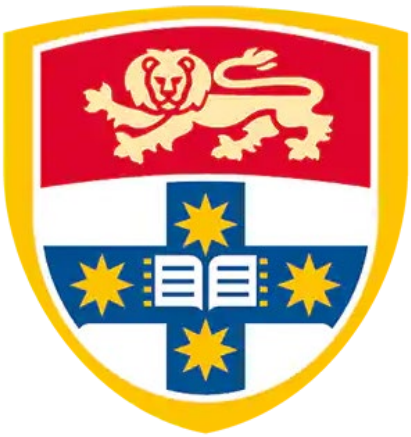
The role of host-associated microbes in host function



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This work was conducted on the lands of the Gadigal people of the Eora nation. I pay my respects to their elders past and present. Sovereignty was never ceded, always was and always will be Aboriginal land.

Declaration

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Alexander Harry McGrath

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Abstract

Host-associated microbiota are increasingly being recognised as critical for eukaryotic host functioning. This is true even for important habitat forming organisms such as macroalgae, whose functioning and persistence often underpins wider ecosystem function. To improve our understanding of the role of host-associated microbiota on macroalgal host function and components of host fitness, I have combined manipulative field and mesocosm experiments with 16S rRNA gene sequencing to examine host measures that included reproduction and settlement of the dominant intertidal macroalga *Hormosira banksii*.

Firstly, in Chapter 2 I developed and tested an experimental framework for determining causation in host-microbiota interactions. The experimental protocols were designed to try and disentangle microbially mediated effects on hosts from direct effects on hosts associated with the methods employed to manipulate host-microbiota. This was done through a combination of antimicrobial treatments, which have widespread use in holobiont research, and inoculations, in mesocosms and in the field. This experimental framework allows for causal relationships to be determined within ecologically important macroalgal holobionts. This should allow for better predictions of how these systems will respond to environmental disturbances in their natural context.

In addition, in Chapter 3 I performed a series of experiments testing the role of host-associated microbiota in not only host function, but also components of fitness. I manipulated host- and benthic-associated microbiota in order to disentangle the relative importance of microorganisms on reproductive output and settlement (i.e. attachment and morphogenesis of algal zygotes). Our results demonstrate the importance of host-associated microbiota on host

reproduction and the interactive effects of host- and benthic-associated microbiota on settlement – both key ecological processes – with potentially important implications for host fitness, ecosystem persistence and management.

Lastly, to investigate how host-microbiota interactions change under environmental stress, in Chapter 4, I conducted a series of manipulative experiments following a series of unusually extreme rainfall events along the coast of Sydney, Australia. I examined the effects of rainfall (in the field) and lowered salinity (in the laboratory) on host reproductive output and performance. Manipulative field experiments using a combination of antimicrobial treatments applied once (pulse) or regularly (press) showed that disruption of *Hormosira*'s microbiome after a rain event inhibited the post-event recovery of host reproductive output and photosynthesis. Press disruption of host-microbiome prevented recovery of normal levels of reproductive output and photosynthesis for over four months. This experiment demonstrates the role host-associated microbiomes play in mediating responses of important habitat forming algae to environmental stress.

Overall, this work has provided novel ecological insights into the relationship between a dominant habitat forming macroalga and its bacterial consortia and provides a baseline for future work into holobiont ecology.

Acknowledgements

This thesis is the culmination of the last three and a half years of my life, and it is impossible to acknowledge and thank everyone who have helped me along the way as much as they deserve. To all of you who have made this PhD possible through your support and have made the last couple of years some of the most rewarding in my life, I am eternally grateful.

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Finally, to my Family – Mum, Dad and Soph – more than anyone else, this thesis is dedicated to you. Without your unwavering support, belief and enthusiasm I would have never dreamed of achieving this. To Pumps, thank you for always asking me questions and teaching me to question the world around me. This thesis is the result of some of the questions you asked.

Thank you all for sharing this journey with me.

I love you all.

Alex, 2023.

Statement of Co-Authorship

Chapter 2 of this thesis is being considered for publication as "McGrath, A.H, Lema, K. Egan, S. Wood, G. Vadillo-Gonzalez, S. Kjelleberg, S. Steinberg, P.D, Marzinelli, E.M, **Disentangling direct vs indirect effects of microbiome manipulations in a habitat-forming marine holobiont"** npj Biofilms and Microbiomes (In Review)

Study conception and design: AHM, KL, EMM, PDS, SE, SK. Sample collection and processing, isolation, and cultivation of bacterial isolates: AHM, KL, GW. Bioinformatic and statistical analyses and data visualisation: AHM and SVG. Drafting of manuscript: AHM, KL, GW, SE, SVG, PS, EM. All authors edited and approved the final manuscript.

Chapter 3 of this thesis has been prepared for publication as "McGrath, A.H, Egan, S.S. Kjelleberg, S. Steinberg, P.D, Marzinelli, E.M, **Disruption of host-associated and benthic microbial communities affects reproductive output and settlement of a habitat forming macroalga"**

AHM, PDS and EMM conceived the project. AHM collected, processed, and extracted samples. AHM conducted bioinformatic and statistical analyses and data visualisation. AHM, PDS and EMM drafted the manuscript.

Chapter 4 of this thesis has been prepared for publication as "McGrath, A.H, Egan, S.S. Kjelleberg, S. Steinberg, P.D, Marzinelli, E.M, Chapter 4 - **Effects of extreme rainfall on a dominant seaweed are mitigated by its microbiota"**

AHM, PDS and EMM conceived the project. AHM collected, processed, and extracted samples. AHM conducted bioinformatic and statistical analyses and data visualisation. AHM, PDS and EMM drafted the manuscript.

Alexander Harry McGrath 9th August 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Ezequiel Miguel Marzinelli 9th August 2023

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Chapter 1.

General introduction

1.1 Holobionts as an emergent form of life

Evidence from systems as diverse as grasslands to the human gut indicates that close associations with microorganisms are fundamentally important to eukaryotic life (Rosenberg & Zilber-Rosenberg, 2018a). The new understanding of these relationships is causing a paradigm shift in biology, such that plants and animals are no longer viewed as singular entities but rather as "holobionts", composed of the eukaryotic host plus its associated microbiota (McFall-Ngai et al., 2013; Theis et al., 2016). Whilst there is debate over how far this paradigm shift extends, e.g., whether holobionts should be considered as superorganisms, with selective pressure acting on the holobiont as a whole or on individual members of the holobiont (see Douglas & Werren, 2016), there is clear evidence that host-associated microbial communities are profoundly important for the normal development and functioning of the host (Agler et al., 2016; Barott et al., 2011; Bourne et al., 2016).

Host-associated microbial communities have been characterized and shown to be important to systems as diverse as the human gut (Donaldson et al., 2016; Lloyd-Price et al., 2019), plants (Berendsen et al., 2012; Wagg et al., 2015), corals (Bourne et al., 2016; Glasl et al., 2016), insects (Ferguson et al., 2018; Gupta & Nair, 2020; Kapheim et al., 2015) and sponges (Fan et al., 2013; Hentschel et al., 2012). These systems – holobionts – by their nature are often very complex. One response of researchers to this complexity is to do research on holobionts in model systems under non-natural or even axenic conditions, removed from their natural environment (Fontaine et al., 2015; Kennedy et al., 2018; KleinJan et al., 2021).

Alternatively, researchers have adopted mostly descriptive approaches (as opposed to experimental) when studying holobionts *in situ*. While much insight into holobionts has been gained from these studies, not studying organisms *in situ* hinders our capacity to understand the functioning of holobionts in their natural environment, and to predict their response to disturbances or changing environmental conditions (Prosser, 2022; Trevathan-Tackett et al., 2019).

Most holobionts harbor thousands of microbial taxa (Bourne et al., 2016; Donaldson et al., 2016; Marzinelli et al., 2016) and the inherent variability in natural systems adds additional complexity to any experimental approaches to studying holobionts *in situ*. The challenge of how to determine host-microbiota interactions in the field is a central theme in modern microbial ecology (Prosser, 2022; Trevathan-Tackett et al., 2019). Many studies have used descriptive approaches where microbial communities are characterized or responses to environmental variation are measured (Fan et al., 2013; Marzinelli et al., 2015; Pfister et al., 2018; Weigel & Pfister, 2019). Typically, these studies place hosts are under different environmental conditions and characterize host and microbial changes, from which relationships are inferred (e.g., Pedicini et al 2023). Whilst descriptive approaches are useful to assess diversity (who is there?) and potential function (what can/do they do?), such approaches have led to a bottleneck in holobiont ecology wherein cause-effect relationships are still not determined for most natural systems (Prosser, 2022; Prosser & Martiny, 2020). To overcome this, experimental approaches which examine causation must be used. This challenge for microbial ecology in some ways mirrors the development of macrobial ecology which was transformed half a century ago from a descriptive field to a strong experimental discipline (e.g. Connell, 1961; Paine, 1966; Simberloff & Wilson, 1969). This was the impetus for Chapter 2 in which an experimental framework was developed for disentangling cause-effect relationships in non-model holobionts in natural systems.

Another key factor for predicting the response of holobionts to varying environmental conditions or disturbances is understanding the role of host-associated microbial communities throughout the life histories of hosts. Microbiomes can exert influence at different stages of a hosts' life cycle (Carrier et al., 2022; Pantoja-Feliciano et al., 2013). For example, in the human gut, early gut colonization by commensal bacteria plays a significant role in mediating the risk of non-communicable diseases such as obesity and metabolic dysfunction in adulthood (Blaser, 2006; Gilbert et al., 2018; Wallace et al., 2016). These and other findings have led to the idea of vertical transmission of the microbiome from adults to their offspring (Blaser, 2006; Grieneisen et al., 2021; McFall-Ngai, 2002). Vertical transmission of microbiota occurs in multiple mammalian hosts, although most examples are typically based on studies done in highly controlled settings (Grieneisen et al., 2021; Moeller et al., 2018; Tochitani, 2021). The majority of non-mammalian work on vertical transmission has used hosts such as insects (Fukatsu & Hosokawa, 2002; Mclaren et al., 1975), sponges (Grice et al., 2017; Hentschel et al., 2012) and corals (Ainsworth et al., 2015; Bourne et al., 2016). Evidence for vertical transmission on microbiomes has been shown to be inconsistent, suggesting that common predictions from model systems are not necessarily true when considering complex microbiomes in natural systems (Björk et al., 2019). Transmission of microbiota from adults to their offspring can also occur horizontally, i.e. via the environment (Rosenberg & Zilber-Rosenberg, 2018). Examples of this include the well-studied bobtailed squid and its symbiont *Vibrio fischeri* (Nyholm et al., 2009), and grasslands where beneficial microbes enhance early the life stages of habitat forming organisms (Frank et al., 2017; Tadych et al., 2014; Wasai & Minamisawa, 2018).

In natural systems, habitat-forming organisms such as trees, grasses, corals and macrophytes are increasingly being recognized in the literature as highly complex holobionts (Adak et al., 2016; Egan et al., 2013a; van der Loos et al., 2019). These habitat-forming organisms host

abundant epiphytic microbiota on the surface layer (Lemay et al., 2018; Weigel & Pfister, 2019), their rhizomes (Berendsen et al., 2012; Knief et al., 2012) and leaves (Enriquez et al., 2002; Laforest-Lapointe et al., 2017), with some species (e.g. *Sargassum muticum, Himanthalia elongata,* and *Chondrus crispus*) hosting an internal endophytic microbial community (Bonthond et al., 2022). These host-associated microbial communities can enhance host functions ranging from nutrient supply (Miranda et al., 2022; Penhale & Capone, 1981; Weigel & Pfister, 2019), developmental regulation (Nakanishi et al., 1996a; Stappenbeck et al., 2002), disease resistance (Li et al., 2022; Qiu et al., 2019), to assistance in acclimating to environmental conditions such as salinity (Dittami et al., 2016; KleinJan et al., 2021). For marine macrophytes - a broad ranging group of habitat-forming macroalgae (e.g. kelps, fucoids) and plants (e.g. seagrasses, mangroves) which form extensive forests/meadows in coastal seas - some of the clearest demonstrations of the importance of microbial communities in host function and development have been shown in the macroalga *Ulva sp.* (Nakanishi et al., 1996; Provasoli & Pintner, 1980) *Ectocarpus sp* (Dittami et al., 2014, 2016; KleinJan et al., 2021) and *Delisea pulchra* (Campbell et al., 2011; Kumar et al., 2016). But the importance of the microbiome in other, arguably more ecologically important macrophytes is also increasingly emerging (Bondoso et al., 2017; Fuggle et al., 2023; Gribben et al., 2017; Martin et al., 2020, 2020; Marzinelli et al., 2015; Miranda et al., 2022; Pfister et al., 2018; Qiu et al., 2019; Weigel & Pfister, 2019).

1.2 Holobionts in the environment

One key reason why it is necessary to study holobionts in their natural environment is that it is where they evolved, and host-microbiome interactions are a consequence of that history (Bosch et al., 2019; Rosenberg & Zilber-Rosenberg, 2018). For example, deficiency in specific gene pathways has led a number of species to rely on co-evolved microbial communities for key metabolic functions such as ammino acid synthesis and carbon

breakdown (Ley et al., 2008; Warnecke et al., 2007; Xia et al., 2017). Holobionts also respond to, and in turn are affected by ecological interactions with other organisms, changes in environmental conditions and the interaction between these factors. Herbivory and predation have been shown to have strong effects on the structure of host-associated microbiome which, in turn, has implications for host function (Carthey et al., 2020; Gilbert, 2020). Several studies have tested the relationship between variation in the physical environment and holobionts, primarily through comparing the composition of microbial communities in different habitats (Marzinelli et al., 2015; Weigel & Pfister, 2019). Environmental conditions such as temperature (Case et al., 2011; Greenspan et al., 2020), light intensity (Coelho-Souza et al., 2017; Pineda et al., 2016), salinity (Dittami et al., 2016; KleinJan et al., 2021) and nutrient availability (Adak et al., 2016; Den Haan et al., 2016; Seabloom et al., 2019; Szitenberg et al., 2022) all can affect holobiont community composition with implications for their function. Furthermore, ecological interactions may change with varying environmental conditions. For example, many bacteria and fungi present in holobionts exhibit the genomic potential to degrade cell walls, whilst many remain commensal or even mutualistic, and environmental change has been shown to shift these interactions to pathogenic (Aiyar et al., 2017; Campbell et al., 2011).

Environmental variability is a natural part of any ecosystem, and holobionts must be resilient to that variability. In marine systems, environmental conditions such as nutrient availability, and salinity vary on multiple temporal and spatial scales (Ridgeway, 2009; Wernberg, Thomsen, et al., 2013). However, the world's oceans are also undergoing unprecedented change driven in part by anthropogenic climate change (Solomon, 2023). Climate change is having a multifaceted effect on marine ecosystems. Long-term stressors such as ocean acidification (Beman et al., 2011) and warming (Lima et al., 2007) are changing the distribution and function of ecological communities. Alongside these chronic stressors,

climate change is also increasing the frequency and severity of extreme events such as heatwaves, droughts and extreme rainfall (Hoegh-Guldberg & Bruno, 2010; Masson-Delmotte et al., 2022).

In marine environments, heatwaves are the most studied extreme environmental events. This is in part due to several recent extreme marine heatwaves (Oliver et al., 2019; Smale, 2020), whose intensity (CSIRO et al., 2018; Smith et al., 2023) and length (Oliver et al., 2018) have significantly increased over the past two decades. Other extreme events in coastal environments include severe rainfall and flooding events, which can have a substantial effect on the structure (O'Gorman et al., 2012) and functioning of these ecosystems (Duvat et al., 2021; O'Connor et al., 2015). This is in part due to the multiple effects which are characteristic of extreme rainfall events, that is, rapid salinity fluxes coupled with nutrient enrichment and often chemical pollution from onshore (Kuntz et al., 2005; O'Connor et al., 2015; White et al., 2018).Whilst there is experimental evidence for the effect of abiotic stressors, such as elevated temperatures (Campbell et al., 2011; Case et al., 2011; Minich et al., 2018) or changing salinities on microbial community composition (Dittami et al., 2016; KleinJan et al., 2021), there is limited evidence as to the effect these microbial changes have in host function (but see Dittami et al 2016; Martinez-Arias et al., 2022). This thesis addresses this within the context of extreme rainfall in the field.

1.2 The study system – Hormosira banksii

As noted above, marine macroalgae are critical biogenic habitats in rocky coastal marine environments worldwide (Bellgrove et al., 2010; Clark et al., 2018; Duffy et al., 2019; Marzinelli et al., 2016; Pfister et al., 2018). On intertidal rocky shores, the canopy provided

by their physical structures ameliorates the often harsh physical conditions and provides important refuge for intertidal invertebrates and other species of non-canopy forming seaweed (Bishop et al., 2009; Gemelli et al., 2019; Wright et al., 2014).

Hormosira banksii (Turner) Decainse (*Hormosira* hereafter) is a perennial fucoidan macroalga which forms habitat on temperate intertidal rocky shores in Australia and New Zealand (Bellgrove et al., 2010; Lilley & Schiel, 2006; Schiel et al., 2006). *Hormosira* is a monospecific genus whose structure is characterized by its distinctive branched chains of vesicles, leading to its common name of "Neptune's necklace" (Getman, 1914; Osborn, 1948). This morphology, however, is highly variable and is dependent on environmental conditions (Bishop et al., 2009; Clarke & Womersley, 1981; Taylor & Schiel, 2003). *Hormosira* is distributed from northern New South Wales through to Albany in Western Australia and is present in both Tasmania and New Zealand (Miller et al., 2020), although its distribution in New Zealand has severely contracted following the earthquakes in 2016 (Schiel et al., 2019; Thomsen et al., 2020).

Intertidal macroalgae such as *Hormosira* are susceptible to both natural and anthropogenic disturbances including climate change (Benedetti-Cecchi et al., 2001; Bulleri et al., 2018), trampling (Povey & Keough, 2023; Schiel & Taylor, 1999a), increased nutrients and pollutants due to sewage (Bellgrove et al., 2010; Doblin & Clayton, 1995; Seery et al., 2006), storms and increased wave activity (Taylor & Schiel, 2003; Underwood, 1998). This is in part due to the sessile nature of habitat forming macrophytes and increased urbanization in coastal ecosystems (Benedetti-Cecchi et al., 2001).

1.3 Thesis subject: The role of host-associated microbes in host function

This thesis examines the role of host-associated microbiota (primarily bacteria) in host function for the important intertidal habitat-forming macroalga, *Hormosira.* The structure of the thesis is summarized in Fig. 1. Three aspects of holobiont functioning are the focus here: understanding the interaction of *Hormosira* and its bacterial microbiota in its natural environment, including methodological approaches necessary for an *in-situ* approach, the importance of microbial communities at various stages of the lifecycle of the host, and the role of the microbiota in the response of the holobiont to extreme environmental events (rainfall). The data chapters in this thesis, Chapter 2-4, have been prepared as stand-alone manuscripts formatted for publication.

Chapter 2 develops an experimental framework for understanding the role of host-associated microbial communities for non-model hosts in both the laboratory and field, using the background of eukaryotic experimental marine ecology as a template (e.g. Underwood et al., 1997, 2000). I conducted three separate manipulative experiments, two in mesocosms in a flow-through aquarium and one in the field. These experiments addressed some of the methodological challenges involved in using antimicrobials as protocols for understanding causality in host-microbiota interactions.

Chapter 3 explores the independent and interactive effects of host- and benthic substratum-associated microbiota on two key events in the life cycle of *Hormosira*: reproductive output (propagule release and quantity) and settlement of zygotes (attachment and morphogenesis).

Chapter 4 investigated the role of host-associated microbial communities in mediating the *Hormosira* holobiont response to extreme rainfall events. This chapter includes two manipulative experiments (laboratory and field), firstly testing for the effect of salinity on the

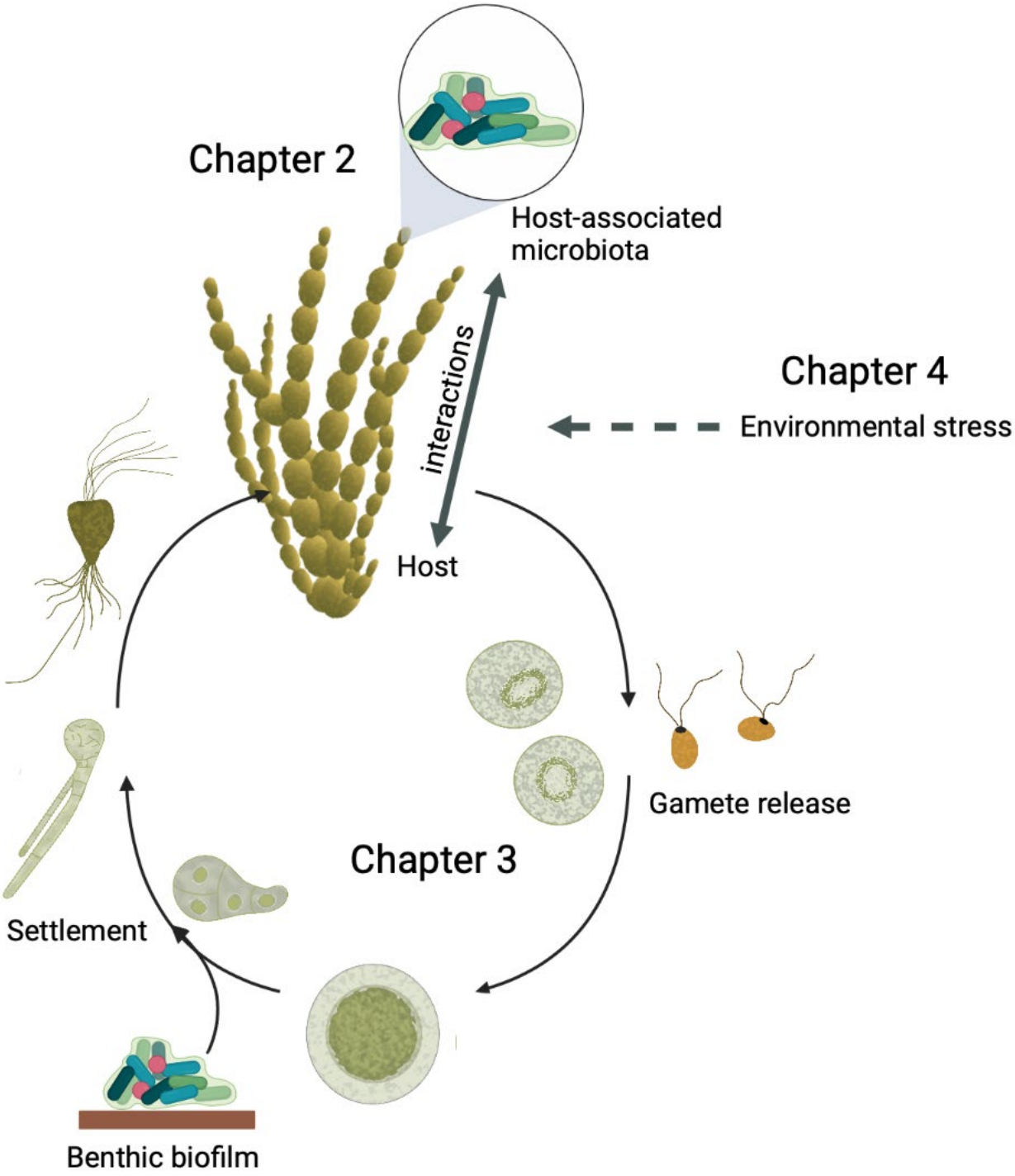


Figure 1.1 Schematic overview of the thesis. Chapter 2 addresses how we approach determining causality in non-model holobionts in the field. Chapter 3 determines the role of host-associated microbiota across ontogeny and the role of benthic biofilms in settlement. Chapter 4 explores the role of host-associated microbiota in host function, and potential fitness during extreme rainfall events.

holobiont, and secondly, exploring how microbial communities influence the response of the holobiont to extreme rainfall which occurred in Sydney in February-April 2022.

The final chapter (Chapter 5, General Discussion) synthesizes this research and draws together the outcomes and ideas presented in this thesis. It discusses the current knowledge of holobiont ecology and integrates ideas from both ecological and microbiological fields. Limitations and links with potential solutions are also discussed. Furthermore, this final chapter presents how this work may be applied to conserving and managing highly important habitat forming organisms in the face of climate change.

Chapter 2.

Disentangling direct versus indirect effects of microbiome manipulations in a habitat-forming marine holobiont.

2.1 Abstract

Host-associated microorganisms are now recognised as being critical for eukaryotic host functioning; however, most studies to date have focused on descriptive approaches or have used model systems, usually in the laboratory, to understand host-microbiome interactions. To advance our understanding of host-microbiome interactions and their wider ecological impacts, we need (i) robust experimental frameworks to explore causality in host-microbiome interactions and (ii) protocols that apply to model systems but also to often highly diverse natural systems. We used a dominant habitat-forming seaweed, *Hormosira banksii*, to explore a widely applicable framework for experimentally testing host-microbiome interactions. The experimental protocols were particularly designed to try and disentangle microbially mediated effects on hosts from direct effects on hosts associated with the methods employed to manipulate host-microbiota. This was done through a combination of antimicrobial treatments, which have widespread use in holobiont research, and inoculations, in mesocosms and in the field. Three different antibiotic treatments were used to disrupt seaweed-associated microbial communities to test whether such microbiome disturbances would negatively affect host performance. Responses of microbiomes to these disturbances were complex and differed substantially among treatments. However, by comparing the temporal sequence of antibiotic treatments, changes in microbial diversity, and decreases in host performance, a consistent effect of the microbiome on host performance was observed in some treatments. To further test these effects, we used 16S rRNA gene sequencing to identify microbial taxa that were both correlated and uncorrelated with poor host

performance following antibiotic treatment. These were then isolated and used in inoculation experiments, independently or in combination with the previously used antibiotic treatments. Negative effects on host condition were strongest where specific microbial disturbances (by particular antimicrobials) were combined with inoculations of strains correlated with poor host performance. For these treatments, negative host effects persisted the entire experimental period (12 days), even though treatments were only applied at the beginning of the experiment. Host condition recovered in all other treatments. This experimental framework allows for causal relationships to be determined within ecologically important holobionts. This should allow for better predictions of how these systems will respond to, and potentially mitigate, environmental disturbances in their natural context.

2.2 Introduction

Evidence from systems as diverse as coral reefs and the human gut indicates that host-associated microbial communities are fundamentally important for the normal development and functioning of eukaryotic hosts (Lozupone et al., 2012; McFall-Ngai et al., 2013; Rosenberg et al., 2007; Wilkins et al., 2019). This is leading to a paradigm shift in biology, where eukaryotes are no longer viewed as single entities, but rather as part of a coherent biological association comprised of the host and their microbiome, i.e. as "holobionts" (Dittami et al., 2021; Rosenberg & Zilber-Rosenberg, 2018).

Our knowledge of holobiont biology is mostly based on observational approaches, that is, descriptions of host or microbial characteristics of the holobiont, often measured in association with variation in environmental parameters or host performance (e.g Nielsen et al., 2016; Qiu et al., 2019). Such approaches typically involve sequencing and 'omics'

techniques that have allowed detailed characterisations of microbial diversity and, in some cases, predicted function of holobionts (Gilbert & Dupont, 2011; Hug et al., 2016; Lloyd-Price et al., 2019).

Descriptive studies are important in providing knowledge of the diversity (who is there?) and the functional potential (what can/do they do?) of host-associated microorganisms and how microbial diversity and function vary with host performance. These studies, however, do not untangle causal relationships, where the effects of complex microbial communities on hosts (and *vice versa*) can be distinguished and tested (Dittami et al., 2014; Prosser & Martiny, 2020).

While description of patterns is a fundamental first step in the scientific framework (Underwood et al., 2000), to understand complex systems we need to move beyond descriptions to experimental manipulations that determine causality and reveal potential mechanisms (Jansson & Prosser, 2013; Prosser, 2022; Underwood et al., 1997). Relying on inferred causation only from observations hinders, for example, our capacity to predict the responses of holobionts to disturbances or changing environmental conditions (Prosser & Martiny, 2020; Trevathan-Tackett et al., 2019). This issue has been long recognised in macrobial ecology, when half a century ago a largely descriptive field was transformed into a strong experimental discipline (e.g. Connell, 1961; Paine, 1966; Simberloff & Wilson, 1969). Unlike in macrobial ecology, the question of how to experimentally approach holobiont ecology remains a bottleneck and has become a major focus of modern microbial ecology (Prosser & Martiny, 2020). This is part due to the complexity of most holobionts which harbour thousands of taxa (Bourne et al., 2016; Donaldson et al., 2016; Marzinelli et al., 2016), giving rise to the potential for millions of interactions among these taxa and between the microbiome and the host. The most notable, if rare, example of an experimental system for the manipulation of complex microbial communities is the use of "germ-free" mice in

biomedical systems, which require intensive, whole-of-life-cycle facilities to raise mice without their normal microbiome (Fontaine et al., 2015; Kennedy et al., 2018).

Other experimental manipulations with holobionts have predominately been performed in "model" organisms, which have enabled us to determine causation and mechanisms, particularly where model eukaryotic hosts are associated with one or a few microbial symbionts, such as for the bobtail squid *Euphrymna scolopes* and its bacterial symbiont *Aliivibrio fisheri* (Koch & McFall-Ngai, 2018). Within algal systems, the clearest demonstrations of the role of complex microbial communities on host function (Reviewed in Egan et al., 2013a) are those of *Ulva spp* (see Nakanishi et al., 1996; Provasoli & Pintner, 1980) and *Ectocarpus spp* (see Dittami et al., 2016; KleinJan et al., 2021).

The process of unravelling the complex associations and interactions between hosts and their microbiome is at a very early stage for most non-model holobionts living in open, "real-world" environmental systems where large, structurally complex, habitat-forming holobionts such as trees, kelps or corals create the biogenic structure of ecosystems. Understanding of host-microbiome interactions for such foundational holobionts is particularly significant because the impacts on the interaction between these hosts and their microbiome can cascade throughout entire ecosystems (e.g. Laforest-Lapointe et al., 2017).

Experimental frameworks that allow establishing causation of host-microbe interactions in holobionts are beginning to be developed (Carrier & Reitzel, 2017; Dittami et al., 2014). In general, to determine the causative effects of the microbiome on host functioning, we need experimental approaches that manipulate the microbiome in a controlled manner (where there are no effects of the manipulations other than on the microbiome) and, if possible, in a specific fashion using targeted manipulations of specific microorganisms correlated with host performance. Moreover, ideally for the environmental systems of concern here,

manipulations would be conducted in the field (e.g., Campbell et al., 2011, 2014; Peixoto et al., 2021) to avoid unwanted effects that are linked to the laboratory environment, as has been the case for many other biotic interactions in ecology (Underwood et al., 2000).

One particular challenge for such manipulations is that many of the experimental protocols do not disentangle direct effects of treatments on the hosts from effects of manipulations of the microbiome on hosts. Experimental manipulations on holobionts can involve the selective removal/reduction of microorganisms of interest (e.g., those found to correlate either positively/negatively with host performance), the addition of such microorganisms or a combination of both. The removal or reduction/disruption of host-associated microorganisms using antibiotics is a common approach (reviewed in Kennedy et al., 2018) that has been used to study, for example, effects on host immunity, metabolism and disease (Blacher et al., 2019; Glasl et al., 2016; Peixoto et al., 2017). However, the key question which arises from their use is: how do we decouple the potential direct physical/chemical effects of antimicrobials on host performance (e.g. Kohanski et al., 2010; Rubin & Tamaoki, 2005) from indirect, microbially-mediated effects? This is a complex and ongoing issue as different antimicrobials cause vastly different effects, both within the host and on its microbiome (Looft et al., 2012; Luo & Song, 2021). Resolving this issue is critical to understand causation in holobionts and provides the foundation of the work presented here.

We used a dominant, habitat-forming marine holobiont to develop and test a general experimental framework for exploring cause-effect relationships between microbiome and host performance in non-model holobionts. We combined microbial reduction/disruption, using antibiotics, with inoculations of specific microbial taxa associated with a widespread marine habitat-forming fucoid alga, *Hormosira banksii* (hereafter *Hormosira*). *Hormosira* forms extensive intertidal forests along Australian and New Zealand rocky shores but has declined in many urbanised areas due to multiple anthropogenic stressors (e.g. Bellgrove,

McKenzie, et al., 2017; Doblin & Clayton, 1995). As with other similar seaweeds, such declines may be mediated by disruptions of their associated microbiome (Qiu et al., 2019).

We first used different antibiotic treatments that reduced/disrupted different components of *Hormosira*'s surface-associated microbiome to test the hypothesis that such microbial disruption would negatively affect host performance. To distinguish direct impacts of antibiotics from indirect, microbially-mediated effects, we isolated, and subsequently inoculated, microbial taxa whose abundance were either correlated (and thus predicted to affect the host when added) or uncorrelated (no effect predicted) with effects on the host. By inoculating such taxa, independently or in combination with microbiome reductions over an ecologically relevant temporal scale, we were able to establish causation within the microbiome. The benefits and challenges of these approaches, individually and in concert, are explored.

2.3 Methodology

2.3.1 Relationships between microbiome and host performance

Mesocosm experiment

Hormosira individuals of similar frond size (mean diameter: 7.8 +/- S.E 2.2 cm, length: 11.2 +/- S.E 0.81 cm; N=120) were haphazardly collected by carefully detaching their holdfast from intertidal rocks during low tide at Cronulla beach, Sydney, Australia (34°03'22.8" S 151°09'19.7" E) on 4 December 2018. *Hormosira* were transported in seawater within one hour to the flow-through aquarium facility at the Sydney Institute of Marine Science (SIMS) where they were thoroughly rinsed in autoclaved filtered (0.2 µm) seawater to remove fouling organisms and loosely associated microorganisms. *Hormosira* individuals were then placed into 12, 2-litre tanks (10 individuals 2.5cm apart per tank) by cable-tying their

holdfasts to weighted mesh (e.g. Marzinelli et al., 2009) and left overnight to acclimatize. Tanks were supplied by filtered (5 µm), UV-treated, running seawater. The following day, algae were rinsed with autoclaved filtered seawater (AFSW) and randomly assigned to one of three antibiotic combinations (AB1, AB2 or AB3; see details in Table S2.1) or an AFSW control. Algae were left to soak in the different antibiotic treatments or in AFSW (control) for 24 hours, then rinsed with AFSW and placed back into the 12 independent tanks (n=3 tanks per treatment; 10 individuals per tank) with UV treated, 5 µm filtered seawater.

One *Hormosira* individual was randomly selected and removed from each tank prior to the addition of any antibiotics (day 0), just after their treatment with antibiotics (day 1) and every second day for 1 week and on day 12 to characterise surface-associated microbiome and host performance (see details in Supplementary Information). Temporal sampling allows for the detection of unwanted direct effects as it is predicted that if antibiotics had any direct (e.g., chemical) effects on the host, such effects would be immediate (see Li et al 2022). On the other hand, if effects on host are microbially-mediated, it is predicted that there would be a lag between the application of the treatment and the response of the host (i.e. time for the microbiota to respond to the treatment and consequentially elicit a response on the host). Whilst temporal sampling provides evidence of direct effects if present, it is fundamentally important to also use suitable controls, i.e., in this case multiple antibiotics with different MOAs and chemical structures.

To characterise the associated microbiome, a consistent area of the algal surface (23 +/- SE 2.7 cm^2; adjacent to, but independent from the area where photosynthesis was measured, see below) was swabbed for 30 seconds with sterile cotton swabs, which were immediately placed in sterile cryogenic tubes in liquid nitrogen and then stored at -80°C until DNA extraction (as per Marzinelli et al., 2015). Host performance was assessed by quantifying the maximum photosynthetic quantum yield (after individuals were dark-adapted for 15 min)

using a Pulse Amplitude Modulated (PAM) fluorometer (WALZ, Germany), a metric of host performance previously used for macroalgae that generally reflects poor host condition under stress (Qiu et al., 2019; Straub et al., 2019).

Field experiment

Forty-two *Hormosira* individuals of similar size (approximately 6.8 +/- 2.3 cm in diameter and 8.5 +/- SE 1.3 cm in length) approximately ~1m apart were tagged at Cape Banks, Sydney, Australia (33°59'55.3" S 151°14'53.6" E) during low tide on 13 May 2018. Algae were rinsed with AFSW for 1 minute and then subjected to one of 6 different treatments: 1) the antibiotic combination AB2 (chosen due to its effect on the microbiome and host performance in the mesocosm experiment above) applied once for 2 hours during exposure at low tide, 2) Povidone Iodine 10% w/v ('iodine' hereafter) applied once for 2 hours (IO), 3) iodine applied every ~ 2 days for 15 minutes (IM), 4) a procedural control for the treatment application once (IMO; applying AFSW once for 2 hours), 5) a procedural control for the continuous treatment application (IMC; applying AFSW for 15 mins every ~ 2 days), 6) control (C, undisturbed individual; see details in Supplementary Table S1).

Half of the surface area of each individual (~12 +/- 2.7 cm^2) was swabbed for 30 seconds to quantify the surface-associated microbiome as described for the mesocosm experiment. Swab controls (i.e., used similarly to those for swabbing alga except no algae was swabbed) were collected. The other half was swabbed for bacterial culturing (details below); these swabs were stored in 1.5ml Eppendorf filled with 1ml of AFSW and placed in ice until arrival to the laboratory. Additional swab controls were collected to test for potential contamination in cultures due to the placement in AFSW. Samples were collected every 2^{nd} day for a total of 46 days (see Supplementary Table S2 for specific dates).

Bacterial isolation and culture

For both the laboratory and field experiments, swabs were in 1.5 ml Eppendorf tubes filled with 1ml of AFSW were immediately taken to the laboratory for spreading on plates. Briefly, the suspended swab was vortexed and 10 ul of the solution was then plated on standard half strength marine broth agar (MP Biomedicals LLC). Individual morphologically distinct colonies were picked after 24hrs. These colonies were suspended in liquid marine broth and left to grow for 48 hrs at room temperature on a shaker plate. 10 ul of the liquid culture was then plated as above on a new sterile agar plate. Colonies were replated until only 1 morphological form remained which was then extracted and sequenced (details below). Isolates were stored in glycerol at -80^{o}C until resuspension in marine broth for inoculations (see Supplementary Information for growth conditions).

Isolates were sequenced (details below) and the identity of the taxa were matched with those from the mesocosm and field experiment. Microbial amplicon sequence variant (hereafter, "ASV") abundance was correlated with host photosynthetic yield to identify isolates for the inoculation experiments. Two strains were used for inoculations: *Vibrio genomosp (F10 str. 9*ZC157) and *Vibrio chagasii (str* ECSMB 14107). *V. genomosp* was chosen as its increase in abundance post-antibiotic treatment was directly correlated with poor host performance (PAM) and had the greatest change in total abundance over the experiment (Figure S1). *V. chagasii* was chosen as it was phylogenetically similar to *V. genomosp* but remained at a constant abundance throughout the experiment and was thus uncorrelated to variation in host performance. It was therefore used as a control inoculant (Figure S1).

2.3.2 Testing for direct effects of components of the microbiome

Inoculation experiment

Hormosira individuals of similar length (7.5cm +/- SE 1.2; N=110) were haphazardly collected as described above from the rocky shore at Cape Banks on 25 January 2021 and

transported to the SIMS aquarium within an hour, where they were rinsed with AFSW. *Hormosira* individuals were then placed into 15ml falcon tubes (1 individual per tube) and attached via their holdfast to a stainless-steel rod at the bottom of the tube (Fig. S3). Each tube was individually fed filtered (50 μm) seawater through aquarium airlines from four replicate header tanks. Once all samples were attached in the aquarium, they were left overnight to acclimatise. On the next day, all tubes were rinsed with AFSW and the algae inside was randomly assigned to one of seven treatments (Table S1):

1) Microbiome disruption with antibiotic combination AB2 applied once for 25 hours (as in the mesocosm experiment; Table S1);

2) Microbiome disruption as in (1) followed by inoculation with bacteria whose relative abundances correlated with poor host condition in the experiments above (*Vibrio genomosp,* see Fig S1; cell density 5×10^8 CFU);

3) Microbiome disruption as in (1) followed by inoculation with bacteria unrelated with host performance in the experiments above (negative control: *Vibrio chagsii sp.*; see Fig S1; cell density 5×10^8 CFU);

4) Microbiome left undisturbed (AFSW) for 24 hours followed by inoculation with *V. genomosp* as in (2);

5) Microbiome left undisturbed (AFSW) for 24 hours followed by inoculation with the negative control strain as in (3);

6) Procedural control, AFSW for 25 hours;

7) Control (undisturbed algae).

Inoculations were done by immersing *Hormosira* for 1 hour within the high-density cell culture which allowed sufficient time for the inoculated bacteria to attach to algal surfaces

(See supplementary figure S1). This timing was confirmed by leaving individuals within high cell density washed cultures for varying times before being swabbed as above. DNA was extracted and custom qPCR primers for the inoculants were used to determine abundance (see Supplementary Information for further details)

Samples were collected following the same timepoints as in the previous experiment: 5 individuals were collected at day 0 before treatments were applied and 3 independent individuals were randomly selected from each treatment on days 1, 2, 5, 8 and 12, at the same time of day on each sampling occasion. These time-points and the overall duration of this experiment were based on results from the previous experiments above which showed effects on microbiome and hosts within ~3-5 days (see Results) and our capacity to maintain independent replicate algae for each treatment x time combination. Surface-associated bacteria and algal photosynthetic efficiency was then characterised for each sample as described above (See mesocosm experiment above).

DNA extraction and sequencing

For characterisation of microbial communities in all experiments, microbial DNA was extracted from each swab sample in a randomised order using a PowerSoil DNA Isolation kit (Qiagen) following the manufacturers protocol. DNA extracts were quantified using spectrophotometry (NanoDrop 1000) and stored at -20ºC until sequencing.

The extracted DNA samples were amplified with Polymerase Chain Reaction (PCR) using the 16S rRNA gene primers 341 (F) (5'- CCTACGGGNGGCWGCAG-3') and 805(R) – (5'-GACTACHVGGGTATCTAATCC-'3), covering the V3-V4 regions of the bacterial and archaeal 16S rRNA gene (Klindworth et al., 2013). The PCR conditions involved a pre-heating step to 95 ºC for 3 min followed by 35 cycles of 95ºC for 15 s, 55ºC for 1 min and 73ºC for 30 s. Both positive (with known DNA sequence) and negative controls (nuclease-

free water, control swabs) were used. The negative controls did not amplify DNA, suggesting no contamination on swabs/materials or during extraction and amplification. Agarose gel electrophoresis and Nanodrop 1000 were used to ensure the quantity and quality of the amplicons before they were sent for sequencing via the Illumina MiSeq 2000 platform at the Ramaciotti Centre for Genomics (UNSW, Sydney).

Bioinformatics

Raw sequencing data was quality filtered using Trimmomatic (Bolger et al., 2014) with a sliding window trim of 4:15 base pairs (bp) and removal of sequences with <36bp. Paired-end reads were merged with a minimum length of 400bp and maximum of 500bp using USEARCH (Edgar, 2017). UNOISE was then used to remove chimeras and produce amplicon sequence variants (ASVs), i.e., operational taxonomic units at a unique sequence level (0% distance) (Edgar, 2017). USEARCH was used to map the original reads back to ASVs, generating a table of 5812, 7637 and 9138 ASVs for the mesocosm, field and inoculation experiments, respectively. ASV sequences were searched with BlastN against the SILVA SSU Ref NR99 database for taxonomic classification to classify and remove chloroplasts, GTDB was then used for taxonomic assignment. Singletons and low abundance taxa (<0.01% of reads) were removed from the dataset for statistical analyses, resulting in 4112, 6488 and 7028 ASVs (mesocosm, field and inoculation experiments respectively).

Estimation of absolute bacterial abundance

Total abundance of the 16S rRNA gene was quantified for each sample by qPCR using the primers 341F/805R (Thijs et al., 2017). Gene amplification and analysis were performed using the QuantStudio 3 thermocycler (Thermo Fisher with PrimeTime® Gene Expression Master Mix, Integrated DNA Technologies) and associated software. The reaction conditions for amplification of DNA were 56ºC for 2 min, 95 ºC for 10 min and 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The final gene copy number per sample was corrected for the total

extraction volume, the surface area and the dilution factor and DNA yield per sample (see (Nappi et al., 2022) for further details on the normalisation) and were used to estimate absolute abundances of ASVs and inoculants.

Statistical Analyses

To account for uneven sequencing depth among samples, data were normalised using the counts per million reads method in the R package DESeq2 (Love et al., 2014). This resulted in a total of 4,423,807, 10,443,709 and 9,921,043 sequences for the mesocosm, field experiment and inoculation datasets, respectively.

To test for effects of microbiome manipulations on host photosynthetic efficiency, we used a linear model with the factors treatment (fixed) and time (fixed, crossed). For the first mesocosm experiment where multiple *Hormosira* individuals were sampled from each tank, 'tank' was fitted as a random factor nested within treatment using the lme4 R package (v4.0.3). To meet the model's assumptions, data were square root transformed. p-values were obtained using the anova function within the car package in R with significance being tested using F-tests, or likelihood ratio tests for the mixed model that included tank as a random effect.

Alpha diversity measures of richness (i.e., number of unique sequences) and Simpson's diversity index were calculated using the 'vegan' R package (Oksanen et al., 2013) and differences between treatment (fixed) and time (fixed, crossed) were examined using a linear model in the R GAD package. Where appropriate, tank was included as a random factor.

To determine differences in the structure of the associated bacterial assemblages, the normalised ASV data were analysed using permutational multivariate analysis of variance (PERMANOVA) (Anderson & Walsh, 2013) in the R vegan package (Oksanen et al., 2013), with the factors treatment, time and tank (mesocosm experiment only) as above. Similarity

matrices were calculated using Bray-Curtis measure on square-root transformed data and visualised through non-metric multi-dimensional scaling (nMDS) ordinations. We also calculated and plotted mean Bray-Curtis similarities to the control (undisturbed algae) treatment. To determine which bacterial taxa's abundance differed among treatments and times, we used multivariate generalised linear models (GLMs) using the R package 'mvabund' (Wang et al., 2012), assuming a negative-binomial distribution to account for over-dispersion. In order to determine how the total abundance of the two inoculants changed over the course of the experiment, linear models were fitted with the factors treatment (fixed) and time (fixed, crossed) in R (v4.0.3).

2.4 Results

2.4.1 Mesocosm experiment

Antibiotic treatments significantly changed the bacterial community structure on *Hormosira* in the mesocosm experiment, but there were significant differences among different antibiotics and over time (PERMANOVA pseudo-$F_{9, 32}$=1.29, p<0.001; Table S7; Fig. 2.1.B). The effects of the antibiotics were not immediate, with no effect of treatments at time 1. As of day 2 *Hormosira* treated with antibiotic mix 2 had a significantly different community structure than all other treatments and that difference remained until the end of the experiment at day 12 ($F_{9, 32}$=2.71, p<0.001). Antibiotic mix 3's microbial community differed significantly from controls at day 2 which lasted till day 12 ($F_{9, 32}$ = 1.94, p<0.001; Fig. 1B) and was significantly different from controls and all other treatments at day 12 ($F_{9, 32}$ = 3.63, p<0.001; Fig. 2.1B).

Community richness was significantly lower in the treatment antibiotic mix 2 than the control (ANOVA, $F_{3, 80}$=6.05, p<0.001; Table S8), Antibiotic mix 3 had significantly lower richness

than controls (ANOVA, $F_{3, 80}$=4.84, p<0.016). Simpson's diversity index was significantly lower in the antibiotic mix 3 than all other treatments at the end of the experiment (day 12, ANOVA, $F_{3, 80}$= 5.49, p<0.001; Table S8).

These changes in the microbiome correlated with changes in host photosynthesis, which occurred ~3 days after treatment with AB2. *Hormosira's* photosynthetic yield under antibiotic mix 2 experienced a continuous and significant decrease after 5 days (ANOVA, $F_{9,32}$= 8.56, *p<0.001*). Algae treated with antibiotic mix 3 also had decreased photosynthetic yield compared to controls, but only towards the end of the experiment, with the lowest decrease at day 12 (ANOVA, $F_{9,32}$= 7.16, p<*0.001*; Fig. 2.1A). Algae treated with the antibiotic mix 1 or in the control did not show any change in photosynthetic yield over time (Fig. 2.1A).

2.4.2 Field experiment

In the field, bacterial relative abundance and community structure differed significantly across time and among treatments (PERMANOVA, pseudo-$F_{20,98}$=1.6505, p<0.001, Fig 2.1.C and D; Table S9). Antibiotics and iodine both caused significant changes in community structure from day 7 which lasted for the 46 days of the experiment (PERMANOVA, pseudo-$F_{20,98}$= 3.35, p<0.001). Multiple applications of iodine caused greater changes in community structure than a single application alone (PERMANOVA, $F_{5,98}$= 3.506, p<0.001). Antibiotics, when administered once, had a similar effect to multiple applications of iodine (PERMANOVA, t= 1.706, p>0.05; Table S9). The number of bacterial

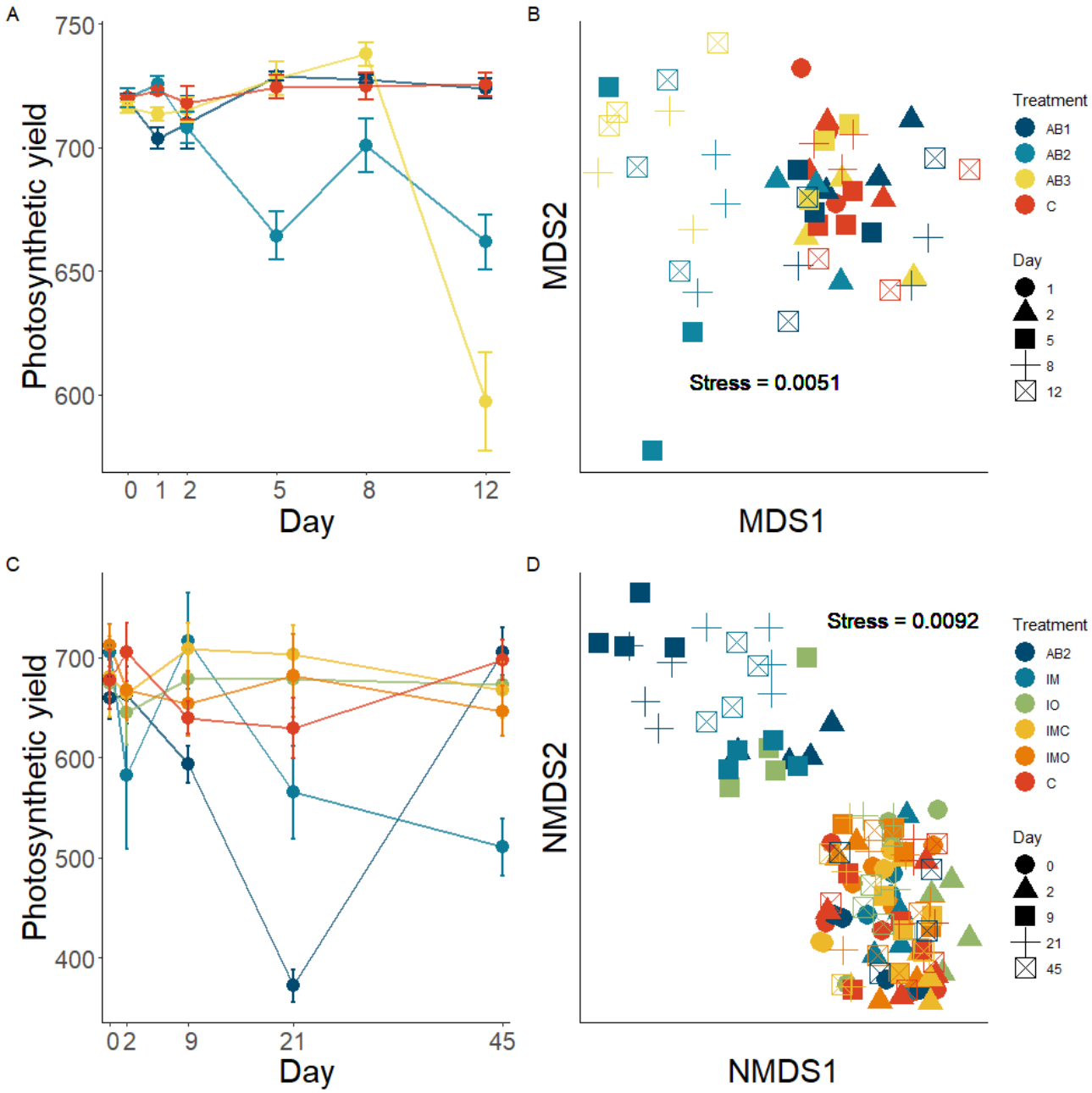


Fig. 2.1. Host photosynthetic efficiency (A, C) means +/- SE, n=3; and microbial community responses Bray-Curtis dissimilarity (B, D) to experimental treatments in the mesocosm (A,B) and field (C,D) experiments. Microbial community data were normalised, and square root transformed prior to calculations of dissimilarities. Treatments are (AB1) Antibiotic mix 1; (AB2) Antibiotic mix 2; (AB3) Antibiotic mix 3; (C) Control; (IM) Iodine press; (IO) Iodine pulse; (IMC) Iodine press control; (PC) Procedural control (AFSW).

ASVs significantly decreased in algae treated with antibiotic and iodine relative to controls (ANOVA, $F_{20,98}$= 4.92, p<0.001)

Photosynthetic yield of the host differed significantly among treatments, with individuals in the antibiotic treatment AB2 showing a significant decline in photosynthetic efficiency over time (ANOVA, $F_{4,162}$=6.145, p<0.001; Table S6), starting at day 9 with the greatest effect occurring at day 21. This effect remained significant until day 46, when photosynthetic efficiency did not differ from controls (Fig. 2.1.C; Table S6). Algae treated multiple times with iodine (IodineM) also showed lower photosynthetic yield than controls and single application of iodine but did not recover by the end of the experiment. Again, this was after a lag period of 21 days (ANOVA, $F_{20,162}$=3.201, p<0.001; Table S6). No other treatments differed from the controls which was coupled with no significant differences in the community composition within these treatments.

2.4.3 Bacterial Isolation

To identify strains correlated with host function we isolated bacteria, leading to over 140 isolates, of which 22 strains were correlated with poor host performance within both the mesocosm and field experiments. The abundance/presence of *Vibrio genomosp* sp was most strongly correlated with poor host performance (r=-0.68, p <0.0001; Fig S2.2) with its abundance significantly increasing in all disruption treatments except AB1 (ANOVA, $F_{9,32}$= 4.39, p<0.001). There were also many bacterial taxa whose abundances were not correlated with host performance, e.g., *Vibrio chagasii* sp (r=0.14, p<0.12; Fig S2.2).

2.4.4 Inoculation experiment

GLM analyses identified 172 ASVs (approx. 1.8% of a total of 9138 ASVs) whose abundances differed significantly among treatments over the duration of the inoculation experiment. Of those, we found a strong main effect of treatment on 10 ASVs in the classes

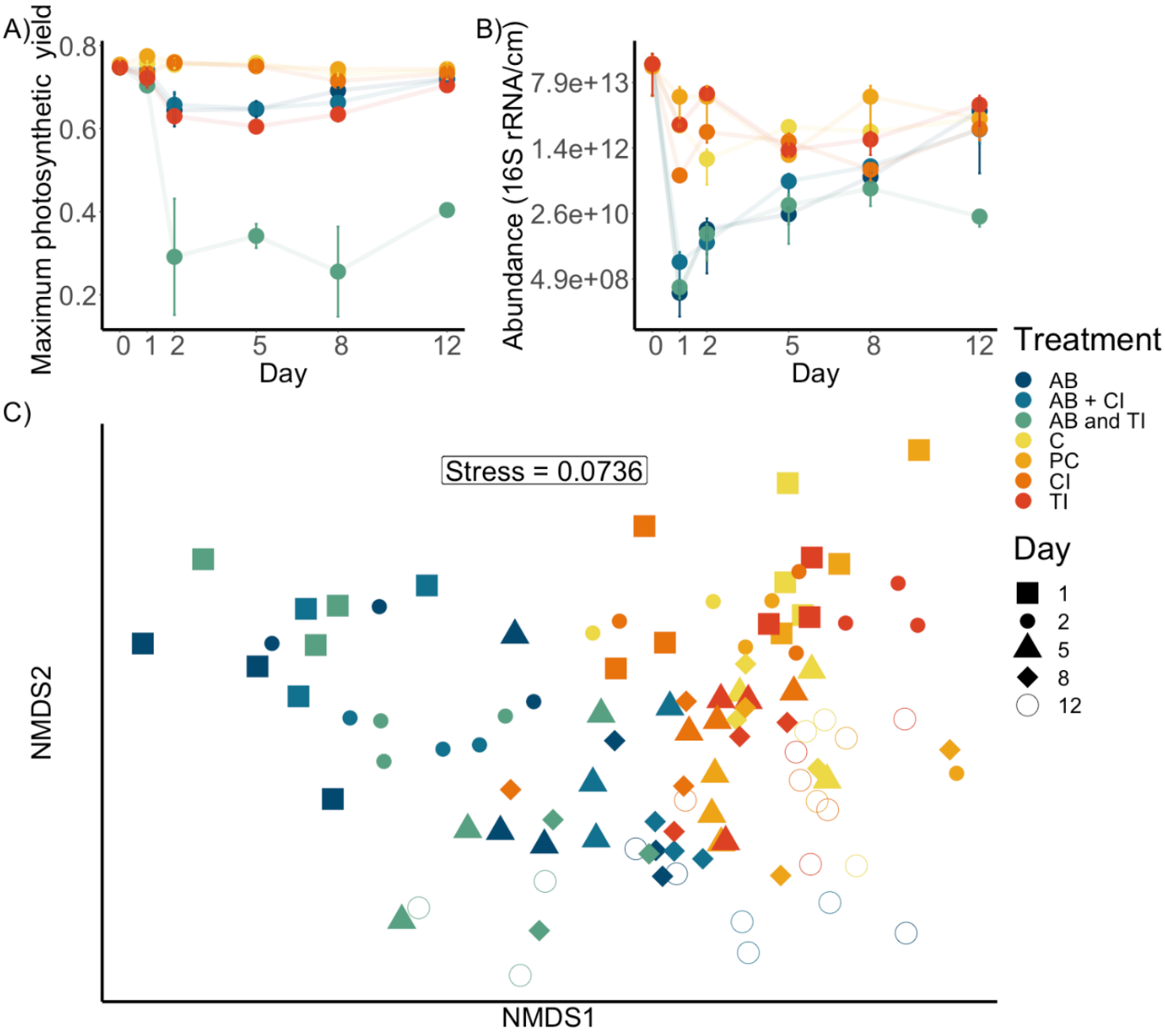


Fig. 2.2. Host and microbial community responses to experimental treatments within the inoculation experiment (Clockwise from top-left); A) Host photosynthetic yield; B) Absolute abundance of the 16S rRNA gene within each treatment over time obtained with qPCR; C) nMDS based on the Bray-Curtis measure on square root transformed absolute abundances of ASVs on the algal surface across treatments (stress =0.0736). Treatments are: (AB2)

Antibiotic mix 2; (AB2 + CI) Antibiotic mix 2 plus control inoculant (*Vibrio chagasii*); (AB2 + TI) Antibiotic mix 2

Gammaproteobacteria, Alphaproteobacteria and Cyanophyceae, with abundances of the latter two being lower in all treatments with microbiome disruption. There was a significant increase in *Vibrio* species alongside a significant increase of the inoculants *V. chagasii* and *V. genomosp* whose abundances were significantly higher within inoculation treatments (ANOVA, $F_{5,24} = 1.77$, $p<0.001$; Fig 2.3).

Inoculation with *V. genomosp* when combined with a disruption of the microbiome had the strongest effect on microbial community structure, which differed from all other treatments at day 2 and lasted until the end of the experiment (ANOVA, $F_{5,24}=4.28$, $p<0.001$; Fig. 2.2.C and D). The microbiome structure of algae disrupted with antibiotics and inoculated with *V. chagasii* did not differ from disruption with antibiotics but was different from controls (ANOVA, $F_{5,24}=1.31$, $p<0.01$; Fig. 2.2.C) indicating that whilst the strain was present it did not influence community structure. No significant differences were found throughout the experiment among the three control treatments (Control, Procedural control, and inoculation control, Table S12). Furthermore, all treatments with antibiotics had significantly lower richness than controls until day 8 where there was no significant difference among treatments (ANOVA, $F_{5,24}= 0.969$, $p=0.21$).

All antibiotic treatments, including both treatments where the algae were further inoculated with both *Vibrio* spp., had significantly lower photosynthetic yield than those where microbiomes were left undisturbed and this effect was observed ~2 days after observed microbial changes (ANOVA, $F_{5,24}= 4.25$, $p<0.001$, Fig 2.2.A). Inoculation with *V. genomosp* had a similar effect on photosynthesis as treatments with antibiotics but without inoculants or inoculated with *V. chagasii* (ANOVA, $F_{5,24}=1.58$, $p<0.03$, Fig 2.2.A). All treatments, except

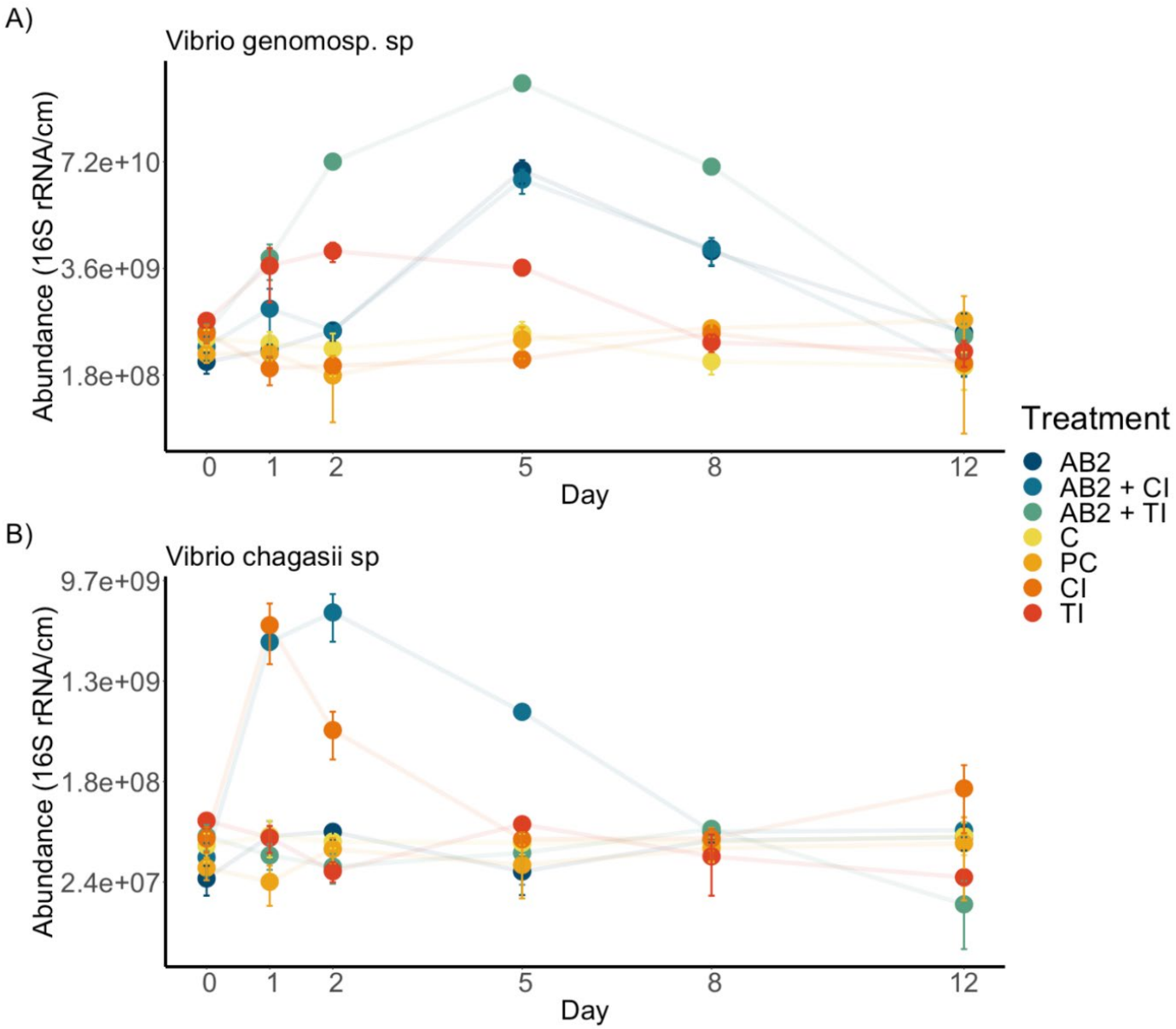


Fig 2.3 Absolute abundances (means +/- SE, n=3) of inoculants used within the inoculation experiment. Data are means and SE (n=3) calculated using sequencing data which was normalised using qPCR of the 16S rRNA gene; (Left) Vibrio *genomosp*; (Right) *Vibrio chagasii sp.* across treatments and time (days). Treatments are (AB2) Antibiotic mix 2; (AB2 + CI) Antibiotic mix 2 plus control inoculant (*Vibrio chagasii*); (AB2 + TI) Antibiotic mix 2 plus test inoculant (*Vibrio genomosp*); (C) Control (Undisturbed algae); (PC) Procedural control (AFSW); (CI) Control inoculant (*Vibrio chagasii*); (TI) Test inoculant (*Vibrio genomosp*).

microbial disruption followed by inoculation with *V. genomosp,* recovered to the same level of photosynthetic yield that was present at the start of the experiment by day 12.

2.5 Discussion

The structure and function of host-associated microbiomes are strongly related with host performance, but as is highlighted in the recent literature (Marchesi et al., 2016), we still have very limited understanding of cause-effect in these interactions in complex, non-model holobionts. Here, we identified the effect of the microbiome, or subsets thereof, on the performance (photosynthesis) of a dominant, marine habitat-forming holobiont via a combination of removals and additions of microbial taxa associated with the host's performance in mesocosms and field experiments. We disrupted the host's microbiome using a series of different antibiotic mixes with several methods of action, also chosen because they are thought to not have direct toxic effects on multicellular algae at the concentrations used (see Fu et al., 2017; Li et al., 2022).We showed that the reduction/disruption of different components of the microbiome affected host performance in different ways, which changed in a non-linear fashion over time. Temporal sampling added further evidence of microbially mediated effects, as any direct chemical effect of the antimicrobials on the host would likely be immediate (Ebert et al., 2011; Fu et al., 2017) whereas microbially-mediated effects would take time following the removal/decline of taxa (Schmidt & Saha, 2021), as was the case in our experiments in both the lab and the field.

2.5.1 Microbiome manipulations using antibiotics

In the mesocosm experiment, when exposed to antibiotics, all microbial communities were significantly altered. This was followed by a decrease in host performance, i.e., there was a lag between the microbial changes and the effect on host performance. In addition, different antibiotic mixes affected the microbiome differently despite having similar microbial targets (Table S2.2). For instance, AB1 influenced the microbiome but not the host, while AB2 and

AB3 affected the microbiome and subsequently the host, but host responses had different lag times (3 vs 5 days after microbial changes, respectively). Because (i) microbial changes occurred before host changes, (ii) the antibiotics had no discernible immediate effect on the host which would be expected from previous work and (iii) the three antibiotic mixes had similar targets but varying MOA, all this suggests no (undesired) direct effects of the antibiotics on the host – changes in host performance were therefore likely caused by changes in the microbiome. Given the challenges associated with controlling for undesired, direct effects of treatments on hosts, we argue that the combination of the use of several antimicrobials with similar targets but varying MOAs and chemical structures, and temporal sampling of the microbiome with host performance in parallel provides a robust approach to understanding microbially mediated effects on host function. Within this work the antibiotics used were selected from previous work which has suggested that they do not have chemical effects on the host (Dittami et al., 2016). However, without direct characterisation of the interaction of the chemicals with the host's tissue we cannot be completely sure that this is the case, thus the need for suitable treatment controls and temporal sampling. Microscopy of the host could be incorporated to determine potential impacts on its cells, in addition to rapid physiological assessments such as done here with maximum photosynthetic yield (e.g. Dittami et al., 2016; Fuggle et al., 2023) .

The antibiotic mix which had the most significant and lasting effect was antibiotic mix 2 (AB2), whose difference from the other antibiotic mixes was the inclusion of the microbial RNA polymerase inhibiting Rifampicin. Antibiotic mix 3 (AB3) also contained a similar antibiotic target (Chloramphenicol) and was close to AB2's efficacy in significantly decreasing relevant microbial taxa from the microbiome. Indeed, the main group negatively affected by these two treatments were nitrogen-fixing cyanobacteria which we were unable to get into a pure culture. The most abundant cyanobacteria ranged from 1-3% (*Pleurocapsa* sp)

of the total abundance. N-fixing bacteria such as these have been implicated in numerous symbioses with terrestrial, and more recently, marine plants (Fiore et al., 2010; Mohr et al., 2021; Nutman, 1969). N is a limiting nutrient within intertidal environments with many algal species often relying on extracellular N fixation to meet their physiological needs (Bracken & Nielsen, 2004; Pfister, 2007). This may explain the observed decline in host performance.

Microbiome manipulations in the field experiment supported the results from our mesocosm experiment as microbial disruption to the host caused significant, negative effects on host performance. As in the lab, the microbial disruption caused by AB2 had a significant negative effect on host function, which eventually recovered over time (45 Days). Antiseptics (Povidone iodine 10%) had a similar, negative effect on host function when applied once (pulse) but caused lasting, negative effects on the host when applied multiple times (press). This approach incorporating both pulse and press stressors allows us to probe the resistance and resilience of *Hormosira*'s microbiome (Bender et al., 1984). This approach also mimics the natural environment closer where *Hormosira* is exposed to both press (e.g., increasing ocean temperatures) and pulse (e.g., desiccation at low tide or extreme rainfall) stressors over varying timescales. Interestingly, as in the lab, cyanobacteria were the main bacteria which remained at low abundances during the press treatments. Whilst our disruptions within both the lab and field show consistent patterns, many others have shown difficulty in relating findings in the lab to real-world settings (see Stachowicz et al., 2008), highlighting the need to run these experiments in the field wherever possible. This is particularly important in the context of environmental change because stressors can interact in complex ways, leading to different responses of organisms depending on the environmental context (Mayer-Pinto et al., 2015; Orr et al., 2020).

2.5.2 Inoculations

Disruption of the microbiome then allowed us to develop the second experimental approach – inoculations of relevant taxa – in an ecologically realistic way. From the lab and field experiments we were able to isolate over 22 unique strains associated with poor host performance. These isolates were dominated by highly culturable taxa from the orders *Altermonadales* and *Vibrionales*, with several ASVs being assigned to taxa that are known pathogens. Although the isolated taxa made up ~1% of total taxonomic diversity within the community, their abundance contributed to ~5% of the total. We used some of these taxa in inoculation experiments combined with microbial disruptions to determine their independent or combined effect on host performance. Importantly though, and a limitation of this method is the fact that we were unable to culture bacterial taxa positively correlated with host function. This would be a logical first step for others to attempt and should be a focus of future work. Alongside this, due to the increasing recognition of the prevalence of marine fungi associated with hosts (see Amend et al., 2019; Tourneroche et al., 2019), future work should also include attempts to isolate these members of the community.

Microbial disruption, when combined with inoculations of bacterial taxa associated with poor host function (*Vibrio genomosp* sp), led to a much stronger negative effect on host performance than disruption or inoculation alone, and their performance did not recover during our experiment. This treatment also led to a shift in the bacterial community structure that did not recover throughout the experiment, suggesting that a threshold may have been reached where the resilience of the microbial community was overcome. Environmental stressors can lead to dysbiosis or an imbalance in the microbiome of holobionts as diverse as corals, kelps and humans, which, in turn, can make them more susceptible to being affected by pathogens (Greenspan et al., 2020; Minich et al., 2018). Thus, the combination of microbial disturbance and inoculations with taxa related to poor host performance can help

understand and better predict responses of holobionts to environmental stressors and the potential mechanisms underlying such responses.

Bacteria belonging to the genera *Vibrio, Pseudoalteromonas* and *Phaeobacter* have been described as antagonistic towards their hosts (Li et al., 2022), although this is species-specific as certain *Phaeobacter* species have been shown to provide antifouling and antilarval abilities (Rao et al., 2007). The specificity of strains is further demonstrated as inoculation with our control inoculant *Vibrio changasii* spp, despite its close taxonomic relation to *Vibrio genomosp*, did not induce a negative effect on host condition – this was expected as this taxon was chosen as an inoculation control because its abundance did not correlate with host performance in our first set of experiments. Sequencing analyses after inoculation showed a significant increase in the abundance of both *Vibrio chagsii* spp and *Vibrio genomosp* indicating that our inoculation was successful in inducing temporary establishment within the host microbiome. However, this establishment did not last throughout the experiment; abundances of both inoculants returned to control levels by the end of the experimental period. Both strains have been reported as opportunists within other host species and are not found in high abundances within the undisturbed microbiome of *H. banksii*, which may be the result of them being outcompeted by other taxa in the microbial assemblage. This initial increase in abundance followed by rapid decrease is supported by recent work showing that commensal and mutualistic bacterial taxa switch to antagonistic pathogens when disrupted (Hoeger et al., 2022).

3.5.3 Understanding holobionts in their natural ecological settings

To enable us to understand how holobionts will respond to environmental change, it is important to study them within their natural ecological settings. This is inherently a difficult problem in natural systems, where thousands of taxa may contribute to millions of potential interactions. Here we have developed an experimental protocol that combines microbiome

disruption using antibiotics, and the identification and subsequent inoculation of presumed harmful bacteria. This was done in both the laboratory and the field. We believe this combined approach, applied (wherever possible) in real-world settings, provides a powerful and necessary approach towards a causal understanding of these interactions. However, there are critical aspects that still need to be considered when interpreting outcomes of these experimental approaches, particularly the complex responses of microorganisms to different antimicrobials, the potential direct (not microbially mediated) effects on the host of the methodology employed to manipulate microbiome, and the temporal sequence of events and the environmental context (i.e. lab vs field). We showed that disruption of the host-associated microbiome using different antibiotics and subsequent temporal sampling of microbial changes and host performance provided critical information on the microorganisms potentially associated with host function, a pattern that was consistent in mesocosms and in the field. We then targeted, isolated and cultured relevant microbial taxa associated with changes in host performance (or lack thereof, as controls) and used them in controlled experiments to formally test their impact on the host. This approach increased the strength of our inferences as we can start to disentangle the effects of disturbance on the broader microbial community (e.g., dysbiosis caused by the antimicrobials) from the effect of individual strains of relevant microbial taxa, or both combined. A logical progression of this work is to incorporate "natural" disruptions, as those influenced by environmental changes, allowing better predictions of how these ecosystems will respond to, and potentially mitigate, future environmental disturbances.

2.6 Supplementary Information – Chapter 2

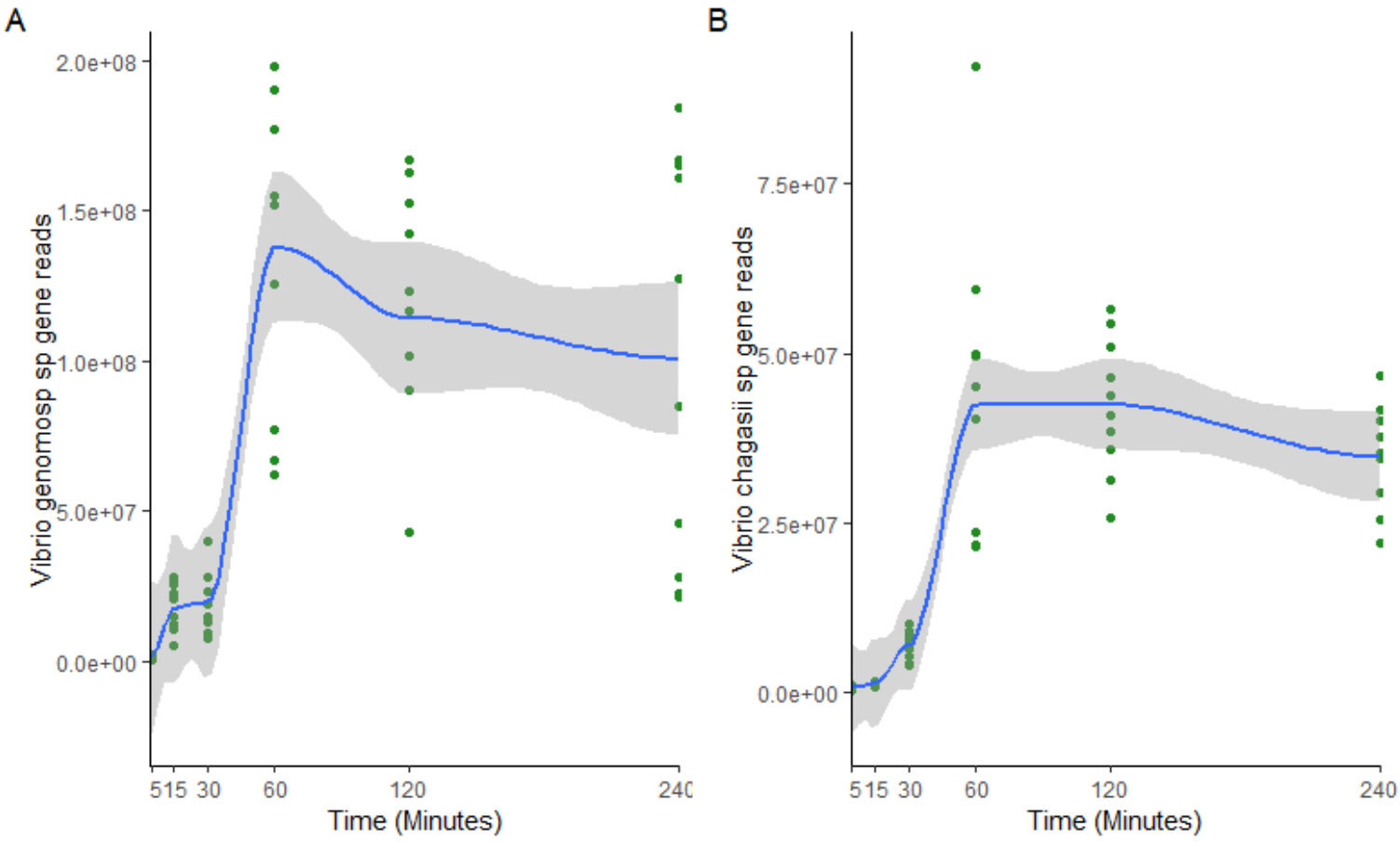
Culture conditions for bacterial inoculants

Bacterial isolates stored within 12.5% glycerol at -80ºC were removed from the freezer and thawed on ice. Once thawed, isolate was gently vortexed for 10s, 10ul of the isolate-glycerol solution was pipetted into a sterile marine broth agar plate and raked. Bacterial stock solution was then return to the freezer and the plate was placed into an incubator set to 25oC overnight. The following day, the plates were checked to ensure that only 1 morphological form had grown, an individual colony was picked using a sterile inoculating loop and placed into 25ml of liquid marine broth. The solution was vortexed and then placed on a shaker tray set to 100rpm. The culture was grown until it reached a cell density of 5×10^8 CFU (i.e., culture was in a stationary phase) which was confirmed using spectroscopy (OD ~ 0.600).

Determination of isolate attachment success and custom primers

Inoculation success was tested for both candidate inoculants (*Vibrio genomosp* and *Vibrio Chagasii)*. This was done by exposing 60 individuals of Hormosira for each inoculant to cell suspensions (cell density 5×10^8 CFU) of *Vibrio genomosp* and *Vibrio Chagasii* for varying lengths of time (5, 15, 30, 60, 120 and 240 minutes). At each timepoint 10 individuals were rinsed with AFSW and swabbed using protocols described in the methods. DNA was extracted using a powersoil pro kit (Qiagen) and qPCR used to determine the abundance of the isolates attached. Custom primers were used; *Vibrio genomosp* (F) (5'-TCAGCGCCTTTGCTATCGGA-3'), (R) (5'-TGCGGATGGCAGTGAGACAT-3') and (PRB) ('5- /56-FAM/TCAGAAGCC/ZEN/ATAGTCGGGAGTAGCC/3IABkFQ/-3'); and *Vibrio chagasii* (F) ('5- TACCGCTGACGAAGTATCGC-3'), (R) ('5-TTCGCATTCCTCGGGTTTCA-3') and (PRB) ('5-/56FAM/TCATGTCCC/ZEN/AGTGTTGTCGCCAAT/3IABkFQ/-3'). 60 minutes was

chosen as the appropriate length of time as within both inoculants there was the greatest attachment at this timepoint (Figure S2.1).



Supplementary figure 2.1 – Abundances of gene reads extracted from qPCR data using custom primers (A) *Vibrio genomosp sp* and (B) *Vibrio chagasii*. Shaded areas indicate smoothing function applied to the data.

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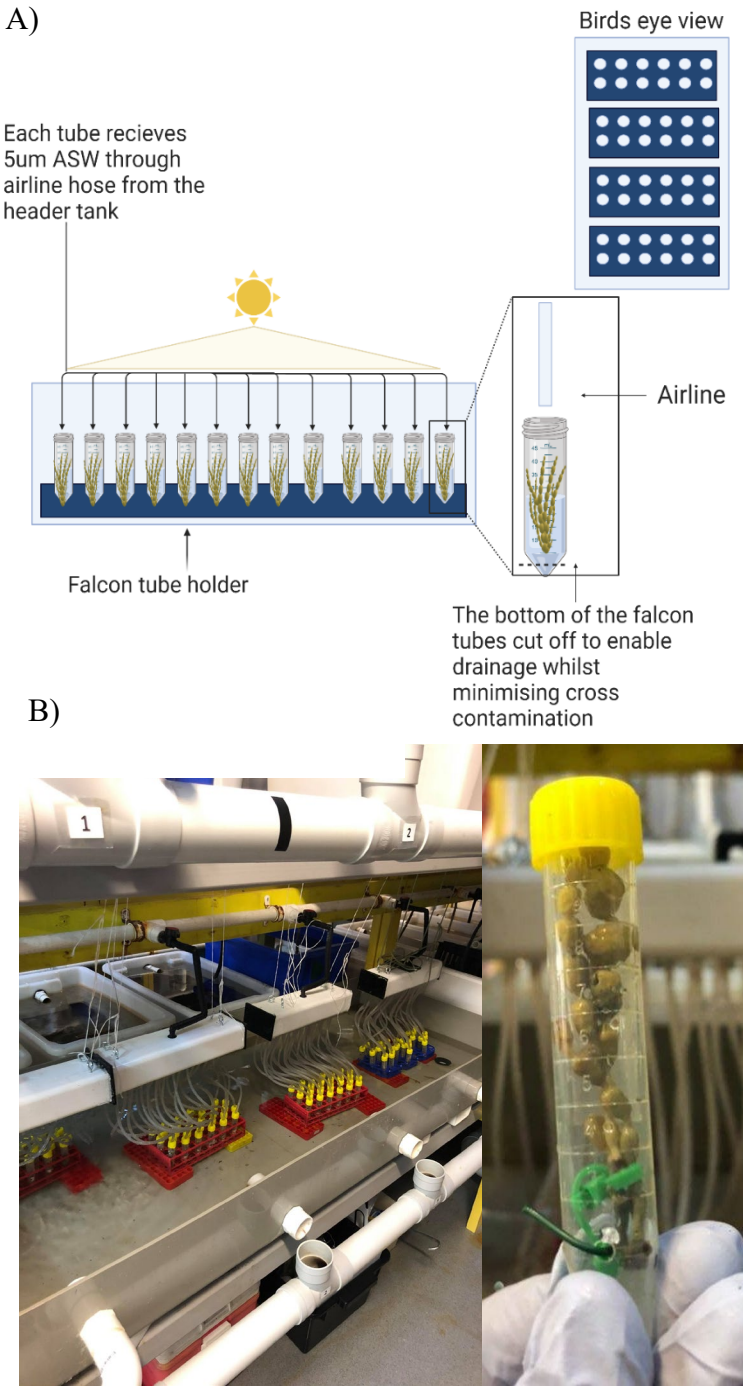


Supplementary figure 3 – Experimental setup showing schematic diagram (A) alongside actual setup (B). Each individual algal was attached by their holdfast to the bottom of a 15ml falcon tube. Each tube is then fed an individual and constant supply of 5um filtered seawater

Table S2.1; Description of treatments used within the 3 experiments (Mesocosm, Field and Inoculation)

Experiment	a) Abbreviation	b) Treatment's specification	c) Treatment application	d) Sampling times for amplicon sequencing	e) Sampling times for Photosynthetic yield
Mesocosm experiment	AB1	Antiobiotic treatement 1: Penincilin (100mg/ml)/Streptomycin(25mg/ml)/Norfloxacin (1mg/ml)/Kanamycin (25mg/ml)	24 hours in antibiotic solution and rinsed with AFSW	Day 1 (24hours after antibiotic treatement-06/12/2018); day 2 (7/12/2018); day 5(10/12/2018);	Day 0 (before antibiotic treatment-05/12/2018); day 1 (24hours after antibiotic treatement-06/12/2018); day 2 (7/12/2018); day 5
	AB2	Antiobiotic treatement 2: Penincilin (100mg/ml)/Neomycin(100mg/ml)/Rifampicin(50mg/ml)	24 hours in antibiotic solution and rinsed with AFSW		
	AB3	Antiobiotic treatement 3: Penincilin (100mg/ml)/Streptomycin(25mg/ml)/Norfloxacin	24 hours in antibiotic solution		

		(1mg/ml)/Kanamycin (25mg/ml)/ Neomycin(100mg/ml)/ Chloramphenicol (10mg/ml)	and rinsed with AFSW	day 12 (17/12/2018)	(10/12/2018); day 7 (12/12/2018); day 12 (17/12/2018); day 16(21
	Control	Control (no antibiotics)	24 hours in AFSW and rinsed with AFSW at the same time then rinsing of antibiotic treatements		
Field experiment	AB2	Antibiotic treatement 2 (as in laboratory experiment but higher concentrations): Penincilin (900mg/ml)/Neomycin(900mg/ml)/Rifampicin(600mg/ml)	Quick AFSW rinse and application of AB2 for 2 hours and thorough rinse with AFSW	Day 0 (before applying any treatment-13/05/19); day 2 (2 days after treatment	Day 0 (before applying any treatment-13/05/19); day 2 (2 days after treatment

	BetO	Betadine applied once (Povidone iodine solution 10%)	Quick AFSW rinse and application of Betadine for 2 hours and thorough rinse with AFSW	application-15/05/19); day 9 (22/05/19); day 21 (03/06/19); day 45 (27/06/19)	application-15/05/19); day 4 (17/05/19); day 7 (20/05/19); day 9 (22/05/19); day 14 (27/05/19); day 16 (29/05/19); day 21 (03/06/19); day 28 (10/06/19); day 36 (18/06/19); day 40 (22/06/19); day 45 (27/06/19)
	BetM	Betadine applied multiple times (Povidone iodine solution 10%)	Quick AFSW rinse and application of Betadine for 15 minutes and rinsed with AFSW. Same process performed approx. every 2		

			days (same days then photosynthetic yield sampling see column e)		
	ProcContO	Procedural control for AB and CBetO	Quick AFSW rinse and application of AFSW for 2 hours and thorough rinse with AFSW		
	ProcContM	Procedural control for BetM	Quick AFSW rinse and application of AFSW for 15 minutes and		

			rinsed with AFSW. Same process performed approx. every 2 days (same days then BetM and photosynthetic yield sampling see column e)		
	Control	General control	Quick AFSW rinse		
Inoculation experiment	AB2	Antibiotic treatment 2: Penincilin (100mg/ml)/Neomycin(100mg/ml)/Rifampicin(50mg/ml)	Quick AFSW rinse and application of AB2 for 25 hours	Day 0 (Before treatment), Day 1, Day 2, Day 5, Day 8, Day 12	

			and thorough rinse with AFSW	
	AB2 V+	Antibiotic treatment with Active inoculant	microbiome disruption with antibiotic combination (AB2) for 24 hours followed by "active" inoculant (*Vibrio genomosp,* Cell density 5×10^8 CFU) for 1 hour,	
	AB2 V-	Antibiotic treatment with Control inoculant	microbiome disruption with antibiotic combination	

			(AB2) for 24 hours followed by control inoculant (*Vibrio chagsii sp.* Cell density 5×10^8 CFU) for 1 hour,	
	Vibrio +	Active inoculant	microbiome left intact (AFSW) for 24 hours followed by "active" inoculant (*Vibrio genomosp,*Cell density 5×10^8 CFU) for 1 hour	

	Vibrio -	Control Inoculant	microbiome left intact (AFSW) for 24 hours followed by control inoculant (*Vibrio chagsii sp.* Cell density 5×10^{8} CFU) for 1 hour	
	PC	Procedural control	Algae left in AFSW for 25Hrs	
	C	Control	Algae left in aquarium during treatment	

Table S2.2 Method of action and key bacterial targets of antibiotics used

Antibiotic	Method of Action	Bacterial target	Half Life	References	Antibiotic mix
Penincilin	Inhibition of transpeptidase - inhibition of cell wall biosynthesis	Gram-positive bacteria	0.4-0.9 hrs	Tipper and Trominger, 1965; Waxman et al 1980; Sass, 2023	1,2,3
Streptomycin	Interference with ribosomal peptide synthesis	Limited aerobic bacteria due to antibiotic resistance	2.5hrs	Demirci et al 2013	1, 3
Norfloxacin	Inhibits DNA gyrase	Most gram-negative and gram-positive bacteria	3-4hrs	Goldstein 1987	1, 3
Kanamycin	Binding to bacterial 30S and 16S ribosomal subunit – causes misreading of t-RNA	Gram-negative bacteria	2.5hrs	Brezden et al 2016	1, 3

Neomycin	Binding to bacterial 30S ribosomal subunit – inhibits elongation of peptide synthesis	Gram-negative bacteria	N/A	Veirup et al 2022; Patidar et al 2013	2
Rifampicin	Inhibits DNA-dependant RNA-polymerase	Gram-positive cocci	3.5hrs	Campbell et al 2001; McClure et al 1978	2
Chloramphenicol	Bacteriostatic inhibitor of protein synthesis – binds to bacterial 50S ribosomal subunit	Gram-positive and gram-negative bacteria	1.5-3.5hrs	Brook et al 2016; Dinos et al 2016	3

Table S2.3. Analysis of Variance (ANOVA) tests comparing maximum photosynthetic yields of *Hormosira banksii* across sampling time points and different treatments for the mesocosm experiment (Fixed, crossed, Treatment 4 levels, AB1, AB2, AB3, C; Time 6 levels, 0, 1, 2, 5, 8, 12)

Source	Df	Sum of Sq	Mean Sq	F	P-Value
Treatment	3	0.041843	0.0139476	20.645	5.30E-10
Sampling time	3	0.42831	0.0142768	21.133	3.54E-10
Treatment*Sampling time	9	0.11584	0.0128712	19.052	<2.2E-16
Residual	80	0.054047	0.0006756		

Table S2.4; Post-hoc analysis comparing maximum photosynthetic yields of *Hormosira banksii* across sampling time points and different treatments for the mesocosm experiment

Days	Treatment comparison	estimate	SE	df	t.ratio	p.value
0d	No Significant differences					
1d						

2d						
5d	AB2-AB1	0.085644	0.015	80	5.707	<.0001
	AB3-AB2	0.082365	0.015	80	5.489	<.0001
	Control-AB2	0.088333	0.015	80	-5.886	<.0001
9d						
12d	AB2-AB1	0.085644	0.015	80	5.707	<.0001
	AB3-AB1	0.168009	0.015	80	11.196	<.0001
	AB3-AB2	0.082365	0.015	80	5.489	<.0001
	Control-AB2	-0.088333	0.015	80	-5.886	<.0001
	Control-AB3	-0.170698	0.015	80	-11.375	<.0001

Table S5; Statistical results. Analysis of Variance (ANOVA) tests comparing maximum photosynthetic yields of *Hormosira banksii* across sampling time points and different treatments for the field experiment

Source	Df	Sum of Sq	Mean Sq	F	P-Value
Treatment	5	0.21121	0.042241	5.6901	7.30E-05
Sampling time	4	0.15841	0.039604	5.3348	4.63E-04
Treatment*Sampling time	20	0.65118	0.032559	4.3859	4.29E-08
Residual	162	1.20263	0.007424		

Table S6; Post-hoc analysis comparing maximum photosynthetic yields of *Hormosira banksii* across sampling time points and different treatments for the field experiment

Days	Treatment comparison	estimate	SE	df	t.ratio	p.value
0d	No Significant differences					
1d						
2d						
5d						
9d	Control-AB	-0.15014	0.0461	162	-3.260	0.0168
	ProcContM-BetM	0.19557	0.0461	162	4.247	0.0005
	ProcContM-BetO	0.21157	0.0461	162	4.594	0.0001
12d						

21d	BetM-AB	-0.18786	0.0461	162	-4.079	0.0010
	BetO-AB	-0.26243	0.0461	162	-5.698	<.0001
	ProcContM-AB	-0.25743	0.0461	162	-5.590	<.0001
	ProcContO-AB	-0.24914	0.0461	162	-5.410	<.0001
	Control-AB	-0.28300	0.0461	162	-6.145	<.0001
45d	BetM-AB	0.19525	0.0609	162	3.205	0.0199
	Control-BetM	-0.186750	0.0583	378	-3.201	0.0185

Table S7 Statistical results. ANOVA results for analysis of bacterial community alpha diversity measures: number of species and Simpson index in Hormosira banksii individuals within the mesocosm experiment.

Source		Df	Sum of Sq	Mean Sq	F	P-value
Treatment		3	73457	24486	6.0464	0.00221
Sampling time		6	132847	44282	10.9348	4.22e-05
Treatment x Sampling time		9	38336	4260	1.0518	0.42293
Residual		32	129589	4050		

Table S8. Statistical results. ANOVA results for analysis of bacterial community alpha diversity measures: number of species and Simpson index in Hormosira banksii subject to different treatments across time in the field). Post-hoc pairwise comparisons are also presented (only showing results of time points and treatments with significant differences).

Source	effect size	Df	Sum of Sq	Mean Sq	F	P-value
Treatment	0.15	5	101780	20356	4.9231	0.0004984
Sampling time	0.21	4	143095	35774	8.6519	5.911e-06
Treatment x Sampling time		20	69236	3462	0.8372	0.6631976
Residual		98	372129	4135		
Pairwise test						
Treatment	AB2 - Control	p<0.005**				
	AB2 - CBetM	p<0.5*				
	AB2 -CBetO	p<0.5*				
	BetM - Control	p<0.5*				
	BetM- CBetO	p<0.5*				

Table S9; PERMANOVAs based on Bray–Curtis Similarity measure for square root-transformed relative abundance (9999 permutations) of bacterial communities (ASVs) in Hormosira banksii across sampling time points and different treatments the mesocosm experiment. Table S1 has the treatments used. Significant p values($p<0.05$) are highlighted in bold and italics.

Source	df	SS	MS	Pseudo-F	P(perm)	perms
Treatment	3	5699.3	1899.8	2.1882	0.0001	9798
Sampling time	3	19463	6487.7	7.4726	0.0001	9884
Treatment x Sampling time	9	10151	1127.9	1.2991	0.0014	9677
Tank	6	1059	176.5	1.862	0.3298	9786
Residual	32	27782	868.19			
Total	47	63095				

Table S10; Pairwise contrasts on PERMANOVAs based on Bray–Curtis Similarity measure for square root-transformed relative abundance (9999 permutations) of bacterial communities (ASVs) in Hormosira banksii across sampling time points and different treatments the mesocosm experiment.

Pairwise test	Days	Treatment comparison	
Treatment x Sampling time	12d	AB2 - AB1	p<0.005**
		AB3 - Control	p<0.5*
		AB3 - AB1	p<0.005**
Treatment	AB2 - Control	p<0.005**	
	AB2 - AB1	p<0.005**	
	AB2 - AB3	p<0.5*	

Table S11. PERMANOVAs based on Bray–Curtis Similarity measure for square root-transformed relative abundance (9999 permutations) of bacterial communities (ASVs) in Hormosira banksii across sampling time points and different treatments the field experiment. Table S1 has the treatments used. Significant p values($p<0.05$) are highlighted in bold and italics.

Source	df	SS	MS	Pseudo-F	P(perm)	perms
Treatment	5	22032	4406.4	7.6284	0.0001	9811
Sampling time	4	33213	8303.2	14.374	0.0001	9846
Treatment x Sampling time	20	26297	1314.9	2.2763	0.0001	9591
Residual	90	51987	577.64			
Total	119	133530				

Table S12. PERMANOVAs based on Bray–Curtis Similarity measure for square root-transformed relative abundance (9999 permutations) of bacterial communities (ASVs) in Hormosira banksii across sampling time points and different treatments the field experiment. Table S1 has the treatments used. Significant p values($p<0.05$) are highlighted in bold and italics.

Days	Treatement comparison	t	P(perm)	perms	P(MC)		
0d	No significant differences						
2d	BetO- ProcContO	2.3292	0.0292	35	0.0076		
	BetO- ProcContM	2.265	0.0292	35	0.0091		
	BetO- Control	2.1701	0.0247	35	0.0135		
	ProcContO- BetM	2.8772	0.0265	35	0.0033		
	ProcContO- AB	1.9269	0.0289	35	0.0142		
	BetM- ProcContM	2.6858	0.0294	35	0.0033		
	BetM- Control	2.446	0.03	35	0.0068		
	BetM- AB	1.7065	0.0279	35	0.0543		
	ProcContM- AB	1.8055	0.0308	35	0.0188		
	Control- AB	1.5982	0.0298	35	0.0419		

9d	BetO- BetM	2.7522	0.0319	35	0.0026	
	BetO- Control	1.7677	0.0292	35	0.026	
	BetO- AB	1.7588	0.0296	35	0.0366	
	ProcContO- BetM	3.2668	0.0287	35	0.0012	
	ProcContO- AB	1.8704	0.0303	35	0.0195	
	BetM- ProcContM	3.5876	0.0312	35	0.0007	
	BetM- Control	3.4544	0.0275	35	0.0008	
	BetM- AB	2.589	0.0313	35	0.0049	
	ProcContM- AB	2.013	0.0273	35	0.0152	
	Control- AB	1.8592	0.0256	35	0.0198	
21d	BetO- BetM	3.0037	0.0292	35	0.0024	
	ProcContO- BetM	3.5358	0.0313	35	0.0009	
	ProcContO- AB	1.6949	0.0285	35	0.0299	
	BetM- ProcContM	4.3121	0.0281	35	0.0008	
	BetM- Control	3.2017	0.0285	35	0.0013	
	BetM- AB	2.7522	0.03	35	0.0019	

	ProcContM- AB	2.016	0.0287	35	0.0104	
	Control- AB	1.5638	0.0267	35	0.0501	
45d	BetO- BetM	2.2648	0.0277	35	0.0061	
	ProcContO- BetM	2.7101	0.0285	35	0.0031	
	BetM- ProcContM	2.4161	0.0261	35	0.0037	
	BetM- Control	2.6674	0.0292	35	0.003	
	BetM- AB	2.5228	0.0286	35	0.004	

Chapter 3.

Disruption of host-associated and benthic microbial communities affects reproductive output and settlement of a habitat forming macroalga

3.1 Abstract

The reproduction and settlement of habitat-forming macroalgae are often key processes affecting the structure of marine benthic communities. Increasingly, macroalgae are being considered as holobionts – the host and associated microbiota – but the role of microorganisms in influencing algal reproduction and settlement, and thus potentially fitness, is poorly understood. Using a dominant habitat forming macroalgae on Australian rocky shores, *Hormosira banksii,* we manipulated host- and benthic-associated microbes in order to determine the relative importance of microbes to reproductive output (number of viable eggs released) and settlement (i.e. attachment and morphogenesis of algal zygotes). Reproductive output decreased after two weeks when the host microbiota was disrupted, this effect however was dependant on the antibiotic used, and as a result the microbiota disrupted. Disruption of both host- and benthic-associated microbes caused a significant and near total decrease in settlement of *Hormosira* zygotes. Interestingly, host-associated microbes had a greater effect on settlement success than benthic-associated microbes. Our results demonstrate the importance of host-associated microbes in host reproduction and interactive effects of host- and benthic microbes on settlement – both key ecological processes – with potentially important implications for host fitness, ecosystem persistence and management.

3.2 Introduction

A central challenge in ecology is to understand the factors affecting dynamics of natural populations throughout their life cycle (Boyce et al, 2006; Lande et al, 2003). This is especially relevant for habitat-forming organisms whose survival and persistence can underpin broader biodiversity and ecosystem function(Brockerhoff et al, 2017; Oliver et al, 2015). In the marine environment, the persistence and success of habitat-forming organisms such as corals, sponges and macroalgae is often dependant on their reproductive output, settlement, morphogenesis, and subsequent survival of their early developmental stages (Ettinger-Epstein et al, 2008; Price, 2010; Veenhof et al 2022). It is therefore imperative to assess the response of all phases of the life-cycle of these organisms to determine where they are most vulnerable. Indeed an understanding of recruitment – the arrival and success of early life history stages into the adult habitat - of marine organisms has been a central feature of our understanding marine ecology and evolution for many years (Bellgrove et al, 2004; Caley et al 1996; Lewis et al, 2021; Price, 2010)

The life cycles of many marine habitat forming organisms are complex and often biphasic, with a pelagic propagule phase (e.g., algal zygotes or spores, invertebrate larvae) followed by a sessile or low-motility post-settlement benthic stage (Hadfield, 2011; Ramirez Llondra, 2002; Suchanek, 1981). Settlement is the process wherein a planktonic propagule transitions from plankton to the benthos (Connell, 1985). This process often includes morphogenesis Hadfield, 2011; Hadfield & Paul, 2001), i.e., cell differentiation and development of the settling propagule. Morphogenesis can be induced by environmental cues such as light intensity and attenuation (Callow & Callow, 2000; Edmunds, 2023; Thomsen et al, 2021), surface chemistry and wettability (Finlay, 2002), acoustics (Pysanczyn et al, 2023), and surface topography (Callow et al, 2002; Hugget et al, 2009; Yang et al, 2017) that may indicate suitable rocky reef or shore habitat (Bak & Engel, 1979; Gleason & Hofmann, 2011;

Nakamura et al, 2011). Furthermore, cues from conspecifics or other macro-organisms can either induce (e.g. Bertness & Grosholz, 1985) or inhibit settlement (Steinberg & Kjelleberg, 2002).

Microbial biofilms can also play an important role in settlement and the early life stages of marine macro-organisms (Dobrestov & Rittschoff, 2020). Settlement can be affected by the density of biofilm bacteria (Huang & Boney, 1983; La Marca et al, 2021; Tu et al, 2023), bacterial quorum sensing molecules (Joint et al, 2007; Tait et al, 2009; Tait & Havenhand, 2013), age (Keough & Raimondi, 1996; Lamare & Baker, 2001; Toupoint et al 2012), specific taxa (Hadfield et al, 2021; Huggett et al, 2009) and other properties of microbial biofilms (e.g. Hadfield et al 2011). The strongest evidence that microbial biofilms induce settlement is for invertebrates such as sponges (Whalan 2014; Abdul et al 2014), cnidarians (Webster et al 2004), bryozoans (Bertrand & Woollacott, 2005) and echinoderms (Huggett et al 2006; Dworjanyn et al 2008).

We know relatively less about the role microbes play in algal propagule settlement and morphogenesis and this evidence is limited to few algal systems such as *Ulva* sp. (Joint et al, 2007; Tait et al, 2009). While there is emerging evidence that early life history phases of other algal species are also affected by surface dwelling microorganisms (Li et al, 2019; Mieszkin et al, 2012, 2013; Pedicini et al, 2023; Weinberger et al, 2007), in general we still know little about how, or if, microbial communities can affect the various early stages of algal histories such as propagule release, settlement, attachment, morphogenesis, and which, if any, microbial taxa are important for these effects.

The concept of microbially mediated development has long been considered within algal systems. For example, algal oogonia and antheridia development has been shown to be in part controlled by exudates released from bacterial consortia (Agrawal, 2012; Machlis, 1973;

Tapia et al, 2016). Furthermore, evidence from several algal species such as Ulva sp., *Ectocarpus* sp*., Chondrus crispus* and *Fucus* sp. has shown that microbial communities are fundamentally important for the normal development and function of the algal host (Davis et al, 2021; Dittami et al, 2016; Nakanishi et al, 1996).

Another reason for focusing on the role host-associated microbiota has in reproduction and early life history stages of holobionts is that these processes or stages are key to the fitness of the organism. Performance of holobionts in response to manipulation of their microbiota is often measured via physiological or molecular parameters, such as photosynthetic yield (Qiu et al, 2019; Straub et al, 2019), nutrient content (Fiore et al, 2010; Weigel & Pfister, 2019) or stress marker genes (Fan et al, 2013; Parkinson et al, 2018). Ultimately, in assessing performance we want to know if the microbiota affects the fitness of the holobiont – i.e., reproduction and/or survival. While fitness measures typically rely on multigenerational data which is difficult to collect for slow growing, long lived species, proxies have been used to accurately predict lifetime fitness, including survival (Ladah & Zertuche-Gonzalez, 2007) and reproductive output and development (Alif et al., 2022; Hendry et al., 2001). Here we focus on reproductive output and success of propagules.

Here, we examined the effect of host associated and benthic microbial communities on the reproduction or early life history stages of *Hormosira* banksii (See Figure 1 for *Hormosira's* life cycle), We using manipulative field and lab-based experiments in which we disrupted these communities, , assessing how the host-associated microbiota may affect reproductive output of the host, and the effect of both host- and benthic-associated microbiota on the settlement and morphogenesis of non-motile algal propagules – zygotes. If microbes are important drivers of host reproduction and settlement, we hypothesised that 1) disruption to the hosts' microbiota would negatively affect reproductive output, and 2) the disruption to the host and/or benthic microbiota would negatively affect settlement success of zygotes.

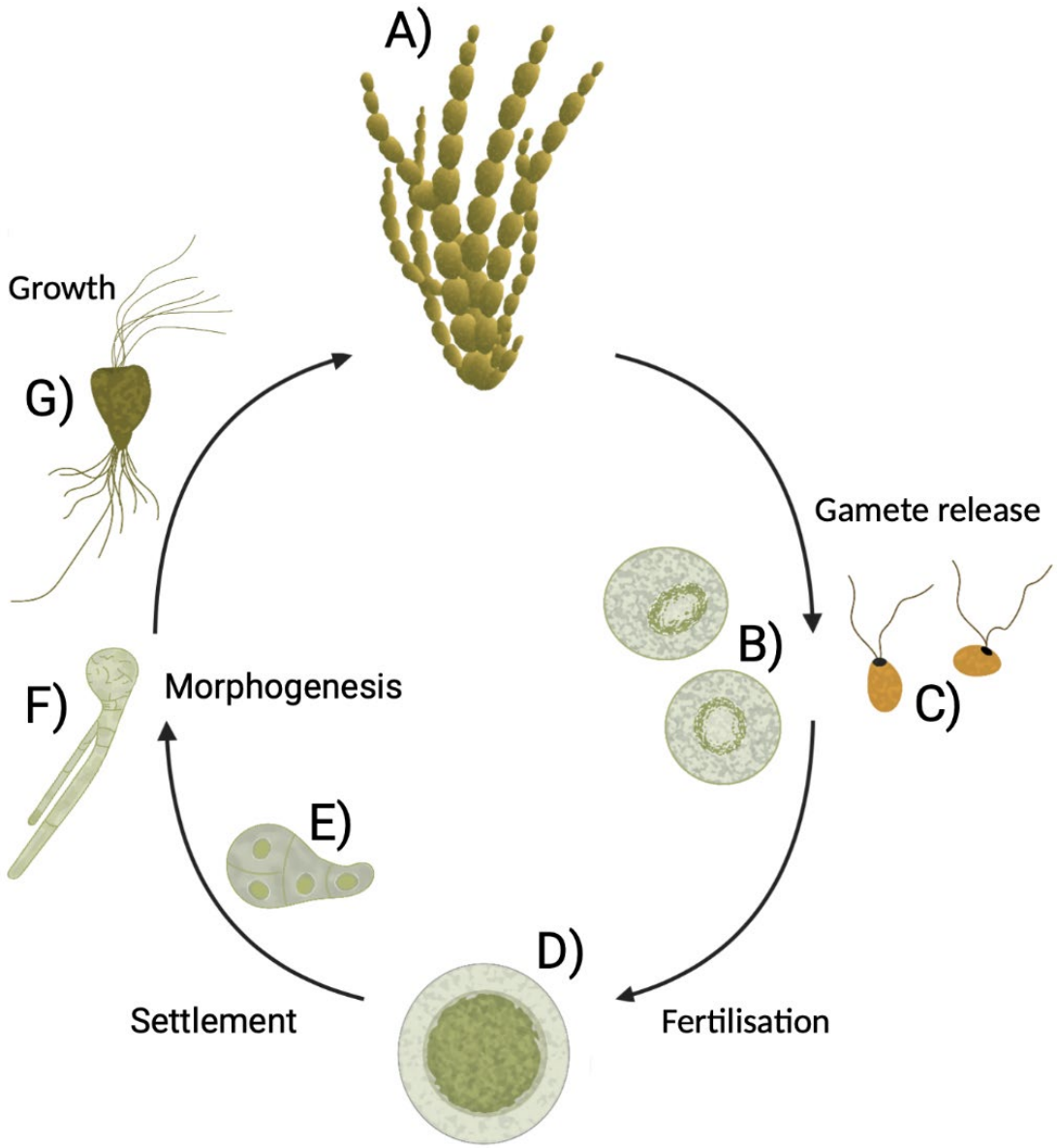


Figure 3.1. Life cycle of *Hormosira banksii*: A) Adult individuals (male or female) release either eggs (B) from ooangia, or sperm from enochites (C). Motile biflagellate sperm have strong chemotactic movement towards eggs and will typically fertilise an egg within 30 minutes of release within the water column (assuming suitable distance between male and female adults) (Osborn, 1948). Fertilised eggs (D) will then attach to suitable habitat and undergo morphogenesis, usually within 12-24 hours, firstly secreting a mucilage to the surface before developing rhizoids (E -3-4 days) to assist in attachment (F – 12 days; G) 86 days) (Dimartino et al, 2016). Full development is described within (Osborn, 1948).

3.3 Methods

3.3.1 Effect of disruption of the host's microbiota on reproductive output

120 *Hormosira banksii* (*Hormosira* hereafter) individuals of approximately 0.8cm +/- 0.3cm in diameter and 8.2 +/- SE 1.7 in length, separated by ~1m, were tagged at Cape Banks Aquatic Reserve, Sydney, Australia (33°59'55.3"S 151°14'53.6"E) during low tide in December 2022. 30 individuals were assigned to each of 4 treatments: 1) Control (HC), *Hormosira* left undisturbed, 2) Procedural control (PC), 50ml of Autoclaved filtered seawater (AFSW) applied once for 4 hours, 3) Antibiotics AB2 (Penicillin (100mg/ml), Neomycin (100mg/ml) and Rifampicin (50mg/ml)), 50ml applied once for 4 hours, and 4) Antibiotics AB3 (Penicillin (100mg/ml), Streptomycin (25mg/ml), Norfloxacin (1mg/ml), Kanamycin (25mg/ml), Neomycin (100mg/ml) and Chloramphenicol (10mg/ml) 50ml applied once for 4 hours. The antibiotic treatments were based on earlier work, (described in Chapter 2) in which I provided evidence that the mixes used in treatments 3 & 4 significantly affected the host microbiota but did not have a discernible direct effect on the host.

All individuals were collected from the field after two weeks, as a previous study showed that the greatest effect of microbial disruption on host function was observed after this time-period (Chapter 2). Individuals were then transported within 40 minutes to the laboratory at The University of Sydney, refrigerated for 12 hours at 4°C and placed under high light (~500 Lux) in individual containers to induce spawning (adapted from Dimartino et al, 2016). 5 individuals from each treatment were removed before spawning to destructively sample the bacterial communities (described below). The number of eggs released from each individual was counted and eggs were fertilised by introducing 1ml of sperm (OD400) suspended in AFSW into each individual's tank before leaving them for 30 minutes to fertilise. Sperm was collected from a common group of 20 males who were separate from the experimental individuals, but which were collected at the same.

3.3.2 Effect of disturbance of microbial communities on zygote settlement

To compare the effect of host- *versus* benthic-associated microbial communities on the settlement and attachment of *Hormosira's* zygotes, 100 sandstone tiles (12 x 12 cm side length, 1cm thickness) were installed at Cape Banks Aquatic Reserve for two weeks prior to treatment. This allowed a natural biofilm to develop (Tan et al, 2015) onto the plates, whilst limiting the time available for colonisation of macro-organisms which could confound analyses. Plates were checked on collection to make sure no zygotes had already attached before being assigned to one of 5 treatments: 1-4 were the same treatments as those applied to the host (described above), and 5) (Plate Sterile, PS) Sterile (Tile autoclaved after collection), this last treatment (PS) was to test whether the bacteria within the biofilm had to be alive to induce settlement. Tiles were then collected after two weeks and transported to the aquarium at the University of Sydney where they were placed within individual tanks.

Zygotes, released within the individuals above were introduced to the tiles as follows with each individuals' zygotes being presented to individual replicate tile. To control for potential density dependant effects, fertilised eggs (zygotes, fertilised as above) were diluted to the lowest concentration obtained (200 +/- SE=23 zygotes per 50 ml) from each individual (n=5/treatment). Zygotes were then introduced to the tiles where all host and tile treatments were crossed (20 treatment combinations; n=5 plates and females per treatment combination). Zygotes were allowed to attach for 4 hours, which represents the time of average low tides at Cape Banks. Then, each plate was flushed using 50ml of AFSW to remove any unattached zygotes and the remaining number of settled zygotes were counted.

Bacterial sampling

To collect surface associated microbial communities for characterisation, the surface of each alga and tile was swabbed for 30 seconds with a sterile cotton swab. Swab controls (treated similarly to those for swabbing surfaces, except no surfaces were swabbed) were also

collected. Swabs were immediately placed intro sterile cryogenic tubes and placed in liquid nitrogen and then stored at -80°C until DNA extraction.

DNA Extraction and sequencing

Microbial DNA was extracted from each swab sample in a randomised order to avoid introducing any bias due to order and time of processing, using a Powersoil DNA Isolation kit (Qiagen) following the manufacturer's protocol. DNA extracts were quantified using spectrophotometry (Nanodrop 1000) and stored at -20°C until sequencing.

The extracted DNA samples were amplified with Polymerase Chain Reaction (PCR) using the 16S primers 341 (F) (5'- CCTACGGGNGGCWGCAG-3') and 805(R) – (5'-GACTACHVGGGTATCTAATCC-'3), containing the V3-V4 regions of the bacterial and archaeal 16S rRNA gene (Klindworth et al., 2013). Both positive (with known DNA sequences) and negative controls (nuclease-free water, control swabs) were used. The negative controls did not amplify DNA, suggesting no contamination of swabs or during extraction and amplification. Agarose gel electrophoresis and Nanodrop 1000 were used to ensure the quantity and quality of the amplicons before they were sent for sequencing via the Illumina MiSeq 2000 platform at the Ramaciotti Centre for Genomics (UNSW, Sydney).

Bioinformatics

Raw sequences were received from the sequencing centre as demultiplexed pair-ended sequences per sampled. UNOISE was used to remove chimeras and produce amplicon sequence variants (ASVs), i.e., operational taxonomic units at a unique sequence level (0% distance) (Edgar, 2016). DADA2 was used to map the original reads back to ASVs, generating a table of 11847 ASVs (Callahan et al., 2016). ASV sequences were searched with BlastN against the SILVA SSU Ref NR99 database for taxonomic classification and to remove chloroplasts, Genome Taxonomy Database (GTDB) was then used for taxonomic

assignment. Singletons and low abundance taxa (<0.01% of reads) were removed from the dataset prior to statistical analyses, resulting in 8075 ASVs.

Estimation of absolute bacterial abundance

Total abundance of the 16S rRNA gene was quantified for each sample by qPCR using the primers 341F/805R (Thijs et al, 2017). Gene amplification and analysis were performed using the QuantStudio 3 thermocycler (Thermo Fisher with PrimeTime® Gene Expression Master Mix, Integrated DNA Technologies) and associated software. The reaction conditions for amplification of DNA were 50°C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15s and 60 °C for 1 min. The final gene copy number per sample was corrected for the total extraction volume, the surface area, the dilution factor and DNA yield per sample (see Nappi et al 2021 for further details) and were used to estimate absolute abundances of ASVs and inoculants.

Statistical Analyses

To test for the effects of microbiota manipulations on host reproductive output, we used a one factor ANOVA (fixed, 4 levels) in R (v4.0.3). To test for the effect of host and tile microbial disruption on settlement we used a two factor ANOVA (Host and Tile factors, fixed, crossed, 4 and 5 levels, respectively) in R using the GAD package.

Alpha diversity measures of bacterial richness (i.e. number of unique sequences) and Simpson's diversity index were calculated using the 'vegan' R package (Oksanen et al., 2013) and differences between host and tile treatment combinations (as above) were examined using a linear model in the R GAD package.

To account for uneven sequencing depth among samples, data were normalised using qPCR reads of the 16S v3-4 region (See Nappi et al 2022). To determine differences in the structure of the associated bacterial assemblages, the normalised ASV data were analysed using

permutational multivariate analysis of variance (PERMANOVA, Anderson & Walsh, 2013) was based on Bray-Curtis dissimilarities between sample pairs calculated on square-root transformed absolute (qPCR normalised) abundances of ASVsin the R vegan package (Okansen et al, 2013). For significant main effects or significant interactions, pairwise comparisons were performed using the pairwise.adonis.2 function in the *pairwise.adonis* package (Martinez Arbizu, 2020).

. Generalised linear latent variable models (GLLVMs) were used to visualise the multivariate models using ordination plots (See Niku et al 2019).

To determine which bacterial taxa differed among treatments, we used multivariate generalised linear models (GLMs, Host and Tile factors as above) using the R package 'mvabund' (Wang et al., 2012), assuming a negative binomial distribution to account for over-dispersion of sequence counts.

3.4 Results

3.4.1 The effect of disruption on the microbial communities

Disruption with to the host and tiles with AB2 caused significant changes in the microbial community structure (PERMANOVA, pseudo-$F_{12,85} = 5.09$, $p<0.001$ Fig, 3.1). Disruption with AB3 had a significant effect on the microbial community structure of both tiles and hosts after 2 weeks (Table S3.3, S3.4). Disruption with autoclaving significantly changed the structure and abundance of the benthic biofilm (Fig 3.1; Table S3.3).

GLM analyses identified 146 ASVs (approx. 1.8% of a total of 8075 ASVs) whose abundances differed significantly among treatments. Of those we found a strong effect of treatment on 13 ASVs in the classes Gammaproteobacteria and Cyanophyceae, with abundances of the latter being lower in AB2 treatments and autoclaving.

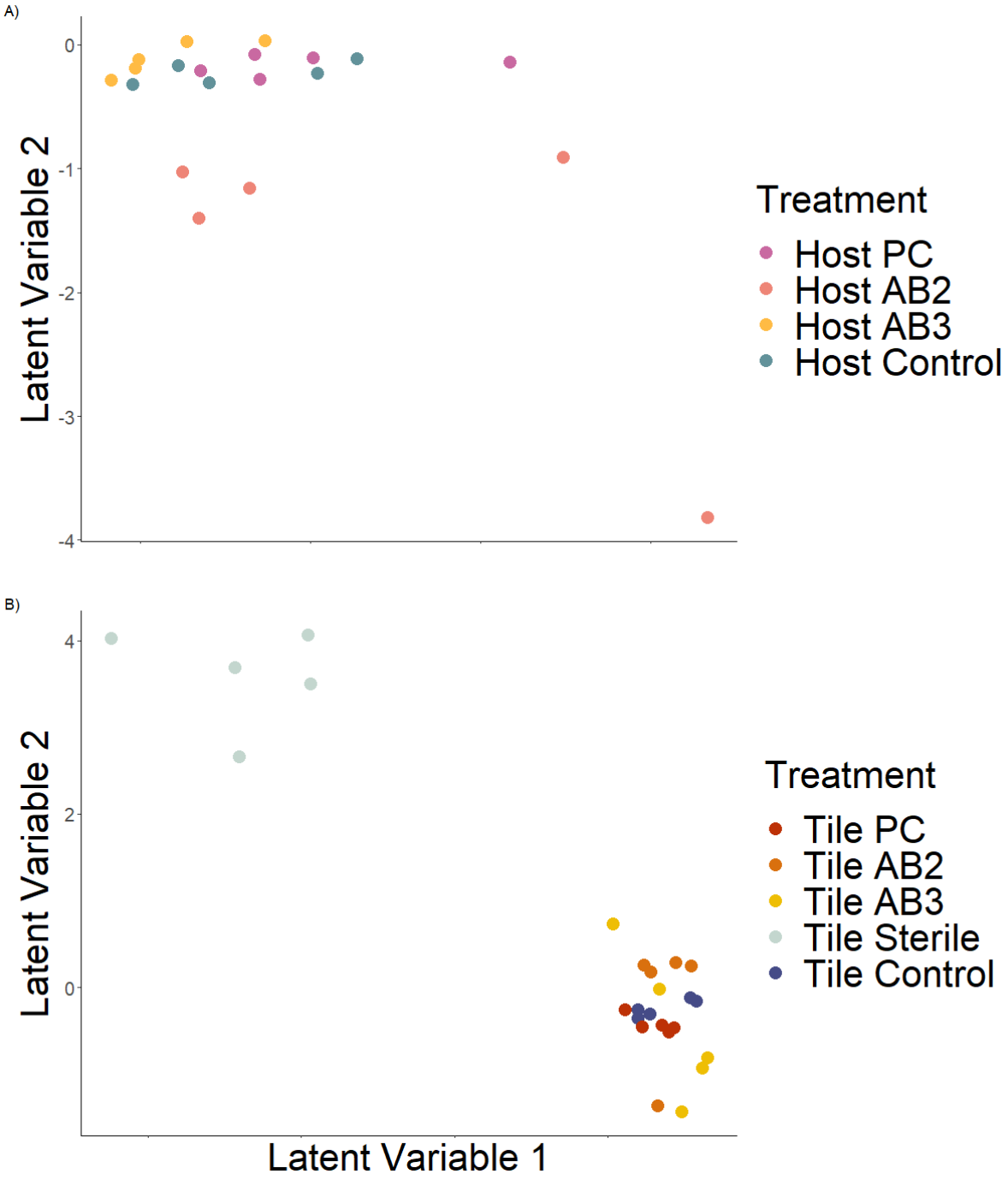


Figure 3.2 Bacterial community structure after 2 weeks. A) Bacterial community associated with the individual hosts from each treatment (n=5) B) Bacterial community structure of the tiles each dot within the panel indicates the community of microbes associated with n=5 independent replicates of each treatment. Data is an ordination based on a GLLVM model (Niku et al, 2019).

3.4.2 The effect of microbial disruption on reproductive output

Disruption of the host microbiota with antibiotic treatment with AB2 caused significant decreases in the number of eggs released (ANOVA, $F_{3,96}$=812.7, p <0.001, Fig.3.3), with egg output in this treatment significantly different from all other treatments (p<0.001, Fig.3.3, Table S3.3, S3.4). AB3 did not have a significant effect on host reproductive output *versus* controls (Fig. 3.3. Table S3.1).

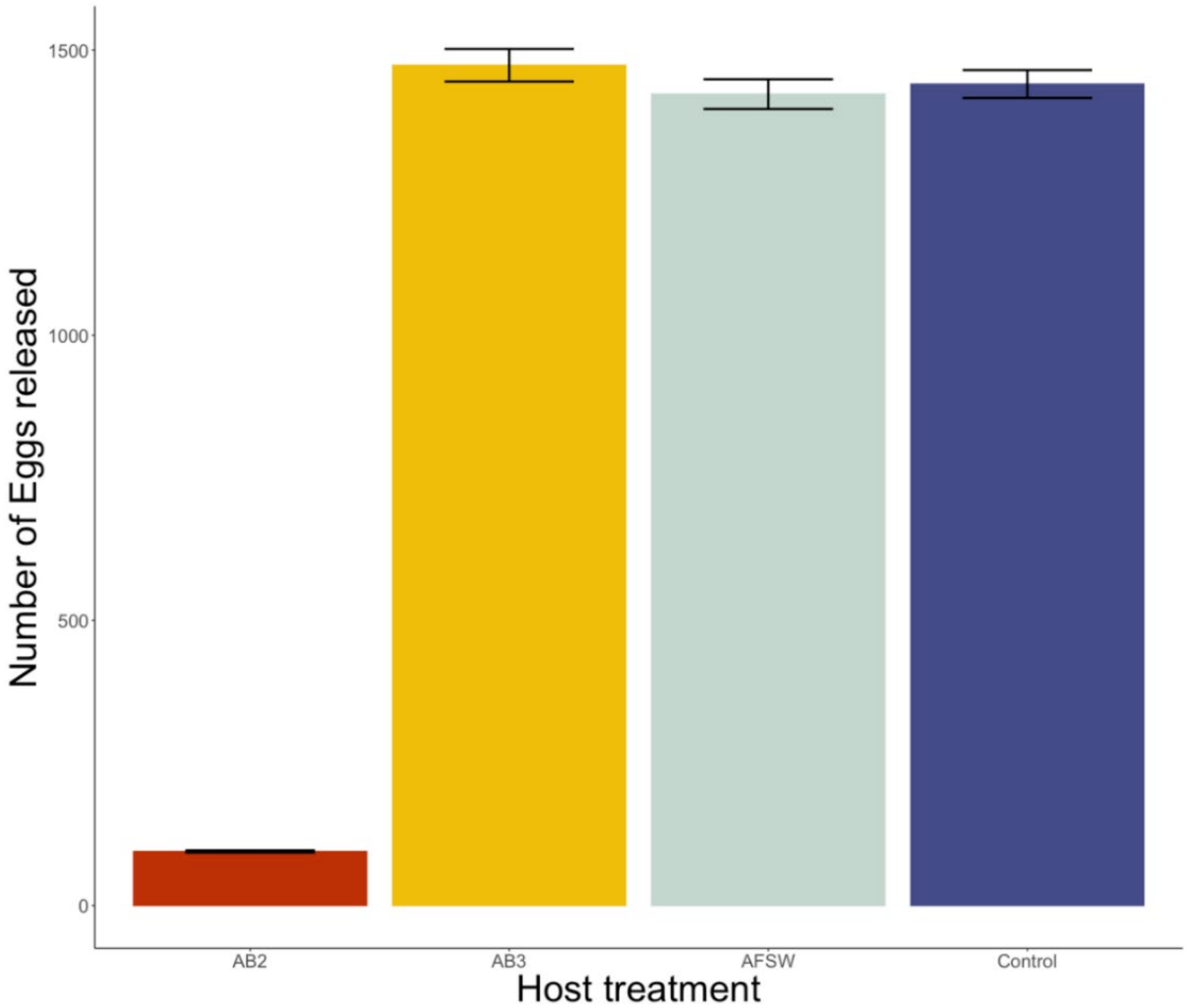


Figure 3.3. Number of eggs released by *Hormosira* from each treatment (means +/- SE; n=25).

3.4.3 Effect of microbial disruption on settlement of zygotes

Disruption to the host- and benthic-associated microbial communities caused significant, but complex and interactive, effects on the settlement of *Hormosira's* zygotes (ANOVA, $F_{12,85}$=7.604, p<0.001. Fig.3.4). AB2, when applied to the host in combination with autoclaving the tile had the lowest settlement percentage of all treatments (0.64% +/-0.43%, n=5, Fig.3). All treatments where the benthic biofilm was reduced by autoclaving had significantly lower levels of settlement (~7%) than those which were not autoclaved (Table S3.2).

All treatments where the host was treated with AB2 had significantly lower numbers of settled zygotes than controls or AB3 (Table S3.2). This negative effect was significantly increased when the benthic biofilm was also disrupted with either AB2 or by autoclaving (Table S3.2). AB3 applied to the host had a significant effect on settlement only in combination with biofilms treated with AB2 (Table S3.2). There was no significant difference in the number of settled zygotes between tiles treated with AB2 and autoclaved tiles treatment except when the host was also treated with AB2 (Table S3.2).

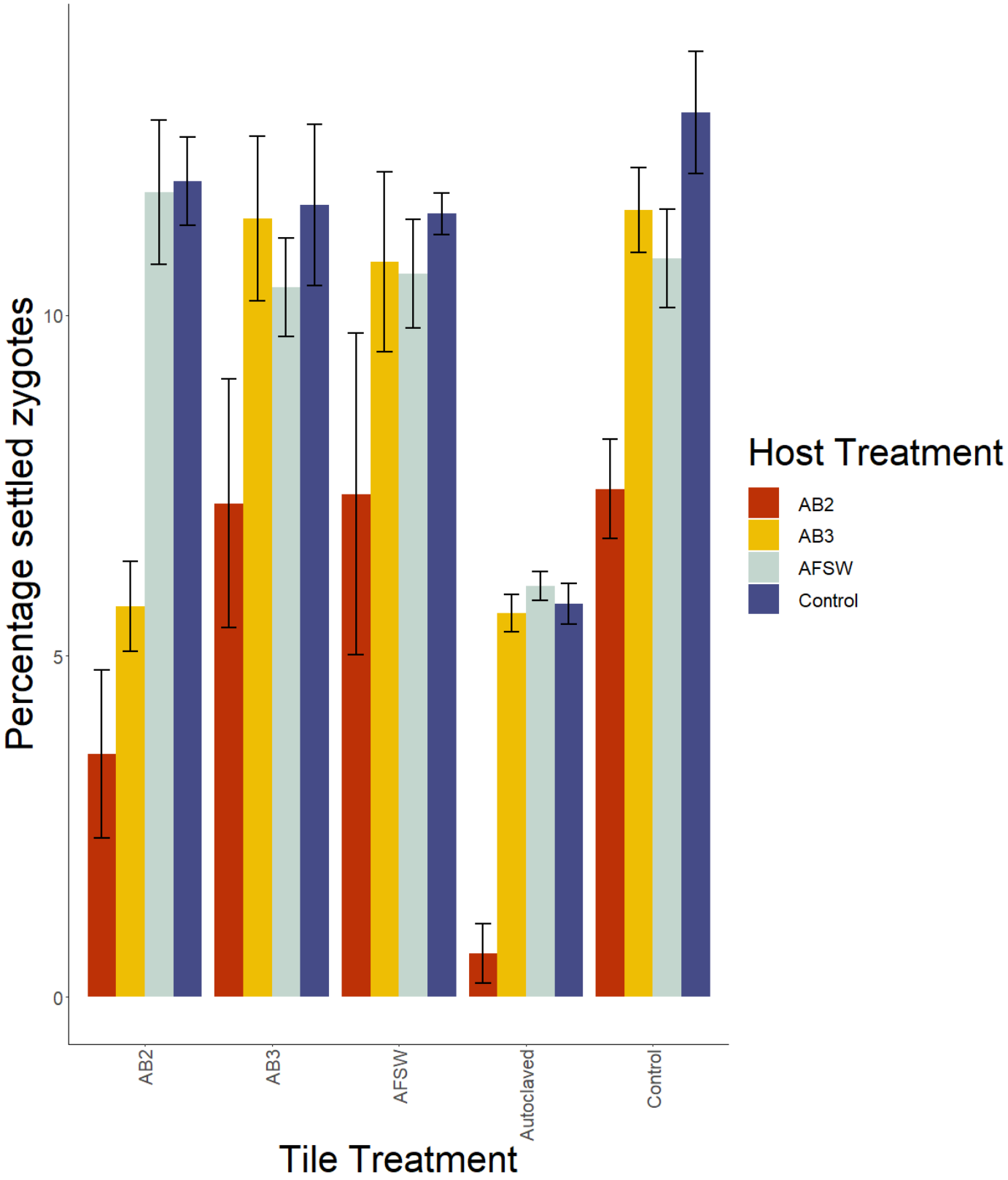


Figure 3.4. Percentage number of eggs that settled out of 200 added to each tile after 4 hours on plates following treatment (means +/- SE, n=5 tiles/treatment combination).

3.5 Discussion

Evidence from a diversity of fields shows that associated microbial communities are fundamentally important for normal host function and performance (Berendsen et al, 2012; Fuggle et al, 2023; Graham et al, 2016), and the work presented here shows that microbial communities are critical for a macroalgal host at several stages of their life cycles. We show that disruption to host-associated microbial communities affected host reproductive output, with likely implications for longer term fitness of this habitat-forming species. Furthermore, microbial communities associated with both the adult host and the benthic substratum had a strong interactive effect on zygote settlement and morphogenesis. This adds support to the broad significance of the "microbial seascape" in the functioning of these communities (Delong, 2007; Karl & Letelier, 2009).

Treatment of the host with AB2 had the greatest effect on the structure of the hosts microbiota. This led to significant decreases in the number of eggs released. As disruption occurred prior to the observed effect and different bacterial groups were affected by the different antibiotic treatments which caused varying host effects and corroborates evidence from previous work (Chapter 2), we can infer that the disruption of the microbiota is causing the observed effect. However, as discussed in Chapter 2, we cannot fully rule out direct effects of antibiotics on the host.

The abundance of 104 ASVs associated with the host-microbiota significantly changed in response to treatment with AB2. Most of the bacteria reduced by this treatment belonged to the class Cyanophyceae. Cyanophyceae are important microbial consorts in a number of marine organism, ranging from sponges (Hinde et al, 1994; Usher, 2008), to macroalgae (Penhale & Capone, 1981), and are often important components of the nitrogen-fixing capabilities of their hosts (Fiore et al, 2010; Mohr et al, 2021). The acquisition pathways of nitrogen is especially important to understand, particularly in intertidal systems where often

nitrogen is limited (Bracken & Nielsen, 2004; Elser et al, 2007; Pfister, 2007). As nitrogen is a key nutrient required for growth and development (Fernandez et al, 2020; Lesser et al, 2007) the loss of nitrogen acquisition pathways from the removal of Cyanophyceae may reduce the nitrogen availability for the host. This could, in turn, lead to a lower investment of nutrients into zygote development, as is often seen within terrestrial plants in nutrient scarce environments (Fujita et al, 2014; McGinley & Charnov, 1988; Wright et al, 2011). Future work could incorporate stable isotope probing to determine the relative role of microbial taxa in the acquisition of nitrogen within this species (Raven & Hurd, 2012).

Whilst AB3 also had a significant effect on microbial community structure, it did not affect the number of viable eggs released. Interestingly, AB3 caused fewer numbers of ASVs to change in abundance (N=37) and many of the reduced taxa belonged to the class Gammaproteobacteria. Gammaproteobacteria have been suggested to be symbionts within several species ranging from snails to sponges. In these associations they have the metabolic pathways to degrade sulphur and as such when not associated with a host are often found in deep sea and sediment microbial communities (Bugnot et al, 2023; Fuggle et al, 2023; Kvennefors et al, 2012). As changes in their abundance did not affect host performance, we can infer that they are not required for host function. However, this relationship may change in different environmental/ ecological contexts as is reported for a number of microbes (Pita et al, 2018; Rosenberg et al, 2007; Wasai & Minamisawa, 2018).

Host-associated microbes had a strong effect on zygote settlement and survival as well as on reproductive output. This was antibiotic specific. Zygotes released from adults whose surface-associated microbiota were disrupted with AB2 had significantly lower levels of settlement. Whereas hosts treated with AB3 did not influence settlement. This is intriguing as

AB3 when applied to the host did cause significant changes in the microbial community. This finding suggests that the effect of the microbiota could be due to the removal of specific microbial taxa which are important for early life stages. For example, specific bacterial taxa belonging to the classes Rhodobacteraceae, Alteromonadaceae, and Oceanospirillaceae have suggested to effect the attraction of sperm to eggs in other invertebrates (Damjanovic et al, 2020; Miller, 1977), in turn influencing fertilisation rates. Whilst we do not have data from the eggs microbial community, these bacterial groups were not found in high abundance in the hosts microbiota. Furthermore, this explanation is unlikely to be the mechanism here as the successful fertilisation of eggs was checked before they were introduced to the plates for settlement. Rather, we posit that disruption to the hosts microbiota may potentially affect the transfer of beneficial microbes from adults to their gametes and eventually zygotes through vertical transmission. This fits with evidence from the field which have shown vertically transmitted microbes being important drivers of early life stage survival and morphogenesis of hosts ranging from corals to sponges (Epstein et al, 2019; Lesser et al, 2007). To explore the mechanisms underlying this observation, further work could examine the microbiota associated with the host, gametes and zygotes - in both disrupted and un-disrupted individuals (e.g. Bjork et al, 2019).

Disruption to the microbiota on the settlement surface also had a significant effect on the morphogenesis of *Hormosira's* zygotes. Autoclaving the settlement plates caused the greatest decrease in biofilm density which was correlated with the greatest reduction in settlement of *Hormosira*, though some settlement still occurred. As autoclaving has been shown to be highly successful in sterilising surfaces and denaturing DNA (Calderon-Franco et al, 2020; Lopez-Andreo et al, 2012), we can suggest that live bacterial cells are important cues for the settlement of this species. However, without using FISH or other cell viability protocols (e.g. Benoit et al, 2010; Shailaja et al, 2022) this cannot be confirmed. Treatment with AB2

significantly reduced the overall abundance of the benthic biofilm along with reducing specific microbial taxa which significantly reduced the number of successfully settled zygotes.

Settlement of marine invertebrates has been strongly correlated with the presence and abundance of overall biofilm density as well as specific microbial taxa such as Proteobacteria and Cyanobacteria species (La Marca et al, 2021; Lee et al, 2014). This suggests that key bacterial taxa are important for the successful settlement of this host unlike other hosts such as bryozoans (Bertrand & Woollacott, 2005) which respond to broader cues. Alongside specific microbial taxa, biofilm density and age have been shown to be key determinants of larval settlement (Hadfield, 2011; Hadfield et al, 2021). As all biofilms were the same age (2 weeks), age cannot explain the observed differences in zygote settlement. Autoclaved tiles had the lowest bacterial densities and rate of successful morphogenesis, which were correlated. Whilst this pattern has been observed in other invertebrates (Dobrestov & Rittschof, 2020; Rittschof et al, 1998), more work is required to determine the mechanisms behind these observations in this system.

Recruitment (propagule settlement, morphogenesis, and survival) can explain much of the spatial or temporal variation in population dynamics in marine organisms (Albertoni et al, 1999; Baird et al, 2003; Raimondi & Keough, 1990). Understanding the drivers of recruitment is an important step in determining lifetime fitness and population persistence (Bassar et al, 2010). Whilst there is a large body of literature showing recruitment patterns for multiple seaweed species (Deysher & Dean, 1986; Lewis et al, 2021; Pfister et al, 2018), the role of microbial communities in mediating these key life history processes is poorly known (but see Pedicini et al, 2023). Here we show that microbial communities associated with both the host and the benthos have important roles in key stages of a hosts' life cycle – reproductive output and settlement. Future work should examine the molecular mechanisms

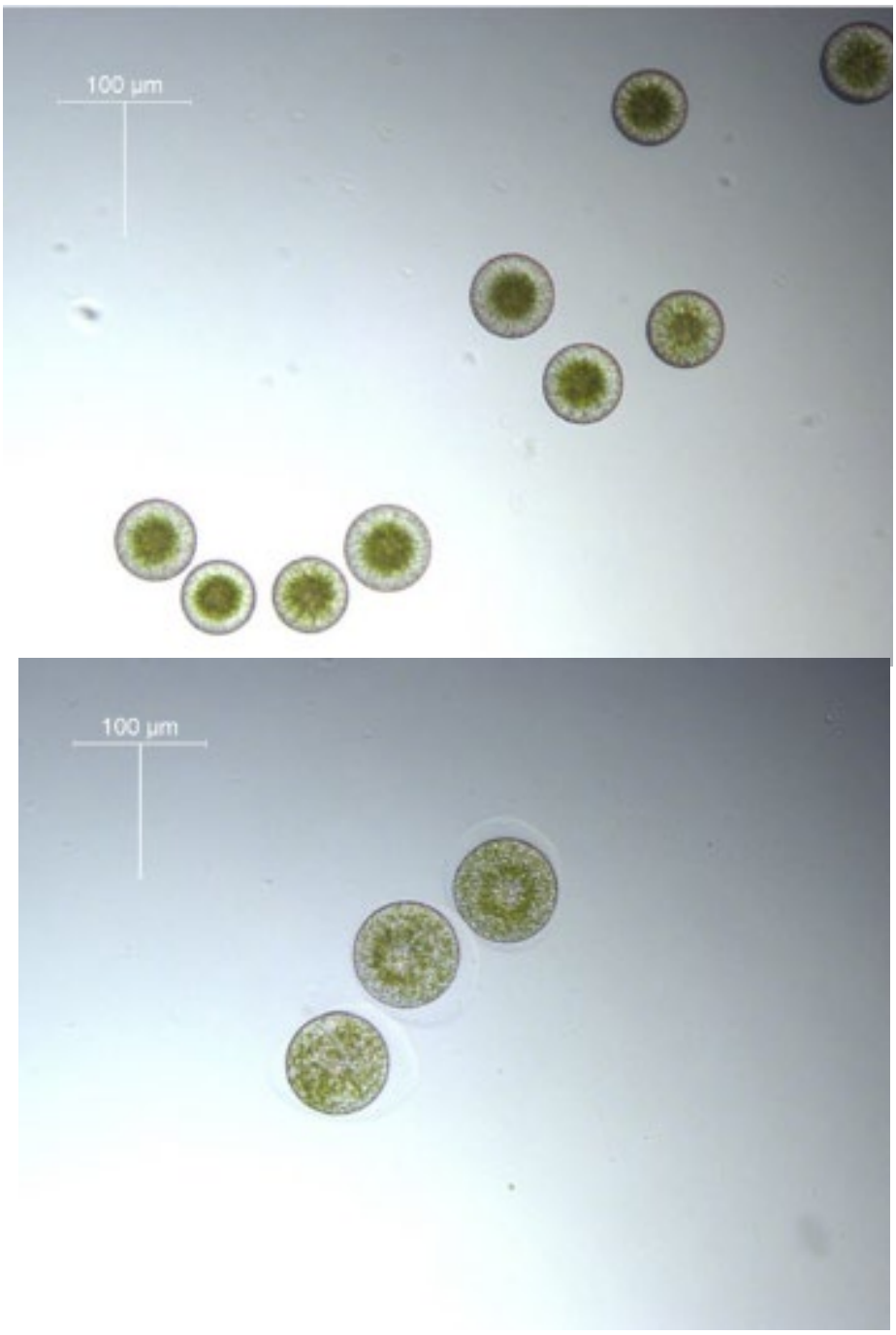
underpinning these observations and incorporate sampling to test for vertical transmission of microbes. Understanding these factors which potentially control habitat forming hosts fitness across their life cycles is important for how we the ecology of these habitat forming organisms in the era of the holobiont.

3.6 Supplementary information – Chapter 3

Supplementary figure 3.1 Settlement plates *in situ* at Cape Banks Aquatic Reserve 2022

Supplementary figure 3.2 female *Hormosira* spawning within the lab.

Supplementary figure 3.3. Female *Hormosira* stress spawning in response to low salinity



Supplementary figure 3.4 Fertilised eggs of *Hormosira* following spawning in the lab

Table S3.1. ANOVA results for analysis of host treatment (fixed, 4 levels, AB2, C, PC, AB3) on reproductive output. Post-hoc pairwise comparisons calculated using emmeans, all treatment differences are based on alpha = 0.05

Source		Df	Sum of Sq	Mean Sq	F	P-value
Host Treatment		3	347244	11574	812.7	<0.001
Residual		96	143853	14243		
Pairwise Comparisons	AB2 < C = PC = AB3					

Table S3.2. Analysis of on settlement rates *versus* treatment (fixed, crossed, 20 levels: Host – AB2, C, PC, AB3; Tile – AB2, C, PC, AB3, Autoclaved). Post-hoc pairwise comparisons calculated using emmeans, all treatment differences are based on alpha = 0.05

Source		Df	Sum of Sq	Mean Sq	F	P-value
Host Treatment		3	34724483	11574828	811.063	<0.001
Tile Treatment		4	93486	23371	1.638	<0.001
Host Treatment * Tile Treatment		12	132002	11000	0.771	<0.001

Residual		81	1213050	14271		
Pairwise Comparisons						
Tile treatment	Host treatment					
Control	AB2< C = PC = AB3					
Autoclaved	AB2< C = PC = AB3					
Procedural control	AB2< C = PC = AB3					
AB2	AB2< AB3 < PC = C					
AB3	AB2< C = PC = AB3					
AB3 = PC = C > AB2 > Autoclaved	AB2					
AB3 = PC = C > AB2 = Autoclaved	AB3					
AB3 = PC = C = AB2 > Autoclaved	Procedural control					

AB3 = PC = C = AB2 > Autoclaved	Control	

Table S3.3. PERMANOVA based on Bray–Curtis similarity measures for qPCR-normalised, square-root transformed ASV abundances on *Hormosira banksii* across different host treatments (fixed, 4 levels: AB2, AB3, C, PC). Significant *p*-values (alpha = 0.05) are highlighted in bold. Post-hoc contrasts were calculated using the pairwise.adonis R function and p-values adjusted for multiple-testing (alpha=0.05).

Source	df	SS	MS	pseudo-*F*	*p*(perm)	perms
Host treatment	3	1524	214	6.74	0.0001	9871
Residual	17	1842	327.4			
Total	20	3366				
Pairwise comparisons						
	AB2>C=PC<AB3					

Table S3.4. PERMANOVA based on Bray–Curtis similarity measures for qPCR-normalised, square-root transformed ASV abundances on *Hormosira banksii* across different Tile treatments (fixed, 4 levels: AB2, AB3, C, PC, Autoclaved). Significant *p*-values (alpha = 0.05) are highlighted in bold. Post-hoc contrasts were calculated using the pairwise.adonis R function and p-values adjusted for multiple-testing (alpha=0.05).

Source	df	SS	MS	pseudo-*F*	*p*(perm)	perms
Tile treatment	4	723.8	118.04	9.60	0.0001	9741
Residual	21	941.97	276.38			
Total	25	1665.77				
Pairwise comparisons						
	Autoclaved > C= PC < AB3 < AB2					

Chapter 4.

Effects of extreme rainfall on a dominant seaweed are mitigated by its microbiota

4.1 Abstract

Extreme weather events are becoming more intense and frequent, driving unprecedented ecological changes globally. In the marine environment, these changes can be exacerbated along coastal habitats due to high levels of urbanisation along the coastlines and its associated anthropogenic impacts (e.g., runoff, eutrophication, human disturbance). Following a series of unusually extreme rainfall events along the coast of Sydney, Australia, I examined the effects of rainfall (in the field) and hypo-saline environments (in the lab) on the reproductive output and performance in female of a dominant habitat forming seaweed, *Hormosira banksii,* and the extent to which its surface microbiota mitigated stress from rainfall/freshwater input. Rainfall and reduced salinity negatively affected host reproductive output and photosynthetic yield. Manipulative field experiments using a combination of antimicrobial treatments applied once (pulse) or regularly (press) showed that the disruption of *Hormosira*'s microbiota after a rain event inhibited the post-event recovery of host reproductive output and photosynthesis. Press disruption of host-microbiota prevented recovery of normal (control) reproductive output and photosynthetic efficiency for over four month. This experiment demonstrates the role host-associated microbiota plays in mediating responses of important habitat forming algae to environmental stress.

4.2 Introduction

Climate change is driving an increase in the frequency and severity of extreme weather events such as heatwaves, droughts and rainfall (Hoegh-Guldberg & Bruno, 2010; Masson-Delmotte et al., 2022; Solomon et al., 2007). The ecological consequences of such extreme events can be severe, particularly when habitat-forming species are affected (Bulleri et al., 2018; Wernberg, Smale, et al., 2013), because this can lead to cascading effects on the ecosystems they underpin (Poloczanska et al., 2016; Smale, 2020; Sydeman et al., 2015).

Within marine environments, marine heatwaves are one of the most studied extreme events, primarily due to the ease of measurement and the recent occurrence of several marine heatwaves that have had strong ecological impacts (Garrabou et al., 2022; Oliver et al., 2019; Smith et al., 2023; Wernberg, Smale, et al., 2013). However, other extreme events which are influenced by a changing climate, such as storms and associated intense rainfall, are also becoming prominent (Bass et al., 2023; Fong et al., 2020). In coastal environments, rainfall can have a substantial effect on the structure (O'Gorman et al., 2012) and functioning of marine ecosystems (Duvat et al., 2021; O'Connor et al., 2015). This can be due to changes in rainfall *per se*, but also due to the synergistic effects which are characteristic of extreme rainfall events, that is, rapid salinity fluxes coupled with nutrient enrichment and chemical pollution from the land, which have the potential for significant negative effects on marine organisms (Kuntz et al., 2005; O'Connor et al., 2015; White et al., 2018). It is important to decouple which of the multiple stressors associated with severe rainfall events cause ecological impacts in order to understand how marine assemblages will respond to future extreme environmental events.

Extreme rainfall events and associated stressors can affect the physiology of marine and estuarine habitat-forming species, negatively affecting their performance and survival (Cornwall et al., 2017; Martínez et al., 2012). Physiological effects such as cellular damage

(Davison & Pearson, 1996), reduced growth rates (Kroeker et al., 2010), reduced recruitment (Deysher & Dean, 1986) and survival have commonly been reported for habitat-forming organisms ranging from corals (Price, 2010) and sponges (Pita et al., 2018; Ribes et al., 2016) to macroalgae (Straub et al., 2022; Wernberg, Smale, et al., 2013). Macroalgae are often more susceptible to rainfall than other habitat forming species due to their sensitivity to nutrient additions from terrestrial run-off (Bellgrove et al., 2010; Bellgrove, McKenzie, et al., 2017; Fong et al., 2020; Worm et al., 1999) and relative intolerance of rapid salinity changes (Fong et al., 1996; Takolander et al., 2017).

Understanding the effects of extreme weather events on macroalgae is challenging because of the numerous life history stages and transitions upon which environmental stressors can act (Schiel & Foster, 2006). For instance, stress tolerance, and thus effects of stressors, may vary across different life history stages within a species (Fain & Murray, 1982; Ladah & Zertuche-González, 2007). *Macrocystis pyrifera* provides a clear example of this variability across ontogeny, with negative effects of temperature on spore production (Buschmann et al., 2004), germination (Buschmann et al., 2004), recruitment (Deysher & Dean, 1986) and sporophyte growth (Rothäusler et al., 2009, 2011) varying spatially, with different components of ontogeny being affected in different populations (Deysher & Dean, 1986; Muñoz et al., 2004). Thus, incorporating multiple life stages is critical to better understand how individual populations of habitat forming organisms will respond to extreme events.

Stressors such as rainfall may act directly on host physiology, but for holobionts, they may also act via disruption of the host's microbiome. There is strong evidence that microbial communities (here broadly referred to as microbiota) associated with macroalgae are important for their normal development and function (Egan et al., 2013; Li et al., 2022; Nakanishi et al., 1996). Microorganisms have been shown to influence host properties or processes such as morphogenesis (Matsuo et al., 2005; Nakanishi et al., 1996),

photosynthesis (Qiu et al., 2019), nutrient cycling and provision (Minich et al., 2018; Pfister et al., 2019) and defence against pathogens (Li et al., 2022; Longford et al., 2019). However, with relatively few exceptions, we still know little about how microbiomes affect core components of fitness – survivorship and reproduction – for macroalgae, particularly in the field. Reproductive output (number of viable offspring produced by an individual) is a potential proxy of fitness (Alif et al., 2022; Hamel et al., 2010; McCoy et al., 2020) and was used in this study as a metric to quantify macroalgal responses to stress. *Hormosira* is dioecious; we focused on females as there are easily identifiable in the field and previous work showed effects of their microbiome on egg release and zygote morphogenesis (Chapter 3).

In this study, I investigated the response of a dominant coastal seaweed *Hormosira banksii* to extreme rainfall events and experimentally tested whether those responses were mitigated by their surface-associated microbiome. Specifically, I hypothesised that extreme rainfall would have significant effects on algal reproductive output and photosynthesis. I predicted that, if effects of rainfall on the host were primarily due to freshwater (as opposed to other rainfall-associated stressors such as nutrients), the exposure of hosts to freshwater levels similar to those observed in rainfall events in the field would lead to similar reductions in reproductive output and photosynthesis comparable to that observed during rainfall events in the field. Using the techniques developed in Chapter 2 to manipulate host associated microbiota, I further examined the model that the reproductive output of the host is mediated by its microbiota. I hypothesised that the disruption of the host's microbiota would reduce the ability of the host to recover its reproductive output after extreme rainfall events and that such responses would be strongest where the host-microbiome was subjected to chronic, press disruption, as opposed to one-off (pulse) disruption.

4.3 Methods

4.3.1 Extreme rainfall events

To identify and examine extreme rainfall events during 2022, monthly rainfall data was collected from the Australian Bureau of Meteorology online data portal using the Sydney Airport weather station (Sydney, NSW, Australia; 151.17 ºE, 33.95 ºS, Bureau of Meteorology, 2023). Rainfall levels in Sydney during 2022 was 209% greater than the average of all years since data has been collected (164 years). Rainfall in March was significantly higher than all other months in 2022, with the highest daily rainfall on the 3rd of March (153mm; see Results). Extreme rainfall events in the dataset were identified when the average monthly rainfall was greater than 99th percentile than the previous 50 years of rainfall data . Furthermore, to determine how salinity changed during the extreme rainfall event, open access datasets were collected through the NSW Government's Department of Planning and Environment Beach Watch program (DPE, 2022).

4.3.2 Effect of extreme rainfall on Hormosira's reproductive output

To determine the effect of extreme rainfall on reproductive output of *Hormosira banksii (Hormosira* hereafter)*,* 20 female *Hormosira* of approximately 1.3 +/- 0.5 cm in diameter and 12.5 +/- SE 0.4 cm in length, ~1 m apart were collected from the rocky platform at Cape Banks Aquatic Reserve, Sydney (33°59'55.3"S 151°14'53.6"E) during low tide each month from October 2021 through to March 2023 (N=340). Spawning was induced by placing algae into a refrigerator at 4ºC for 12 hours (Dimartino et al., 2016; Forbes & Hallam, 1978) and then into individual containers with no water and under a high LUX (~500 Lux) lamp for 2 hours at room temperature. Gametes were washed from the individuals with 50ml of autoclaved filtered seawater (AFSW) and 5 subsamples of 5ml were used to count the number of eggs released by each individual female (n=20).

4.3.3 Effect of salinity on Hormosira's reproductive output

To directly test the impact of lowered salinity on reproduction in *Hormosira*, 80 female individuals (approximately 1.3 +/- 0.5 in diameter; 12.3 +/- SE 0.6 in length) separated by ~1 m from each other were collected at Cape Banks Aquatic Reserve during low tide in March 2023. Individuals were transported in seawater within 30 minutes to The University of Sydney where they were placed into a recirculating aquarium for 24 hours to acclimate. 15 individuals were then placed into separate 1L tanks and assigned to each of five salinity treatments to represent the range of salinity experienced in the field: Freshwater (0ppt), 5ppt, 15ppt, 25ppt and 35ppt. To avoid rapid osmotic stress to the individuals salinity levels were decreased to the desired level over the course of 1hr (Davis et al., 2022). Individuals were left in each of these treatments for 24 hours. To see whether any eggs were released as a stress response, the number of eggs released into the treatment tanks were counted. Spawning was then induced (as above) in 10 individuals from each treatment to give a ratio of eggs released due to stress. The remaining 5 individuals from each treatment were then sampled to characterise their surface-associated bacterial communities.

4.3.4 Effect of disruption of surface microbiota on recovery of host reproduction

180 female *Hormosira* individuals of similar size (approximately 1.2 +/- 0.6 in diameter and 11.9 +/- SE 1.1 in length) separated by ~1 m were tagged at Cape Banks Aquatic Reserve during low tide on the 24th of March 2022. Algae were rinsed with AFSW for 30 seconds to remove loosely attached epiphytes and then subjected to one of 6 treatments: 1) pulse microbial disruption via exposure to an antibiotic combination containing Penicillin (100mg/ml), Neomycin (100mg/ml) and Rifampicin (50mg/ml) applied once for 4 hours; 2) pulse microbial disruption via exposure to iodine (povidone-iodine, Betadine©) applied once for 4 hours; 3) a pulse procedural control (AFSW applied for 4 hours); 4) undisturbed control; 5) press microbial disruption via exposure to iodine applied for 4 hours every month;

6) press procedural control (AFSW applied for 4 hours every month). Samples were collected at day 0 (before treatment application), 7, 21, 35, 60, 90, and 120. Antimicrobial treatments used were based on those described in Chapter 2/McGrath et al. (in review), who explored the effects of these different antimicrobials on *Hormosira* and controls for those treatments. Thus, treatments 1 and 2 here were chosen because previous experimental work showed that disruption by these treatments caused changes to specific bacterial taxa in the microbiome which, in turn, caused significant effects on host function. Importantly, these treatments did not have any discernible direct effect on host function which is critical when trying to disentangle microbially mediated effects from direct chemical effects (see Chapter 2).

Host measurements

Host performance was quantified in several ways. Maximum photosynthetic quantum yield (after dark-adapting individuals for 15min) was measured using a Pulse amplitude Modulated (PAM) fluorometer (Walz, Germany). PAM was used because previous work has shown it to be a metric which generally reflects the condition of photosynthetic hosts under stress (Qiu et al., 2019; Straub et al., 2019). Reproductive development was estimated by taking thin sections of the third vesicle from the apical end of the longest frond of each individual *Hormosira*. To do this, fronds were fixed in 80% ethanol for 24 hours before embedding in paraffin. Sections were then taken on a microtome set to 7μm, they were then stained with Tylenol blue (0.4%) and the number of developing eggs counted in the sexual conceptacles under a light microscope. Reproductive output was quantified as the number of eggs released per ml of AFSW (as detailed above).

Sampling the surface microbiota

The surface of the kelp was swabbed for 30 seconds with a sterile cotton swab to collect surface-associated microbiota at each of the timepoints described above, following methods established in Marzinelli et al., (2015) and Qiu et al., (2019). Swab controls (i.e., treated

similarly to those for swabbing kelp except no kelp was swabbed) to test for contamination during processing were also used. Swabs were immediately placed intro sterile cryogenic tubes and placed in liquid nitrogen and then stored at -80ºC until DNA extraction.

DNA Extraction and sequencing

Microbial DNA was extracted from each swab sample in a randomised order to avoid introducing any bias due to order and time of processing, using a Powersoil DNA Isolation kit (Qiagen) following the manufacturers protocol. DNA extracts were quantified using spectrophotometry (Nanodrop 1000) and stored at -20oC until sequencing.

The extracted DNA samples were amplified with Polymerase Chain Reaction (PCR) using the 16S primers 341 (F) (5'- CCTACGGGNGGCWGCAG-3') and 805(R) – (5'-GACTACHVGGGTATCTAATCC-'3), containing the V3-V4 regions of the bacterial and archaeal 16S rRNA gene (Klindworth et al., 2013). The PCR protocol used is described in Chapter 2. Both positive (with known DNA sequence) and negative controls (nuclease-free water, control swabs) were used. The negative controls did not amplify DNA, suggesting no contamination on swabs or during extraction and amplification. Agarose gel electrophoresis and Nanodrop 1000 were used to ensure the quantity and quality of the amplicons before they were sent for sequencing via the Illumina MiSeq 2000 platform at the Ramaciotti Centre for Genomics (UNSW, Sydney).

Bioinformatics

Raw sequences were received from the sequencing centre as demultiplexed pair-ended sequences per sampled. UNOISE was then used to remove chimeras and produce amplicon sequence variants (ASVs), i.e., operational taxonomic units at a unique sequence level (0% distance) (Edgar, 2016). DADA2 was used to map the original reads back to ASVs, generating a table of 17,387 ASVs (Callahan et al., 2016). ASV sequences were searched

with BlastN against the SILVA SSU Ref NR99 database for taxonomic classification to classify and remove chloroplasts; Global Taxonomy Database (GTDB) was then used for taxonomic assignment. Singletons and low abundance taxa (<0.01% of reads) were removed from the dataset for statistical analyses, resulting in 13,210 microbial taxa.

Estimation of absolute bacterial abundance

Total abundance of the 16S rRNA gene was quantified for each sample by qPCR using the primers 341F/805R developed by Thijs et al., (2017). Gene amplification and analysis were performed using the QuantStudio 3 thermocycler (Thermo Fisher with PrimeTime® Gene Expression Master Mix, Integrated DNA Technologies) and associated software. The reaction conditions for amplification of DNA were 50ºC for 2 min, 95 ºC for 10 min and 40 cycles of 95 ºC for 15s and 60 ºC for 1 min. The final gene copy number per sample was corrected for the total extraction volume, the surface area and the dilution factor and DNA yield per sample (see Nappi et al., 2021 for further details) and were used to estimate absolute abundances of ASVs and inoculants.

Statistical Analyses

All statistical analyses were conducted in the R (v4.0.3) coding language in R-Studio (RStudio Team, 2020). To determine whether rainfall varied among months and between years, ANOVA was run with the fixed, crossed factors Month and Year.

To test for effects of microbial disruption on host function (PAM, number of eggs released, number of developing eggs), we used a linear model with the orthogonal factors' treatment (fixed, 6 levels) and time (fixed, with 7 levels) using the *GAD* package. To meet the model's assumptions, the response variables number of developing eggs and eggs released, were square-root transformed. Post-hoc tests were run on significant interaction terms, or

significant main effects (when no interactions were present) with more than two levels, using the *emmeans* function of the *emmeans* package (Lenth et al., 2019).

To account for uneven sequencing depth among samples, data were normalised using total 16S rRNA reads per sample of the v3-v4 region which was calculated using qPCR (see Nappi et al 2022). Alpha diversity measures of richness (i.e. number of unique sequences) and Simpson's diversity index were calculated using the 'vegan' R package (Oksanen et al., 2013) and differences between treatment (fixed), time (fixed, crossed) and their interaction were examined using a linear model in the GAD package.

To determine differences in the structure of the associated bacterial assemblages, the normalised ASV data were analysed using permutational multivariate analysis of variance (PERMANOVA) (Anderson & Walsh, 2013) in the *vegan* package (Oksanen et al., 2013), with the factors treatment, time and their interaction, as above. PERMANOVA was based on Bray-Curtis dissimilarities between sample pairs calculated on square-root transformed absolute (qPCR normalised) abundances of ASVs. For significant main effects or significant interactions, pairwise comparisons were performed using the pairwise.adonis.2 function in the *pairwise.adonis* package (Martinez Arbizu, 2020).

To determine which bacterial taxa differed the most between treatments and times and their interaction, we used multivariate generalised linear models (GLMs) using the package *mvabund* (Wang et al., 2012), assuming a negative binomial distribution to account for over-dispersion of sequence counts. Generalised linear latent variable models (GLLVMs) were used to visualise the bacterial community structure (see Niku et al 2019).

4.4 Results

4.4.1 The effect of extreme rainfall on the reproductive output of Hormosira

Rainfall significantly varied over the year with March 2022 recording the highest monthly rainfall (>15mm/day; daily max. 156mm; ANOVA, $F_{11,288}$=431.8, p<0.001; Table S4.1). Rainfall was negatively associated with *Hormosira*'s reproductive output, with thalli in March 2022 having a significantly lower reproductive output than all other months (p<0.001; Fig. 4.1 A-B). April had significantly lower reproductive output that all other months except March (p<0.001; Fig. 4.1). February, May, and August had significantly lower reproductive output than the remaining months other than March and April (Table S4.1; Fig. 4.1).

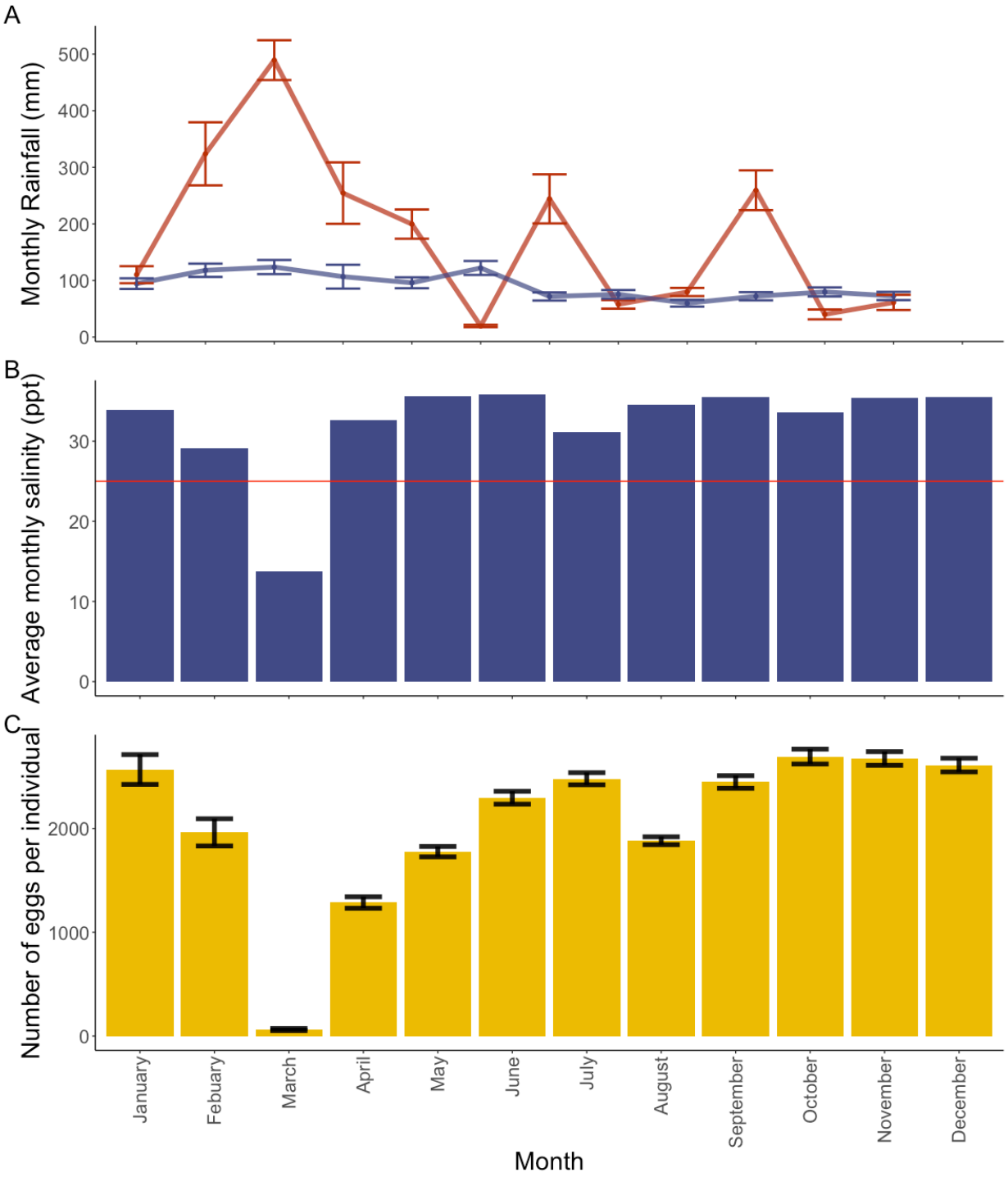


Figure 4.1. A) Monthly rainfall in 2022 (red line) and average from 1972-2022 (blue line, data are means +/- SE; n=50. B) Average monthly salinity in 2022; the red line indicates the threshold where salinity drops below 25ppt (means +/- SE, n=15). C) Mean (+/- SE, n=20) number of eggs released per individual at each monthly sampling time.

4.4.2 Effect of salinity on reproductive output

Lower salinity (0ppt, 5ppt 15ppt.) caused significant increases in the number of eggs *Hormosira* spawned in the laboratory (ANOVA, $F_{4,45}$=376.8, p<0.001, Fig 4.2, Table S3). Freshwater (0ppt) caused the highest level of spawning (98%) and was significantly different from all other salinity levels (Table S4.2, Fig 4.2). 5ppt had significantly higher levels of stress spawning (~55%) than 15, 25 and 35ppt but lower levels than freshwater (Table S4.2). 15ppt had significantly higher levels of stress spawning (~24%) than 25 and 35ppt but lower than freshwater and 5ppt (Table S4.2). There was no significant difference between 25ppt and 35ppt (Table S4.2).

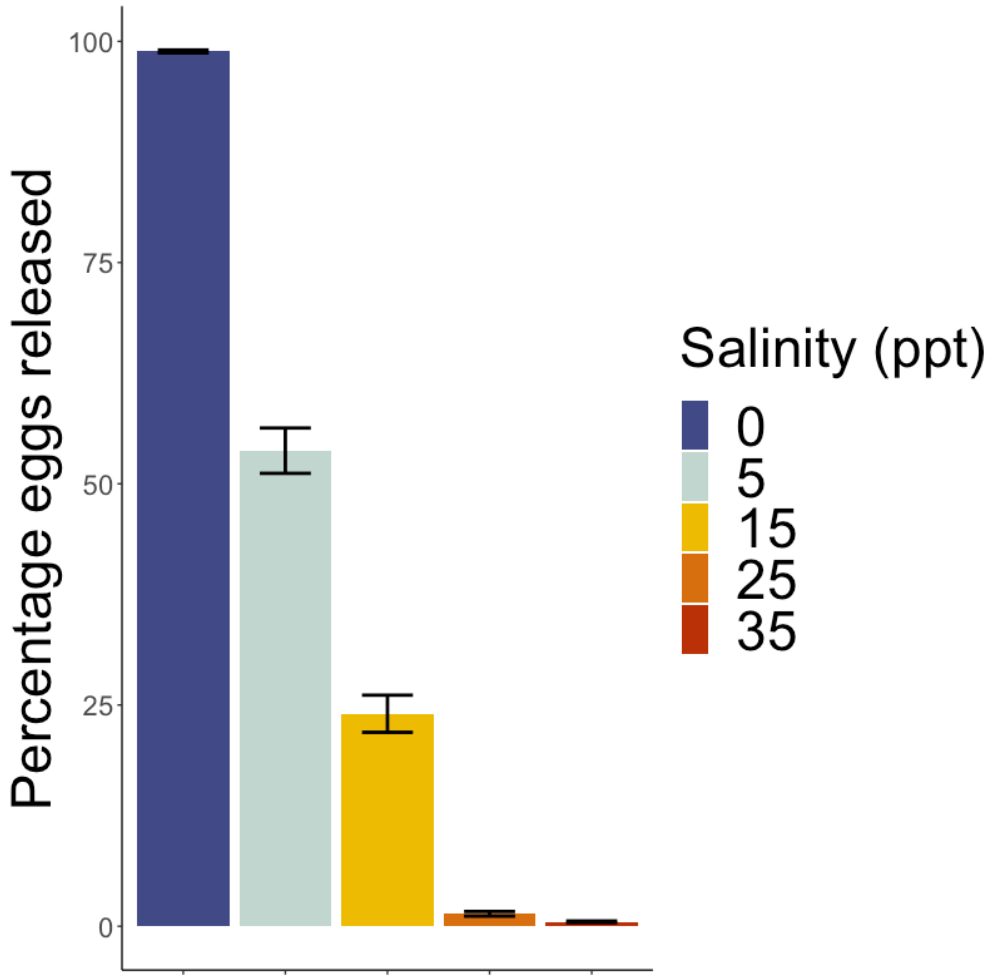


Figure 4.2. Mean (+/-SE, n=10) percentage number of eggs released during the salinity stress experiment.

4.4.3 Effect of microbial disruption on host reproductive output

Microbial community

The surface-associated microbiota of *Hormosira* significantly differed across treatments and times (PERMANOVA, pseudo-$F_{30,168}$ = 6.903, p<0.001, Fig. 4.3, Table S4.3). Antibiotics (AB2) and iodine caused significant changes in the community structure compared to controls from day 7 which lasted till day 60 (Fig. 4.3, Table S4.3). The effect of antibiotics (AB2) on the community structure differed from that of iodine, for both pulse and press application, from days 7 to 60 (Table S4.3). Press application of the Iodine treatment caused significant and lasting effects compared with controls for the entire experimental period of 120 day, and was significantly different from all treatments from day 60 (Fig, 4.3)

The disruption of the surface microbiota with antibiotics (AB2) and antiseptics (iodine) caused significant decreases in ASV richness which recovered to control levels over time (Table S4.4). Antibiotics caused a greater decrease in ASV richness than iodine but richness recovered to control levels by day 60 (Table S4.4). The only treatment which remained significantly different from all others at day 120 was the repeated application of iodine (Table S4.4).

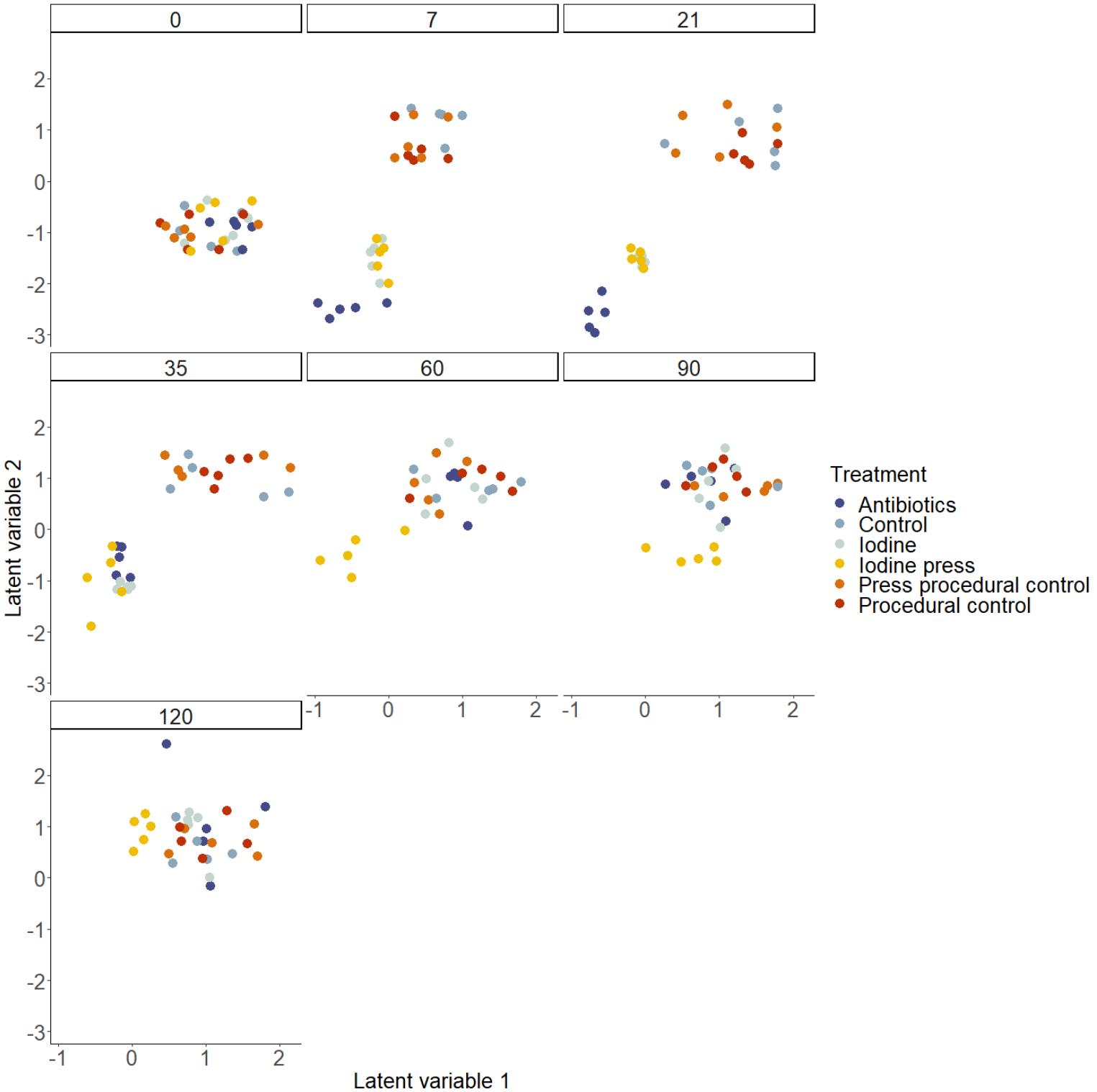


Figure 4.3 Bacterial community structure over time in the field experiment. Data is an ordination based on a GLLVM model with each panel indicating a different day. Each circle represents the bacterial community in a sample (n=5 per treatment and time).

Reproductive output

Host reproductive output from thalli in the field was significantly affected by microbial disruption, and this effect varied over time (ANOVA, $F_{30,164}$=105.7.1, p<0.001; Fig 4.4.B; Table S4.5). Reproductive output in thalli subjected to pulse treatments recovered to pre-rainfall stress levels by day 60 (Fig 4.4.B; Table S4.5). However, press disruption when applied to thalli caused significantly lower reproductive output relative to controls from day one until day 120 and relative to other disruption treatments from day 60 to 120 (Fig 3.B; Table S4). Overall, across all treatments, host reproductive output also increased over time following the freshwater stress at the start of the experiment (Fig 4.4.B; Table S4).

Overall, the number of developing eggs increased over the course of the experiment following rainfall stress (120 days; Fig 4.4.C). Microbial disruption with AB2 and Iodine caused a significant lag in the recovery of *Hormosir*a's egg development (Fig 4.4.C). Individuals treated with antibiotics and iodine had significantly lower numbers of developing eggs *versus* controls until day 60, when there was no significant difference (Table S5).

Photosynthetic efficiency

Disruption to the host microbiota caused significant, negative effects on host photosynthetic efficiency (ANOVA, $F_{5,168}$= 33.1, p<0.001; Fig 4.5.A; Table S4.4). Hosts whose microbiota were treated with pulse disruptions recovered over time and by day 30 there was no significant effect of these treatments (Table S4.4). Press treatments however caused significant and lasting effects on host photosynthetic efficiency which lasted the entire experimental period (120 Days; Fig 4.5.A). Antibiotics had a greater negative effect on host photosynthetic efficiency than Iodine when applied in a pulse treatment; effects lasted until day 60 wherein individuals from both Antibiotics and Iodine treatments returned to control levels (Fig 4.5A; Table S6).

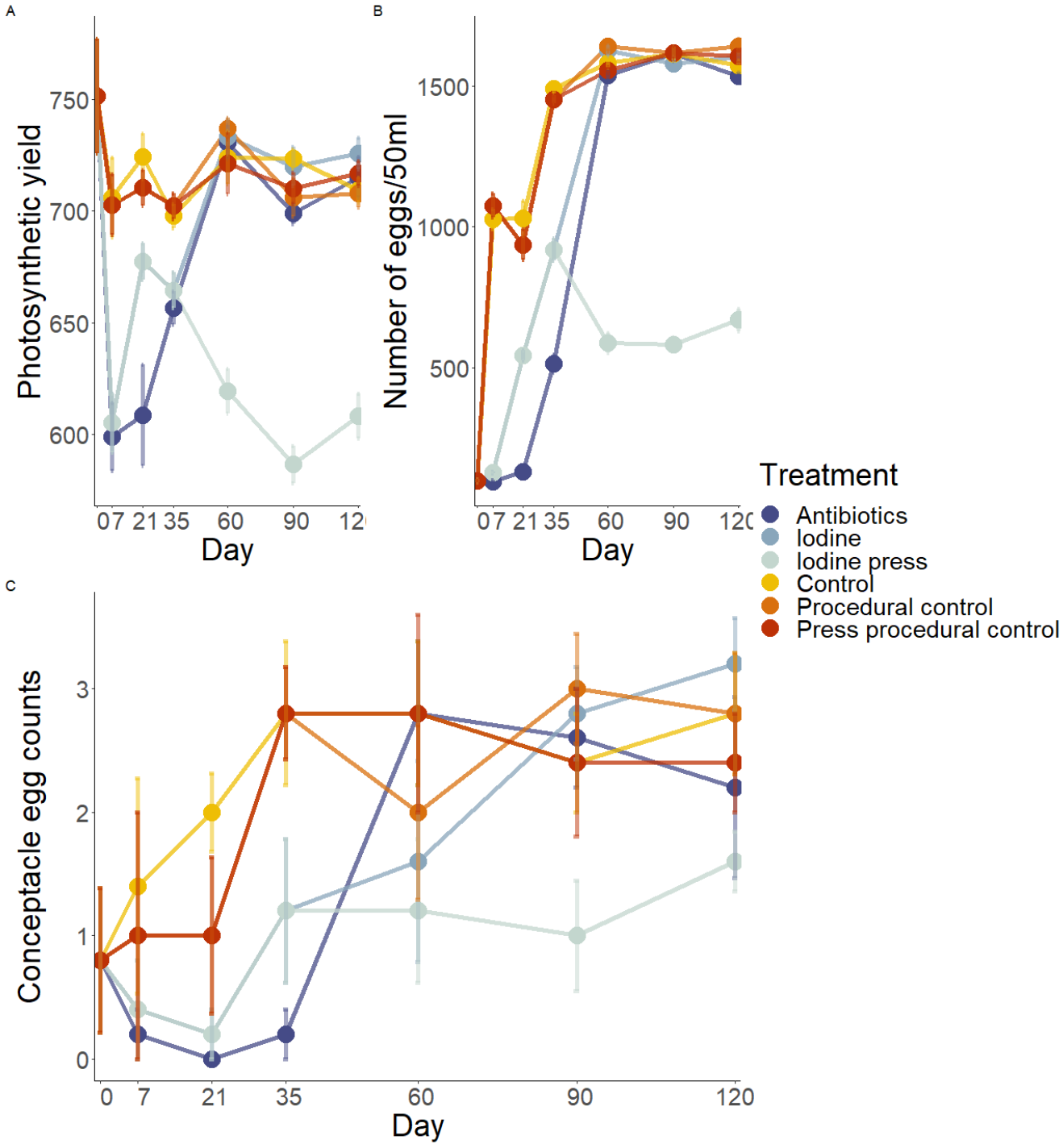


Figure 4.4. Host maximum photosynthetic yield (A), reproductive output (number of eggs per 50ml; B) and number of developing eggs in each conceptacle (C) at each timepoint. Data are means +/- SE; n=5.

4.5 Discussion

In this study, bacteria associated with a dominant habitat-forming seaweed enhanced recovery following extreme rainfall events of both host physiological function and reproductive output. The results show that, disruption of *Hormosira's* microbiome inhibited host recovery: in treatments where the microbiome recovered, host reproductive output also recovered. Conversely, when the microbiota were not allowed to recover (as in the press treatment), there was also no recovery of host reproductive output. Furthermore, individuals whose microbiomes were subjected to a pulse disturbance with antibiotics and antiseptics took two months longer to recover reproductive capacity following a one-in-100-year rainfall event than controls. This work thus provides insights into the importance of microbial communities in mediating host responses to extreme events.

Whilst direct osmotic effects on *Hormosira* could have led to the observed decrease in reproductive output as a result of lower salinity levels, our evidence suggests that it was likely a microbially mediated response because 1) changes in the microbial community occurred prior to changes in reproductive output, and 2) where the microbial community recovered, the reproductive output of the host also recovered. Algal holobionts have been shown to actively alter their microbiome in response to environmental stress through the release of algal metabolites (Saha et al., 2019, 2020).This alteration of the microbial community potentially increases host fitness and resilience against environmental change. The observed temporal change in the microbial communities within our control treatments adds additional evidence that microbial communities enhance the resilience of the host to environmental change. This has been shown within *Ectocarpus sp.* where microbial communities facilitate algal survival within different environmental conditions through mitigation of osmotic stress (Dittami et al., 2016b; KleinJan et al., 2021b).

Physiological measures such as photosynthetic efficiency and nutrient content are commonly used to assess macroalgal performance under stress (Fernandez et al., 2020; Pfister et al., 2019; Qiu et al., 2019; Straub et al., 2019). However here, rainfall did not affect the photosynthetic efficiency of *Hormosira.* This is potentially due to the large variability in the measurements of the efficiency of chlorophyll, as has been described within previous work using PAM fluorometry (Durako & Kunzelman, 2002; Enriquez et al., 2002). Whilst physiological measures are useful for assessing the performance of an organism, relating them to host fitness is challenging, especially in the field. Other measures that relate to reproduction and survival of hosts can provide a better indication of host responses to stress and are better proxies for fitness, and thus should be incorporated when assessing holobiont responses to extreme events.

4.5.1 Effect of extreme rainfall on reproductive output

Extreme rainfall caused significant decreases in the reproductive output of female *Hormosira.* This effect was greatest in March 2022 when rainfall was also the greatest (489.4mm total, 156mm daily max). Interestingly, whilst there were significant spikes in rainfall in July (344.2mm total, 19.4 daily max.) and October (259.4mm total, 24.4 daily max.) 2022, these rainfall events did not induce a similar negative effect to that observed in March. The microbial community associated with *Hormosira* in the months of July and October did not differ from those in other months in 2022; the only difference was between March and all other months. One likely explanation is that the salinity in the bay did not drop below 25 ppt except in March 2022 – this salinity value was a threshold in the salinity experiment in the laboratory, below which *Hormosira* stress spawned. The microbial community associated with individuals at lower salinities (<25ppt) was significantly different than that on individuals at higher salinity levels. All this combined suggests that the observed negative effect on the host reproductive output was potentially induced by changes in the microbial

community. Previous work has shown that microbial communities associated with various seaweed species can mediate abiotic effects such as salinity fluxes through functional enrichments, i.e. the microbiota add additional functional capacities to the host that otherwise would not able to undertake (Dittami et al., 2016; Saha et al., 2020). These functional enrichments enhance host responses to abiotic stress and are potentially the driver of the observed patterns here.

4.5.2 Effect of microbial disruption on recovery of host reproduction

Evidence from the previous chapters shows the complex effect of disrupting the microbiome of hosts with antibiotics (Chapters 2 and 3). That is, the difficulty in determining whether the observed effect on the host is mediated by its microbiota or a consequence of the methodology used to manipulate the microbiota. The work presented here adds additional evidence to suggest the former – a microbially mediated effect. Disruption to different components of the microbiome affected the host in different ways, an effect which was also induced not by chemicals but exposure to varying salinity levels. Furthermore, the observed host effects occurred after the microbial community changed, which is important as any direct chemical effects of the antimicrobials used would likely be immediate (Ebert et al., 2011; Fu et al., 2017).

Disruption with antibiotics and antiseptics caused significant differences in the community structure of the microbiome when compared with controls. These differences were characterised by significant decreases in ASVs belonging to the classes *Cyanophyceae*. The most abundant of these ASVs (*Pleurocaspa* sp) ranged from 3-5% of the total community abundance. Cyanobacteria, e.g. *Pleurocaspa* sp, are suggested to be ubiquitous in the sunlit waters of the ocean and have been implicated in a number of symbioses with eukaryotic hosts (Schvarcz et al., 2022; Taylor, 1973; Webster & Blackall, 2009). As symbionts, Cyanobacteria have been reported to provide hosts with a number of key nutrients including

carbon (Foster et al., 2022), nitrogen (Fiore et al., 2010; Zehr & Kudela, 2011), and sulphur (Jensen et al., 2017). The provision of nutrients is key to host persistence, especially in nutrient limited environments such as intertidal environments (Bracken & Nielsen, 2004; Elser et al., 2007). Thus, disruption to the host's supply of nutrients by the loss of important microbial taxa potentially leads to lower reproductive output, as has been shown in plant systems (Li et al., 2017; McGinley & Charnov, 1988). Press disruption with antiseptics inhibited the recovery of reproductive output for the entire experimental period (120 days). As in the pulse treatments, the main bacterial taxa that were reduced by the press treatment belonged to the class Cyanophyceae, but unlike the pulse treatments the abundance of this taxa did not recover over the entire experimental period. Future work should focus on uncovering the mechanisms behind these observations to determine whether these taxa really are important, and if so what function do they provide the host.

Host-associated microbiomes have critical roles in the normal functioning and development of marine organisms (Brodie et al., 2016; Fuggle et al., 2023; Trevathan-Tackett et al., 2019). There is growing evidence that disruption to the microbiome, and by proxy the interactions between host and its microbiome, can negatively affect host health and function (Qiu et al., 2019). Whilst this reinforces the idea that organisms should be studied as holistically, as holobionts, it is unclear the role that microbes play in host fitness (Carthey et al., 2020; Harley et al., 2006; Prosser & Martiny, 2020). This is particularly important to understand for habitat-forming holobionts which form the biogenic structure of the ecosystems they grow in, as interactions within the holobiont can cascade throughout the entire ecosystem (Laforest-Lapointe et al., 2017). Such habitat forming species are in decline within marine ecosystems in many places in the world, in part due to an increase in extreme events exacerbated by climate change (Bellgrove et al., 2010; Carthey et al., 2020; Straub et al., 2019). Whilst we are gaining an understanding of the consequences of these losses (Beman et al., 2011; M.

Koch et al., 2013), we still know very little as to the importance of host-microbe interactions in mitigating and enhancing responses to climate change. Given the importance of *Hormosira* on intertidal rocky shores along coastal Australasia (Bellgrove, McKenzie, et al., 2017; Schiel & Taylor, 1999), and the growing recognition of the role of microbes in host function, it is critical to understand their role in host fitness to better manage and conserve critical marine ecosystems.

4.6 Supplementary information – Chapter 4

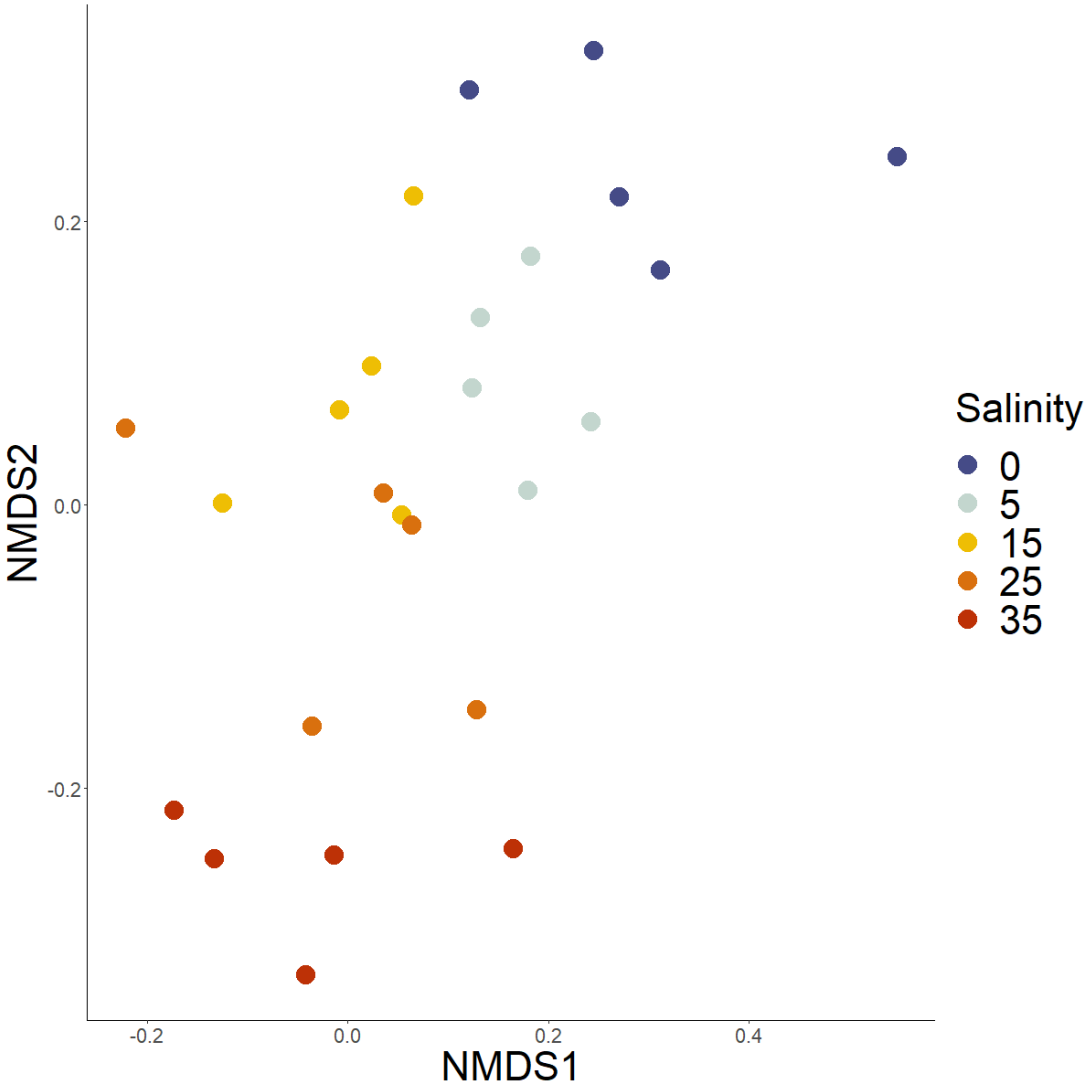


Figure S4.1. nMDS based on the bray-Curtis measure on square-root transformed absolute abundances of ASVs on the algal surface across treatments (stress =0.0186). Colour represents the salinity of the treatment. Each dot within the panel indicates the community of microbes associated with n=5 independent replicates of each treatmen

Table S4.1. Analysis of monthly rainfall across month and year (fixed, 12 levels: January – December) Post-hoc contrasts were calculated using the emmeans R function and p-values adjusted for multiple-testing (alpha=0.05).

Source		df	Sum of Sq	Mean Sq	F	p
Month		11	6332	575.6	431.8	>0.001
Residual		551	384	1.3		
Pairwise contrasts						
Month	March > February > July =October >January = April = June = August = September = November = December					

Table S4.2. Analysis of number of eggs released by *Hormosira* individuals exposed to different salinities (fixed, 5 levels: 0, 5, 15, 25, 35 ppt). Post-hoc contrasts were calculated using the emmeans R function and p-values adjusted for multiple-testing (alpha=0.05).

Source		Df	Sum of Sq	Mean Sq	F	p
Salinity		4	144565	36141	374.8	>0.001
Residual		45	43163	9592		
Pairwise comparison						
Salinity	0<5<15<25=35					

3

Table S4.3. PERMANOVA based on Bray–Curtis similarity measures for qPCR-normalised, square-root transformed ASV abundances on *Hormosira banksii* across different treatments (fixed, 6 levels: AB2, I, IP, C, PC, PPC) and sampling times (fixed, crossed, 7 levels: 120 days). Significant *p*-values (alpha = 0.05) are highlighted in bold. Post-hoc contrasts were calculated using the pairwise.adonis R function and p-values adjusted for multiple-testing (alpha=0.05).

Source	df	SS	MS	pseudo-*F*	*p*(perm)	perms
Treatment	5	4499.3	1795.8	1.988	0.0001	9698
Sampling time	6	94683	6487.7	4.2726	0.0001	9891
Treatment x timepoint	30	145271	26814.9	6.903	0.0014	9787
Residual	168	60587	682.19			
Total	209	305040.3				
Pairwise comparisons						
	Day	Treatment				

	0	AB2=I=IP=C=PC=PPC				
	7	AB2<I-IP<C=PC=PPC				
	21	AB2<I=IP<C=PC=PPC				
	35	AB2=I=IP<C=PC=PPC				
	60	IP<AB2=I=C=PC=PPC				
	90	IP<AB2=I=C=PC=PPC				
	120	IP<AB2=I=C=PC=PPC				
	7=21<35<0=60=90=120	AB2				
	7<21=35<0=60=90=120	I				
	90<7=60=120<21=35<0	IP				
	0<7<21=35=60=90=120	C				
	0<7<21=35=60=90=120	PC				
	0<7<21=35=60=90=120	PPC				

4

Table S4.4 ANOVA results for analysis of bacterial community alpha diversity measures: number of species and Simpson index in Hormosira banksii subject to different treatments (fixed, 6 levels: AB2, I, IP, C, PC, PPC) and sampling times (fixed, crossed, 7 levels: 120 days). Significant *p*-values (alpha = 0.05) are highlighted in bold. Post-hoc pairwise comparisons calculated using emmeans, all treatment differences are based on alpha = 0.05.

Source		Df	Sum of Sq	Mean Sq	F	P-value
Treatment		5	101780	20356	4.9231	0.0004984
Timepoint		6	143095	35774	8.6519	5.911e-06
Treatment: Timepoint		30	69236	3462	0.8372	0.6631976
Residual		168	372129	4135		
Pairwise comparisons						
Day	0=7<21<35=60=90=120					
Treatment	AB2=IP<I<C=PC=PPC					

Table S4.5 ANOVA results for analysis of host photosynthetic yield between different treatments (fixed, 6 levels: AB2, I, IP, C, PC, PPC) and sampling times (fixed, crossed, 7 levels: 120 days). Significant *p*-values (alpha = 0.05) are highlighted in bold. Post-hoc contrasts were calculated using the emmeans R function and p-values adjusted for multiple-testing (alpha=0.05).

Source		Df	Sum of Sq	Mean Sq	F	P-value
Treatment		5	15.013	3.003	33.190	>3.4e-6
Timepoint		6	16.410	2.735	30.257	>2.2e-08
Treatment x Timepoint		30	14.844	4.9448	5.474	>2.72e-16
Residual		168	15.1866	9.04		
Pairwise comparisons						
	Day	Treatment				
	0	AB2=I=IP=C=PC=PPC				
	7	AB2=I-IP<C=PC=PPC				
	21	AB2<I=IP<C=PC=PPC				
	35	AB2=I=IP<C=PC=PPC				
	60	IP<AB2=I=C=PC=PPC				

	90	IP<AB2=I=C=PC=PPC				
	120	IP<AB2=I=C=PC=PPC				
	7=21<35<0=60=90=120	AB2				
	7<21=35<0=60=90=120	I				
	90<7=60=120<21=35<0	IP				
	0>7=21=35=60=90=120	C				
	0>7=21=35=60=90=120	PC				
	0>7=21=35=60=90=120	PPC				

Table S7 ANOVA results for analysis of numbers of developing eggs between different treatments (fixed, 6 levels: AB2, I, IP, C, PC, PPC) and sampling times (fixed, crossed, 7 levels: 120 days). Significant *p*-values (alpha = 0.05) are highlighted in bold post-hoc contrasts were calculated using the emmeans R function and p-values adjusted for multiple-testing (alpha=0.05).

Source		Df	Sum of Sq	Mean Sq	F	P-value
Treatment		5	37.91	7.582	4.937	0.00304
Timepoint		6	118.63	19.771	12.874	>6.04e-12
Treatment x Timepoint		30	46.06	1.535	1.00	0.4744
Residual		168	258	1.536		
Pairwise comparisons						
Day	0=7=21<35=60=90=120					
Treatment	IP<AB2=I<PC=C=PPC					

Chapter 5.

General discussion

There is growing evidence that microbes associated with eukaryotic hosts are critically important for their normal function and development (Blacher et al., 2019; Nakanishi et al., 1996). These microbial associations are nearly ubiquitous, spanning the tree of life and across ecosystems (Greenspan et al., 2020; Laforest-Lapointe et al., 2017; van der Loos et al., 2019). This is leading to a paradigm shift in biology where eukaryotes are no longer viewed as single entities, but rather as a coherent biological association comprised of the host and its microbiome, i.e. as "holobionts" (McFall-Ngai et al., 2013; Rosenberg & Zilber-Rosenberg, 2018).

Work has shown that habitat forming organisms ranging from trees and grasslands to corals and macroalgae are in fact highly complex holobionts (Bourne et al., 2016; Egan et al., 2013; Laforest-Lapointe et al., 2017). Microbial communities within these holobionts have been described to provide functions ranging from nutrient acquisition (Adak et al., 2016; Fiore et al., 2010), developmental assistance (Carrier et al., 2022; Nakanishi et al., 1996; Tochitani, 2021), pathogen resistance (Egan et al., 2014; J. Li et al., 2022; Saha et al., 2019), carbon uptake and sequestration (Bengtsson et al., 2011; Tsubaki et al., 2020), to abiotic amelioration (e.g. salinity acclimation (Agler et al., 2016; Dittami et al., 2016). Understanding the causative roles of these microbial communities is, however, a major bottleneck in holobiont ecology (Prosser & Martiny, 2020). This is especially true for habitat forming organisms whose biogenic structure underpins the ecosystems they live in.

There is a growing number of studies that aim at understanding causation and mechanisms by which microbiota affect host performance. To understand causation and the underlying

mechanisms, manipulations of the microbiota are required; however, such manipulations can be challenging. Examples of previous approaches include experiments on the gut microbiota of mice (Blacher et al., 2019; Kennedy et al., 2018), zebra fish (Bordenstein & Theis, 2015; Fraune & Bosch, 2010; Levraud et al., 2022), microbiota of mosquitos (Cansado-Utrilla et al., 2021; Coon et al., 2016; Dada et al., 2021; Rodríguez-Ruano et al., 2020) and daphnia (Decaestecker et al., 2005; Massol et al., 2021). However, in most cases, these host-microbiota manipulations occur in the laboratory, removed from their natural environment, limiting the extent of our inferences and our capacity to predict responses of holobionts in nature.

This thesis used experimental marine ecology as a framework to develop and test an approach to determine causation for a dominant marine habitat-forming host – the intertidal seaweed *Hormosira banksii* (Chapter 2). This approach allows for the effects of complex microbial communities on hosts (and *vice versa*) to be distinguished and tested within their natural systems (Dittami et al., 2014; Prosser & Martiny, 2020; Underwood et al., 2000). The use of this experimental approach enabled us to test for the effects of microbial communities (host-associated and benthic) on components of fitness across ontogeny (reproductive output, zygote attachment and morphogenesis; Chapter 3). To further understand the role of microbial communities in mediating host function and reproduction under environmental stress – an extreme rainfall event – a series of manipulative experiments were conducted in Chapter 4. The findings of this thesis extend our base understanding of host-microbiota interactions and lay the foundation for further manipulative experimentation in holobiont ecology.

5.1 Determining causation in non-model holobionts

Chapter 2 developed a framework to determine the effect of the host-associated microbiota, or subsets thereof, on the photosynthetic efficiency – a proxy for host performance – of

Hormosira. To do this, I combined microbiota disruptions using antibiotics, with the identification, culturing, isolation, and subsequent inoculation of presumed harmful bacteria. To quantify whether the observed effects were representative of natural systems, these experiments were done in the laboratory and in the field. Whilst antibiotics have been used widely as a method for reducing/disrupting components of a hosts microbiome (Dittami et al., 2014, 2016; Kennedy et al., 2018; KleinJan et al., 2021), few studies have considered that the use of these chemicals might have a direct effect on the host (reviewed in Kennedy et al., 2018). The complex responses of microorganisms to different antimicrobials, the potential direct (not microbially mediated) effects on the host of the methodology employed to manipulate the associated microbiota, as well as the temporal sequence of events and the environmental context (i.e., laboratory vs field) are critical aspects that must be considered when interpreting outcomes of experimental approaches that involve microbial manipulations on hosts.

The combination of both approaches, i.e. the disruption/removal and the addition/inoculation of microorganisms, applied (wherever possible) in real-world settings, coupled with temporal sampling, provides a powerful and necessary approach towards a causal understanding of host-microbiota interactions. This combined approach is, however, not infallible and suffers from one of the great challenges of microbiology: the ability to broadly culture bacteria (Barer & Harwood, 1999; Das et al., 2015; Martiny, 2019). Recent work examining corals and their associated microbiota has provided a potential avenue for future study, that is, the culture independent technique of homogenisation of a host's tissue to generate a bacterial suspension for inoculation (Damjanovic et al., 2019; Doering et al., 2021; Peixoto et al., 2022; Villela et al., 2023). This technique enables the inclusion of slow growing and difficult to culture bacterial taxa such as cyanobacteria, which seemed to be of high importance within our system (Chapters 2-4), in a suspension which can then be used as the inoculant.

Homogenisation would also allow future work to potentially 'conventionalise' hosts. Conventionalisation is a term that refers to the application of the treatment (e.g. antibiotics or antiseptics) before restoring the normal, pre-treatment microbiota. Such approach provides an appropriate procedural control for manipulative experiments involving the removal/disruption of the microbiota (Blacher et al., 2019; Kennedy et al., 2018; Levraud et al., 2022). Whilst the framework used in Chapter 2 to examine causation incorporated the use of several chemically similar antimicrobials but with different effects on the microbiota, which, combined with temporal sampling, allowed us to make inferences about microbial effects on the host, the inclusion of a host-conventionalisation treatment would be ideal as it is a more robust control for potential artefacts introduced by manipulations. This technique is difficult to incorporate outside of germ-free or model systems, but there are a number of studies which have attempted it in mosquitos and daphnia, although, removed from their natural ecological settings (Cansado-Utrilla et al., 2021; Coon et al., 2016; Massol et al., 2021). A logical next step for our framework is to incorporate these culture independent techniques to generate conventionalised hosts where the microbiota are removed/disrupted and/or re-introduced as a whole within natural, open systems (i.e. in the field).

Within this thesis, when considering microbial communities, I have mainly focused on the effect of bacterial taxa on proxies for host function (e.g. photosynthetic efficiency) and fitness (e.g. reproduction, morphogenesis). Bacteria have been the focus here because previous work has suggested that they are important members of seaweed-associated microbiota (Egan et al., 2013, 2014; J. Li et al., 2022; Longford et al., 2019; Marzinelli et al., 2015; Miranda et al., 2022; Weigel & Pfister, 2019). However, fungi (Amend et al., 2012, 2019; Ferrari et al., 2021; Roik et al., 2022; Tourneroche et al., 2019; Yarden, 2014) and viruses (Kim et al., 2016; Lachnit et al., 2016; Ren et al., 2022; van der Loos et al., 2019) are increasingly being recognised as important to eukaryotic host function and may also play a

significant role in *Hormosira*. The antimicrobials used in this thesis included antibiotics, which mainly target bacteria (see Chapter 2), as well as iodine, which can also affect other microorganisms (Manna et al., 1984; Piérard et al., 1997; World Health Organization & others, 2020). The observed effects on host function and potential fitness may therefore be attributed to changes in the fungal or phage communities. Future work should include molecular tools and analyses (e.g., 18S rRNA gene and ITS amplicon sequencing), as well as targeted culturing and isolation approaches, to capture other members of the microbial community and understand their role on host performance.

5.2 Effect of microbiota on hosts across ontogeny and beyond physiology

Most studies testing the effect of microbiomes on hosts function have focused on molecular (e.g. stress marker genes) or physiological parameters (e.g. metabolic rate) (Carrier et al., 2022; Fiore et al., 2010; Kennedy et al., 2018; Lesser et al., 2007; Mohr et al., 2021; Pedersen et al., 2016). The ubiquity of host-microbiota associations and their effects on host function strongly suggest that host-associated microorganisms may affect host fitness (Davitt et al., 2011; Glasl et al., 2016; Graham et al., 2014; Rosengaus et al., 2011), that is, may affect survivorship and reproduction across generations. However, for many marine holobionts, the effect of microbiota on host fitness has not necessarily been established because relating individual physiological functions to fitness components such as survival or reproduction, or even broader ecological concepts such as resilience, can be fraught with difficulty (Björk et al., 2019; Leray et al., 2021; O'Brien et al., 2019; Sison-Mangus et al., 2014). For example, in long-lived and slow-growing species such as *Hormosira* (~5-8 months from zygote to 3 vesicles, Kain, 2015), it may not feasible to accurately measure fitness over short timescales (Bassar et al., 2010). For instance, most research projects and funding cycles are far shorter (often 3 years) than the lifespan of many of the hosts studied. As such, many researchers use proxies of fitness to estimate overall lifetime fitness. These measures include germination

rates (Kaur et al., 2017; Myers et al., 2006, 2007; Ordoñez et al., 2017), size (Earl & Whiteman, 2015; Hendry et al., 2001; McGinley & Charnov, 1988), biomass (Calmes et al., 2020; Dudenhöffer et al., 2018; Hughes et al., 2016) and reproductive investment or output (Alif et al., 2022; Fujita et al., 2014; Hamel et al., 2010; Hendry et al., 2001; Pfister, 1992) which was used in this thesis. Incorporating ontogeny, through the use of measures such as reproductive output and reproductive investment, into proxies of fitness further increases the accuracy of inferences of an organism's lifetime fitness (Alif et al., 2022; Bassar et al., 2010; Wallace et al., 2016). Ultimately though, multi-generational studies are required to truly understand the effect of microbial communities on life histories. This however requires longer project times or other, shorter-lived hosts.

Given how the probability of surviving or reproducing varies across the life cycle or age of an organism, this also highlights how important it is to understand the effects of the microbiome on the host throughout its entire life cycle (Agrawal, 2012; Davitt et al., 2011; Glasl et al., 2016; Hamel et al., 2010; Nakamura et al., 2011). There is evidence that host-associated microbial communities have different effects at different life stages of a holobiont (Carrier et al., 2022; Pantoja-Feliciano et al., 2013; Vijayan et al., 2019). For example, recent work has shown both ticks and helminths have a core microbial community which is present throughout its life, but which varies in function as the host progresses through its life cycle (Hahn et al., 2022; Jorge et al., 2020; Zolnik et al., 2016). Considering the effect of microbial communities over ontogeny is especially important for ecologically dominant organisms such as *Hormosira* because understanding which microorganisms are important at which life history stage may be critical for any management strategy or intervention (Peixoto et al., 2017, 2021).

Chapter 3 provided important insights as to the role of host-associated microbes in mediating reproductive output and attachment success across *Hormosira*'s life history. Host-associated

microorganisms had a strong effect on the reproductive output of female *Hormosira.* As the reproductive output of male individuals was not quantified, the effects of host-associated microorganisms on males is not clear. However, the observed decrease in female reproductive output is critically important for a recruitment-limited species such as *Hormosira* because disruption to propagule supply can have significant, negative effects on recruitment and population persistence (Bellgrove et al., 2004; D'Antonio et al., 2001; Hoffmann & Ugarte, 1985; Lee & Bruno, 2009; Schiel & Foster, 2006; Seabloom, 2011). Furthermore, there was a strong interactive effect of host- and benthic-associated microbial communities on the attachment of the zygotes. Whilst previous studies have reported between 5-8% of zygotes surviving after 12 hours in the field (Bellgrove et al., 2004; Clarke & Womersley, 1981; Taylor & Schiel, 2003), here I found significantly higher zygote survival rates (10-15%), potentially as an artifact of spawning in the laboratory. Despite this potential artefact, I showed that disruption to the microbial community led to a near complete loss of zygote survival (0-2%), with potential strong effects on the establishment and maintenance of populations under stress. Whilst there is significant literature on how benthic-associated microbes induce settlement in marine invertebrates (reviewed in Dobretsov & Rittschof, 2020), as well as development in some algal species (Nakanishi et al., 1996a; Provasoli & Pintner, 1980), this work is one of the first to show that adult host-associated microbial communities can influence successful zygote morphogenesis.

5.3 Host-associated microbes can enhance recovery from extreme rainfall events

Host associated microbial communities have been suggested to ameliorate environmental conditions within a number of systems ranging from grasslands to coral reefs (Adak et al., 2016; Carrier & Reitzel, 2017; Case et al., 2011; Fuggle et al., 2023; Tadych et al., 2014). In Chapter 4, I examined the effect of extreme rainfall on host-microbe interactions and how

these interactions can mediate the response of the host to stress. Whilst *Hormosira* has been shown to be fecund throughout the year, with several studies showing differences in recruitment across seasons (Bellgrove et al., 2004; Clayton et al., 1985; Kain, 2015; Taylor & Schiel, 2003; Underwood, 1998), I found a strong effect of salinity – an important variable that changed during the extreme rainfall event – on the reproductive development of females and release of eggs. Interestingly, I found that when *Hormosira* was subjected to low salinity levels (>20ppt) for a prolonged period (1 month) there was an almost complete collapse of *Hormosira*'s reproductive output (females) in the field. As the microbial community associated with *Hormosira* mediated its reproductive response, changes in this community as a result of increased and more intense rainfall events have the potential to cause declines of the entire populations. To future-proof these populations in the context of climate change and related stressors, there are two potential avenues of inquiry: 1) manipulate the microbial communities to increase the resilience of the hosts and/or 2) utilise the hosts genetics (e.g. out planting/ selective breeding). The experimental approach outlined in Chapter 2 can be used to identify microbes which enhance host resilience to stress, which can then be used as probiotic treatments as is currently done in corals (e.g. Peixoto et al., 2017, 2021, 2022). However, whilst there is promise within these probiotic approaches, their application within the field can be challenging. Additionally, microbial communities with their inherently high adaptive potential may develop tolerance to stress without the need for manipulation (Bosch et al., 2019; Fraune & Bosch, 2010; Lowe et al., 2021).

Alternatively, future-proofing approaches may focus on the host, or combine microbial manipulations as described above with specific host genotypes, deemed tolerant to stress. *Hormosira* has two unique ecotypes (as described by King, 1981) which are exposed to varying salinity regimes. The free-living ecotype of *Hormosira* is consistently subjected to low salinities in its estuarine environment and remains detached for its entire life cycle

(Coleman et al., 2019; King, 1981). The coastal ecotype is consistently subjected to a higher average salinity regime that the free-living ecotype due to its exposure to the open sea (den Hartog, 1968). Work from other systems suggest that ecotypes exposed to more variable environmental regimes have greater plasticity in their responses and may thus explain why the low salinity ecotype can reproduce in such conditions (De Jong, 2005; Gibbons et al., 2017; Hollander & Butlin, 2010; Huang & Boney, 1983). Recent genetic evidence shows a clear separation of the ecotypes which may suggest their differential responses to salinity may be inheritable (Bellgrove, van Rooyen, et al., 2017; Coleman et al., 2019). As extreme rainfall events are predicted to increase, crossbreeding of these two ecotypes may provide a genetic lifeline for this species given the strong effects rainfall had on reproduction of the coastal ecotype. Genetic lifelines such as crossbreeding have shown promising results in increasing the resilience and persistence in organisms at their ranges edges (Abicair et al., 2020; Attard et al., 2016; Hails et al., 1993; Van Oppen et al., 2017; Van Oppen & Aranda Lastra, 2022).

5.4 Conclusion and future directions

This work explored how to determine causation in non-model holobionts, particularly seaweed holobionts, and lays the foundation for future work into holobiont research. Whilst the use of 16S rRNA gene amplicon sequencing has allowed us to understand the diversity of the bacterial communities associated with this seaweed host, and which taxa are involved in cause-effect relationships, it does not allow us to determine the function of these communities. Using this manipulative approach with functional measures of the microbiome and the host, such as metagenomics, meta-transcriptomics and metabolomics coupled with host transcriptomics, would allow better mechanistic understanding of host-microbiota interactions. Whilst these genomic tools are by no means without issue, primarily low numbers of annotated functional identity (Sunagawa et al., 2015), their use would also allow

determination of critical functions and also whether there is redundancy in the microbiome. There are a number of studies which have shown that specific microbial functions are of greater importance to host function and survival than the identity of the microbial taxa (Balser et al., 2002; Glasl et al., 2016; Spoerner et al., 2012). However, these studies have shown that these interactions are highly species-specific, which suggests that drawing predictions from these systems and applying them to others should be done with caution (Tadych et al., 2014).

Results from Chapter 3 indicate that microbial communities are important for seaweed function and survival across ontogeny. I showed that host-associated microbiota are important for both reproductive output and settlement in this species. A question which arises from this is: where do hosts acquire the microbes which are important for these processes – reproduction and settlement? Habitat forming organisms have been shown to host unique microbial communities from their surrounding environment (Adak et al., 2016; Marzinelli et al., 2015; Miranda et al., 2022; Palit et al., 2022; Weigel & Pfister, 2019). However, whilst several studies have established that vertical transmission of the microbiome is commonplace within natural systems (Carrier et al., 2022; Davitt et al., 2011; Donaldson et al., 2016; Tochitani, 2021), the evidence for this within habitat forming seaweeds is limited. Future work could determine the prevalence and importance of the origin of a host-microbiota.

Holobionts live in variable ecosystems and can be exposed to rapid environmental changes. It is important to understand the ecology of holobionts within their natural environment (Rosenberg & Zilber-Rosenberg, 2018b) as the response of holobionts to environmental conditions is, at least partially, determined by the microbial communities (Seymour et al., 2017; Simon et al., 2019). In this thesis, host-associated microbial communities mediated host responses following extreme rainfall events. Future work should aim at determining key functions within the microbiome and the mechanisms by which they mediate this response.

Furthermore, as is currently done within corals (Peixoto et al., 2017, 2021), the development of probiotic microbial treatments to assist habitat forming organism could prove to be a lifeline in a changing environment. Developing suitable probiotics is challenging as microbial taxa may vary from commensal and mutualistic to parasitic within varying conditions (Singh & Reddy, 2016). Therefore, the function of the microbes of interest must be taken in a number of different environmental and ecological contexts (Epstein et al., 2019; Peixoto et al., 2021, 2022). If the culturability of the microbes on interest is low or difficult, homogenisation of the a health host could be used as a 'microbiome donor' as has been done with faecal transplants (Kaakoush, 2020; E. K. A. Schmidt et al., 2020; Secombe et al., 2021; Vrieze et al., 2013).

Overall, this thesis shows that host-associated microbes are fundamentally important for host function and potential fitness and exert influence through an organism's life history. This is an important step for holobiont ecology and has implications for how we consider holobionts' persistence within a changing ocean. To further our understanding of an organism ecology within a variable world, we must consider the whole ecological unit – the holobiont – if we are to actively conserve and manage ecologically important species.

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