete within the microbiome and to sustain the host under prevailing environmental conditions (Suggett et al., 2017). Indeed, it has been shown that cnidarian-associated bacteria play an important role in holobiont health, contributing to pathogen defence, metabolism and nutrient cycling (Raina et al., 2009; Krediet et al., 2013; Pogoreutz et al., 2022). An extension of the coral probiotic hypothesis, the 'microbiome flexibility hypothesis', posits that corals can adapt to a changing environment by altering their microbiome (Voolstra & Ziegler, 2020). Indeed, it has been shown that the microbiome in the model cnidarian Aiptasia shifts in response to symbiotic state (i.e., symbiotic *versus* aposymbiotic; Röthig et al., 2016). Due to their rapid generation time, bacterial populations can quickly shift, selecting for communities beneficial to the host (Voolstra & Ziegler, 2020; Voolstra et al., 2021). For example, microbiome flexibility is thought to play a role in the tolerance of the coral *Fungia granulosa* to high salinity levels (Rothig et al., 2016) and the coral *Acropora hyacinthus* to thermal stress (Ziegler et al., 2017). Microbiome composition is thought to be so important that microbiome transplants among cnidarians may aid a holobiont's ability to adapt to changing environmental conditions (Costa et al., 2021).

Biogenic volatile organic compounds (BVOCs) are low molecular weight (<200 Da) chemicals with high vapour pressure (Mansurova et al., 2018) that are produced by a broad diversity of organisms. BVOCs have been detected from bacteria (Tahir et al., 2017), fungi (Werner et al., 2016), algae (Lawson et al., 2019), plants (Jürgens et al., 2013), insects (Wyatt, 2003), corals (Lawson et al., 2021), sea anemones (Chapter 2), and mammals (Gallagher et al., 2008). At a global scale, the emission of organically produced VOCs exceeds that of VOCs from anthropogenic sources (Guenther et al., 2000). BVOCs can be metabolic by-products (Tyc et al., 2017), end-products (e.g., serving as pollination attractants (Raguso, 2008)) or precursors to other molecules (e.g., plant hormones; Dani & Loreto, 2022). While the majority of BVOC research has focused on compounds produced by terrestrial ecosystems (Monson, 2002), aquatic ecosystems also produce a diversity of BVOCs, collectively characterised as 'volatilomes' (Steinke et al., 2018). The composition and abundance of

BVOC production has been shown to vary among environmental conditions and across different species of Symbiodiniaceae (Lawson et al., 2019) and corals (Lawson et al., 2021), but the physiological roles of many of these compounds remain unknown. I hypothesise that, as with previously described classes of organic compounds, BVOCs will show altered abundance in symbiosis with heterologous partners.

The symbiotic sea anemone *Exaiptasia diaphana* ('Aiptasia') is a model system for studying the cnidarian-dinoflagellate symbiosis (Baumgarten et al., 2015; Rädecker et al., 2018). Like corals, Aiptasia forms a stable symbiosis with dinoflagellates in the family Symbiodiniaceae (Wolfowicz et al., 2016). Aiptasia can be rendered aposymbiotic by laboratory-induced bleaching, maintained in this state for years through heterotrophic feeding, and repopulated by a variety of symbiont species (Matthews et al., 2016; Gabay et al., 2018). Furthermore, Aiptasia can reproduce asexually, allowing the maintenance of clonal populations (Lehnert et al., 2012). While previous work has shown how the microbiome and volatilome change in Aiptasia in response to symbiosis with a homologous symbiont (Röthig et al., 2016; Chapter 2), and in response to thermal stress (Hartman et al., 2020b), there is a gap in the research regarding the impact of symbiont identity on the microbiome and BVOC production. This work sought to describe the ways in which the microbiome, and composition of emitted volatile gases change in response to a switch in the dinoflagellate endosymbiont community of Aiptasia.

I profiled Aiptasia associated BVOCs in three different symbiotic states: 1) in symbiosis with the native partner *Breviolum minutum* (homologous symbiont); 2) in symbiosis with a non-native but thermally tolerant partner, *Durusdinium trenchii* (heterologous symbiont); and 3) in the absence of symbionts (aposymbiosis). Microbial communities present in the holobiont of these three states were also assessed to further elucidate how altering the predominant algal symbiont influences holobiont composition, and to provide new insight into the production of BVOCs and patterns observed. This study adds to our understanding of the ways in which holobionts may respond to changing environmental conditions and provides a platform for the future elucidation of the roles of BVOCs in symbiotic cnidarians.

3.3 Methods

3.3.1 Experimental Organisms

A long-term (15+ years) clonal culture of the sea anemone Aiptasia (culture ID: NZ1) of unknown Pacific origin (Matthews et al., 2017) was maintained in the laboratory in 0.22 µm filtered seawater (FSW) at 25 °C and approximately 70 µmol photons m^{-2} s^{-1} on a 12 : 12 h light dark cycle. Clonal anemones (n = 100) were rendered aposymbiotic (i.e., symbiont–free) using menthol-induced bleaching; exposure to menthol (20% w/v in ethanol; Sigma-Aldrich, Auckland, NZ) at a final concentration of 0.19 mmol L^{-1} in 0.22 µm FSW (Matthews et al., 2016). Anemones were incubated in menthol for 8 h during the 12-h light period, after which photosynthesis was inhibited by replacing menthol/FSW with FSW containing 5 µmol L^{-1} 3-(3,4–dichlorophenyl)-1,1-dimethylurea (DCMU; 100 mmol L^{-1} dissolved in EtOH, Sigma-Aldrich) for 16 h to prevent repopulation by inhibiting photosynthesis of the remaining symbionts. After repeating this 24-h cycle for four consecutive days, anemones were maintained in 0.22 µm FSW for three days. Anemones were fed once weekly with *Artemia* sp. nauplii, with fresh FSW changes 8 h post-feeding. This protocol was continued for six weeks, and aposymbiotic anemones were maintained in the dark at 25 °C for 1.5 years prior to BVOC sampling.

Aiptasia can associate with various species of Symbiodiniaceae, both homologous and heterologous. *Breviolum minutum* (ITS2 type B1, culture ID 'FLAp2') was used as the homologous symbiont and *Durusdinium trenchii* (ITS2 D1a, culture ID 'Ap2') the heterologous symbiont. *B. minutum* and *D. trenchii* isolates were grown in 0.22 µm FSW enriched with f/2-medium maintained at 25 °C in a climate-controlled incubator. Symbiodiniaceae cultures were grown under light provided by fluorescent lamps (Osram Dulux 36/W890) at approximately 70 µmol photons m^{-2} s^{-1} on a 12 : 12 h light : dark cycle. One week prior to inoculation, cultures were diluted with fresh f/2 medium to ensure that they were in exponential growth.

Symbiotic anemones were generated by recombining aposymbiotic anemones with cultured Symbiodiniaceae isolates. Prior to this inoculation, aposymbiosis was confirmed using fluorescence microscopy (Olympus IX53 inverted microscope; 100× magnification). A subset of aposymbiotic anemones were starved for seven days prior to inoculation with either cultured *B. minutum* or *D. trenchii* (n = 25 for both species). An aliquot (~20 µL) of Symbiodiniaceae culture, concentrated by centrifugation to a density of 3×10^6 cells mL^{-1}, was pipetted directly onto the oral disc of individual aposymbiotic anemones. *Artemia* sp. nauplii were mixed into this suspension to encourage phagocytosis of algal cells (Davy et al., 1997). Inoculated anemones were fed twice weekly with

Artemia sp. nauplii and maintained at 25 °C and approximately 70 µmol photons m^{-2} s^{-1} (GE Lighting T5 F54W/840) on a 12:12 h light : dark cycle. Anemones were fully symbiotic for six months prior to BVOC sampling. The presence of intracellular symbionts was confirmed by fluorescence microscopy three months prior to sampling, as described above. Symbiosis was maintained and inspected visually weekly until sampling, after which symbiont cell densities were determined as described below.

Symbiotic anemones were maintained at a constant temperature of 25 °C and light intensity of 70 µmol photons m^{-2} s^{-2} on a 12:12 h light : dark cycle as described above. Aposymbiotic anemones were maintained at a constant temperature of 25 °C and kept in the dark. All anemones were fed twice weekly, with water changes the day after feeding, using 0.22 µm FSW. All animals were retained in these states for six months prior to BVOC sampling. On the day of sampling, the maximum quantum yield of photosystem II (F_v/F_m, dimensionless) was used as a relative indicator of photosynthetic competency in symbiotic anemones (Fig. S1a). Symbiotic anemones were dark-acclimated for 15 min before performing measurements with an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3 and damping = 3).

3.3.2 Microbe sampling and microbiome analysis

Frozen Aiptasia samples from which BVOCs were analysed (see below) were thawed, sterile artificial seawater (ASW) was added to produce 1 mL samples, and pooled anemones were mechanically homogenised for 30 s on ice. I performed DNA extraction, and subsequently analysed 16S rRNA diversity from 15 samples across three symbiotic states: aposymbiotic anemones (n = 5; ~15 anemones/vial), anemones symbiotic with their homologous symbiont, *B. minutum* (n = 5; ~10 anemones/vial), and anemones symbiotic with a heterologous symbiont, *D. trenchii* (n = 5; ~12 anemones/vial).

DNA extraction from 300 µL of anemone homogenate was performed using the DNeasy Plant Mini Kit (Quiagen), according to the manufacturer's instructions. DNA concentrations were quantified on a NanoDrop spectrophotometer (Impen NanoPhotometer™ NP80, Thermo Fisher). Variable regions 5 and 6 of the 16s rRNA gene were targeted using the primer pair 784F [5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG–AGGATTAGATACCCTGGTA 3'] and 1061 R [5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG–CRRCACGAGCTGACGAC 3′] with Illumina adaptor overhangs (underlined above). This primer pair has been used in previous cnidarian microbiome studies (Rothig et al., 2016; Voolstra & Ziegler, 2020). Extracted DNA was

sent to the University of Auckland Genomics Facility for analysis (Auckland, NZ). gDNA was normalised to 50 ng μL^{-1} and 200 ng used as an input for 25 µL PCR reaction. The PCR mix included 12.5 µL of 2x Platinum SuperFi Master Mix. The concentration of each primer in the reaction was 0.2 µM. The first round of PCR thermal cycling conditions was 3 min initial denaturation at 95 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final elongation step at 72 °C for 5 min. After the first round PCR, 1 µL of each sample was checked for quality on a BioAnalyzer (2100-Agilent, USA) with the HS DNA chip. A 0.8× volume of AMPure XP beads was used to clean up the reactions (20 µL PCR product, 16 µL bead suspension), with two ethanol washes and elution in 12 µL water. A total of 1.5 µL of the cleaned first round PCR product were used as input to the second-round indexing PCR, which used Nextera V2 indexing primers and the Platinum SuperFi MasterMix. Thermal cycling conditions were 3 min at 95 °C, followed by 8 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. After this round of amplification, dsDNA was quantified using a Qubit dsDNA HS (high sensitivity) Assay Kit. A total of 10 µL of each sample were pooled and cleaned up with two rounds of AMPure beads using a 1:1 ratio of beads to sample, with 2 × 80% ethanol washes each round before loading onto the Illumina MiSeq platform for sequencing.

3.3.3 BVOC sampling and volatilome characterisation

Volatile gases were collected and analysed from anemones in three different symbiotic states: aposymbiotic anemones (n = 8; ~15 anemones *per* vial); anemones symbiotic with *B. minutum* (n = 8; ~10 anemones *per* vial); and anemones symbiotic with *D. trenchii* (n = 8; ~12 anemones *per* vial). Different numbers of anemones were used across treatments to account for variability in anemone size across symbiotic states. The experimental setup and BVOC retrieval were performed as in Chapter 2, using previously established methods modified from Lawson et al. (2019, 2021). The night before BVOC sampling, experimental organisms were transferred into sterile 150 mL glass vials (Wheaton, Millville, NJ, USA) containing 75 mL 0.22 µm FSW, where they were retained under conditions identical to those used for growth. Immediately prior to sampling, FSW was refreshed, and vials sealed using 20 mm PTFE/Si crimp caps (Agilent, USA). BVOCs were collected by passing instrument grade air (100 mL min^{-1}; BOC Gases, Wellington, NZ) into gas-tight sampling vials for 20 min, whereby the outgoing air was passed through open-ended thermal desorption tubes (TDTs; Markes International Ltd, Llantrisant, UK) containing the sorbent Tenax TA, onto which BVOCs adhered. After 20 min, TDTs were immediately sealed with brass storage caps and stored at 4 °C until processing. After BVOC retrieval, anemones were immediately frozen at -80 °C and stored at this temperature until DNA extraction, symbiont cell density determination and protein quantification. All TDTs were analysed within two weeks of sampling to minimise sample degradation using gas

chromatography coupled with single quadrupole mass spectrometry (GC×MS) as *per* Olander *et al*. (2021). TDT desorption was performed using a Marks Unity 2 Series Thermal Desorber and ULTRA multi-tube autosampler. Desorption occurred at 300 °C for 6 min, after which BVOCs were concentrated on a cold trap at -30 °C. The cold trap was subsequently flash-heated to 300 °C, injecting the concentrated sample onto a 7890A GC (Agilent Technologies, Ltd., Melbourne) *via* a transfer line maintained at 150 °C. The GC was equipped with a 30 m × 0.25 mm Rxi-624Sil MS column (Restek Corp.) with a film thickness of 1.40 µm. To encourage complete desorption of BVOCs, the GC oven was heated for 35 °C for 5 min, then increased to 240 °C by increasing 5 °C min^{-1}, and held at 240 °C for 5 min. All samples were run spitless at a flowrate of 1 mL min^{-1}. The GC was attached to a Model 5974 mass-selective detector (Agilent Technologies, Ltd., Melbourne), with the scanning range set to 35-300 amu.

Spectral data from the GC-MS were run through the open-source MS data processing program OpenChrom (Wenig & Odermatt, 2010) to remove common contaminating ions (amu: 73, 84, 147, 149, 207, 221). Output files were then imported to Galaxy (Guitton et al., 2017), and processed using the metaMS.runGC package (Galaxy version 2.1.1; Wehrens et al., 2014; Giacomoni et al., 2015; Afgan et al., 2018) on Workflow4Metabolomics. Peaks were tentatively identified (hereafter, 'identified') against a database in the National Institute of Standards and Technology (NIST) Mass Spectral library (NIST 14 library in NIST MS Search v2.2; NIST, Gaithersburg, MD). A compound match factor of at least 60% was required for compound identity to be recorded; otherwise, the compound was listed as 'unknown'.

Prior to desorption, each TDT was injected with 0.2 µL of 150 ppm bromobenzene (GC grade, Sigma Aldrich, Castle Hill, NSW, Australia) in methanol (HPLC grade, Sigma Aldrich) as an internal standard. BVOCs were also collected from filtered (0.22 µm) seawater blanks (n = 8) using the same methods, at the time of sample collection. Average values for BVOCs present in the blanks were subtracted from all samples. Finally, peak abundance was normalised to the protein content of each replicate. Protein was released from the samples by lysis using ultrasonication (VCX500; Sonics & Materials Inc., Newtown, CT, USA) and the protein content measured using the fluorometric Qubit Protein Assay Kit (Thermo Fisher Scientific; Vergauwen et al., 2017). Compounds identified as likely methodological artifacts, e.g. silicon-containing compounds, were also removed from the dataset as they were suspected contaminants from dimethylpolysiloxane hydrolysis (Cella & Carpenter, 1994). Only BVOCs present in a minimum of four biological replicates in at least one symbiotic state were classed as 'present' in a state. All BVOCs were grouped according to their chemical class (Fig. 3.3c).

3.3.4 Symbiont cell density and protein determination

Anemones (n = 8, with 10-15 pooled anemones *per* replicate), frozen as described above, were thawed on ice and homogenised using a saw-tooth homogeniser in 500 µL double distilled water (EASYpure II RF/UV ultrapure water system). Host and symbiont fractions were separated by centrifugation at 400 × g for 2 min after which the symbiont fraction was washed of residual host material by one further resuspension and centrifugation with double distilled water. The supernatant fraction was analysed for protein content using the fluorometric Qubit Protein Assay Kit. Cell counts were performed using a haemocytometer (Improved Neubauer) with eight replicate counts *per* sample and normalised to protein content. Cell density (symbionts *per* mg host protein) was then calculated (Fig. S1b).

3.3.5 Data Analysis

For microbial analysis, the MiSeq output fastq file was de-multiplexed in R (R Core Team, 2021) using the insect package (v 1.4.0; Wilkinson et al., 2018) and trimmed sequences were filtered to produce a table of exact amplicon sequence variants (ASVs) using the DADA2 R package (Callahan et al., 2016). ASVs were identified to the lowest possible taxonomic rank using a two-step classification process. This involved: 1) exact matching against the RDP v18 reference database (accessed 15 June 2022 from https://doi.org/10.5281/zenodo.4310150) and assigned taxonomy at the lowest common ancestor level (LCA; i.e. assigning to family level if sequence matches with 100% identity to more than one genus); and 2) querying any unwanted sequences against the same RDP reference database using the SINTAX classification algorithm (Edgar, 2016) with a conservative assignment threshold of > 0.99. In both cases, maximum assignment resolution was set to one genus level due to the high over-classification rates associated with species-level assignment (Edgar, 2016).

Differential abundance of BVOCs was estimated using the *limma* R package (Ritchie et al., 2015); the *voom* function was used to convert counts to $\log_2$-counts-*per*-million and to assign weights to each observation based on the mean-variance trend. The counts assigned to each observation roughly corresponds to abundance of the BVOC, which can then be used to compare BVOC production between treatments. Functions *lmFit*, *eBayes*, and *topTable* were used to fit weighted linear regression models, and calculate empirical Bayes moderated t-statistic and q-values (Phipson et al., 2016). Box plots, bar graphs and pie charts were created with *ggplot2* (Wickham, 2016) in RStudio. Pairs of biological replicates, standardised using the *decostand* function in the *vegan* package (Oksanen et al., 2020) in R (version 1.2.5033), were compared using the Bray-Curtis similarity measure, and this output was subjected to non-metric multidimensional scaling to visualise differences among groups

based on BVOCs. To compare dispersion of biological replicates using a distance measure, PERMANOVA was performed to test if the distance between biological replicates was greater between treatments than within treatments. PERMANOVA was performed on NMDS scores using the *adonis* function, and *post hoc* tests were performed using the *pairwise.adonis* function in the vegan package in R (version 1.2.5033) to distinguish differences among treatments. Differential abundance of microbial taxa was measured using the relative abundance of each microbial taxon within a sample and estimated using the *limma* R package as described above.

3.4 Results

3.4.1 Bacterial Community of Aiptasia in different symbiotic states

Bacterial taxa identified by 16S diversity data were grouped at the family level, revealing notable differences among all three symbiotic states (Fig. 3.1). Aposymbiotic anemones were dominated by Vibrionaceae (average 22% of the microbiome) and Campylobacteraceae (16%). In contrast, anemones symbiotic with *D. trenchii* were dominated by Crocinitomicaceae (24%) and the class Gammaproteobacteria (mean 17%), whereas anemones symbiotic with *B. minutum* were dominated by the class Gammaproteobacteria (20%) and by 'other' bacteria (unclassified or rare taxa; 28%).

Ten microbial ASVs were identified in all samples across the dataset, designated as the 'core microbiome' (i.e., ASVs present in all biological replicates). These taxa included: *Alteromonas* sp.; Crocinitomicaceae; three taxa within the Gammaproteobacteria; *Maricaulis maris*; *Owenweeksia* sp.; *Pseudoalteromonas arabiensis*; *Rhizobium subbaraonis*; and Rhodobacteraceae (Table S4).

3.4.2 Contrasting microbiota between symbiotic states

A total of 57 bacterial taxa were differentially abundant between the three symbiotic states (Table S2).

(i) Aposymbiotic anemones *versus* anemones harbouring homologous symbionts

A total of 41 differentially abundant bacterial taxa were recorded between aposymbiotic anemones and anemones symbiotic with *B. minutum*. Of these, 16 were more abundant in anemones symbiotic with *B. minutum*, including 1 taxon of the genus *Labrenzia*, 2 taxa of the genus *Chlamydia*, and 2 taxa of the family Alteromonadaceae. A total of 25 ASVs were more abundant in aposymbiotic anemones, including 8 taxa of the family Vibrionaceae, and 2 species of the family Rhodobacteraceae (*Tepidibacter mesophilus* and *Polaribacter huanghezhanensis*).

(ii) Aposymbiotic anemones *versus* anemones harbouring heterologous symbionts

A total of 38 differentially abundant bacterial taxa were recorded between aposymbiotic anemones and anemones symbiotic with *D. trenchii*. Of these, 11 were more abundant in anemones symbiotic with *D. trenchii*, including *Erythrobacter gaetbuli*, 2 taxa of the genus *Limimaricola,* and 1 taxon from each of the orders Cytophagales and Rhizobiales. A total of 27 ASVs were more abundant in aposymbiotic anemones, including 8 taxa of the family Vibrionaceae, 3 taxa of the phylum Bacteroides and 1 taxon of the family Campylobacteraceae.

(iii) Anemones harbouring homologous symbionts *versus* heterologous symbionts

A total of 14 differentially abundant bacterial taxa were recorded between anemones symbiotic with *B. minutum versus D. trenchii*. Of these, 3 taxa were more abundant in anemones symbiotic with *D. trenchii*, including 2 taxa of the family Rhodobacteraceae and 1 taxon of the family Vibrionaceae. In contrast, 11 were more abundant in anemones symbiotic with *B. minutum*, including 2 taxa of the family Alteromonadaceae, 2 taxa of the genus *Chlamydia* and 1 taxon of the genus *Labrenzia*.

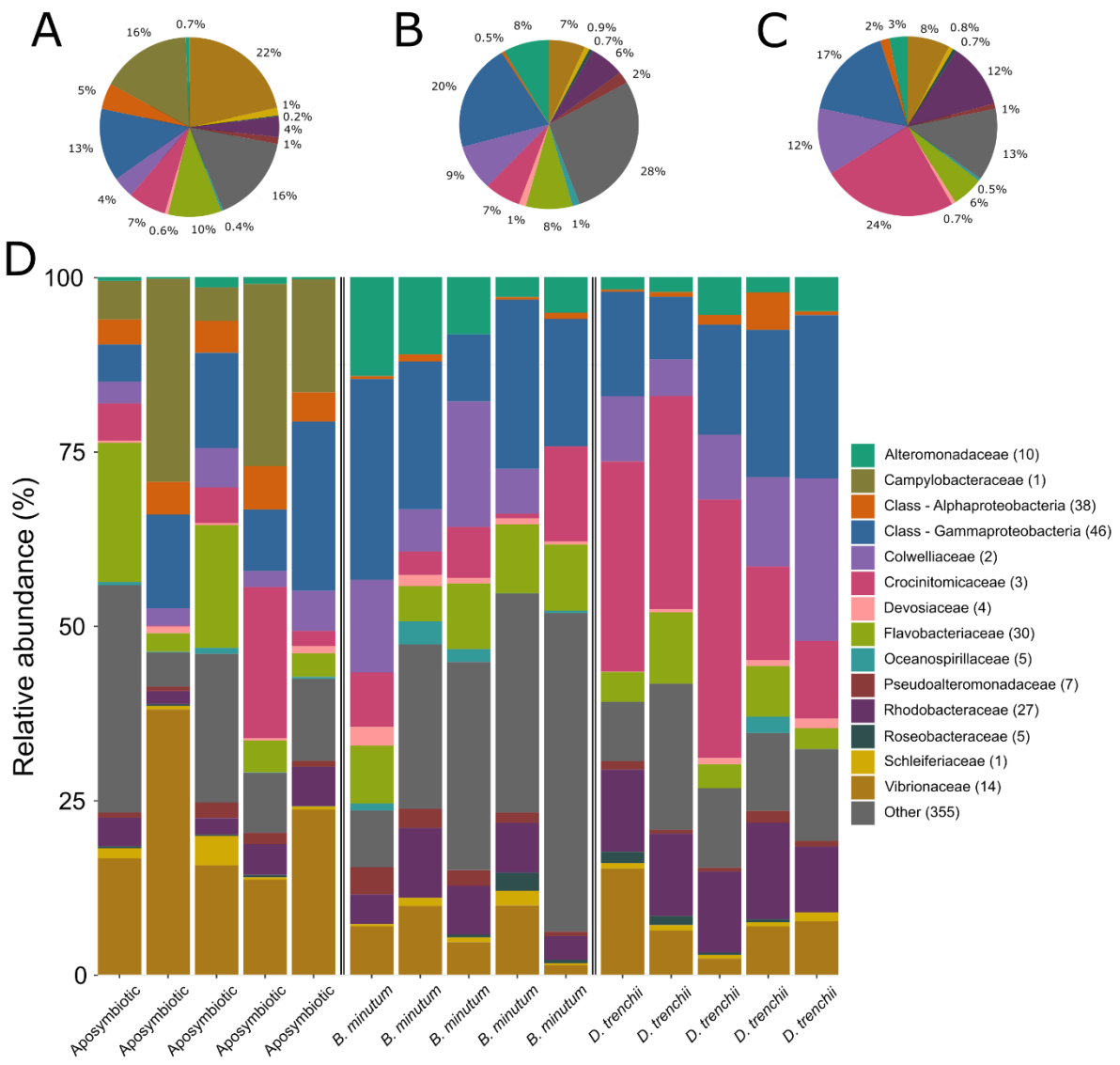

Figure 3.1: Bacterial community composition grouped by family. Each colour represents one of the most abundant 14 families identified across the dataset. Sequences unclassified on the family level were denoted at the class level. Less abundant families were designated as 'other'. Numbers in parenthesis denote the number of unique taxa within that family. Mean abundances across biological replicates shown in: A) aposymbiotic anemones; B) anemones symbiotic with homologous symbiont *B. minutum*; C) anemones symbiotic with heterologous symbiont *Durusdinium trenchii*; and D) relative abundances of bacterial taxa in each biological replicate.

Non-metric multidimensional scaling further demonstrated a clear distinction between the microbial communities associated with each of the symbiotic states (Fig. 3.2). PERMANOVA ($F_{2,12} = 3.704$, $p < 0.01$) *post hoc* analysis indicated that all three symbiotic states were distinct from each other; aposymbiotic anemones harboured microbiota distinct from hosts symbiotic with *D. trenchii* ($p < 0.05$) and those symbiotic with *B. minutum* ($p < 0.05$), while anemones symbiotic with *B. minutum* were distinct from those symbiotic with *D. trenchii* ($p < 0.05$).

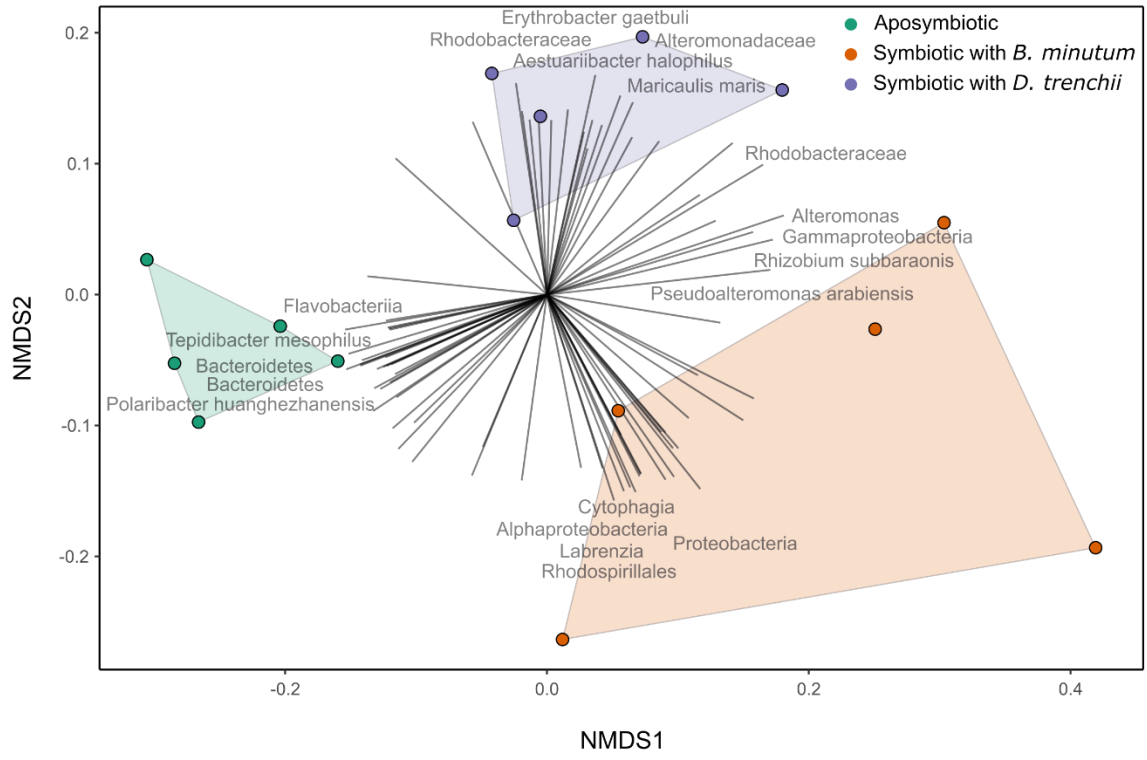

Figure 3.2: Non–metric multidimensional scaling (stress = 0.088) plot of bacterial amplicon sequence variants (ASVs) in three different symbiotic states in the Aiptasia model system. Displayed taxa were chosen based on the top 5 and bottom 5 loading scores for each NMDS dimension. PERMANOVA ($p < 0.01$); *post hoc* results indicated that all symbiotic states were distinct from each other.

3.4.3 BVOC emissions are affected by symbiont identity

Changes to volatilomes among symbiotic states were visualised using non-metric multidimensional scaling, with a clear distinction between aposymbiotic anemones and anemones symbiotic with *B. minutum*, whereas anemones symbiotic with *D. trenchii* overlapped with the two other groups (Fig. 3.3a). PERMANOVA ($F_{2,21} = 1.9501$, $p < 0.01$) was performed and *post hoc* results indicated that aposymbiotic anemones and anemones symbiotic with *B. minutum* were distinct ($p < 0.01$), while anemones symbiotic with *D. trenchii* produced volatilomes that were not significantly different from either aposymbiotic anemones ($p = 1$) nor anemones symbiotic with *B. minutum* ($p = 0.123$). A total of 142 BVOCs were detected across the three symbiotic states (Fig. 3.3b). Of these, 29 were produced solely by aposymbiotic anemones, 13 by anemones in symbiosis with *B. minutum* and 13 by anemones in symbiosis with *D. trenchii*. A substantial number of BVOCs (45) were present in all samples (and hence irrespective of anemone state) and therefore designated as 'core compounds'. Of these core compounds, 35 were identified, with the most abundant being aromatic compounds, though BVOCs containing multiple different elements or functional groups (classified as diverse functional groups, DFGs) were also identified. The only halogenated hydrocarbon core compound identified was

dibromomethane. Interestingly, the volatilome of aposymbiotic anemones was the most diverse, with 107 BVOCs, whereas the volatilome of anemones symbiotic with *B. minutum* was the least diverse, with only 70 BVOCs. Anemones symbiotic with *D. trenchii* produced a volatilome comprised of 97 BVOCs. The most abundant chemical class within the various volatilomes was DFG for aposymbiotic anemones (22) and *B. minutum*-populated anemones (17), and unclassified compounds (24) for *D. trenchii*-populated anemones (Fig. 3.3c).

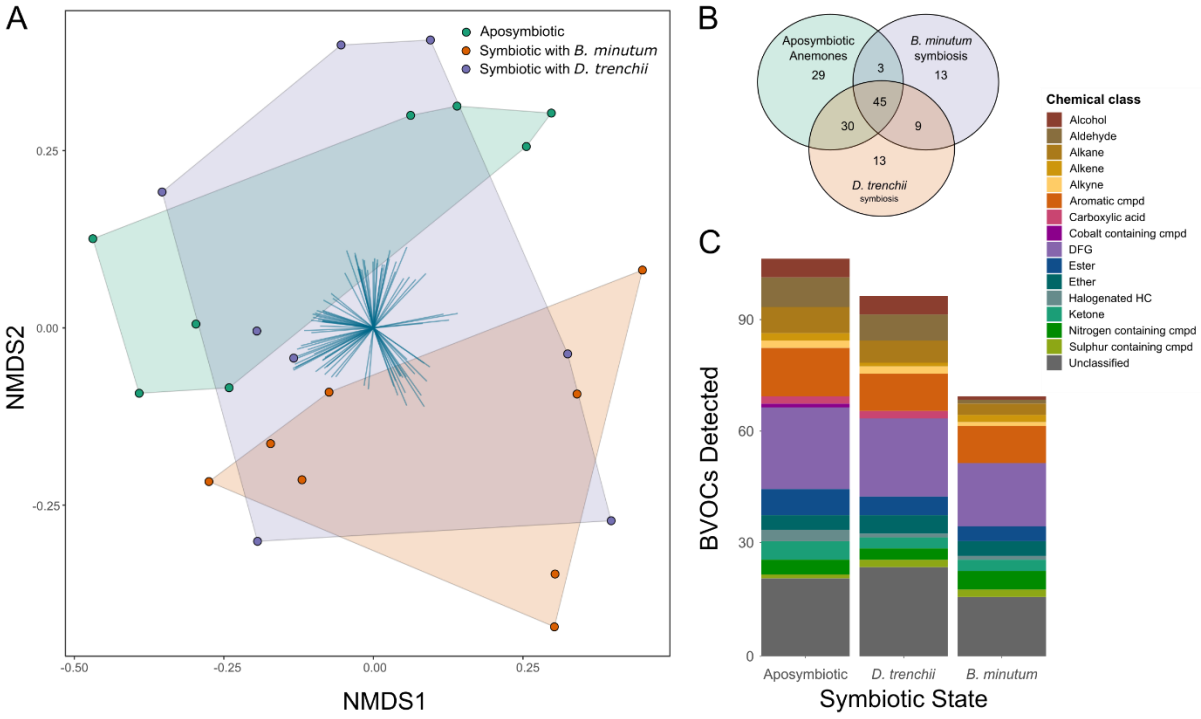

Figure 3.3: BVOC diversity among three symbiotic states in Aiptasia: aposymbiotic anemones, anemones symbiotic with the homologous symbiont (*Breviolum minutum*) and anemones symbiotic with a heterologous symbiont (*Durusdinium trenchii*). A) Non–metric multidimensional scaling (NMDS) ordination plot of BVOCs detected across symbiotic states; B) Venn diagram showing presence of 142 BVOCs detected across dataset and C) BVOCs grouped by chemical class. BVOCs had to be present in at least four of eight biological replicates within one symbiotic state to be included. DFG = diverse functional group; halogenated HC = halogenated hydrocarbon; cmpd = compound.

3.4.3 Contrasting volatilomes between symbiotic states

A total of 8 BVOCs were differentially abundant between the three symbiotic states, as determined by pairwise differential abundance testing (Table S1, Fig. 3.4). Only 6 differentially abundant BVOCs were recorded between aposymbiotic anemones and Aiptasia symbiotic with *B. minutum*. Of these, isoprene and dimethyl sulphide were detected in higher quantities in the volatilome of the *B. minutum*-populated anemone ($p < 0.001$ and < 0.05, respectively), while octanal, dodecanal, nonanal and cis-6-nonenol were detected in higher quantities in aposymbiotic anemones ($p < 0.05$ for all comparisons). A further 3 differentially abundant BVOCs were identified between aposymbiotic anemones and anemones containing *D. trenchii*. Dimethyl sulphide and 2-methoxy-thiazole were detected in higher quantities in anemones symbiotic with *D. trenchii* ($p < 0.001$ and < 0.05, respectively), while 1,1,2,2,3,3-hexamethylindane was detected in higher quantities in aposymbiotic anemones ($p < 0.01$). Only 1 differentially abundant BVOC was detected between anemones symbiotic with *B. minutum vs. D. trenchii*; specifically, isoprene was detected in higher quantities in holobionts associated with *B. minutum* ($p < 0.05$).

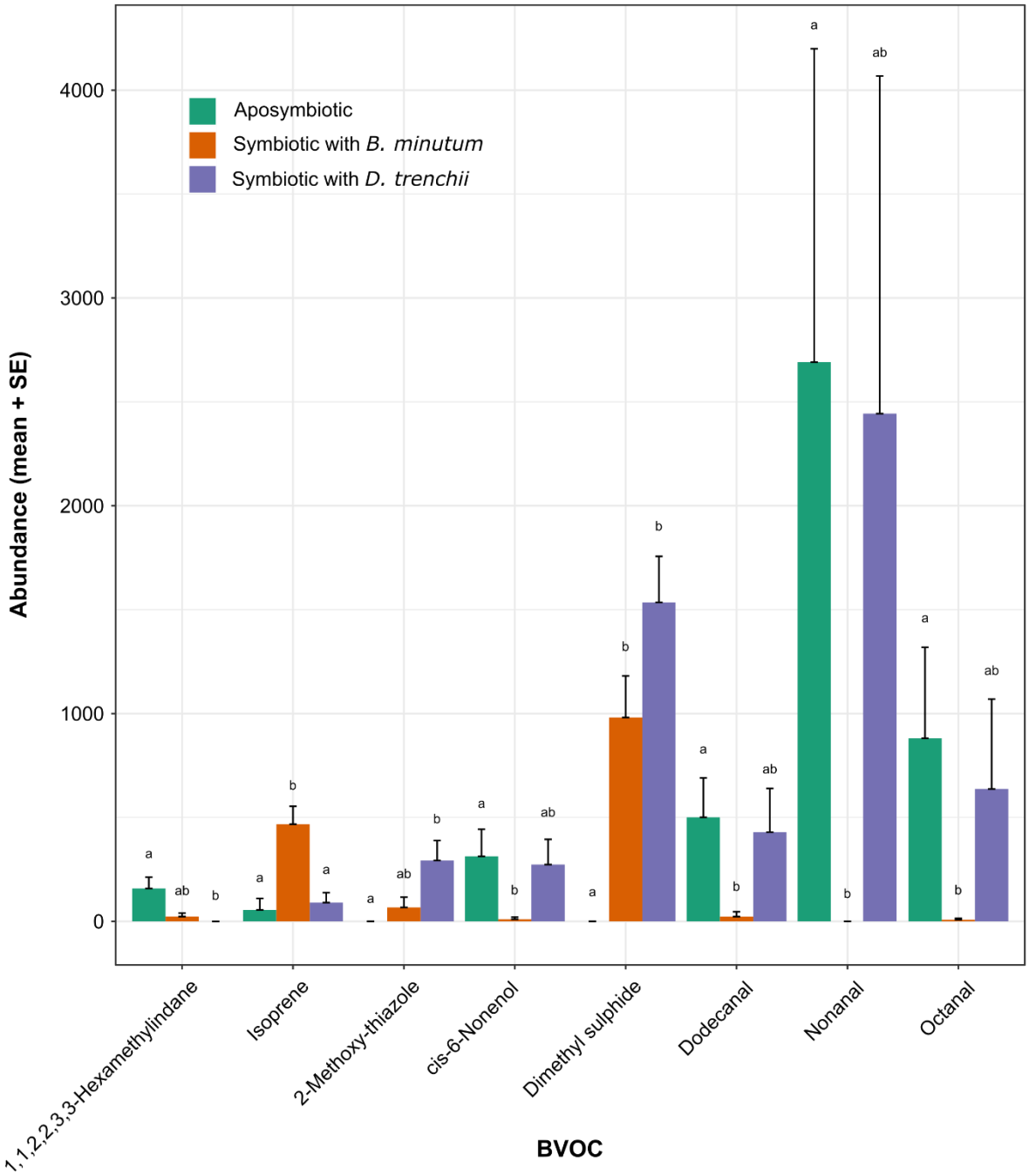

Figure 3.4: Differentially abundant BVOCs (+ standard error) produced by aposymbiotic anemones, anemones symbiotic with *Breviolum minutum* and anemones symbiotic with *Durusdinium trenchii*. Abundance data represent dimensionless areas under a peak normalised to chemical standard, seawater blanks and protein content. Differential abundance was performed to determine differences in abundance of BVOCs between treatments. Statistical significance is indicated by letters above error bars on the plot.

3.5 Discussion

Cnidarian metabolism changes in response to symbiosis with different symbiont species (Matthews et al., 2017), and BVOC analysis can provide a non-invasive technique with which to explore the underlying metabolic regulation and restructuring associated with this change (see Lawson et al., 2021). I examined microbial composition and BVOC output of the model cnidarian Aiptasia in three different symbiotic states: 1) aposymbiosis; 2) symbiosis with the homologous symbiont, *B. minutum*; and 3) symbiosis with a heterologous symbiont, *D. trenchii*. Previous studies with Aiptasia have shown that symbiosis with this same heterologous symbiont results in an intermediate, physiologically sub-optimal state with respect to gene expression (Matthews et al., 2017), proteomics (Sproles et al., 2019) and metabolite profile (Matthews et al., 2017), in comparison to the aposymbiotic state and symbiosis with *B. minutum*. My analysis of BVOC emission profiles was consistent with these earlier observations, again showing that the heterologous *D. trenchii* induces a Physiological response that is indistinct from either aposymbiosis or symbiosis with the homologous *B. minutum*. Here, I explore the shift in holobiont composition in response to symbiosis with different species of Symbiodiniaceae, potential sources and functions of released BVOCs, and the relative contributions of the different members of the holobiont, including the microbiome, to the patterns seen.

3.5.1 Symbiosis and symbiont type induce changes to the holobiont microbiome

Microbial communities shift in response to environmental change in a variety of systems, including humans (Ahn & Hayes, 2021), plants (Hacquard et al., 2022), corals (Pootakham et al., 2019), and anemones (Hartman et al., 2020a). Indeed, the Aiptasia microbiome, which has been defined in multiple studies (Dungan et al., 2020; Costa et al., 2021), has previously been shown to respond to both symbiotic state (Rothig et al., 2016) and thermal stress (Hartman et al., 2020b). Here, I demonstrated that the microbiome also shifts in response to symbiosis with a non-native, physiologically sub-optimal species of Symbiodiniaceae.

Across the three symbiotic states, a core microbiome (ASVs present in all biological replicates) was revealed that was comprised of ten bacterial taxa, including *Maricaulis maris* and *Rhizobium subbaraonis*. The ubiquity of these taxa may highlight their importance to the cnidarian host, regardless of symbiotic state. An analysis of human-associated microbes (The Human Microbiome Project) revealed few common bacterial species, and it was proposed that the 'core' microbiome be interpreted in terms of the functionality of metabolic pathways rather than the specific species present (Gevers et al., 2012). Although I observed a difference at the species level between mine and that of Röthig et al. (2016), the respective microbiomes may not be functionally different from each other.

Thus, these observed differences may have little consequence to holobiont function in the model systems between these two experimental groups. The only bacterial taxon common to the two studies was *Alteromonas* sp., with this dissimilarity perhaps being unsurprising given that both studies used laboratory Aiptasia cultures reared over many generations in an isolated environment. The repeated presence of *Alteromonas* sp. in the microbiome of Aiptasia is consistent with this bacterium playing an important role in the holobiont community and warrants further exploration.

My data also show that anemones symbiotic with the homologous symbiont associate with a more diverse microbiota relative to aposymbiotic anemones and those containing heterologous symbionts. This diversity could be indicative of a healthier, more balanced holobiont. Indeed, in humans, a diverse gut microbiome correlates with improved personal health, with links to a more nutritional diet (Rook et al., 2014; Deng et al., 2019). A link between the microbiome and holobiont health is also suggested by the greater abundance of bacteria belonging to the family Vibrionaceae in aposymbiotic anemones relative to anemones containing either symbiont type. *Vibrio* species have been identified as prominent coral pathogens and implicated in coral disease. For example, *V. coralliilyticus* is a known pathogen of the coral *Pocillopora damicornis*, in which infection causes cell lysis in infected corals at high temperatures (Ben-Haim & Rosenberg, 2002; Ben-Haim et al., 2003). Similarly, *V. shiloi* is thought to cause bleaching in the coral *Oculina patagonica* by secreting extracellular materials that inhibit photosynthesis (Ben-Haim et al., 1999), while *V. owensii* is particularly abundant in corals suffering from *Acropora* white syndrome (Wilson et al., 2012). The reasons for a microbiome shift in aposymbiotic anemones towards reduced diversity and putatively more pathogenic bacteria is unknown but could relate to a poor nutritional state due to the absence of the photosynthate supply provided by the dinoflagellate symbionts, and compromised defences against pathogens. For example, surface microbes of corals are regulated in part through the periodic sloughing of mucus (Rivera-Ortega & Thomé, 2018), yet much of this mucus is synthesised from the products of symbiont photosynthesis (Crossland et al., 1980) so it seems reasonable to assume that aposymbiotic anemones will produce less mucus than symbiotic ones. Furthermore, there is both transcriptomic and proteomic evidence to suggest that aposymbiotic anemones may exhibit higher levels of cellular oxidative stress than symbiotic anemones (Oakley et al., 2016; Matthews et al., 2017). Likewise, the observed decrease in microbial species richness between anemones symbiotic with homologous symbionts *versus* those with heterologous symbionts may indicate the presence of a sub-optimal dinoflagellate symbiont. Crocinitomicaceae, previously classified within the Cryomorphaceae family (Munoz et al., 2016), were particularly common in the presence of *D. trenchii*; these bacteria are often found in areas rich in organic carbon, including in seawater, marine sediment and coral mucus (Munoz et al., 2016). Why they were more abundant in the presence of the

heterologous dinoflagellate is unknown, but it is plausible that they simply benefitted from reduced competition from other members of the microbiome.

3.5.2 Symbiosis and symbiont type induce changes to the holobiont volatilome

BVOC emissions are being increasingly explored as indicators of organism health in both human medicine (Ratiu et al., 2021) and environmental studies (Laothawornkitkul et al., 2009), including on coral reefs (Lawson et al., 2021). Additionally, BVOCs are recognised as agents of signalling molecules between phylogenetically distinct organisms (Bouwmeester et al., 2019; Rosset et al., 2021). For example, root-associated bacteria have been shown to stimulate the growth of neighbouring plants (Cordovez et al., 2018), and green leaf volatiles can act as a warning signal to neighbouring plants to upregulate defence mechanisms in the presence of herbivores (Li et al., 2012). Indeed, as has been proposed for other organically produced molecules like primary metabolites (Matthews et al., 2017) and proteins (Sproles et al., 2019), BVOCs may play a role in cellular signalling in the cnidarian-dinoflagellate symbiosis. For example, BVOCs could act as messengers to other individuals, perhaps to signal stress to neighbouring cnidarians, to induce an immune response, or to attract a particular symbiont species. Conversely, neighbouring cnidarians could be 'warned' against a less desirable algal partner, acting to induce an immune response in neighbouring cnidarians, as has been observed with parasitic insects *via* BVOC production by terrestrial plants (Yi et al., 2009; Fu & Dong, 2013).

Dimethyl sulphide (DMS) was found in high abundance in the volatilome of both Aiptasia-Symbiodiniaceae combinations, irrespective of symbiont identity, but was absent in the volatilome of aposymbiotic anemones. This is somewhat in contrast to my previous study, where I *did* identify relatively small amounts of DMS in the volatilome of aposymbiotic anemones (Chapter 2), potentially arising from bacterial metabolism of dimethylsulphoniopropionate (DMSP) to DMS and acrylate (Kuek et al., 2022); nevertheless, in all reported cases, the presence of Symbiodiniaceae is associated with much more prolific release of DMS from the holobiont, highlighting a central role for the algal partner in its synthesis (Hopkins et al., 2016). Moreover, I found DMS production to be greatest in the presence of the heterologous *D. trenchii*. One explanation for this could relate to an innate physiological difference between the two symbiont types, as previous work on DMS production by cultured Symbiodiniaceae showed that *D. trenchii* is a more prolific producer of DMS than *Breviolum* sp. (Lawson et al., 2019). A second explanation however, which is not necessarily mutually exclusive – could relate to the fact that *D. trenchii* is known to induce more cellular oxidative stress in Aiptasia than *B. minutum* (Matthews et al. 2017). DMSP, the precursor of DMS, has multiple roles in other ecosystems, including osmoregulation (Otte et al., 2004), cryoprotection (Trevena et al., 2000), and as

a foraging cue for fishes and attractant for a diversity of marine bacteria (e.g., Miller et al., 2004), but of particular interest here, it is known as an effective scavenger of reactive oxygen species (ROS). Furthermore, its breakdown products, DMS and acrylate, are 20–60 times more reactive than DMSP (Sunda et al., 2002) and so even more effective at scavenging ROS. Together, these three compounds (DMSP, DMS, acrylate) act as a powerful antioxidant system, and could help combat cellular stresses induced by the non-native symbiont.

Isoprene was detected in higher abundance in anemones symbiotic with *B. minutum* relative to those in aposymbiosis or in symbiosis with *D. trenchii*. A prolifically emitted terrestrial BVOC, isoprene has functions in thermal tolerance in the tropical tree *Vismia guianensis* (Rodrigues et al., 2020), scavenging ROS in the tall grass *Phragmites australis* (Velikova et al., 2004) and providing protection against herbivory in tobacco plant *Nicotiana tabacum* (Laothawornkitkul et al., 2008) by deterring potential plant pests. Although isoprene has indeed been detected from microalgae previously (Exton et al., 2013), to my knowledge this is the first report of its production in Aiptasia. Should the protective function of isoprene be conferred to the cnidarian host, my results may indicate that symbiosis with *B. minutum* conveys higher thermal tolerance to this native symbiosis relative to symbiosis with non-native *D. trenchii* or aposymbiosis.

A further compound of note, nonanal was detected at higher levels in the volatilome of both aposymbiotic anemones and anemones containing heterologous symbionts relative to those with homologous symbionts. It is possible that nonanal could act as an infochemical in cnidarians signalling a non-optimal symbiotic state. Nonanal is produced by a variety of plant species and has been shown to elicit a response in both insects and neighbouring plants. For example, nonanal production by the cactus *Opuntia stricta* acts as an attractant to the South American cactus moth *Cactoblastis cactorum* (Pophof et al., 2005). Furthermore, emission of nonanal by barley (*Hordeum vulgare*) and lima beans (*Phaseolus lunatus*) infected with *Blumeria graminis* and *Pseudomonas syringae* respectively, induced systemic acquired resistance in neighbouring plants (Yi et al., 2009; Brambilla et al., 2022), priming the immune system of uninfected plants by acting as a chemical warning signal. Nonanal is also emitted during courtship by the female vector for chagas disease *Triatoma infestans* to attract male suitors (Fontan et al., 2002) and acts as an aggression pheromone in the bed bug *Cimex lectularius* (Siljander et al., 2008). There is therefore considerable potential for nonanal to play a comparable signalling role in the Aiptasia system, making it an interesting candidate for future research.

Other chemicals detected in higher abundance in aposymbiotic anemones relative to those in symbiosis with *B. minutum* include dodecanal and octanal. Dodecanal has been identified as a sex pheromone in ring–tailed lemurs (Shirasu et al., 2020), while in the moth *Zygaena filipendulae* it is a by-product of metabolism or stress (Zagrobelny et al., 2015). Its release by aposymbiotic anemones could therefore be consistent with either a role in chemoattraction of symbionts or host stress metabolism. By comparison, octanal is released from insect-damaged potato tubers and has putative roles in the attraction of nematode predators (Laznik & Trdan, 2016). If this compound similarly plays a role in pathogen defence in Aiptasia, then this pattern could reflect the putative shift towards a more pathogenic microbiome in aposymbiotic anemones discussed above, and which is also observed in bleached corals (Muller et al., 2018).

Collectively, I found that the volatilome of Aiptasia had a reduced diversity of compounds in the presence of the homologous *B. minutum*. This perhaps indicates a tighter recycling of metabolic products between cnidarian host, dinoflagellate symbiont, and the associated microbiome, as may be expected for an optimally functional holobiont. By contrast, the greater diversity of the volatilome of anemones containing the heterologous *D. trenchii* and especially aposymbiotic anemones perhaps arises from a lesser degree or lack of inter-partner metabolic integration, respectively, and even cellular stress. Previous work on nutritional interactions in the Aiptasia-dinoflagellate symbiosis have shown that heterologous symbionts may form a metabolically sub-optimal, less well integrated association when compared to homologous symbionts (Leal et al., 2015; Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2020; Mashini et al., 2022). Nevertheless, much more work is needed to elucidate the BVOC pathways involved and how the various partners of the holobiont, including members of the microbiome, interact to metabolise and modify different volatiles, so generating the emission patterns observed.

3.6 Conclusion

In conclusion, I have shown that symbiosis with the non-native dinoflagellate symbiont *D. trenchii* induces a distinct shift in the microbiome that may contribute to the observed shift in the volatilome. This heterologous symbiont induces a Volatilome indistinct from either aposymbiosis or symbiosis with their homologous partner *B. minutum*. This physiological state is consistent with previous molecular, cellular, and physiological studies, and has been suggested to reflect a suboptimal nutritional state. How the different members of the holobiont contribute to this shift is unknown given the complex inter-partner interactions that are no doubt involved, and we need to know much more about the functional roles of the specific volatiles generated to appreciate the organismal and

ecological implications of such changes. This study provides a foundation for these future investigations, as we aim to understand the implications of climate change on the cnidarian-dinoflagellate symbiosis, and coral reef function and health.

Chapter 4 Thermal stress induces restructuring of volatile gas emissions by the model symbiotic cnidarian Aiptasia

4.1 Abstract

With a changing climate and increasing stressors imposed upon coral reefs, coral bleaching events, where the symbionts are lost from host tissues, have become more frequent and severe. This study characterised the suite of biogenic volatile organic compounds (BVOCs) that comprise the volatilome of the model sea anemone *Exaiptasia diaphana* ('Aiptasia') when exposed to three temperature treatments (control: 25 °C, sub-bleaching: 30 °C, and bleaching: 33.5 °C) both in symbiosis with its native dinoflagellate symbiont *Breviolum minutum* ('symbiotic') and in the absence of dinoflagellate symbionts ('aposymbiotic'). To further characterise the entire symbiotic entity (i.e., 'holobiont') contributing to the volatilome, the microbiome of symbiotic and aposymbiotic anemone was also characterised, demonstrating a marked community shift under thermal stress irrespective of symbiotic state. The volatilome of symbiotic anemones at bleaching temperature was distinct from that at the lower temperatures, mostly driven by the high quantities of dimethyl sulphide (DMS), eucalyptol and 1-iodododecane, produced under thermal stress. By comparison, the volatilome of aposymbiotic anemones was most distinct at the sub–bleaching temperature, when most significantly different BVOCs were detected at the highest abundance, including 2-phenyl-3-methyl-pyrrolo(2,3-b)pyrazine, acetone, and naphthalene. Assessment of BVOCs present in all biological replicates across temperature treatments ('core volatiles') revealed that symbiotic anemones had a 12-fold larger core volatilome than aposymbiotic anemones (48 *vs*. 4 compounds), and that the symbiotic anemone volatilome retained its richness of BVOC compounds under thermal stress while the richness of the aposymbiotic anemone volatilome decreased. This observation suggests that, despite the obvious physiological signs of thermal stress (e.g., elevated respiratory rate), the presence of symbiotic dinoflagellates may confer a degree of metabolic stability to the holobiont. These observed changes in metabolic output can inform our understanding of the ways in which coral holobionts may respond to increasing seawater temperatures and allow us to target specific BVOCs for further studies of BVOC function and biomarker development on coral reefs.

4.2 Introduction

Coral reefs are exceptionally diverse and valuable ecosystems, providing a habitat for at least 25% of all marine species in the small percentage of ocean space that they occupy (~0.1%; Hoegh-Guldberg et al., 2017). At the foundation of this remarkable diversity is the endosymbiosis between the cnidarian host and dinoflagellate symbionts in the family Symbiodiniaceae (LaJeunesse et al., 2018). Tight nutrient cycling between these organisms allows for the growth of reef-building corals in an

otherwise oligotrophic environment (Muscatine & Porter, 1977), but as a process is particularly vulnerable to thermal stress (Beyer et al., 2018). When exposed to high temperature stress, the symbiosis becomes dysfunctional and dinoflagellate symbionts are lost from host tissues (Nielsen et al., 2018), resulting in a 'bleached' coral, that quickly transitions to coral death if stress is prolonged (Oakley & Davy, 2018). While it is acknowledged that coral bleaching is the result of the perturbation of complex biological pathways (Suggett & Smith, 2020), the accumulation of reactive oxygen species (ROS) is still considered the prevailing driver (Nielsen et al., 2018). Synthesis of ROS is a natural by-product of photosynthetic and respiratory metabolic pathways (Foyer, 2018), but high temperature damages the photosynthetic apparatus and hence capacity to process light energy, causing an increase in ROS generation. Additionally, ROS are generated during cellular respiration in mitochondria, the production of which are increased during heat stress (Heise et al., 2003; Slimen et al., 2014). Once ROS concentrations overwhelm oxidative defence mechanisms, tissues are damaged through the oxidation of cellular components (Weis, 2008; Oakley and Davy, 2018; Mittler et al., 2022). Thermal stress ultimately results in a range of impacts, including restructuring of the actin cytoskeleton and extracellular matrices (Desalvo et al., 2008, 2010), and reduced nutritional flux between the symbiotic partners (Hillyer et al., 2016, 2017; Rädecker et al., 2021). In turn, sustained oxidative stress leads to the loss of symbionts from the host's tissues, potentially through mechanisms of apoptosis, autophagy, and exocytosis, although the relative importance of these different mechanisms remains unclear (Oakley and Davy, 2018).

In addition to the Symbiodiniaceae, corals associate with a diversity of bacteria, fungi and viruses that may influence their physiology, growth and stress tolerance (McFall-Ngai et al., 2013), a community collectively referred to as the 'microbiome' (Rosenberg et al., 2007). It is postulated that the whole organism collective (the 'holobiont') can restructure its microbiota as a mechanism to quickly adapt to a changing environment (Voolstra & Ziegler, 2020). Indeed, this hypothesis has been supported by coral transplant experiments in which microbes have been shown to change to reflect their new environment (Ziegler et al., 2019), and by the successful inoculation of corals by beneficial bacteria for the purpose of successfully increasing their stress tolerance (Doering et al., 2021). In addition to the microbiome (Oppen & Blackall, 2019), other omics platforms – spanning transcriptome (Pinzón et al., 2015), proteome (Ricaurte et al., 2016), lipidome (Sikorskaya & Imbs, 2020), steady-state metabolomics analysis (Hillyer et al., 2017; Farag et al., 2018), and volatilomics (Lawson et al., 2021) – have been applied to better understand the process and regulatory factors underpinning coral bleaching. However, a full picture of holobiont physiology is altered in response to the ever-increasing threat of global climate change remains unclear.

Biogenic volatile organic compounds (BVOCs) are low molecular weight (<200 Da) chemicals with high vapour pressure (Mansurova et al., 2018) and are produced by all organisms on Earth (e.g., Laothawornkitkul et al., 2009; Tahir et al., 2017; Lawson et al., 2019). BVOC functions are diverse and span local cloud formation, postulated to increase protection from UV light (Swan et al., 2016), to lipid membrane stabilization during thermal stress (Siwko et al., 2007) and stimulation of an immune response in neighbouring plants in response to herbivory (Ton et al., 2007). Once thought to be simply by-products of primary organism metabolism, some BVOCs are now recognised as physiological agents in themselves, acting across individuals (Wenig et al., 2019) or species (Minerdi et al., 2021) to convey a chemical message. The ways in which BVOC emissions from an organism are affected by environmental stressors is a new area of research (Lawson et al., 2021, 2022; Okereke et al., 2022), and potentially highlights a route by which climate change alters interactions among organisms.

The symbiotic sea anemone *Exaiptasia diaphana* (hereafter, 'Aiptasia') is a model organism adopted for studying cnidarian-dinoflagellate symbiosis (Weis et al., 2008; Baumgarten et al., 2015; Rädecker et al., 2018). As with corals, Aiptasia forms a stable symbiosis with algae from the family Symbiodiniaceae (Wolfowicz et al., 2016) and similarly responds to environmental stressors with the expulsion of algae from host tissues (Sawyer & Muscatine, 2001). Furthermore, Aiptasia can reproduce asexually, allowing the maintenance of genetically identical populations, such that any physiological differences can be attributed to differences in environmental conditions (Lehnert et al., 2012). While previous work has shown that thermal stress alters volatile gas production in corals (Lawson et al., 2021), there is a gap in the literature regarding how thermal stress alters BVOC emissions across symbiotic states. Aiptasia provides a unique model system in which in explore BVOC synthesis in association with, and without, dinoflagellate symbionts.

The aim of this study was to determine the impact of thermal stress on BVOC production by the Aiptasia holobiont. I profiled BVOCs in two different symbiotic states (i.e., symbiotic *versus* symbiont-free) at control, sub-bleaching, and bleaching temperatures. The microbiome under these various treatments was also characterised, to define the holobiont composition responsible more clearly for the volatilome patterns observed. I hypothesised that thermal stress would shift both holobiont composition and the volatilome, adding to our understanding of the physiological responses associated with the breakdown of symbiosis in this important model system, and helping to identify potential BVOC biomarkers of sub-lethal stress.

4.3 Materials and Methods

4.3.1 Experimental organisms

A long-term (15+ years) clonal culture of the sea anemone Aiptasia (culture ID: NZ1) of unknown Pacific origin (Matthews et al., 2017) was maintained in the laboratory in filtered seawater (FSW) at 25 °C and 70 µmol photons m^{-2} s^{-1} on a 12:12 h light-dark cycle (GE Lighting T5 F54W/840). Clonal anemones (n = 100) were rendered aposymbiotic (i.e., symbiont-free) using menthol-induced bleaching, *via* exposure to menthol (20% w/v in ethanol; Sigma-Aldrich, Auckland, NZ) at a final concentration of 0.19 mmol L^{-1} in 0.22 µm FSW (following Matthews et al., 2016). Anemones were incubated in menthol for 8 h during the 12-h light period, after which photosynthesis was inhibited by replacing menthol/FSW with FSW containing 5 µmol L^{-1} 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 100 mmol L^{-1} dissolved in EtOH, Sigma-Aldrich) for 16 h to prevent repopulation by inhibiting photosynthesis of the remaining symbionts. After repeating this 24-h cycle for four consecutive days, anemones were maintained in 0.22 µm FSW without menthol or DCMU for three days. Anemones were fed once weekly with *Artemia* sp. nauplii, with fresh FSW changes 8 h post-feeding, with this protocol being continued for six weeks. Aposymbiosis was confirmed using fluorescence microscopy (Olympus IX53 inverted microscope; 100× magnification). All aposymbiotic anemones were then maintained in 0.22 µm FSW in the dark at 25 °C for 1.5 years prior to BVOC sampling, during which time they were fed with *Artemia* sp. nauplii and given fresh FSW twice weekly.

A long-term stock of symbiotic anemones (> 1 year) that had been repopulated with a homologous symbiont (*Breviolum minutum*), as described in Chapters 2 and 3, was used for consistency among thesis chapters. Symbiotic anemones were maintained at a constant temperature of 25 °C and irradiance of approximately 70 µmol photons m^{-2} s^{-1} on a 12 : 12 h light-dark cycle. This anemone stock culture was fed twice weekly, as described above.

4.3.2 Thermal stress treatment

Aiptasia were fed three times weekly with freshly-hatched *Artemia* sp. nauplii for the duration of the experiment to maintain consistency between aposymbiotic and symbiotic anemones. Both aposymbiotic and symbiotic anemones were maintained at 130 µmol photons m^{-2} s^{-1} (light saturation for photosynthesis, Table S1) during the heat-ramping period. The control group was kept at 25 °C throughout the experiment; the sub-bleaching group was subjected to a temperature increase of 1 °C *per* day to a maximum temperature of 30 °C, which was maintained for seven days until sampling; the bleaching group was subjected to a temperature increase of 1 °C *per* day until 30 °C, after which the

temperature was increased by 0.5 °C *per* day to a maximum temperature of 33.5 °C, which was maintained for 24 h prior to sampling (Fig. 4.1a).

Every day during the thermal ramping period, maximum quantum yield (F_v/F_m, dimensionless) was used as an indicator of photosynthetic health (Krause & Weis, 1984; Levin et al., 2016). Symbiotic anemones were dark-acclimated for 15 min before measurement with an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3 and damping = 3) as *per* Oakley et al. (2017) and Hawkins and Davy (2013).

4.3.3 Physiological measurements

To assess thermal impacts on holobiont physiology, oxygen consumption of both aposymbiotic and symbiotic anemones (and photosynthetic oxygen production by symbiotic anemones) was measured by incubating anemones in a sealed Perspex chamber (13 mL) fitted with an internal stir bar and an oxygen optode (Fibox 4, PreSens Gmbh, Regensburg, Germany). The magnetic stir bar was underneath a perforated mesh floor on which the anemones sat and rotated at a speed at which the anemones did not appear visually stressed (their tentacles were out). Chamber vials were immersed in one of three temperature baths according to the treatment temperature (25 °C, 30 °C or 33.5 °C), atop a magnetic stir table (Multistirrer Digital 6, VELP Scientifica Srl, Italy) where anemones (2 *per* chamber, n = 5 *per* temperature *per* symbiotic state) were allowed to settle and attach in the chambers for ~1 h prior to measurement. Lights were initially turned off and oxygen consumption was measured for 15 min. For symbiotic anemones, illumination was subsequently administered for 20 min at 130 µmol photons m^{-2} s^{-1} (saturating irradiance, see above), with all measurements taken during the photic period. Rates of holobiont dark respiration were calculated as moles O_2 consumed h^{-1}. Dark respiration rates were then subtracted from net photosynthetic rates for symbiotic anemones (moles O_2 produced h^{-1} during the light phase) to determine gross photosynthesis values. Respiration and gross photosynthetic rates were normalised to soluble animal protein content of each anemone and symbiont cell density, respectively. Subsequent to respirometry sampling, anemones were immediately frozen at -80 °C and stored for algal cell density and protein analysis.

Frozen anemones (n = 10 for symbiont cell density calculations; n = 5 for respirometry and photosynthesis calculations, with 10-15 anemones *per* biological replicate) were thawed on ice and homogenised using a saw-tooth homogeniser in 500 µL milliQ water. Host and symbiont fractions were separated by centrifugation at 400 x g for 2 min after which the symbiont fraction was washed of

residual host material by one further resuspension and centrifugation with milliQ water. The supernatant fraction was analysed for protein content using the fluorometric Qubit Protein Assay Kit (Vergauwen et al., 2017). Cell counts were performed using a haemocytometer (Improved Neubauer) with eight replicate counts *per* sample and normalised to protein content. Cell density (symbionts *per* mg host protein) was then calculated. Different numbers of biological replicates are reported for cell density *versus* respirometry measurements since more biological replicates were available for cell density calculations.

4.3.4 BVOC sampling and volatilome characterisation

Biogenic Volatile Organic Compound (BVOC) gases were collected and analysed from anemones in two different symbiotic states at three temperatures: aposymbiotic anemones (n = 6 at 25 °C and 33.5 °C, n = 5 at 30 °C; ~15 anemones *per* vial) and symbiotic anemones (n = 6 at 25 °C and 33.5 °C, n = 5 at 30 °C; ~10 anemones *per* vial) at 25 °C (control), 30 °C (sub-bleaching) and 33.5 °C (heat-stressed). Different numbers of anemones were used between symbiotic states to equalise biomass among groups. The experimental setup and BVOC retrieval were performed using previously established methods as described in Chapters 2 and 3, modified from Lawson et al. (2019, 2021). To allow anemones to settle and attach onto sampling vials, the night before BVOC sampling, experimental organisms were transferred into sterile 150 mL, unsealed, glass crimp-cap serum glass vials (Wheaton, Millville, NJ, USA) containing 75 mL FSW, where they were retained under conditions identical to those used for growth. Immediately prior to sampling, FSW was refreshed, and vials were sealed using 20 mm PTFE/Si crimp caps (Agilent, USA). BVOCs were collected by passing instrument grade air (100 mL min^{-1}; BOV Gases, Wellington, NZ) into gas-tight sampling vials for 20 min, whereby the outgoing air was passed through open-ended thermal desorption tubes (TDTs; Markes International Ltd, Llantrisant, UK) containing the sorbent Tenax TA, onto which BVOCs adhered as described in Chapters 2 and 3. After a 20 min sampling time, TDTs were immediately sealed with brass storage caps and stored at 4 °C until processing. All TDTs were analysed within two weeks of sampling to minimise sample degradation, using gas chromatography coupled with single quadrupole mass spectrometry (GC×MS) as *per* Olander *et al*. (2021). TDT desorption was performed using a Marks Unity 2 Series Thermal Desorber and ULTRA multi-tube autosampler. Desorption occurred at 300 °C for 6 min, after which BVOCs were concentrated on a cold trap at -30 °C. The cold trap was subsequently flash-heated to 300 °C, injecting the concentrated sample onto a 7890A GC (Agilent Technologies, Ltd., Melbourne) *via* a transfer line maintained at 150 °C. The GC was equipped with a 30 m x 0.25 mm Rxi-624Sil MS column (Restek Corp.) with a film thickness of 1.40 µm. To encourage complete desorption of BVOCs, the GC oven was heated for 35 °C for 5 min, then increased to 240 °C by increasing 5 °C min^{-1}, and held at 240 °C for 5 min. All

samples were run splitless at a flowrate of 1 mL min^{-1}. The GC was attached to a Model 5974 mass-selective detector (Agilent Technologies, Ltd., Melbourne), with the scanning range set to 29 – 450 amu.

Spectral data from the GC-MS were run through the open-source MS data processing program OpenChrom (Wenig & Odermatt, 2010) to remove common contaminating ions (amu: 73, 84, 147, 149, 207, 221). Output files were then imported to Galaxy (Guitton et al., 2017), and processed using the metaMS.runGC package (Galaxy version 2.1.1; Wehrens et al., 2014; Giacomoni et al., 2015; Afgan et al., 2018) on Workflow4Metabolomics. Peaks were tentatively identified against a database in the National Institute of Standards and Technology (NIST) Mass Spectral library (NIST 14 library in NIST MS Search v2.2; NIST, Gaithersburg, MD). A compound match factor of at least 60% was required for compound identity to be recorded; otherwise, the compound was listed as 'unclassified' (UC). The number following 'UC' indicates the retention time of the compound.

Prior to desorption, each TDT was injected with 0.2 µL of 150 ppm bromobenzene (GC grade, Sigma Aldrich, Castle Hill, NSW, Australia) in methanol (HPLC grade, Sigma Aldrich) as an internal standard. BVOCs were also collected from filtered (0.22 µm) seawater blanks (n = 6 at 25 °C and 33.5 °C; n = 5 at 30 °C) using the same methods, at the time of sample collection. Average values for BVOCs present in the blanks were subtracted from all samples. Finally, peak abundance was normalised to the protein content of each replicate. Protein content was released from samples using an ultrasonication probe (VCX500; Sonics & Materials Inc., Newtown, CT, USA) to lyse cells. Protein content was measured using the fluorometric Qubit Protein Assay Kit (Vergauwen et al., 2017). Compounds identified as likely methodological artifacts, e.g. silicon-containing compounds, were also removed from the dataset as they were suspected contaminants from GC column dimethylpolysiloxane hydrolysis (Cella & Carpenter, 1994). Only BVOCs present in a minimum of four biological replicates in at least one symbiotic state were classed as 'present' in a state. All BVOCs were grouped according to their chemical class. BVOC abundance data is dimensionless and was normalised to a chemical standard, seawater blanks and protein content.

4.3.5 Microbiome analysis

Anemones from which BVOCs were collected were also used for microbiome analysis. After BVOC retrieval, anemones were transferred with sterile glass Pasteur pipettes into 1.5 mL microtubes and remaining water was carefully removed. All microtubes were immediately frozen at -80 °C and stored at this temperature until DNA extraction. Aiptasia samples were then thawed, sterile artificial

seawater (ASW) was added to produce 1 mL samples, and pooled anemones were mechanically homogenised for 30 s with a tissue homogeniser on ice. 16S rRNA diversity data were obtained from 5 biological replicates *per* symbiotic state *per* temperature treatment.

DNA extraction was performed using manufacturer's instructions for the DNeasy Plant Mini Kit (Quiagen), using 300 µL of anemone homogenate as starting material. DNA concentrations were quantified on a NanoDrop spectrophotometer (Impen NanoPhotometer^{TM} NP80, Thermo Fisher). Variable regions 5 and 6 of the 16S rRNA gene were targeted using the primer pair 784F [5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-AGGATTAGATACCCTGGTA 3'] and 1061 R [5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-CRRCACGAGCTGACGAC 3′] with Illumina adaptor overhangs (underlined above; Rothig et al., 2016; Voolstra & Ziegler, 2020). Extracted DNA was sent to the University of Auckland Genomics Facility (Auckland, NZ). Here, gDNA was normalised to 50 ng/µL and 200 ng were used as an input for 25 µL PCR reaction. The PCR mix included 12.5 µL of 2x Platinum SuperFi Master Mix. Primer concentration in the reaction was 0.2 µM. The first round of PCR thermal cycling conditions was 3 min initial denaturation at 95 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final elongation stage at 72 °C for 5 min. After the first round of PCR, 1 µL of each sample was QC'd on a BioAnalyzer with the HS DNA chip. A 0.8x volume of AMPure XP beads was used to clean up the reactions (20 µL PCR product, 16 µL bead suspension), with 2 × ethanol washes and elution in 12 µL water. A 1.5 µL aliquot of the cleaned first round PCR product were used as input to the second-round indexing PCR, which used Nextera V2 indexing primers and the Platinum SuperFi MasterMix. Thermal cycling conditions were 3 min at 95 °C, followed by 8 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. After this round of amplification, dsDNA was quantified using a Qubit dsDNA HS (high sensitivity) Assay Kit. A total of 10 µL of each sample were pooled and cleaned up with two rounds of AMPure beads using a 1:1 ratio of beads to sample, with 2 × 80% ethanol washes during each round before loading onto the Illumina MiSeq platform for NGS sequencing. Bacteria were grouped at the family level; in cases where identification to the family level was not possible, taxa were grouped by class. Low abundance bacterial taxa (comprising 1% or less of the dataset) and unclassified taxa were binned into 'other'.

4.3.6 Data analysis

Symbiont density and oxygen production/consumption were tested for normality and homoscedasticity, and then tested with a two-way ANOVA in R (version 4.0.3; R Core Team, 2020). Quantum yield measurements did not meet assumptions of normality and homoscedasticity, so were analysed using the non-parametric Wilcoxon test in R (version 4.0.3; R Core Team, 2020). All plots

were created with ggplot2 (Wickham, 2016) in RStudio. For microbiome data, the MiSeq output fastq file was de-multiplexed in R (R Core Team, 2021) using the insect package (v 1.4.0; Wilkinson et al., 2018) and trimmed sequences were filtered to produce a table of exact amplicon sequence variants (ASVs) using the DADA2 R package (Callahan et al., 2016). ASVs were identified to the lowest possible taxonomic rank using a two-step classification process. This involved: 1) exact matching against the RDP v18 reference database (accessed 15 June 2022 from https://doi.org/10.5281/zenodo.4310150) and assigned taxonomy at the lowest common ancestor level (LCA; i.e. assigning to family level if sequence matches with 100% identity to more than one genus); and 2) querying any unwanted sequences against the same RDP reference database using the SINTAX classification algorithm (Edgar, 2016) with a conservative assignment threshold of > 0.99. In both cases, maximum assignment resolution was set to one genus level due to the high over-classification rates associated with species-level assignment (Edgar, 2016).

Differential abundance of BVOCs was estimated using the limma R package (Ritchie et al., 2015); the *voom* function was used to convert counts to $\log_2$-counts-*per*-million and to assign weights to each observation based on the mean-variance trend. The counts assigned to each observation roughly corresponds to abundance of the BVOC, which can then be used to compare BVOC production between treatments Functions *lmFit*, *eBayes*, and *topTable* were used to fit weighted linear regression models, calculate empirical Bayes moderated t-statistic, and calculate FDR-corrected p-values (Phipson et al., 2016). Biological replicates, standardised using the *decostand* function in the vegan package (Oksanen et al., 2020) in R (version 1.2.5033), were compared using the Bray-Curtis similarity measure, and this output was subjected to non-metric multidimensional scaling (NMDS) to visualise differences among groups based on BVOCs. To compare dispersion of biological replicates using a distance measure, PERMANOVA was performed to test if the distance between biological replicates was greater between treatments than within treatments. PERMANOVA was performed on NMDS scores using the *adonis* function, and *post hoc* tests were performed using the *pairwise.adonis* function in the vegan package in R (version 1.2.5033) to distinguish differences among treatments. Differential abundance of microbial taxa was measured using the relative abundance of each microbial taxon within a sample and estimated using the limma R package as described above.

4.4 Results

4.4.1 Cell density, respiration, and photosynthesis

Whilst quantum yield of anemones in the bleaching treatment generally declined from 0.631 +/- 0.04 to 0.552 +/- 0.03 (Fig. 4.1b), throughout the thermal ramping period, this decline was insignificant (Wilcoxon test; $p > 0.05$ for all timepoints).

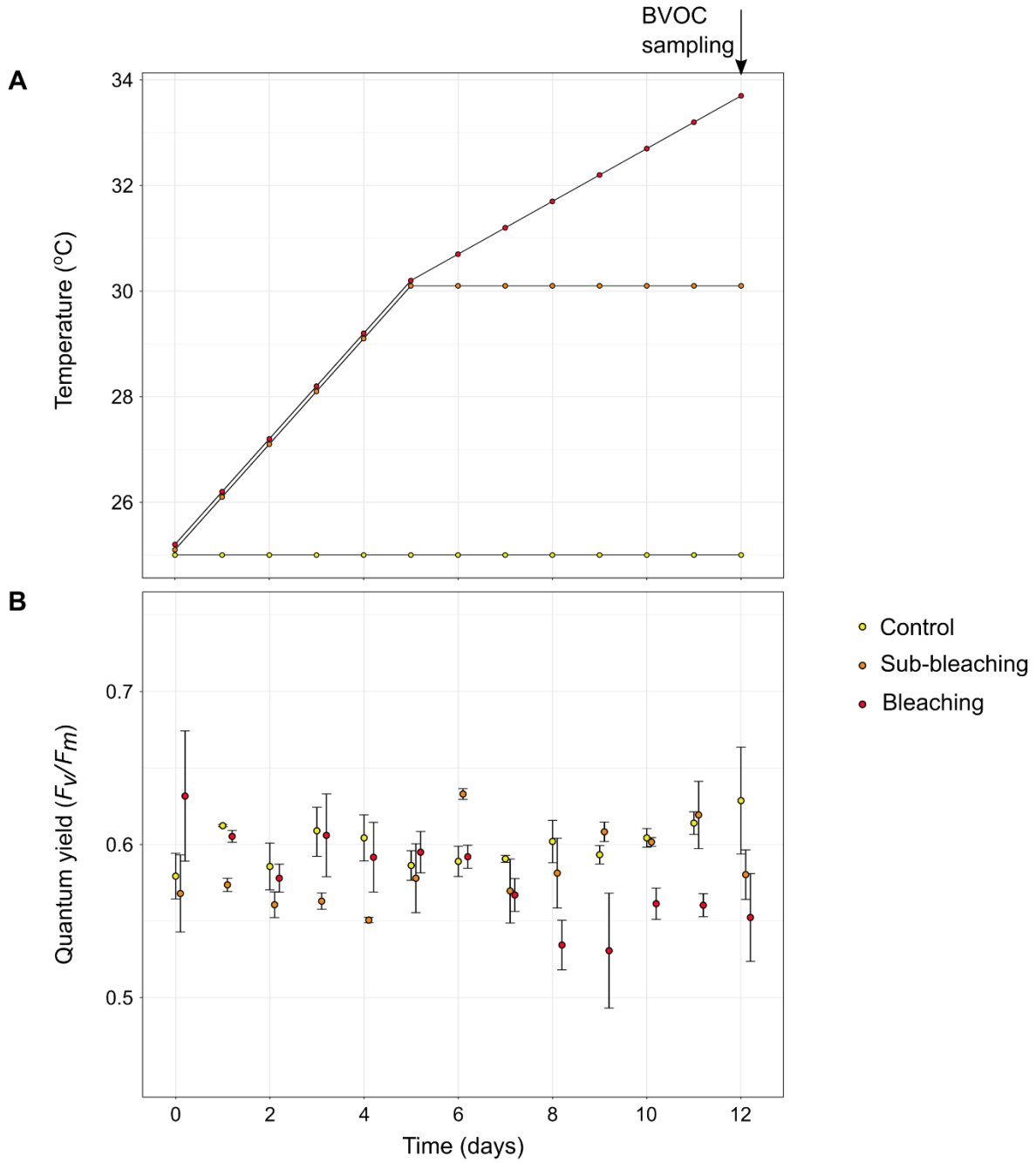

Figure 4.1: Heat ramping, sampling schedule and quantum yield (F_v/F_m measurements; dimensionless) measurements for Aiptasia symbiotic with *Breviolum minutum* at three temperature treatments (control: 25 °C, sub-bleaching: 30 °C; bleaching: 33.5 °C). A) Thermal ramping schedule. Control anemones were maintained at 25 °C for the duration of the experiment; the sub-bleaching treatment was increased by 1 °C *per* day to a final temperature of 30 °C, at which it was held for seven days; the bleaching treatment was increased by 1 °C *per* day until reaching 30 °C, after which it was increased by 0.5 °C *per* day to a peak temperature of 33.5 °C. Sampling occurred after the bleaching treatment was maintained for 24 h. B) Quantum yield measurements (F_v/F_m, dimensionless; +/- standard error bars) taken from symbiotic anemones prior to BVOC sampling. Anemones (n = 3 for each treatment *per* day) were dark acclimated for 15 min before measurement. Data points are slightly offset for clarity.

Symbiont density was significantly impacted by elevated temperature (Fig. 4.2a, one-way ANOVA, $F_{2,27} = 10.84$, p = 0.0003). Tukey *post hoc* testing revealed a difference in cell density between anemones at 33.5 °C (1.72 million cells *per* mg host protein) compared to those at 25 °C (2.29 million cells *per* mg host protein; $p = 0.019$) and those at 30 °C (2.63 million cells *per* mg host protein p = 0.0003). Cell densities were statistically indistinct for anemones at 25° C and those at 30 °C ($p = 0.227$). The cell-specific gross photosynthetic rate was not significantly impacted by thermal treatment (one-way ANOVA, $F_{2,11} = 2.844$, p = 0.101), where values across all treatments were 0.34, 0.36 and 0.49 mg O_2 *per* million symbiont cells *per* hour for anemones in control, sub-bleaching and bleaching treatments, despite the noticeably higher value at the highest temperature (Fig. 4.2b). Thus, gross photosynthesis for the holobiont did not change significantly in response to temperature (Fig. 4.2c), despite the loss of symbiont cells (Fig. 4.2c; one-way ANOVA, $F_{2,12} = 2.843$, $p = 0.098$). In contrast to photosynthesis, the dark respiration rate of aposymbiotic anemones increased at 33.5 °C (0.28 mg O_2 *per* mg host protein *per* hour) relative to both lower temperatures (0.14 mg O_2 *per* mg host protein at both at 30 °C and 33.5 °C; Fig 2d; one-way ANOVA, $F_{2,11} = 6.94$, p = 0.0112; *post hoc* $p < 0.05$ for both comparisons). Similarly, the dark respiration rate of symbiotic anemones increased from 0.26 mg O_2 *per* mg host protein *per* hour at 30 °C to 0.39 mg O_2 *per* mg host protein *per* hour at 33.5 °C (Fig 4.2e; one-way ANOVA, $F_{2,10} = 8.746$, p = 0.006; *post hoc* p <0.005), with no difference in respiration rate between symbiotic anemones at 25 °C (0.33 mg O_2 *per* mg host protein *per* hour) relative to those at 30 °C or 33.5 °C (*post hoc* $p > 0.05$ for both comparisons). From these various responses, 33.5 °C caused symbiont cell loss, and increased respiration in both aposymbiotic and symbiotic anemones.

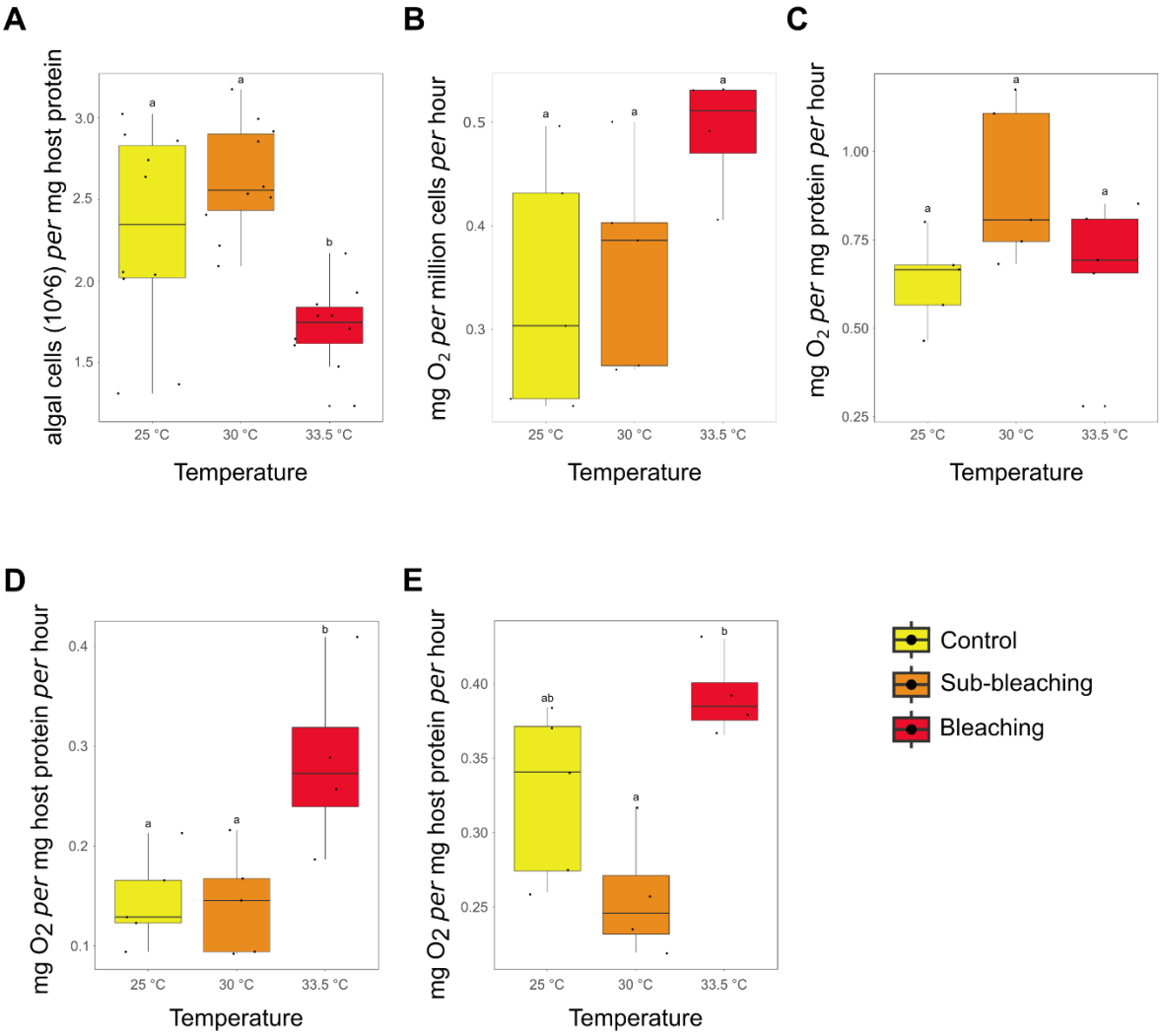

Figure 4.2: Effects of temperature on symbiosis stability and physiology. Anemones were exposed to control (25 °C), sub-bleaching (30 °C) and bleaching (33.5 °C) temperatures. A) Symbiont cell densities in Aiptasia (n = 10 *per* temperature); B) Symbiont cell-specific rate of gross photosynthesis (n = 5 *per* temperature); C) Overall photosynthetic rate of symbiotic anemones (n = 5 *per* temperature); D) Dark respiration rate of aposymbiotic anemones (n = 5 *per* temperature); E) Dark respiration rate of symbiotic anemones. For clarity, respiration rates are shown as positive values. Lines around box plot indicate interquartile ranges from 25-75%; statistical significance among temperature treatments is indicated by letters above error bars.

4.4.2 Temperature affects the Aiptasia microbiome

Non-metric multidimensional scaling was used to visualise differences in the microbiome associated with both aposymbiotic and symbiotic anemones at different temperatures. Regardless of symbiotic state, anemones followed the same trend, whereby groups were significantly separated by temperature treatment (PERMANOVA: $F_{2,12} = 2.324$, $p = 0.002$; PERMANOVA: $F_{2,14} = 2.214$, $p = 0.001$ for aposymbiotic (Fig. 4.3a) and symbiotic anemones (Fig. 4.3c), respectively); microbiomes of anemones at 25 °C were significantly different from those at 33.5 °C (*post hoc*, $p < 0.05$ for both symbiotic states). Microbiota of anemones at 30 °C were not distinct from those at 25 °C or 33.5 °C for either symbiotic state ($p > 0.05$ for all comparisons).

Bacterial community composition differed at the three temperatures and between symbiotic states (Fig. 4.3b, 4.3d). Overall, aposymbiotic anemones at 25 °C were dominated by the class Gammaproteobacteria (15.9%). In contrast, anemones at 30 °C were dominated by bacteria in the families Vibrionaceae (18.2%) and Crocinitomicaceae (14.1%), whereas anemones at 33.5 °C primarily harboured bacteria from the Vibrionaceae (24.8%) and Flavobacteriaceae (18.6%). Symbiotic anemones harboured microbiomes dominated by the family Flavobacteriaceae (32.2%) at 25 °C, but dominated by bacteria in the class Gammaproteobacteria at 30 °C and 33.5 °C (23.8% and 25.3% respectively).

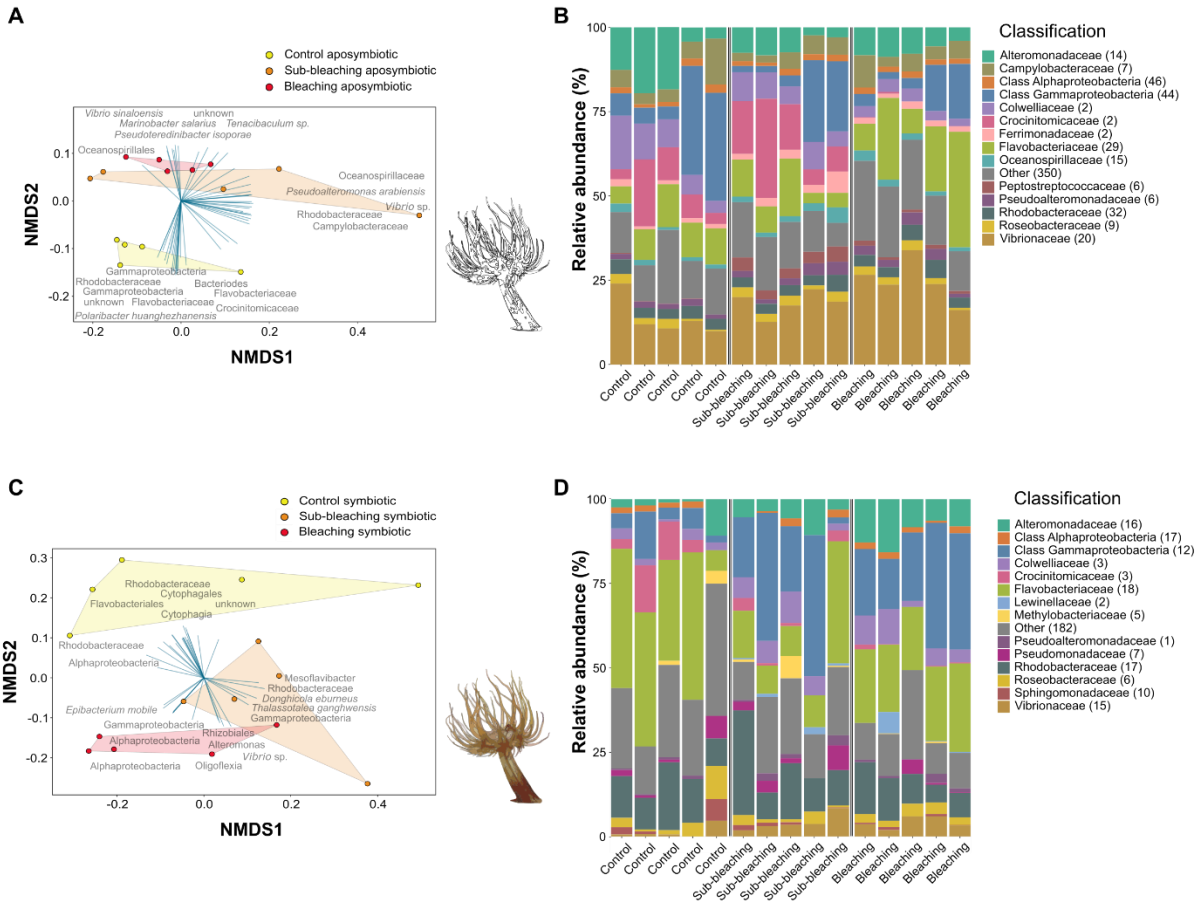

Figure 4.3: Bacterial community composition of aposymbiotic and symbiotic anemones in response to control (25 °C), sub-bleaching (30 °C) and bleaching (33.5 °C) temperatures. A) Non-metric multidimensional scaling (NMDS) plot (stress = 0.071) of aposymbiotic anemone microbiota; B) Relative abundances of bacterial taxa associated with aposymbiotic anemones in each biological replicate, grouped by family; C) Non-metric multidimensional scaling (NMDS) plot (stress = 0.071) or symbiotic anemones; D) Relative abundances of bacterial taxa associated with symbiotic anemones in each biological replicate, grouped by family. Each colour represents one of the most abundant 14 families identified across the dataset. Sequences unresolved at the family level were denoted at the class level. Less abundant families (comprising 1% or 0.7% or less of the dataset for aposymbiotic or symbiotic anemones, respectively) were designated as 'other'. Numbers in parentheses denote the number of unique taxa within that family.

4.4.3 BVOC emissions by aposymbiotic anemones are influenced by temperature

Non-metric multidimensional scaling demonstrated a shift in the volatilome of aposymbiotic Aiptasia across temperature treatments (Fig. 4.4a). Specifically, the volatilome for anemones at the sub-bleaching temperature was different to that for anemones at the highest temperature (PERMANOVA: $F_{2,14} = 2.49$, $p = 0.001$; *post hoc*: p = 0.003). By comparison, the volatilome of control aposymbiotic anemones overlapped with those at both high temperatures ($p > 0.05$ for both comparisons).

A total of 148 BVOCs were detected across aposymbiotic anemones at the three temperatures (Fig. 4.4b). Of these, 70 were produced solely at the control temperature, 15 at the sub-bleaching temperature, and 11 at the bleaching temperature. A total of 40 BVOCs were common between aposymbiotic anemones at control and sub-bleaching temperatures – of which 5 were common between sub-bleaching and bleaching temperatures, and 3 were common between control and bleaching temperatures. Only 4 BVOCs were present in all aposymbiotic samples irrespective of thermal treatment and are designated as 'core aposymbiotic compounds': two are aromatic compounds (styrene and (1-methylethyl)-benzene), one has diverse functional groups (3-ethenyl-2-ethoxypyrazine), and one is an unclassified compound (UC45.72).

Exposure to elevated temperature decreased the number of BVOCs detected in the volatilome of aposymbiotic anemones. Aposymbiotic anemones produced 117 BVOCs at 25 °C, the most common of which were aromatic compounds (29); 64 BVOCs were produced at 30 °C, predominantly aromatic compounds; and only 23 BVOCs at 33.5 °C, predominantly unclassified compounds (5; Fig. 4.4c). A total of 14 BVOCs were differentially abundant across temperature treatments in the aposymbiotic anemone volatilome (Table S2, Fig. 4.5a). Only one BVOC, 2,7,10-trimethyldodecane, was differentially abundant between control and heat-stressed anemones (detected in higher abundance at 33.5 °C than either at 25 °C or 30 °C). The other 13 differentially abundant BVOCs were significantly different between 33.5 °C and 30 °C, with 12 detected at higher abundance in the sub-bleaching treatment, including dibromomethane, 1,2-dichloroethane and 2-butanone. The one exception was hexadecane, which was detected in higher quantities at 33.5 °C *vs.* 30 °C. All other differentially abundant BVOCs are listed in Supplementary Table S2.

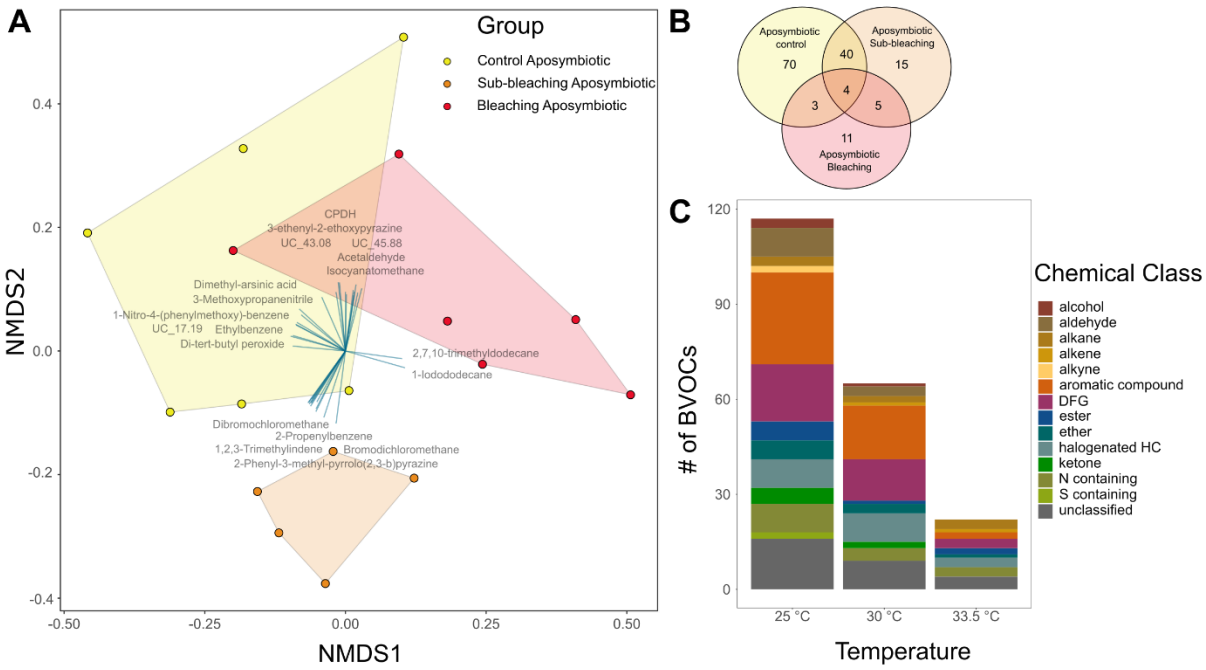

Figure 4.4: Distribution of BVOCs among the volatilomes of aposymbiotic anemones in three thermal treatments (25 °C, 30 °C, 33.5 °C). BVOCs had to be present in at least three biological replicates in at least one thermal treatment to be included. (A) Non-metric multidimensional scaling (NMDS; stress = 0.187) plot of BVOCs produced by aposymbiotic anemones. Displayed BVOCs were chosen based on the top 5 loading scores for each NMDS dimension. Volatilomes for anemones at 30 °C and 33.5 °C were significantly different from one another (p = 0.003); volatilomes for anemones at 25 °C were significant from either of the higher temperature treatments (p > 0.05 for both temperatures). (B) Venn diagram showing presence of 148 BVOCs detected across aposymbiotic anemone dataset. (C) Abundance of BVOCs grouped by chemical class. CPDH = Cyclopropa[3,4]pentaleno[1,2-d][1,3]dioxole, 2a,2b,2c,5a,5b,5c-hexahydro-, (2aa,2ba,2ca,5aa,5ba,5ca)-; DFG = diverse functional group; HC = hydrocarbon; N = nitrogen; S = sulphur.

A total of 14 BVOCs were differentially abundant in response to temperature in the volatilome of aposymbiotic anemones (Table S2, Fig. 4.5a). Only one BVOC, 2,7,10-trimethyldodecane, was differentially abundant between control and heat-stressed anemones. This volatile was detected at higher abundance at 33.5 °C than either the control or medium temperature. The other 13 differentially abundant BVOCs were significantly different between 33.5 °C and 30 °C, with 12 detected at higher abundance in the sub-bleaching treatment, including dibromomethane, 1,2-dichloroethane and 2-butanone. The one exception was hexadecane, which was detected in higher quantities at 33.5 °C *vs.* 30 °C. All other differentially abundant BVOCs are listed in Supplementary Table S2.

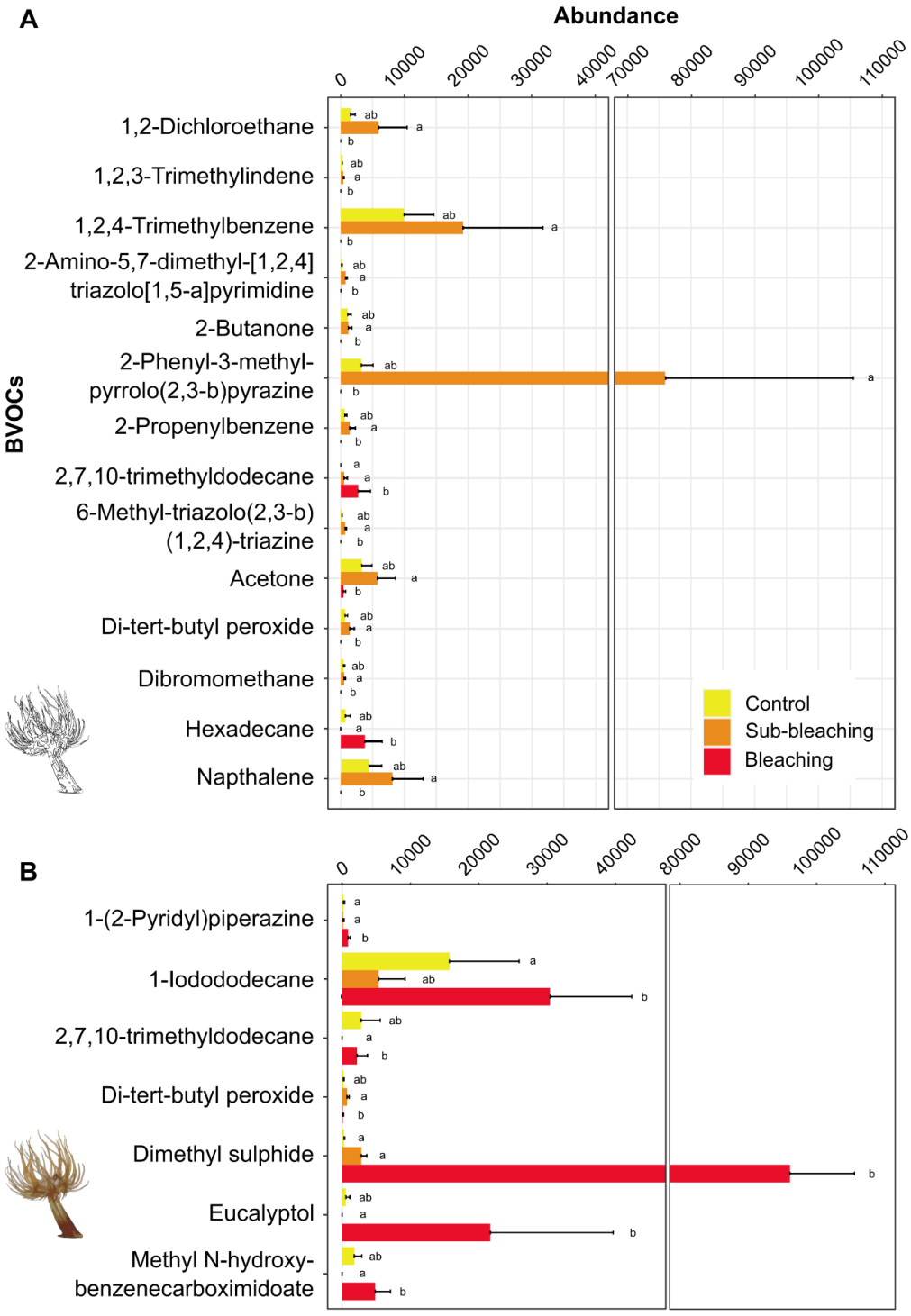

Figure 4.5: Differentially abundant BVOCs (+ standard error) in aposymbiotic anemones in three thermal treatments (control: 25 °C; sub-bleaching: 30 °C; bleaching: 33.5 °C). BVOCs had to be present in at least three biological replicates in at least one thermal treatment to be included in the volatilome of: A) aposymbiotic Aiptasia; and B) symbiotic Aiptasia. Letters beside error bars indicate statistical differences among temperature treatments ($p < 0.05$). Abundance data are dimensionless and were normalised to a chemical standard, seawater blanks and holobiont protein content.

4.4.4 BVOC emissions by symbiotic anemones are also influenced by temperature

Non-metric multidimensional scaling also demonstrated changes in volatilomes produced by symbiotic anemones across temperature treatments (Fig. 4.6a). Anemones exposed to the highest temperature (33.5 °C) produced a volatilome that differed to those for anemones exposed to 25 °C or 30 °C (PERMANOVA $F_{2,14} = 3.25$, $p = 0.001$; *post hoc*, $p = 0.009$ for both comparisons), and where 25 °C and 30 °C volatilomes were largely the same (*post hoc*, $p = 0.987$).

A total of 170 BVOCs were detected in the volatilomes of symbiotic anemones across the three temperature treatments (Fig. 4.6b). Of these, 21 were produced solely at the control temperature, 8 at the sub-bleaching temperature, and 39 at the bleaching temperature. A total of 17 BVOCs were common between symbiotic anemones at control and sub-bleaching temperatures, 16 were common between sub-bleaching and bleaching temperatures, and 21 were common between control and bleaching temperatures. A substantial number of BVOCs (48) were present in the volatilome of all symbiotic anemones – irrespective of temperature treatment – and are therefore designated as 'core symbiotic compounds'. Of these 48 of these core BVOCs, the most abundant chemical group were aromatic compounds (14), followed by halogenated hydrocarbons (8), including dibromomethane, bromodichloromethane, dibromochloromethane and bromochlorodifluoromethane). As observed for the aposymbiotic anemones, temperature influenced the number of BVOCs detected in symbiotic anemones. However, intriguingly – and in contrast to the aposymbiotic anemones – the richest volatilome (124) was detected at the highest temperature (33.5 °C), with aromatic compounds (29) comprising the most abundant compounds. The fewest BVOCs (89) were observed at 30 °C, with the most being aromatic compounds (21), whereas symbiotic anemones at 25 °C produced 107 BVOCs comprised primarily of volatile with diverse functional groups (20; Fig. 4.6c).

Only 7 BVOCs were differentially abundant between symbiotic anemones exposed to the different temperatures (Table S3; Fig. 4.6b). The only BVOC that was more abundant at 30 °C relative to 33.5 °C was di-tert-butyl peroxide. The other six BVOCs were more abundant in anemones exposed to the highest temperature (33.5 °C) compared to 30 °C, and included dimethyl sulphide, eucalyptol, methyl N-hydroxybenzenecarboximidoate, 1-(2-pyridyl)piperazine, 1-iodododecane, and 2,7,10-trimethyldodecane. DMS was also more abundant in the volatilome at 33.5 °C compared to 25 °C.

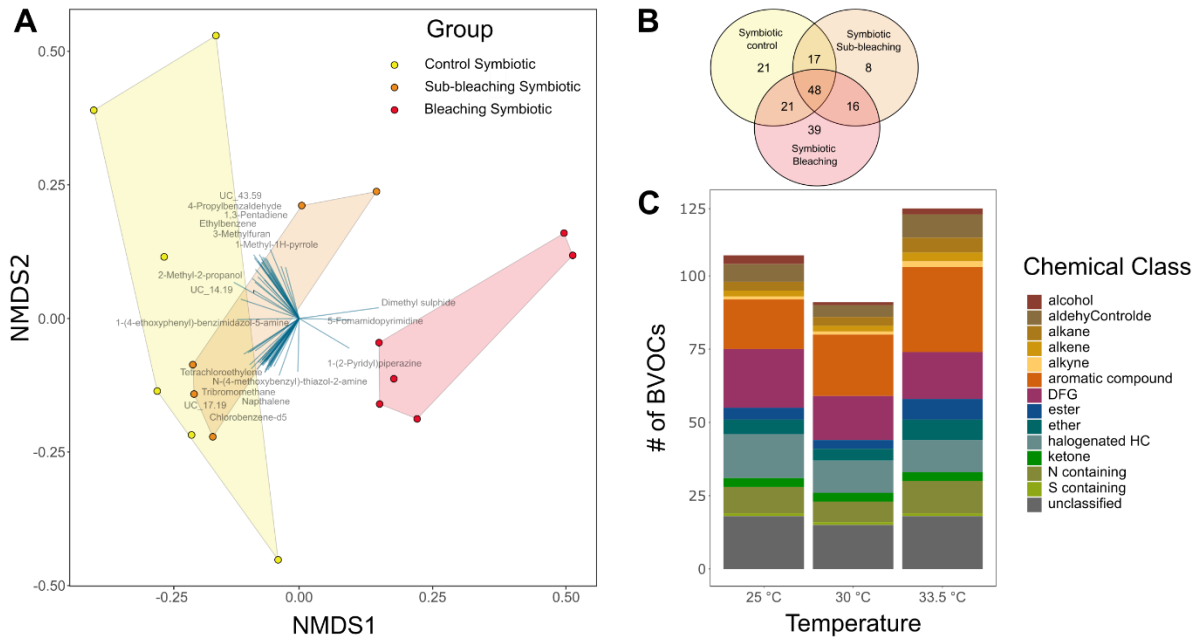

Figure 4.6: Distribution of BVOCs in the volatilomes of symbiotic anemones in response to thermal treatments (25 °C, 30 °C, 33.5 °C). BVOCs had to be present in at least three biological replicates in at least one temperature to be included. (A) Non-metric multidimensional scaling (NMDS; stress = 0.152) plot of BVOCs produced by symbiotic anemones. Displayed BVOCs were chosen based on the top 5 loading scores for each NMDS dimension. Volatilomes for anemones at 33.5 °C were distinct from those at 25 °C and those at 30 °C (p = 0.009 for both temperatures). Volatilomes for anemones at 25 °C were not distinct from those at 30 °C (p = 0.987). (B) Venn diagram showing presence of 170 BVOCs detected across the symbiotic anemone datasets. (C) Abundance of BVOCs grouped by chemical class at each temperature. DFG = diverse functional group; HC = hydrocarbon; N = nitrogen; S = sulphur.

4.5 Discussion

BVOC emissions are increasingly recognised as important physiological agents in themselves as agents of chemical communication (Baldwin et al., 2006), while also providing a method by which to assess organism health using a non-invasive detection (Jud et al., 2018). Corals, and in turn coral reef ecosystems, are at risk of extinction as global temperatures continue to rise and become more variable (Boström-Einarsson et al., 2018; Hughes et al., 2018). As coral reefs are major producers of BVOCs (Swan et al., 2016; Jones et al., 2018; Lawson et al., 2021), and BVOCs have shown promise in other systems to provide a non-invasive snapshot into organism physiology (Mansurova et al., 2018), BVOC analysis provides a promising avenue with which to understand how coral reef metabolism is altered in response to climate change. To date, the volatilomes of cnidarians have been retrieved from a single heat-stress experiment on corals (Lawson et al., 2021) and from anemones across symbiotic states (Chapter 2), though this study did not explore how cnidarian volatilomes are altered in response

to thermal stress under different symbiotic states. The purpose of this study was therefore to characterise BVOC production under thermal stress by the Aiptasia model system, both in symbiosis and when aposymbiotic.

4.5.1 Aiptasia microbiome is affected by temperature

Both aposymbiotic and symbiotic anemones exhibited a restructuring of their microbiome between 25 °C and 33.5 °C, with an intermediate microbiome state at 30 °C. Such an outcome is consistent with the hypothesis that cnidarian microbiomes can shift in response to changing environmental conditions (Ahmed et al., 2019; Pootakham et al., 2019; Hartman et al., 2020), while it is likely the case that the altered host metabolism has altered exudate release, thus selecting for different microbial associates. Microbiomes are well described in terrestrial systems (Averill et al., 2022) such as those associated with arthropods (Esposti & Romero, 2017) and that associated with human skin (Byrd et al., 2018), as well as in coral communities (Hernandez-Agreda et al., 2017; Oppen & Blackall, 2019). Bacteria additionally produce BVOCs on their own (De Vrieze et al., 2015; Ebadzadsahrai et al., 2020), and consume BVOCs as a carbon source to support their own metabolism (Kramshøj et al., 2018; Albers et al., 2018). While it is currently unknown which BVOCs are synthesised or consumed by these microbes, it is important to keep in mind that they undoubtedly influence the volatilomes described here.

The aposymbiotic Aiptasia microbiome shifted in dominance with bacteria in the class Gammaproteobacteria at 25 °C to bacteria in the family Vibrionaceae at 33.5 °C. Multiple species in the family Vibrionaceae have been identified as pathogens of corals; for example, *V. shilonii* has been shown to cause seasonal bleaching in *Oculina patagonica* (Rosenberg & Falkovitz, 2004), and *V. coralliilyticus* causes bleaching and tissue lysis in *Pocillopora damicornis* (Y. Ben-Haim & Rosenberg, 2002). Considered opportunistic pathogens, endemic populations of *Vibrio* spp. become capable of overwhelming host defences during periods of environmental stress, quickly leading to *Vibrio* proliferation and tissue deterioration (Munn, 2015). The observed shift towards a higher proportion of Vibrionaceae at higher temperatures is perhaps indicative of the susceptibility of anemones to disease at higher temperatures. At lower temperatures in contrast, these opportunists were limited in a community characterised by high microbial diversity; indeed, a decrease in bacterial community diversity is a precursor to the onset of disease in corals (Pollock et al., 2019). Additionally, there is evidence of *Vibrio* sp. infection altering metabolic cycling of infected individuals (Tan et al., 2021), thus it is plausible that *Vibrio* induced infection in anemones alters their metabolism and thus influences BVOC output. A shift in microbial composition was also observed for symbiotic anemones across temperatures, from dominance by bacteria from the family

Flavobacteriaceae at 25 °C to bacteria from the class Gammaproteobacteria at 33.5 °C. It may be that this community shift signifies a precursor to a disease state, but at this broad taxonomic resolution it is impossible to have certainty. Further work will also be needed to uncover the potential role of Gammaproteobacteria in the production, or consumption, of BVOCs observed at this temperature.

4.5.2 Core BVOCs

Between symbiotic states and across temperature treatments, 3 BVOCs were common to all Aiptasia samples across all temperatures: (1-methylethyl)-benzene, 3-ethenyl-2-ethoxypyrazine and styrene. None of these core compounds were detected at differentially abundant concentrations between temperature treatments, suggesting that they play an important role in central metabolism. One unclassified BVOC, UC45.72, was identified as a core BVOC in aposymbiotic anemones, but it was only present in symbiotic anemones when undergoing bleaching. This may suggest that this BVOC is produced in response to stress. Indeed, upregulation of pathways for mediating oxidative stress and apoptosis in aposymbiotic Aiptasia has been reported (Rodriguez-Lanetty et al., 2006; Oakley et al. 2016; Gorman et al., 2022), with the suggestion that symbiosis somehow protects the host from oxidative stress and cellular damage (Oakley et al. 2016). Oxidative stress is a well-known feature of coral bleaching (reviewed by Oakley and Davy 2018), so the production of UC45.72 in the volatilome of thermally-stressed anemones could conceivably have arisen from the direct impacts of high temperature and/or the marked loss of symbionts and hence a shift towards a state closer to that of aposymbiosis. Either way, this unclassified BVOC is an important candidate for future study.

Interestingly, only 4 BVOCs were detected consistently in the volatilome of the aposymbiotic anemones irrespective of thermal treatment, while for symbiotic anemones, 48 BVOCs met this criterion. This perhaps indicates greater physiological stability of symbiotic anemones than aposymbiotic ones, once again suggesting that symbiosis may confer a degree of protection to the host, and the holobiont as a whole. In particular, given that aposymbiotic anemones are already under a degree of cellular stress (Oakley et al. 2016), additional environmental stress may cause a more rapid shutdown of central metabolism, hence releasing fewer BVOCs as by-products. By contrast, despite previously observed transcriptomic, proteomic and metabolomic impacts of thermal stress on symbiotic cnidarians (Desalvo et al., 2010; Hillyer et al., 2017b; Oakley et al., 2017; Cziesielski et al., 2018), the relative consistency in the volatilome at high temperature observed here suggests that central metabolism is altered less dramatically than in the aposymbiotic state. What the underlying cellular processes are for this, and how the different members of the holobiont interact to facilitate metabolic homeostasis, are interesting questions for future research.

4.5.3 Temperature affects the Aiptasia volatilome

4.5.3.1 Aposymbiotic anemones

In the aposymbiotic state, progressively higher temperatures generate increasingly diminished volatilome richness, with a consistent core volatilome of just 4 BVOCs. As seen in other organisms (e.g., Atkin and Tjoelker, 2003), respiration rate in aposymbiotic anemones increased at the highest temperature. As respiration and metabolism are intrinsically linked processes (Weis, 2014), an observation of increased metabolism, and therefore BVOC output, may be expected. My results show the contrary, that there was a decline in BVOC richness at 33.5 °C which may indicate the onset of metabolic dysregulation, as has been observed in stressed and bleaching corals (Roach et al., 2021; Sun et al., 2022). Although it is unclear which (if either) of these processes is reflected in the observed decline in BVOC richness, it may be that this observation is a reflection of stress or bleaching cascades. It is, however, important to exercise some caution when drawing such conclusions, as nothing is known about the physiology of the associated microbiome (see above).

Fourteen BVOCs were differentially expressed in the volatilome of aposymbiotic anemones in response to thermal change. Interestingly, twelve of these were detected at higher abundance at the sub-bleaching temperature of 30 °C relative to the bleaching temperature of 33.5 °C. The most abundant BVOCs in the volatilome of aposymbiotic anemones at elevated, but sub-bleaching temperature (30 °C), are known to have diverse functions in other systems and include: naphthalene and dibromomethane. Naphthalene is produced by termites (Chen et al., 1998; Wilcke et al., 2000), inhibiting the growth of pathogenic fungi and so forming part of the termite's defence system (Wright et al., 2022). Naphthalene has also been shown to act as an apoptosis inhibitor in the model nematode *Caenorhabditis elegans*, suggesting that it may promote the survival and proliferation of tumour cells (Kokel et al., 2006). Dibromomethane has been shown to induce metamorphosis in the mollusc *Crepidula fornicata* (Taris et al., 2010). These functions all suggest that these BVOCs have the potential to also play a role in cellular signalling or restructuring in Aiptasia and other symbiotic cnidarians, though this of course requires detailed confirmation.

Just two BVOCs, hexadecane and 2,7,10-trimethyldodecane, were produced more by anemones at 33.5 °C than 30 °C. Given the physiological stress that occurs in Aiptasia at such a high temperature, as seen both in the current study and previous work (e.g., Hawkins et al., 2013; Hillyer et al., 2016; Oakley et al., 2017; Cleves et al., 2020), it is very possible that these BVOCs are downstream products of stress pathways. Moreover, hexadecane has been previously shown to induce cell death in both rat and human cell cultures (Herman et al., 2012). Apoptosis is thought to be an important

cellular end-point of the bleaching cascade (Weis 2008; Oakley and Davy, 2018), so this compound could play a comparable role here.

4.5.3.2 Symbiotic anemones

In contrast to aposymbiotic anemones, the volatilome of symbiotic anemones appeared more stable in response to thermal change, with the core volatilome consisting of 48 BVOCs and there being just 7 BVOCs that were differentially abundant at the different temperatures. The volatilome was the most diverse at the highest temperature, suggesting a proliferation of metabolic downstream products associated with cellular dysfunction in the host, symbionts, and /or altered microbiome metabolism. It is impossible from this approach to determine which member of the holobiont was responsible for each BVOC, or how the metabolism of various partners interacts under stress, but future work with isolated partners (e.g., axenic Symbiodiniaceae) would help to shed some light on this matter. In contrast, the volatilome was least diverse at the intermediate, sub-bleaching temperature. One interpretation of this is that metabolic integration of the holobiont was most efficient at this temperature resulting in the release of fewer BVOCs to the environment. It is somewhat surprising that this would be the case at 30 °C rather than 25 °C, but it should be noted that there were no obvious signs of physiological stress at 30 °C in this study.

Of the 7 differentially abundant BVOCs in symbiotic anemones, most were detected at higher abundance at 33.5 °C than at 30 °C (5 compounds) or control (1 compound). Of these BVOCs, dimethyl sulphide (DMS) and eucalyptol have roles as antioxidants in other systems (Guan et al., 2017; Xu et al., 2021), suggesting that their production may be involved in the amelioration of free radicals or toxic metabolic by-products in the current system too. The sources of these various compounds (i.e., host, symbiont or microbiome) are hard to determine, but it is notable that 2,7,10-trimethyldodecane was also conspicuous in the volatilome of heat-stressed aposymbiotic anemones, so it could well originate from the host or common members of the microbiome. Only one BVOC was more abundant at 30 °C than 33.5 °C: di-tert-butyl peroxide. This suggests that this compound is involved in, or is a by-product of, a fully functional symbiosis, though why it was more abundant at the intermediate temperature than the control temperature is unclear.

Notably, DMS was the BVOC produced in highest abundance by symbiotic anemones, at 33.5 °C. This is a widely studied and multi–functional BVOC with established roles in the global sulphur cycle (Brimblecombe, 2013), and an ability to modulate an organism's response to stress (Sunda et al., 2002). At the cellular level, dimethylsulphoniopropionate (DMSP) and its breakdown products (DMS,

acrylate, dimethyl sulphoxide and methane sulphinic acid) act as a powerful antioxidant system (Sunda et al., 2002; Hopkins et al., 2016), and have been shown to increase in both corals (Hopkins et al., 2016a; Lawson et al., 2021) and Symbiodiniaceae (Deschaseaux et al., 2014) in response to thermal stress. This pattern of DMS production can be considered in the context of physiological stress, which was apparent at the highest temperature with respect to increased holobiont respiratory rate, and decreased symbiont density. Such physiological changes typically lead to metabolic shifts in both the host and symbiont, for example to meet nutritional losses by mobilising carbohydrate and lipid stores (Hillyer et al., 2017a; Hillyer et al., 2017b). Furthermore, high temperature can induce cellular oxidative stress and, potentially, the upregulation of immunity and inter-partner signalling pathways involved in symbiosis dysfunction (Dunn et al., 2007; Krueger et al., 2015; Hillyer et al. 2017b). In symbiotic Aiptasia, it therefore seems most likely that the observed increase in DMS at high temperature acts to ameliorate ROS produced in response to thermal stress (Perez & Weis, 2006). Although the total number of symbionts in the holobiont decreased at 33.5 °C, DMS production was higher such that the remaining symbionts would have produced much more DMS *per* cell than at lower temperatures. Perhaps the ability to produce copious amounts of DMS is a particularly advantageous trait during periods of thermal stress, and individual symbiont cells with the ability to produce DMS in abundance are more likely to stay in symbiosis due to the crucial antioxidant role that DMS provides. A similarly protective role could be provided by eucalyptol, which was also most abundant here at the highest temperature. This volatile has demonstrated antioxidant (Kennedy-Feitosa et al., 2016), anti-inflammatory (Kim et al., 2015), and anti-bacterial (Li et al., 2016) properties in mammal models, though it can also induce DNA damage in mammalian cells (Dörsam et al., 2015). While the identification of these BVOCs in other systems provides a framework from which to explore their role in cnidarian-dinoflagellate symbiosis, much more work is needed to elucidate their function in the breakdown of this symbiosis.

4.6 Conclusion

Elevated temperature impacts both the microbiome and volatilome of both aposymbiotic and symbiotic anemones. The largest temperature-induced response was observed in aposymbiotic anemones, suggesting that the presence of Symbiodiniaceae provides a stabilising effect on holobiont function, at least until the upper thermal threshold is reached. Much more work is now needed to identify all BVOCs, and the sources and functions of BVOCs within the symbiosis. Crucial next steps will be to characterise the volatilome of the host cnidarian, dinoflagellate symbionts and associated microbes in true isolation from one another, and to elucidate the biosynthetic pathways involved. Nevertheless, the observations here provide a first step towards understanding how heat stress, and corresponding microbial shifts, can influence BVOC emissions across symbiotic states. This work has

additionally identified various BVOCs (e.g., DMS, eucalyptol) that may serve as non-invasive biomarkers for thermal and cellular stress and provides a foundation from which to understand the ways in which coral reef ecosystems may shift in response to climate change.

Chapter 5 Chemotactic responses to DMSP vary among Symbiodiniaceae species

5.1 Abstract

Central to the success of reef building corals is the symbiotic relationship between cnidarians and dinoflagellate microalgae. Whilst these microalgae can proliferate within the host tissues, cells are typically acquired anew, enabling plasticity to environmental change, and including stress events that results in coral bleaching. As such, the ability for these microalgal symbionts to find prospective hosts is a crucial first step for the onset of this relationship. While this process of symbiont acquisition is crucial for symbiosis establishment, little is known about this process, but chemotaxis is hypothesised as a means by which symbionts locate prospective hosts. Here, I describe a method by which to assess chemotaxis in Symbiodiniaceae, using *in situ* chemotactic assays (ISCAs). I describe the differing chemotactic responses of four Symbiodiniaceae species to a variety of prospective chemoattractants, and ultimately focused on chemotactic response to dimethylsulphoniopropionate (DMSP), a pervasive metabolite in the ocean. I found that *Breviolum minutum* was repelled by DMSP, while *Cladocopium* sp. exhibited no chemotactic response to this molecule. These differing responses by distinct species of Symbiodiniaceae may reflect differing chemical cues used by Symbiodiniaceae to locate and ultimately establish symbiosis with new cnidarian hosts. Additionally, halogenated methane BVOCs identified in the volatilomes of aposymbiotic Aiptasia in Chapter 2, bromodichloromethane and diiodomethane were tested for chemotactic response by *B. minutum* and *Cladocopium* sp. I didn't observe a chemotactic response by either species to these halogenated methane's, indicating that another chemical cue is responsible for attracting symbionts to a prospective host.

5.2 Introduction

The endosymbiotic association between cnidarian hosts and dinoflagellates from the family Symbiodiniaceae is central to the growth and survival of reef-building corals (Davy et al., 2012). This association allows for the recycling of limited nutrients and metabolites in an oligotrophic environment, ultimately powering corals' calcium carbonate accretion and building the framework that supports one of the most productive ecosystems on Earth (Muscatine & Porter, 1977; Hatcher, 1997). Symbiodiniaceae taxa are genetically diverse, comprised of multiple genera and species (LaJeunesse et al., 2018; Nitschke et al., 2020, 2022; LaJeunesse et al., 2022) that display remarkable phenotypic diversity with respect to a variety of traits including motility (Yamashita & Koike, 2016), thermal tolerance (Russnak et al., 2021), and compatibility with cnidarian hosts (Suggett et al., 2017; Schoenberg & Trench, 1980).

An important feature of Symbiodiniaceae is their ability to switch between free-living and symbiotic lifecycles (Maor-Landaw et al., 2020), and also between motile and coccoid (non-motile) cell types during the free-living stage (Yamashita & Koike, 2016). At night, Symbiodiniaceae are benthic and coccoid in morphology (Yamashita & Koike, 2016). Upon mitotic (and potentially meiotic) cell division in the benthos (Figueroa et al., 2021), motile zoospores emerge, during which time cells can forage for heterotrophic sources of food or a host (Yamashita & Koike, 2016). Upon encountering a cnidarian host, these microalgae can enter the gastric cavity through the mouth and are phagocytosed by host endodermal cells (Schwarz et al., 1999). These microalgae can evade digestion by suppressing host immune response (Jacobovitz et al., 2021), and reside within host-derived membranes (i.e., the 'symbiosome') in the cnidarian gastrodermal layer (Wakefield & Kempf, 2001). Once endosymbiotic, host cells acquire photosynthetic products, such as glucose, from Symbiodiniaceae (Burriesci et al., 2012; Hillyer et al., 2017), who in turn receive host waste products, such as carbon dioxide and ammonium, fuelling their photosynthesis (Falkowski et al., 1984).

The ability for prospective symbionts and hosts to find each other is often a crucial first step in the establishment of this symbiosis, as many cnidarians have to acquire their symbionts *de novo* with each generation (Raina et al., 2019). Moreover, following thermal stress, cnidarian hosts may reassociate with Symbiodiniaceae from the surrounding seawater (Buddemeier & Fautin, 1993; Huang et al., 2020; Scharfenstein et al., 2022). Following thermal stress, previously compatible symbionts may be expelled from host tissue, as they are no longer metabolically compatible with the host, allowing re-population with another species of Symbiodiniaceae more suited to the new environment (Stat & Gates, 2011). Forming a successful symbiosis relies on chemical communication (Rosset et al., 2021), with chemotaxis potentially playing an important role in finding a compatible symbiotic partner. Chemotaxis is a widespread trait of microbial cells that allows them to bias their movements in response to a chemical gradient. It is employed by many organisms – such as tube worms, squid, mussels, legumes, insects, protists or phytoplankton – for the acquisition of microbial symbionts from the environment (Raina et al., 2019). Indeed, from the perspective of a microorganism, the ocean is a remarkably heterogeneous habitat (Blackburn et al., 1998; Stocker, 2012), with patches of dissolved organic and inorganic material (Moore & Crimaldi, 2004) arising from the exudation of photosynthates from photosynthetic microorganisms (Seymour et al., 2009; Smriga et al., 2016), cell lysis (Blackburn et al., 1998) or zooplankton faecal pellets (Turner, 2002). Microorganisms can exploit this heterogeneity by sensing chemicals in their environment and swim towards or away from cues based on their potential utility.

Symbiodiniaceae are also thought to use chemical cues to find their hosts (Fitt, 1984; Takeuchi et al., 2017). For example, several Symbiodiniaceae species have been shown to be attracted to chemicals released by coral larvae (Yamashita et al., 2014), sea anemones and jellyfish (Fitt, 1984). However, while these studies suggest that chemotaxis is a likely mechanism by which symbionts find future cnidarian hosts, with Fitt (1984) suggesting that excretory ammonium could play a role, the responsible chemical cues remain uncertain. Indeed, I speculate that a universal signal molecule attracting Symbiodiniaceae to their host may not exist, but that instead different Symbiodiniaceae respond to different molecules, aiding their navigation towards metabolically compatible hosts.

Dimethylsulphoniopropionate (DMSP) is a well-studied sulphur compound produced in huge quantities by marine organisms (~ 10^9 tonnes annually, worldwide; Curson et al., 2011). Coral reefs are a hotspot for the production of DMSP, with microalgae (including Symbiodiniaceae), coral associated bacteria (Kuek et al., 2022), macroalgae (Van Alstyne & Puglisi, 2007), and corals (Raina et al., 2013) all producing this molecule. DMSP is an important signalling molecule attracting bacteria (Miller et al., 2004; Zimmer-Faust et al., 1996), fish (Foretich et al., 2017) and birds (Nevitt & Bonadonna, 2005). Additionally, I hypothesised that BVOCs produced by anemones may play a role in attracting Symbiodiniaceae to a prospective host; halogenated methane's, bromodichloromethane and diiodomethane, identified in Chapter 2 were examined for chemotactic response. Here, I quantified the chemotactic response of different Symbiodiniaceae species to DMSP, bromodichloromethane and diiodomethane *via* a microfluidics chemotaxis assay (Lambert et al., 2017), to determine whether this molecule could allow free-living symbionts to locate prospective cnidarian hosts. I hypothesised that different Symbiodiniaceae species would exhibit different chemotactic responses to DMSP, DMS, bromodichloromethane, and diiodomethane, thus elucidating a mechanism that could ultimately contribute to the specific host-symbiont partnerships seen on reefs.

5.3 Methods

5.3.1 Experimental organisms – Algal cultures

Due to COVID travel restrictions, this study was carried out in two parts, in different countries. The first part was performed in Australia at the University of Technology, Sydney (UTS); the second part was performed in New Zealand at Victoria University of Wellington (VUW). Collectively, all experimental work performed at UTS is referred to as 'Experiment 1'; work performed at VUW collectively referred to as 'Experiment 2'. Individual experiments are referred to as 'trials'.

A first set of experiments (hereafter, 'Experiment 1') were performed on four species of Symbiodiniaceae cultured in Diago's IMK medium for Marine Microalgae (Nihon Pharmaceutical Co. Ltd, Tokyo, Japan) as *per* Suggett et al (2015) at 25 °C under irradiance of 85 ± 15 µmol photons $m^{-2}s^{-1}$ (Philips TLD 18W/54 fluorescent tubes, 10,000 K) set to a 12 h : 12 h light :dark cycle. The cultures either originate from the collection of Robert Trench (RT; Trench & Blank, 1987; LaJeunesse, 2017), or the Australian Institute of Marine Science Symbiont Culture Facility (SCF): *Symbiodinium microadriaticum* (ITS2 majority sequence A1, culture ID: RT61), *Breviolum minutum* (ITS2 majority sequence B1, culture ID: RT2), *Cladocopium C1*^{acro} (ITS2 majority sequence C1, culture ID: SCF058-04) and *Durusdinium trenchii* (ITS2 majority sequence D1a, culture ID: SCF082).

A second set of experiments (hereafter, 'Experiment 2') were performed on three species of Symbiodiniaceae grown in 35 ppt 0.22 µm filtered seawater (FSW) enriched with f/2-medium (AusAqua Pty Ltd., SA, Australia) and maintained at 25 °C in a climate-controlled incubator. Cultures were grown under fluorescent lamps (Osram Dulux 36/W890 fluorescent bulbs) at approximately 70 µmol photons $m^{-2}s^{-1}$ on a 12 h : 12 h light : dark cycle. The following cultures were used: *Breviolum minutum* (ITS2 type B1, culture ID FLAp2), *Cladocopium* sp. (ITS2 type C1, culture ID LHI-33), and *Durusdinium trenchii* (ITS2 type D1a, culture ID Ap2).

Two weeks prior to experimentation, cultures for chemotaxis assays were diluted with fresh medium to maintain the state of exponential growth. All trials in Experiment 2 were performed in low-nutrient medium – since previous work has shown that high nitrogen levels in the surround medium decreased the ability of Symbiodiniaceae cells to respond chemotactically to nitrogen levels (Fitt, 1985). Additionally, preliminary trials (Fig. S5.4) suggested that Symbiodiniaceae responded to my established positive control, tryptone, more strongly being in low nutrient media. All trials in Experiment 2 were thus performed in low-nutrient media – cultures were transferred to 50 mL Falcon tubes and centrifuged at 500 × g for 5 min. Supernatant was removed and refreshed with fresh sterile artificial seawater (ASW: Aquaforest Marine sea salt dissolved in distilled water to 35 ppt) 5 days prior to chemotaxis experiments. These steps were performed at night during the non-motile portion of the Symbiodiniaceae lifecycle, as confirmed by light microscopy (Zeiss, Germany; 10× magnification).

5.3.2 Maximum quantum yield of Photosystem II

Measurements of the maximum quantum yields of Photosystem II light harvesting were used to verify if the health of Symbiodiniaceae cells transferred from f/2 media to low nutrient conditions was compromised (Suggett et al., 2009) for *B. minutum*, *Cladocopium* sp., and *D. trenchii* (Table S1). Cultures were dark acclimated in the Falcon tubes in which they were reared for 15 min before measurement with an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3, damping = 3). Equivalent quantum yield measurements (ANOVA; $F_{2, 12} = 0.255$, $p = 0.779$) among species ensured that short-term nutrient deprivation had not compromised photosynthetic health (Table S1).

5.3.3 Confirmation of symbiont identity

Genomic DNA of each Symbiodiniaceae cell culture was extracted using a CTAB/phenol-chloroform protocol (Baker & Cunning, 2016). PCR was performed on extracted DNA using primers ITSD (fwd, 5'- GTGAATTGCAGAACTCCGTG-3') and ITS2rev2 (rev, 5'-CCTCCGCTTACTTATATGCTT-3'). The PCR amplicons were purified, and Sanger sequenced (Macrogen, Seoul, Korea). Sequence results were checked for quality with Geneious Prime v. 2019.2.3 (Biomatters Ltd., Auckland, NZ), and then BLAST searched against databases GeoSymbio and NCBI to identify the majority ITS2 sequence.

5.3.4 Chemotaxis assays

The *in situ* chemotaxis assay (ISCA) (Lambert et al., 2017) was used to investigate the chemotactic abilities of *S. microadriaticum*, *B. minutum*, *Cladocopium C1^{acro}*, *Cladocopium* sp. and *D. trenchii* (Lambert et al., 2017). This polycarbonate microfluidic device contains 20 wells, each with a 110 µL volume and 0.8 mm diameter port linking the well to the outside environment. Experiments were conducted with a minimum of four ISCA replicates deployed in parallel, with each ISCA containing five technical replicates of four different treatments (Fig. S5.1, S5.2). Putative chemoattractants were dissolved in algal growth medium and inoculated into wells; each ISCA was then immersed in algal growth medium containing actively swimming Symbiodiniaceae cells. Putative chemoattractants can diffuse out of wells from the port, creating concentration gradients that chemotactic cells can respond to, migrating into, or away from the wells. Chemotaxis assays were carried out according to previously published protocols (Clerc et al., 2020) with some specific modifications outlined below.

All experiments were performed in the dark, to minimise potential confounding effects from phototaxis (Aihara et al., 2019; Hollingsworth et al., 2005), during the motile portion of the algal cells' lifecycle, as determined by light microscopy (Zeiss, Germany; 10× magnification). Every ISCA contained negative control wells loaded with spent algal growth medium. These negative control wells accounted for the effect of random motility, which would cause a small number of Symbiodiniaceae cells to enter wells even in the absence of chemical gradients. The spent medium was prepared by filtering out Symbiodiniaceae cells < 1 hr prior to chemotaxis assays (pore size: 5 µm). Chemotaxis assays were performed by securing ISCAs loaded with putative chemoattractants inside sterile plastic trays, followed by the slow addition of 80 mL of Symbiodiniaceae cell suspension to the trays and an incubation of 20 min in the dark. At the end of the incubation period, cell suspension was removed slowly with a pipette and the ISCA well contents were collected using 1 mL syringes and 27G needles (Terumo, Sydney, Australia). Within each ISCA, the contents of five wells were pooled and fixed with 0.2 µm-filtered glutaraldehyde (2% final concentration) for flow cytometry analysis (Experiment 1). Samples for flow cytometry were analysed on a CytoFLEX (model LX and S) flow cytometer (Beckman Coulter, Brea, CA, USA) with filtered MilliQ water as sheath fluid. For each sample, forward scatter (FSC), side scatter (SSC), and red fluorescence (650 nm; targeting chlorophyll autofluorescence) were recorded; samples were analysed at a flow rate of 25 µL min^{-1}. Samples derived from Experiment 2 were fixed with 37% formalin (4% final concentration) and counted using cells' autofluorescence on a high throughput confocal microscope (HTCM). Samples were pipetted into a 384-well plate (Perkin Elmer Ultra, USA) and imaged by the HTCM (n = 10 wells *per* sample, 20 µL *per* well; IN Cell Analyzer 6500 HS, GE Healthcare, USA; settings: magnification ×10, excitation 642, emission Far Red 488, exposure 40 ms). Images were analysed using IN Carta Image Analysis Software (GE Healthcare, USA; settings 72% sensitivity, 10 µm minimum diameter) and validated using Image J (Abràmoff et al., 2005). In ImageJ, images were processed as batches, using a macro with the following settings: 'threshold was set to 'moments dark no-reset'; black background was enabled, and particle analysis was set as size = 10 µm-infinity'. Preliminary trials (Experiment 1) used non-randomised treatments within each ISCA with chemoattractants in rows. Subsequent experiments (Experiment 2) were performed again using randomisation and the same trends were found between randomised and non-randomised ISCAs (Fig. S5.2).

To ensure that Symbiodiniaceae were chemotactically active in every experiment, I tested a range of molecules (Experiment 1) to act as a positive control for use in all subsequent experiments. Specifically, I tested mannitol (sugar alcohol) at 10^{-4} M, glutamate (amino acid) at 10^{-4} M, and tryptone (mixture of small peptides; Condalab, Madrid) at 20% concentration. All these chemicals were chosen based on the attraction of other microorganisms towards sugars (Adler et al., 1973),

amino acids (Yang et al., 2015) and peptides (Govorunova & Sineshchekov, 2005). Two halogenated methane compounds, bromodichloromethane ($BrCl_2CH$) and diiodomethane (I_2CH_2), based on their presence in volatilomes of aposymbiotic anemones in my earlier work (Chapter 2), were also trialled at 10^{-4} M. Of these chemicals, tryptone was the only one to elicit a chemotactic response across all the species tested (Fig. 5.1) and was therefore selected as a positive control for the remainder of the experiments. The primary compound of interest tested in this study was DMSP; 10^{-4} M was the primary concentration used for DMSP and DMS since this concentration had been previously shown to induce chemotaxis in other microalgae (Seymour et al., 2010).

5.3.5 Chemotactic index and statistical analyses

The chemotactic index (*Ic*) was determined by dividing the number of Symbiodiniaceae cells present in each sample by the averaged Symbiodiniaceae counts in the corresponding negative control samples. A positive and significant ($p < 0.05$) *Ic* indicates attraction towards a chemical, while a negative *Ic* value indicates repulsion to a given chemical.

Statistical analyses were performed in RStudio (version 4.0.3; R Core Team, 2020) using the rstatix package (Kassambara, 2020). All results were tested for homogeneity of variance using the function "levene_test". A Shapiro-Wilk's test was performed to test normality using the function "shapiro_test". A linear model was fitted using the function "lm" (stats v. 4.0.3) and a one-way analysis of variance (ANOVA) was performed using the function "aov"; *post hoc* testing was performed with a Tukey test using the function "TukeyHSD". In cases where assumptions were not met, pairwise Wilcoxon non-parametric tests were performed using the function "pairwise.wilcox.test".

5.4 Results

5.4.1 Identification of a positive control and trials of volatile compounds – Experiment 1

B. minutum and *C. C1acro* were both significantly attracted to tryptone; *B. minutum* had 2.1× more cells in the tryptone wells, and *C. C1*^{acro} had 6.24× more cells in the tryptone wells (Wilcoxon test, $p < 0.05$ for both species; Fig. 5.1, Table S2). *B. minutum* and *C. C1acro* exhibited different behavioural responses to both dimethylsulphoniopropionate (DMSP) and dimethyl sulphide (DMS; Fig. 5.1). Indeed, *B. minutum* was significantly repelled by DMSP and DMS (Wilcoxon test, $p < 0.005$; Table S3), with 1.9-times fewer cells in the DMSP treatment and 2.48-times fewer cells in the DMS treatment compared to negative controls. In contrast, *C. C1*^{acro} exhibited no significant response to

these chemicals (Wilcoxon test, $p < 0.05$; Table S3). There were no significant responses to the volatiles bromodichloromethane or diiodomethane in *B. minutum* or *C. C1^{acro}*, or to sugar alcohol mannitol in *B. minutum* or amino acid glutamate in *C. C1^{acro}*, *D. trenchii* or *S. microadriaticum*; both of these chemicals were trialled as positive controls.

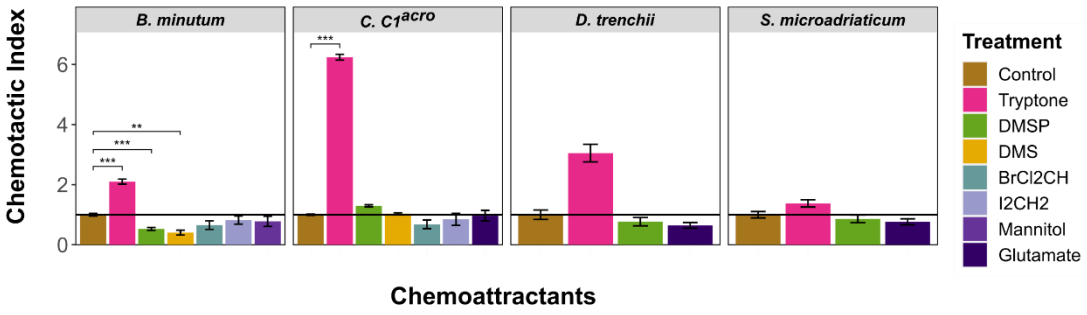

Figure 5.1: Chemotactic indices of putative chemicals for use as positive control across four species of Symbiodiniaceae: *Breviolum minutum*, *Cladocopium C1acro*, *Durusdinium trenchii* and *Symbiodinium microadriaticum*. Values represent mean +/- standard error; n = 4-28, see Table S2.

5.4.2 Chemotactic response to tryptone and DMSP under low nutrient conditions in three species of Symbiodiniaceae

Under low nutrient conditions, *B. minutum*, *Cladocopium* sp. and *D. trenchii* continued to exhibit positive chemoattraction to tryptone (Fig. 5.2). *Cladocopium* sp. exhibited the strongest positive response to tryptone, with 99.8-times more cells in the tryptone condition compared to negative control wells. *D. trenchii* had 4.64-times more cells in tryptone than controls, while *B. minutum* had 1.73-times more cells in tryptone than controls (Wilcoxon test, $p < 0.05$ for all comparisons; Table S5). *B. minutum* was the only species to again exhibit negative chemotaxis away from DMSP, with 3.45-times fewer cells in DMSP than control wells (Wilcoxon test, $p < 0.05$; Table S5) while neither *Cladocopium* sp. nor *D. trenchii* exhibited a significant response to DMSP (Wilcoxon test, $p > 0.05$; Table S5).

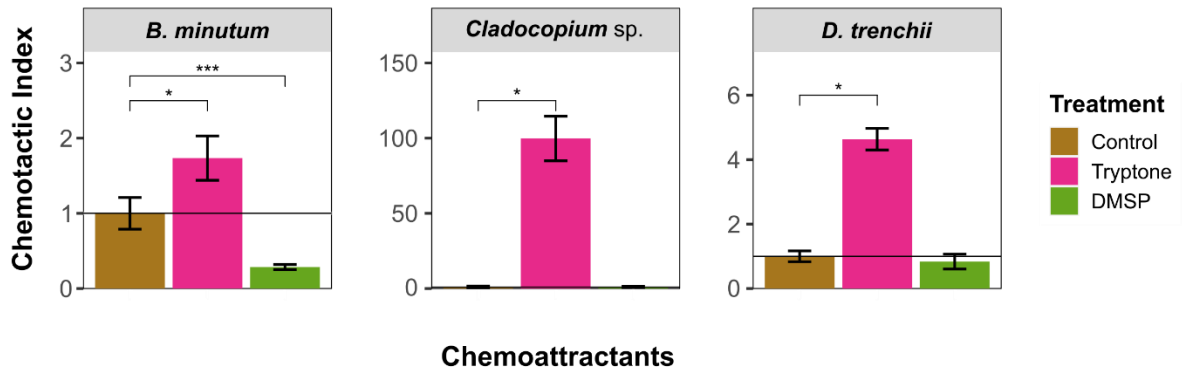

Figure 5.2: Chemotactic indices (+/- SE, n = 8 for *B. minutum*, n = 4 for *Cladocopium* sp. and *D. trenchii*) of *Breviolum minutum*, *Cladocopium* sp. and *Durusdinium trenchii* in low nutrient growth media. Statistical significance denoted by asterisks: * <0.05; ** <0.01; *** <0.001. Values represent mean +/- standard error; n = 8 for *B. minutum*, n = 4 for *Cladocopium* sp. and *D. trenchii*.

5.4.3 Chemotactic response to different concentrations of DMSP in B. minutum and Cladocopium sp.

Under low nutrient conditions, *B. minutum* and *Cladocopium* sp. exhibited different behavioural responses to DMSP (Fig. 5.3). At all concentrations tested, *B. minutum* was significantly repelled by DMSP (Tukey *post hoc* test, $p < 0.05$; Table S6), with 5.2-times, 2.7-times and 3.3-times fewer cells in the DMSP treatment relative to the negative controls at 10^{-3} M, 10^{-4} M and 10^{-5} M, respectively. In contrast, *Cladocopium* sp. exhibited no significant response to DMSP at any concentration (Wilcoxon test, $p > 0.05$ for all comparisons; Table S6).

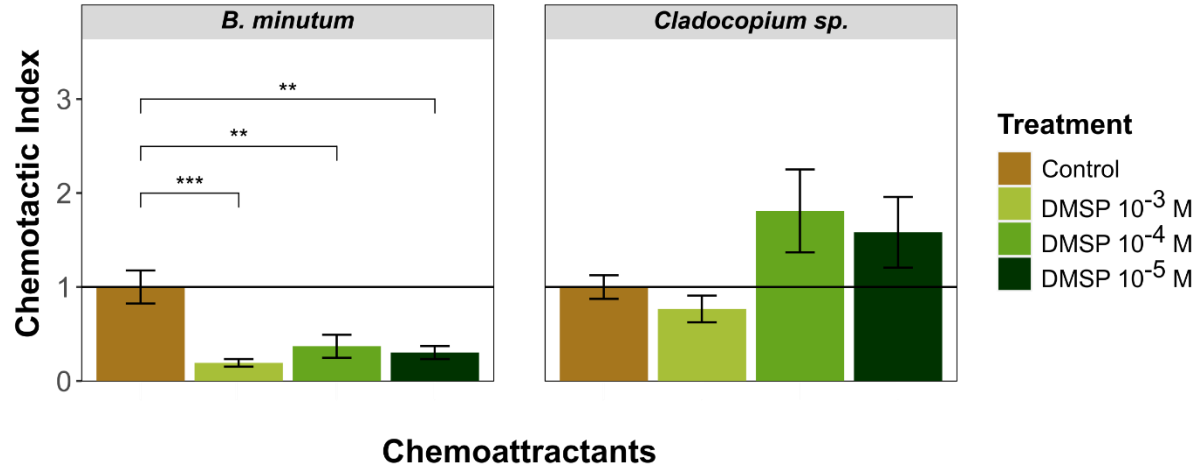

Figure 5.3: Chemotactic indices (+/- SE; n = 5 for *Breviolum minutum*, n = 16 for *Cladocopium* sp.) testing different concentrations of DMSP in *Breviolum minutum* and *Cladocopium* sp. Statistical significance denoted by asterisks: * <0.05; ** <0.01; *** <0.001. Values represent means +/- standard error; n = 5 for *B. minutum* and n = 16 for *Cladocopium* sp.

5.5 Discussion

Mechanisms by which corals acquire algal symbionts have not been fully elucidated; however, here I hypothesize that symbiont chemotaxis towards prospective hosts' is a crucial first step. I describe a technique to measure the chemotactic response of Symbiodiniaceae using *in situ* chemotactic assays (ISCAs), initially designed for use with bacteria (Lambert et al., 2017). I have demonstrated the utility of this technique for use with Symbiodiniaceae, identified tryptone as a positive control, and evaluated the response of cultured Symbiodiniaceae to dimethylsulphoniopropionate (DMSP), a pervasive metabolite in the marine environment. Comparison between *Breviolum minutum* and other species of Symbiodiniaceae revealed different chemotactic responses: *B. minutum* was repelled by DMSP while *Cladocopium* spp., *Symbiodinium microadriaticum*, and *Durusdinium trenchii* exhibited no chemotactic response to this molecule. These results add to the literature regarding the functional differences among Symbiodiniaceae genera in response to a ubiquitous marine chemical.

5.5.1 Symbiodiniaceae chemotaxis

Establishment of cnidarian-dinoflagellate symbiosis is a multi-step process, the initiation of which involves mechanisms yet to be elucidated. Densities of Symbiodiniaceae in the natural environment are low in the water column (Littman et al., 2008), yet corals effectively acquire symbionts, leading to the possibility that cnidarians can attract Symbiodiniaceae using chemical chemoattractants. The literature on chemoattraction in Symbiodiniaceae is scarce, but there is evidence of chemoattraction towards juvenile corals (Pasternak et al., 2004), and coral extract (Takeuchi et al., 2017), specifically towards *N*-acetyl-D-glucosamine-binding lectin (Takeuchi et al., 2021), in a species-specific manner. Collectively, these studies support the notion that chemotaxis is a crucial step in establishing a symbiosis between cnidarians and dinoflagellates, as evidenced through several chemicals as follows.

5.5.2 Tryptone as a positive chemoattractant

As a product of casein digestion, tryptone contains a mix of oligopeptides, individual amino acids, carbohydrates, and vitamins. This mixture of biological molecules is widely used to supplement growth media for microorganism cultivation (Anacker & Ordal, 1955; Puhm et al., 2022). It is a chemoattractant of multiple microorganisms including the marine bacterium *Caulobacter crescentus* (Berne & Brun, 2019), the nitrogen-fixing bacterium *Azospirillum brasilense* (Okon et al., 1980), the green alga *Chlamydomonas* sp. (Govorunova & Sineshchekov, 2005), and the waterborne pathogen *Vibrio cholerae* (Freter & O'Brien, 1981), highlighting its utility across aquatic taxa. Despite its ubiquity as an attractant in other microorganisms, to my knowledge, this is the first demonstration of tryptone as a positive chemoattractant for any dinoflagellate. Amino acids produced and excreted by

bacteria as waste products (Green & Mecsas, 2016), may be detected by Symbiodiniaceae searching for heterotrophic food. Likewise, cnidarians produce amino acids as part of their central metabolism (Wang & Douglas, 1999), so it is plausible that they are released into the surrounding seawater and act as attractants to Symbiodiniaceae. Thus, tryptone as a chemoattractant could mimic natural cues reflecting searching behaviour for either heterotrophic food sources or cnidarian hosts. Future studies should elucidate which components of tryptone act as an attractant to Symbiodiniaceae. Moreover, all species tested in this study are capable of forming symbioses with cnidarians; whether such responses to tryptone are conserved among exclusively free-living members of the family (e.g., *Effrenium voratum*, *Symbiodinium pilosum*) is worthy of future experimentation and may reveal fundamental aspects of traits related to Symbiodiniaceae lifestyles (Nitschke et al., 2022).

5.5.3 Chemotaxis away from DMSP

Many organisms are attracted to DMSP and its breakdown product dimethyl sulphide (DMS). For example, macrofauna use DMSP when aggregating on breeding and hunting grounds in coral reef habitats (DeBose et al., 2008; Steinke et al., 2006), while opportunistic pathogenic bacteria like *Vibrio coralliilyticus* use DMSP as a cue to locate heat-stressed and immunocompromised corals like *Pocillopora damicornis* (Garren et al., 2014). To my knowledge, reports of organism repulsion by DMSP are absent from the literature, but repulsion by metabolic compounds has been documented in nature and is a response that indicates something important physiologically. For example, toxins produced by blue-green algae *Microcystis aeruginosa* are known to cause negative chemotaxis in *Daphnia magna* (Jüttner et al., 2010), illuminating the ecological importance of chemical repellents.

DMSP is a stress metabolite produced in high quantities by corals experiencing thermal abnormalities (Gardner et al., 2022). As such, DMSP may act as a warning to potential symbionts, indicating an uninhabitable environment. In this case, I would hypothesise repulsion by DMSP, as was observed in *B. minutum*. However, this is not consistent with the failure of DMSP to repel *Cladocopium* spp. Instead, this could suggest that this genus is capable of associating with a broader range of hosts including those such as *Acropora* spp., which are known to release DMSP (Davies et al., 2020). Indeed, *Breviolum* spp. have previously been described as host-specialists (Pettay & LaJeunesse, 2007; Parkinson et al., 2015), while *Cladocopium* spp. are regarded as host-generalists (Thornhill et al., 2014).

5.6 Conclusion

Here, I have shown significantly different responses towards DMSP in two species of Symbiodiniaceae. While *B. minutum* was actively repelled by DMSP, *Cladocopium* sp. did not show a chemotactic response to this molecule. These results contribute to the understanding of the physiological and functional diversity within the family Symbiodiniaceae, and suggest that differential responses to waterborne chemoattractants could play a role in determining patters of both host-symbiont specificity and bleaching recovery. Additionally, I showed that neither *B. minutum* nor *C. C1*^{acro} were attracted to the volatiles bromodichloromethane or diiodomethane, suggesting that a different chemical cue is used to attract dinoflagellates to prospective cnidarian hosts. These results have ecological implications for the survival of coral reefs in a changing global environment and warrants further exploration to determine the generality and physiological basis of these responses.

Chapter 6 General Discussion

The coral holobiont is a diverse metaorganism, including the cnidarian host, endosymbiotic dinoflagellates and a diversity of microorganisms. With increasing environmental extremes and a warming climate, a greater in-depth knowledge of the ways in which these partners interact with one another is crucial to understand how corals may respond and adapt to future environmental change. This thesis expands our knowledge of how a subset of metabolites (biogenic volatile organic compounds; BVOCs) are altered in response to symbiotic state and thermal stress. Non-invasive analysis of volatile metabolites was used to explore their release across symbiotic states (**Chapter 2**), in symbiosis with a non-native algal symbiont (**Chapter 3**), and upon exposure to thermal stress (**Chapter 4**), as a step towards understanding the metabolic and signalling pathways involved in symbiosis function and maintenance, and dysfunction in response to environmental stress. This thesis also describes a method by which to assess chemotactic responses in cultured Symbiodiniaceae (**Chapter 5**). This work can be used for future experiments looking to understand the cues used by Symbiodiniaceae to find prospective hosts. Further to this, I found a species-specific response to the pervasive volatile precursor dimethylsulphoniopropionate (DMSP; **Chapter 5**). With changing environmental conditions, the ability of prospective symbionts to locate new cnidarian hosts, or heterotrophic food sources, may become increasingly important due to more frequent bleaching events.

BVOC analysis of the cnidarian host and algal symbiont in isolation, and in symbiosis with each other, revealed a more similar volatilome between Aiptasia in and out of symbiosis, relative to *B. minutum* in and out of symbiosis (**Chapter 2**). These results suggest that symbiotic state may modify symbiont physiology more dramatically than host physiology, though caution must be taken in the interpretation of these results given the impacts of long-term culture on symbiont physiology and morphology. BVOC analysis of Aiptasia hosting a non-native symbiont (*D. trenchii*) revealed a volatilome indistinct from either aposymbiosis or symbiosis with a native symbiont (*B. minutum*; **Chapter 3**). This result is consistent with previous metabolic studies, which have shown that symbiosis with a heterologous symbiont may cause shifts in metabolic and signalling pathways, and even cellular oxidative stress (Stat & Gates, 2011; Cunning et al., 2015; Matthews et al., 2017). Thermal stress also impacted the volatilome, with different responses in the two symbiotic states, i.e. aposymbiotic *vs.* symbiotic (**Chapter 4**). Under thermal stress, the BVOC diversity of aposymbiotic anemones was noticably less diverse, suggesting metabolic shutdown. By comparison, symbiotic anemones did not exhibit this reduced BVOC richness at high temperature, perhaps indicating a stabilising impact of dinoflagellate symbionts on holobiont health (**Chapter 4**), consistent with the previous observations of Oakley et al. (2016).

Future targeted studies are needed to unravel the roles of BVOCs identified as being differentially detected between symbiotic states (**Chapter 2**), in symbiosis with a sub-optimal partner (**Chapter 3**) and across temperature treatments (**Chapter 4**). For example, amylene hydrate was detected in both aposymbiotic anemones and Symbiodiniaceae cultures but was absent from symbiotic anemones (**Chapter 2**), and thus may be consumed in symbiosis. Isoprene was detected in higher abundance in anemones symbiotic with the native symbiont *versus* both a non-native symbiont and the aposymbiotic state (**Chapter 3**), and thus may act as a marker for a fully optimal symbiosis. The aldehyde's dodecanal, nonanal and octanal were all detected in similar abundances in both aposymbiosis and in symbiosis with heterologous symbionts (**Chapter 3**), and so may be involved in a stress response pathway. Under exposure to high temperature, two key BVOCs were upregulated under thermal stress – DMS in the holobiont and 2-phenyl-3-methyl-pyrrolo(2,3-b)pyrazine in aposymbiotic anemones (**Chapter 4**) – and these may be used as non-invasive indicators for sub-lethal stress in Aiptasia. Elucidating the functional role of these BVOCs will contribute further to our understanding of molecular and nutritional exchange in symbiosis. Now that an established method with which to assess chemotactic response of Symbiodiniaceae has been established (**Chapter 5**), BVOCs identified in **Chapters 2, 3 & 4** can also be used to test chemotactic activity of diverse species of Symbiodiniaceae and elucidate the roles of these compounds on an ecological scale. Other methods with which to assess BVOC functions will be discussed below.

I further characterised the microbiome of the holobiont through analysis of 16S rRNA diversity in symbiosis with non-native symbiont species, in comparison to aposymbiotic anemones and those populated with native symbionts (**Chapter 3**), and in Aiptasia under thermal stress both in symbiosis and out of symbiosis with *B. minutum* (**Chapter 4**). This investigation provided insight into the ways that the holobiont may shift in nature with changing algal symbionts, and under higher environmental temperatures. I observed a shift in the Aiptasia microbiome towards a higher proportion of *Vibrio* spp., known cnidarian pathogens, both when hosting a non-native symbiont (**Chapter 3**), and under periods of thermal stress (**Chapter 4**). These results suggest, when stressed, the microbiota of Aiptasia shifts to favour the proliferation of pathogenic microorganisms, as has been described in previous work (Marhaver et al., 2008).

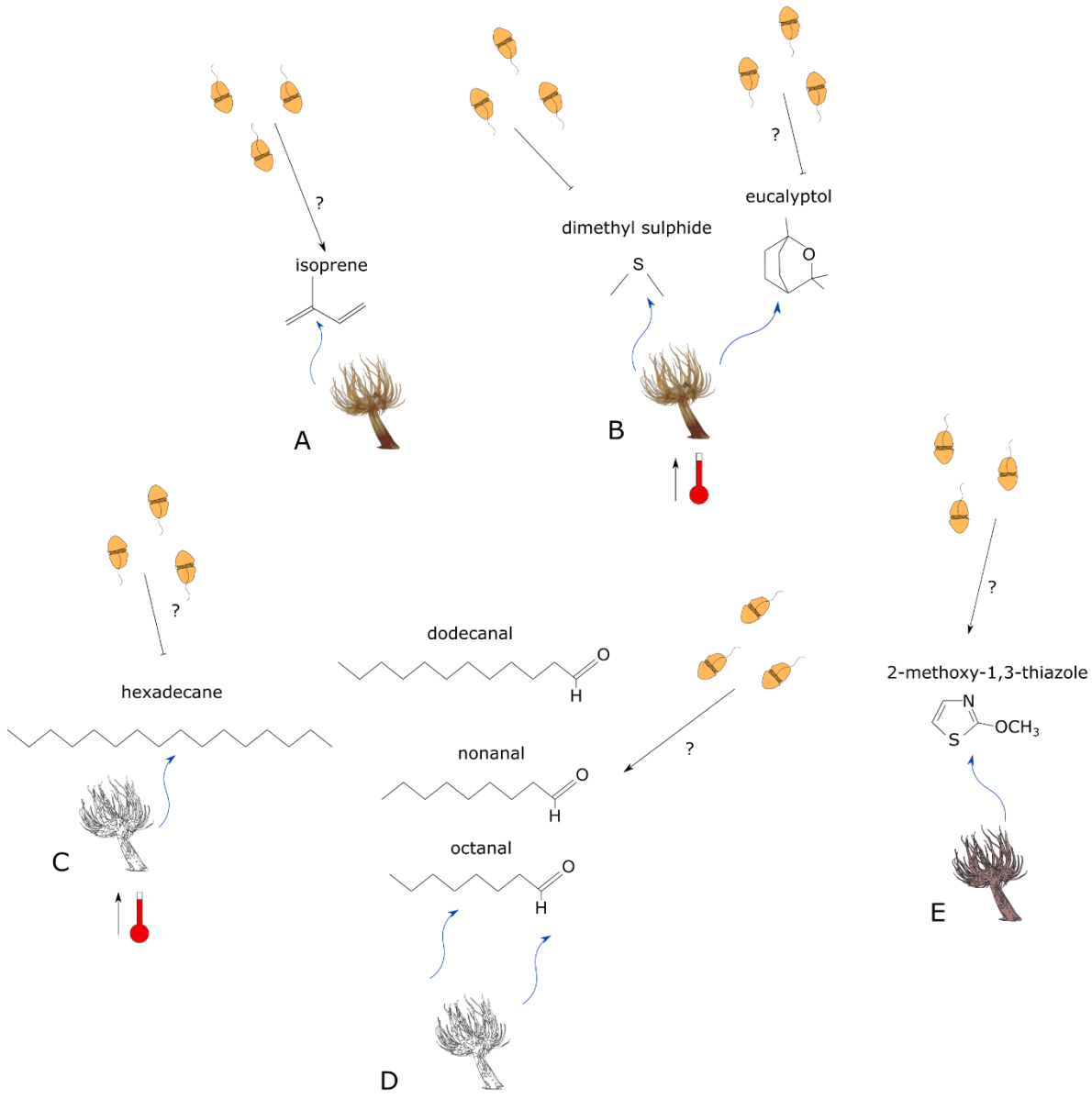

Figure 6.1: Key BVOCs produced by anemones in various symbiotic states and under thermal stress, with proposed chemotactic response of prospective symbionts. A) Anemones with native symbiont *Breviolum minutum* produce isoprene, which may attract Symbiodiniaceae; B) symbiotic anemones under thermal stress (33.5 °C) – native symbiont *B. minutum* produces dimethyl sulphide, which has been shown to repel *B. minutum*, and eucalyptol, which may also repel Symbiodiniaceae; C) aposymbiotic anemones produce hexadiene under thermal stress (33.5 °C) which may repel prospective symbionts; D) aposymbiotic anemones produce the aldehydes octanal, nonanal, and dodecanal, which may attract Symbiodiniaceae; E) anemones harbouring the heterologous symbiont *Durusdinium trenchii* produce 2-methyl-1,3-thiazole, which may attract Symbiodiniaceae. Black arrows indicate swimming behaviour of Symbiodiniaceae; blue wavy arrows indicate the release of a BVOC from Aiptasia.

The work from this thesis provides an important baseline from which to explore the functional role of BVOCs identified differentially among symbiotic states and environmental conditions. The findings in this work raises two particularly important questions regarding the function and utility of these

BVOCs. Two main themes will be explored here: 1) What kind of future experimental work can be carried out to determine the role of these BVOCs in symbiosis?; and 2) How can volatilomics be applied in ecosystem analysis of coral reef health?

6.1 Future methods to uncover functional roles of BVOCs

6.1.1 Identification of BVOCs involved in known symbiosis pathways

While this work has successfully identified BVOCs that are differentially detected across symbiotic states and environmental conditions, future studies are needed to elucidate the functional roles that they play in symbiosis. Furthermore, it will be important to determine if specific combinations of BVOCs are required to elicit a physiological response, or if individual BVOCs have specific responses. I have shown that the abundance of many BVOCs varies with symbiotic state (**Chapter 2**), association with a sub-optimal symbiont (**Chapter 3**) and upon exposure to thermal stress (**Chapter 4**). It may be that some of these BVOCs are produced because of cellular or symbiosis breakdown; but their downstream roles, which could range from antioxidant functions to signalling molecules, potentially attracting pathogenic microbes to the detriment of the holobiont, need to be elucidated.

With the onset of genetic sequencing, the identification of many uncharacterised genes were defined, without initial knowledge of their function (Lander et al., 2001). A common method with which to decode the function of genes of interest has been to disrupt the normal gene expression and examine the resulting phenotype. There are multiple methods with which to manipulate a genetic pathway, including general inhibition through pharmaceutical administration, to more targeted gene silencing techniques (Tsai et al., 2022). The medical field has been using pharmaceuticals to silence the expression of genes as treatments for high blood pressure (Krishnan et al., 2019), Alzheimer's disease (Griñán-Ferré et al., 2020), and cancer (Wingelhofer et al., 2018; Zhu et al., 2021). A targeted approach called RNA interference (RNAi) has been used to knockdown the expression of specific genes through post-transcriptional control by binding to transcripts *in vivo* (Setten et al., 2019). This approach gained popularity and has been widely applied to a diversity of fields including as an environmentally-friendly method to control mosquito populations (Whyard et al., 2015) and as treatment for lung cancer, which can be administered through inhalation (Fujita et al., 2013). While RNAi reduces the transient expression of targeted genes, the newer technology CRISPR works to silence genes at the DNA level, and promises higher specificity with longer-lasting genetic manipulation (Tsai et al., 2022).

The CRISPR-Cas9 technology is targetable to specific regions of DNA by using an endonuclease protein programmed by a short guide RNA, to either knock out or upregulate genes, a phenomenon that has had demonstrated effects on secondary metabolites (Cleves et al., 2020; Shkryl et al., 2021), including isoprene (Shaikh & Odaneth, 2021). The CRISPR-Cas9 system has already been applied to sea anemones (Ikmi et al., 2014; Nakanishi & Martindale, 2018) and corals (Cleves et al., 2020), thus this technology could be used to target and silence potential genes involved in volatile production. Multiple genes have been targeted in cnidarians using the CRISPR-Cas9 system including a region encoding a red fluorescent protein (Ikmi et al., 2014), and a gene involved in metamorphosis (Nakanishi & Martindale, 2018) in sea anemones. Notably, CRISPR knockout of a gene encoding a heat shock protein has shown reduced thermal tolerance in a coral (Cleves et al., 2020). These studies highlight the utility of CRISPR technology to understanding the function of genes in cnidarians. Manipulating select genes in cnidarians, and the subsequent meaurement of BVOCs may be one method with which to illuminate genetic regulation and therefore function of BVOCs in anemone and coral holobionts.

Physiological inhibition has also been applied to the study of BVOC production in other systems; the pathways for select BVOCs, such as isoprene (Logan et al., 2000) and DMS (Taylor & Visscher, 1995; Stefels, 2000) have been identified, allowing the targeted pathway manipulation (Holopainen et al., 2013). For example, isoprene production has been successfully upregulated in *Bacillus subtilis* with the administration of the drug paclitaxel (Xue, 2018), repressed in poplar trees using RNAi (Behnke et al., 2010), and upregulated in *Escherichia coli* using CRISPR (Kim et al., 2016). The successful manipulation of isoprene is indeed promising for the functional elucidation of other BVOCs in the future, however the pathways of synthesis for many BVOCs are unknown. Because of this, targeting pathways known to be involved in symbiosis may be a logical first step in elucidating which BVOCs are involved in symbiosis. For example, oxylipins have roles in inflammation and stress responses in symbiosis (Matthews et. al., 2017), and sphingolipids have roles in apoptosis and cell survival (Detournay & Weis, 2011). The CRISPR-mediated knock out of enzymes in either of these pathways, and subsequent volatilome comparison between knock out and wild type cnidarians could begin to tease apart the synthesis pathways of some BVOCs observed in this work.

6.1.2 Symbiosis establishment experiments

BVOCs identified in aposymbiotic anemones may act as cues for symbiosis establishment; symbiosis establishment and chemotaxis experiments could be used together to determine their role in the onset of symbiosis. Select BVOCs detected in higher abundance by aposymbiotic anemones relative to symbiotic anemones, like the aldehydes dodecanal, nonanal and octanal (**Chapter 3**), could be

acquired from a commercial supplier, in the liquid phase and tested for chemoattractant properties as described in **Chapter 5**. Additionally, the exogenous exposure of these BVOCs could be administered, alongside cultured Symbiodiniaceae, to aposymbiotic anemones, and symbiosis establishment experiments performed to assess whether these BVOCs have a role in the establishment of a successful symbiosis. Following on from work in **Chapter 5**, subsequent experiments could be performed to determine the specific chemoattractant cue(s) used by Symbiodiniaceae to find potential hosts. Homogenate from aposymbiotic anemones could be separated through fractionation (Davy & Cook, 2001; Raina et al., 2016), and those fractions tested using the ISCA technique described in **Chapter 5**. The fraction exhibiting the highest rate of chemoattraction could subsequently be subjected to nuclear magnetic resonance spectroscopy to determine the individual components of this homogenate (Raina et al., 2016; Andersson et al., 2021).

Studying the onset of symbiosis has previously been shown to elucidate underlying molecular mechanisms in cnidarian-dinoflagellate symbiosis, such as the immune system modulation, known to be a hallmark of a successful symbiosis (Parisi et al., 2020). Transforming growth factor beta (TGFβ) members are a large family of cytokines with known roles in immunity across the animal kingdom (Travis & Sheppard, 2014), and whose expression was shown to decrease with the onset of symbiosis between the coral *Fungia scutaria* and *Cladocopium* sp. (Berthelier et al., 2017). Since BVOC production may be influenced over the course of symbiosis establishment it may be interesting to track BVOC production at several stages over the establishment of symbiosis between cnidarians and dinoflagellates. To do this, BVOC production could be analysed prior to inoculation with Symbiodiniaceae, and at multiple stages post-inoculation.

6.1.3 Synthesis of BVOCs from all symbiotic partners

It has been previously shown that BVOCs originating from distinct species of bacteria can independently produce BVOCs intracellularly that are released into the environment and combine in the air to produce novel BVOCs (Kai et al., 2018). While it was previously accepted that BVOCs were metabolic end-products, this work shows that BVOCs can be precursors that non-enzymatically interact to form a final compound in the air. The production of BVOC precursors by cnidarians and dinoflagellates could be an exciting development to further our understanding of the integrated metabolism between these two partners. It would be interesting to explore the interaction between BVOCs independently produced by aposymbiotic Aiptasia and cultured *B. minutum*. Additionally, creating axenic *B. minutum* cultures will help isolate BVOCs produced by each partner individually, as algal-associated bacteria undoubtedly contribute to detected BVOCs in this thesis. BVOCs would be taken from: 1) aposymbiotic Aiptasia; 2) cultured *B. minutum*; 3) axenic cultures of *B. minutum*; 4)

Aiptasia symbiotic with *B. minutum*; and 5) a combination of gases emitted from aposymbiotic Aiptasia and cultured *B. minutum*. BVOCs would be released from each partner in tandem, and any BVOCs released would mix with BVOCs from the other partner in an enclosed chamber, potentially interacting such that new BVOCs are produced. Any novel BVOCs detected from treatment 5 may be consumed in a successful symbiosis or may be synthesised *de novo* from BVOCs emitted by each partner independently.

Since it is not known to what extent BVOCs of bacterial origin contributed to the detected BVOCs in this thesis, more work is needed to disentangle which BVOCs are produced by which partners in this dynamic holobiont. Isolating bacterial species and analysing their BVOC output would give us information about which BVOCs are produced by cnidarian-associated bacteria in isolation, however these data would need to be interpreted cautiously since bacterially produced BVOCs could be consumed by other partners in symbiosis. Individual bacterial species associated with the Aiptasia microbiome can be isolated on Marine Agar, transferred into Marine Broth, from which BVOCs can be trapped and analysed as described by Lawson et. al (2019) and in **Chapters 2**, **3** and **4**.

6.2 Use of BVOCs as biomarkers for underlying metabolism

The potential power of volatilomics in the assessment of organism and ecosystem health lies in this technique being non-destructive to biological tissues, while still getting a snapshot of the underlying metabolic processes. The application of volatilomics to the medical field has the potential to overcome typical screening barriers associated with potentially damaging invasive screening tests and the related, but not insignificant, patient compliance with such tests. Invasive, expensive, and uncomfortable screening tests are often met with patient resistance, thus the application of BVOC biomarkers to human health may increase patient compliance and thus make screening of diseases more accessible. Once BVOC biomarkers have been established, volatilomics can deliver a non-invasive and pain-free method with which to screen for early biomarkers of potentially deadly diseases. As a rapidly evolving field, volatilomics in medical research has shown that BVOCs in urine or exhaled breath can act as biomarkers for the health conditions Alzheimer's disease (Kimball et al., 2016) and lung cancer (Phillips et al., 2019; Ratiu et al., 2021), and has shown promise in prostate cancer research (Berenguer et al., 2022).

As with medical research, biomarkers can be used to assess ecosystem health (Hook et al., 2014), and volatile compounds provide a conservative and non-invasive approach with which to assess natural habitats. Indeed, BVOC biomarkers with which to assess the health of terrestrial organisms have been

identified (Tiwari et al., 2020; Hamow et al., 2021). In agriculture research, BVOC markers have been utilised to detect stresses in crops. For example, the lipoxygenase, shikimic acid, methylerythritol and melvalonic acid pathways are all susceptible to perturbation during post-harvest storage and unhygienic processes (Holopainen et al., 2013; Cellini et al., 2018). VOCs such as linalool, methyl salicylate and methyl benzoate are emitted by plants in stressful environments (Niinemets, 2010b), thus may serve as biomarkers. Additionally, volatilomics have been applied to phenotypic screening in healthy plants as well to reveal plant phenotypic traits such as net CO_2 assimilation and transpiration rates in barley varieties (Jud et al., 2018).

Lagging behind research into terrestrial BVOC biomarkers, research into biomarkers for aquatic ecosystems is still in its infancy (Saha et al., 2019). Some work has gone into investigations of drinking water quality, for example, volatiles geosmin and 2-methylisoborneol (2-MIB) can be produced by cyanobacteria (Fujise et al., 2010) and produce an unsavoury taste thus causing problems in drinking water supplies (Watson et al., 2016). β-cyclocitral, a by-product of stress metabolism in freshwater alga *Microchloropsis salina*, has additionally been identified as an early indicator of aquatic ecosystem decline (Reese et al., 2019). Apart from VOCs of biological origin, VOCs in the aquatic environment can be of anthropogenic origin, like volatile methyl siloxanes, derived from industrial sectors (Warner et al., 2010), or be downstream products from wastewater or nutrient-rich agricultural runoff (Akpor & Muchie, 2011).

Considering the multitude of ways in which BVOC production can change in response to external stimuli, aquatic BVOCs here have an enormous potential as bioindicators for the state of aquatic organisms or communities. The ability to non-invasively analyse the volatilomes of an ecosystem before, during and after a disturbance event could provide a sensitive method with which to understand the ways that the underlying metabolism of organisms' in this habitat are altered. Biomonitoring ecosystems through BVOC analysis could also be useful to understand how a habitat may respond to climate change. The work presented in this thesis can aid in the identification of biomarkers for the assessment of coral reef health in the field. For example, dimethyl sulphide and eucalyptol were both detected in significantly higher abundance in the volatilome of thermally stressed symbiotic anemones relative to non-thermally stressed anemones, prior to signs of visual stress (**Chapter 4**). The aldehydes dodecanal, nonanal and octanal were all detected in significantly higher abundance in aposymbiosis relative to symbiotic anemones (**Chapter 3**), and thus may indicate bleaching or sub-optimal symbiosis. Any of these BVOCs may act as a biomarker for a stressed cnidarian, but further work is needed to determine how other environmental stressors interact to alter the production of these BVOCs. A variety of environmental cues have been shown to influence

BVOC production, such as light availability, UV radiation, temperature, salinity, and oxidative stress (Korpi et al., 2009; Venuleo et al., 2017; Saha & Fink, 2022). Additionally, BVOC production varies with coral species (Lawson et al., 2021), which undoubtedly interacts with these stressors to produce unique volatilomes; this indeed necessitates the investigation of BVOC production in light of multiple environmental stressors.

While laboratory-based experiments can inform BVOC release from individual, or groups of organisms, the next steps in this research would include the development of an *in situ* sampling chamber to get a more complete picture of BVOC release from organisms in their natural habitat (Lawson et al., 2022). The sampling of organisms in the marine environment comes with a unique set of limitations (Davie-Martin et al., 2020), including the collection of BVOCs onto thermal desorption tubes that need to remain in air, and the subsequent storage and transport of water samples. Ideally, sample storage should occur in a dark container to prevent photosynthesis and at 4 °C, to low degradation and respiration of any microorganisms, unless BVOCs will be collected within 1 hour (Pozzer et al., 2022). Non-invasive analysis of *in situ* aquatic organisms can still be achieved without damaging the organism by collecting water surrounding the reef and analysing BVOC output on land, as described in **Chapters 2**, **3** and **4**. For example, (inert) clear plastic bags could be secured to the base of a reef, fitted with flow through valves and connected to a water collection vessel. Collected water from surrounding the organism of interest would be transported to the surface in a water-tight, sealed container for BVOC collection as soon as possible.

A snapshot of reef health could be taken by collecting water from an area of interest, or a flow-through system could be created by surrounding corals in semi-open chambers, allowing water flow while BVOCs are not being collected. An automated or remote system could close chambers at regular intervals and collect water into a sealed container. At set intervals, the chambers close, and water is pumped through the coral-containing chamber and collected into a sealed container. In this way, we could analyse how BVOCs change over a diel cycle, seasons, or during a bleaching event.

6.3 Concluding remarks

The emerging field of volatilomics provides enormous potential as a non-invasive real-time biological monitoring tool both in natural habitats and controlled environment. This thesis has described for the first time the influence of symbiotic state and thermal stress on the production of BVOCs in an important model organism at a time when coral reefs are threatened with extinction. I have shown that BVOC production is influenced by symbiotic state, symbiont identity and in response to thermal

stress. Additionally, my work sought to assess the potential role of chemotaxis in response to BVOCs during symbiosis establishment, with the pervasive marine info-chemical DMSP seemingly a particularly interesting candidate for further exploration. Ultimately, this work can serve as a baseline from which to explore the role of BVOCs in inter-partner signalling, and hence symbiosis function and dysfunction. As volatilomics research becomes more widely adopted, BVOCs identified here may serve as biomarkers for aquatic ecosystem health in nature.

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Supplementary data

The following supplementary data can be found below:

Figure S2.1: BVOC sampling setup, use for Chapters 2, 3, & 4. Anemones (~10-15 *per* vial) settled in sampling vials 24 h prior to BVOC sampling.

Other supplementary data can be found at https://github.com/maggiewuerz/PhD_thesis_supplementary and contains the following information:

Supplementary Material: Chapter 2

Table S2.1: Fv/Fm values for *B. minutum* cultures. Cultures were dark-adapted for 15, prior to measurement. Values were collected using an Imaging Pulse Amplitude Modulated Fluorometer (I-PAM, Walz, Effeltrich, Germany; settings: measuring light = 4, saturation intensity = 8, saturation width = 0.8 s, gain = 3, damping = 3). (Chapter 2)

Table S2.2: BVOCs detected throughout dataset. All BVOCs (peak normalised to protein content) and their chemical classes that were detected in aposymbiotic anemones, symbiotic anemones and cultures of *B. minutum*. Compounds had to be detected in at least two replicates in at least one symbiotic state. Chemical class was determined based on the molecule's functional group(s). Significance was determined using differential abundance testing and the number of asterisks denotes the size of the adjusted p-value: *<0.05, **<0.01, ***<0.001. (Chapter 2)

Supplementary Material: Chapter 3

Figure S3.1: A) Maximum photosystem II (PSII) photochemical efficiency (F_v/F_m) and B) symbiont cell densities of holobionts colonised by symbiont species *Breviolum minutum* and *Durusdinium trenchii* taken on day of BVOC and microbial sampling. Whiskers represent minimum and maximum values for this dataset, dots represent individual datapoints. Anemones were dark adapted for 15 min in sampling vials prior to quantum yield measurements.

Table S3.1: Differentially abundant BVOCs (<0.05) detected across 1) aposymbiotic anemones; 2) anemones symbiotic with *D. trenchii* (heterologous) and 3) anemones symbiotic with *B. minutum* (homologous).

Table S3.2: Differentially abundant microbes ($p < 0.05$) detected across 1) aposymbiotic anemones; 2) anemones symbiotic with *D. trenchii* (heterologous) and 3) anemones symbiotic with *B. minutum symbiosis* (homologous).

Table S3.3: BVOCs detected throughout dataset. All BVOCs (peak normalised to protein content) and their chemical classes that were detected in aposymbiotic anemones, anemones symbiotic with *B. minutum symbiosis* (homologous symbiosis) and anemones symbiotic with *D. trenchii* (heterologous symbiosis). Compounds had to be detected in at least four replicates in at least one symbiotic state. Chemical class was determined based on the molecule's functional group(s). Significance was determined using differential abundance testing and the number of asterisks denotes the size of the adjusted p-value: *<0.05, **<0.01, ***<0.001.

Table S3.4: Bacterial taxa (ASV counts) detected throughout dataset. All bacteria that were detected in aposymbiotic anemones, anemones symbiotic with *B. minutum symbiosis* (homologous symbiosis) and anemones symbiotic with *D. trenchii* (heterologous symbiosis). Bacteria had to be detected in at least three replicates in at least one symbiotic state to be included. Significance was determined using differential abundance testing and the number of asterisks denotes the size of the adjusted p-value: *<0.05, **<0.01, ***<0.001.

Supplementary Material: Chapter 4

Table S4.1: Oxygen evolution at seven irradiances to determine saturating irradiance for symbiotic anemones

Table S4.2: Differentially abundant BVOCs (< 0.05) detected across aposymbiotic anemones at 1) 25 °C (control); 2) 30°C (mid) and 3) 33.5 °C (high)

Table S4.3: Differentially abundant BVOCs (<0.05) detected across symbiotic anemones at 1) 25 °C (control); 2) 30°C (mid) and 3) 33.5 °C (high)

Table S4.4: Differentially abundant microbes (<0.05) detected across aposymbiotic anemones at 1) 25 °C (control); 2) 30°C (mid) and 3) 33.5 °C (high)

Table S4.5: Differentially abundant microbes (<0.05) detected across symbiotic anemones at 1) 25 °C (control); 2) 30°C (mid) and 3) 33.5 °C (high)

Table S4.6: BVOCs detected throughout aposymbiotic dataset. All BVOCs (peak normalised to protein content) and their chemical classes that were detected in aposymbiotic anemones at three different temperature treatments (control: 25 °C, sub-bleaching: 30 °C and bleaching: 33.5 °C). BVOCs had to be detected in at least three replicates in at least one temperature treatment to be included. Chemical class was determined based on the molecule's functional group(s). Significance was determined using differential abundance testing and the number of asterisks denotes the size of the adjusted p-value: *<0.05, **<0.01, ***<0.001.

Table S4.7: BVOCs detected throughout symbiotic dataset. All BVOCs (peak normalised to protein content) and their chemical classes that were detected in symbiotic anemones at three different temperature treatments (control: 25 °C, sub-bleaching: 30 °C and bleaching: 33.5 °C). BVOCs had to be detected in at least three replicates in at least one temperature condition to be included. Chemical class was determined based on the molecule's functional group(s). Significance was determined using differential abundance testing and the number of asterisks denotes the size of the adjusted p-value: *<0.05, **<0.01, ***<0.001.

Supplementary Material: Chapter 5

Figure S5.1: Schematic of ISCA diagram showing non-randomised distribution of treatments within each ISCA used for preliminary trials. This setup was used in high-nutrient trials, in Experiment 1.

Figure S5.2: Example of ISCA chemical treatments using randomised treatments. This setup was used in low-nutrient trials, in Experiment 2.

Figure S5.3: *Breviolum minutum* and *Cladocopium C1acro* chemotactic response to tryptone, DMSP and DMS in high nutrient media. Statistical significance denoted by asterisks: * <0.05; ** <0.01; *** <0.001. Values represent means +/- standard error; n = 4-28, see Table S3

Figure S5.4: *Breviolum minutum* and *Cladocopium goreaui* response to tryptone and DMSP in high and low nutrient media. One replicate *per* datapoint.

Table S5.1: Quantum yield measurements (+/- standard error) of *B. minutum*, *C. goreaui* and *D. trenchii* after incubation in low nutrient artificial seawater for 5 days (n = 5 for each species), prior to chemotaxis trials. Symbiodiniaceae were dark adapted for 15 minutes before quantum yield measurements were taken.

Table S5.2: Chemotactic indices of putative chemicals for use as positive control across four species of Symbiodiniaceae: *Breviolum minutum*, *Cladocopium C1^{acro}*, *Durusdinium trenchii* and *Symbiodinium microadriaticum*. Ic = chemotactic index; SD = standard deviation; SE = standard error; n = number of biological replicates. Trials from Experiment 1.

Table S5.3: Chemotactic response to tryptone, DMSP and DMS by *B. minutum* and *C. C1^{acro}* in high nutrient media. Ic = chemotactic index; SD = standard deviation; SE = standard error; n = number of biological replicates. Experiment 1.

Table S5.4: Preliminary trial testing chemotactic response of *B. minutum* and *C. goreaui* to tryptone and DMSP in two different nutrient conditions in Experiment 2.

Table S5.6: Chemotactic response to DMSP at three different concentrations by *B. minutum* and *C. goreauii* in low nutrient media. Ic = chemotactic index; SD = standard deviation; SE = standard error; n = number of biological replicates.

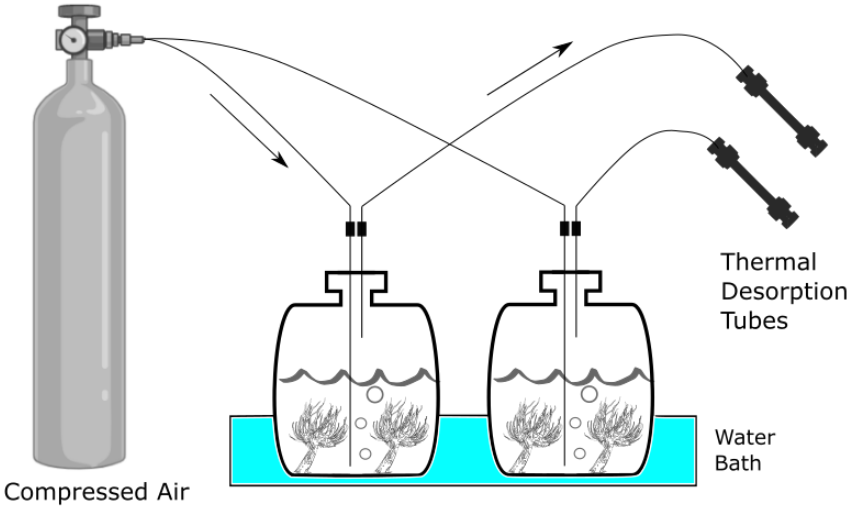

Figure S1: BVOC sampling setup. Anemones (~10-15 *per* vial) settled in sampling vials 24 h prior to BVOC sampling. BVOCs were collected under growth conditions in gas-tight vials over 20 min, during which time instrument-grade air was passed through and BVOCs trapped onto the stationary phase inside Markes thermal desorption tubes. Arrows indicate direction of airflow.