UTS UNIVERSITY OF TECHNOLOGY SYDNEY

# Dynamics of coral-associated bacterial communities induced by reef restoration practices

**by Paige Victoria Strudwick**

Thesis submitted in fulfilment of the requirements for the degree of

**Doctor of Philosophy**

under the supervision of Professor David Suggett, Professor Justin Seymour and Dr Emma Camp

University of Technology Sydney
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# Certificate of Original Authorship

I, Paige Victoria Strudwick declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research was supported by the Australian Government Research Training Program.

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Date: 3rd July 2023

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# Thesis Abstract

Development and implementation of coral reef restoration approaches are rapidly advancing worldwide to safeguard vital biodiversity and ecosystem services. However, the 'success' of propagation and out-planting restoration programs is variable. Efforts to refine restoration protocols have neglected to consider coral microbiology. Hence, how restoration protocols and materials influence essential coral-associated bacterial communities, and ultimately restoration success has remained unresolved. Therefore, the goal of this thesis was to describe the dynamics of coral-associated bacterial communities induced by propagation and out-planting across diverse reef sites, using various materials, to inform and optimise restoration approaches.

I first examined the impacts of propagation and out-planting on coral-associated bacterial communities for two commonly propagated coral species. I identified that coral-associated bacterial communities undergo significant restructuring during propagation and initial out-planting, which varies depending on the coral species. These results highlight the importance of adjusting protocols to account for species-specific bacterial community dynamics, e.g., avoiding propagating coral species with variable bacterial communities in monostands where disease risk is higher.

I next characterised the long-term dynamics of *Acropora millepora* associated bacterial communities after out-planting at three reef sites with contrasting environmental conditions. Across sites, *A. millepora* exhibited distinct rates of change in associated bacterial communities after out-planting, and different survival trajectories, despite longer term similarities in associated bacterial communities. These findings indicate that factors beyond coral-associated bacterial community changes – such as corallivore presence, epilithic algal matrix or fish communities – may influence out-plant survival and require further attention.

I next evaluated whether materials used to secure corals during propagation differentially impact coral-associated bacterial communities. I found that tie materials do not differentially impact coral-associated bacterial communities for *A. millepora,* and notably biodegradable plastics did not cause proliferation of putatively pathogenic bacteria. One biodegradable material had the same coral retention rate as conventional plastic. Thus, these results highlighted that transitioning to biodegradable alternatives is possible and should be encouraged, but coral retention rate should be quantified prior to widespread application of new materials.

I finally evaluated the role of metal propagation structures used for *in situ* coral propagation and/or rubble stabilisation in shaping the taxonomic composition and functional potential of coral-associated bacterial communities. Differences in individual bacterial taxa and functional potential of coral-associated bacterial communities emerged after six months of growth on different metal propagation structures. However, significant restructuring of coral-associated bacterial communities across different metal propagation structures did not occur. Consequently, these findings have the potential to reassure practitioners that propagation structure material does not significantly impact coral-associated bacterial communities.

Collectively, I have uncovered dynamics of coral-associated bacterial communities induced by reef restoration practices for different coral species, propagation structures, and reef sites. I have provided a framework for incorporating coral microbiome knowledge into day-to-day operations and decision making within coral restoration programs. I offer recommendations to guide future research and further optimise restoration practices. My work highlights the importance of considering and conserving coral-associated bacterial communities during reef restoration to improve management outcomes and to preserve coral reefs into the future.

# Thesis Structure

This thesis is comprised of an introductory chapter (**Chapter 1**), four data chapters (**Chapters 2** to **5**) in the form of journal manuscripts for peer-review and a synthesis chapter (**Chapter 6**). At the time of this thesis submission, two data chapters (**Chapter 2** and **Chapter 3**) were published, one chapter was under review (**Chapter 4**) and the final data chapter was in preparation for submission (**Chapter 5**).

**Chapter 1:**

General introduction of background literature.

**Chapter 2:**

This chapter has been published: **Strudwick, P.**, Seymour, J., Camp, E. F., Edmondson, J., Haydon, T., Howlett, L., Le Reun, N., Siboni, N., & Suggett, D. J. (2022). Impacts of nursery-based propagation and out-planting on coral-associated bacterial communities. *Coral Reefs,* 41, 95-112. https://doi.org/10.1007/s00338-021-02207-6

**Chapter 3:**

This chapter has been published: **Strudwick, P.,** Seymour, J., Camp, E. F., Roper, C., Edmondson, J., Howlett, L., & Suggett, D.J. (2023). Bacterial communities associated with corals out-planted on the Great Barrier Reef are inherently dynamic over space and time. *170*(7), 1-17. *Marine Biology*, https://doi.org/10.1007/s00227-023-04235-y

**Chapter 4:**

This chapter is in review: **Strudwick, P.**, Camp, E. F., Seymour, J., Roper, C., Edmondson, J., Howlett, L., & Suggett, D. J. Assessing efficacy of plastic-free alternative ties for coral propagation in reef restoration. (2023). *Marine Biotechnology*, https://doi.org/10.21203/rs.3.rs-2729419/v1.

**Chapter 5:**

This chapter is presented as a full article prepared for journal submission.

**Strudwick, P.**, Suggett, D. J. , Seymour, J, DeMeare, M. Z. , Grima, A., Edmondson, J., McCardle, A., Nicholson, F., & Camp, E. F. Assessing how metal reef restoration structures shape the functional and taxonomic profile of coral-associated bacterial communities.

**Chapter 6:** General discussion, synthesis of results from all data chapters and recommendations for future research.

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# Chapter 1: General introduction and thesis roadmap

## 1.1 Coral reefs of the Anthropocene

Tropical coral reefs can be considered metaphorical oases in blue deserts. Globally, coral reefs host over a quarter of marine biodiversity (Plaisance et al. 2011) and hence present an ecological paradox given their relatively small oceanic footprint and primary existence in nutrient devoid waters (Rädecker et al. 2017). Hundreds of millions of people across tropical and sub-tropical coastal communities are supported by coral reefs (Kleypas et al. 2021; Wong et al. 2022), particularly in small-island developing states that rely on coral reef fisheries for food security (Rogers et al. 2018; Eddy et al. 2021). Further, coral reefs carry significant indigenous heritage values, provide vital ecosystem services such as protection against damaging wave action, and provide high socio-ecological and economic value (Hughes et al. 2017). Whilst the health of coral reef ecosystems inherently fluctuates on millennial timescales, it is being diminished within decades under compounding local and global stressors of the Anthropocene (Eddy et al. 2021).

The significant overall value of coral reefs is under increasing threat from the stressors they face. Globally more than 50% of living coral cover has been lost since the 1950s (Eddy et al. 2021). More than half of all remaining coral reefs are critically threatened by a collective of environmental stressors (Suggett and Smith, 2020), but primarily by increasingly frequent and severe sea surface temperature thermal anomalies induced by climate change (Hoegh-Guldberg et al. 2018). It has been predicted that by 2030 over 95% of reefs will be exposed to environmental conditions beyond thresholds needed to maintain ecosystem functions (Hoegh-Guldberg et al. 2018; Voolstra et al. 2021). Major concerns centre around thermal stress that causes the breakdown of coral-algae symbioses and eventual loss of algal endosymbionts, which in

the absence of alternative energy sources, leads to 'coral bleaching' induced mortality (Suggett and Smith, 2020). Widespread coral bleaching events date back to 1982 (Coffroth et al. 1990); however, the most significant events have been observed in the last decade and are largely attributed to globally unprecedented high temperatures during 2014-2017, which caused the most severe, long-lasting, and wide-spread coral bleaching event on record (Eakin et al. 2019).

Mass coral-mortality from bleaching events initiates ecological cascades that ripple far beyond the corals immediately affected (e.g., shifts in the functional diversity of reef-associated fish communities, Graham et al. 2015; Elma et al. 2023), and ultimately compromises the ecological functioning of entire reef ecosystems and their capacity to provide associated values and services (Hughes et al. 2019; Eddy et al. 2021). Whilst coral reefs can naturally recover between major stress events, this can take up to 10-15 years (Romero-Torres et al. 2020), and global climate change has resulted in increased frequency of stress events (Smale et al. 2019), such that the periods of relief – required for recovery – are becoming shorter. Unfortunately, even under best-case-scenarios, climate change is forecast to worsen before improving (Anthony et al. 2017; Ruela et al. 2020). The impacts of such global stressors on reefs are often exacerbated by locally concentrated anthropogenic stressors such as boat groundings, over-fishing, pollution, and biotic stressors like crown-of-thorns starfish out-breaks (Donovan et al. 2021), which severely minimises windows of opportunity for natural recovery. Currently, it is predicted that, without amelioration of global and local stressors and reef restoration, 90% of coral cover could be lost by 2050 (Hughes et al. 2018; Kleypas et al. 2021). As such, the persistence of coral reefs during – and let alone beyond – the Anthropocene remains uncertain (Eddy et al. 2021; Sully et al. 2022).

## 1.2 Reef management

Traditionally 'proactive' management approaches such as Marine Protected Areas (MPAs), have been applied to reduce local stressors and prevent further localised degradation of coral reefs (Rinkevich, 2008; Strain et al. 2019). However, there is growing evidence that natural recovery facilitated by MPAs (or similar proactive approaches aimed at mitigating local or global stressors) is not rapid enough to match current trajectories of degradation (Anthony et al. 2017). Consequently, proactive approaches no longer offer adequate levels of protection for coral reefs to withstand current or future climate stress (van Oppen et al. 2017; Bruno et al. 2019; Rinkevich, 2019). Reef management bodies are now actively expanding their toolbox beyond MPAs and mitigation alone as the sole methods to safeguard coral reef health (Rinkevich, 2019; GBRMPA, 2019). For example, in 2019, the Great Barrier Reef Marine Park Authority (GBRMPA) the governing body for the world's largest coral reef, highlighted the urgent need for action addressing climate change *alongside* immediate implementation or expansion of local 'active' management (GBRMPA, 2019; Fig. 1.1a-b.).

Active management approaches, also referred to as 'reactive' approaches (Hein et al. 2021a), involve direct human interventions to expedite natural recovery and repair ecosystem functions (van Oppen et al. 2017; Rinkevich, 2019). It has been suggested that the window of opportunity for reactive approaches to have meaningful impacts will close by 2050 without action on climate emissions (Knowlton et al. 2021). This sentiment was echoed by the United Nations (UN) – with increased urgency – via the announcement of 2021-2030 as the 'UN Decade on Ecosystem Restoration', with the commencement of the decade representing humankind's last chance to successfully apply strategies to counter the degradation of natural habitats worldwide (United

Nations General Assembly, 2019). In 2022, a landmark declaration was adopted at COP17 (Kunming-Montreal biodiversity deal) by 200 countries to mobilise $200B in finance (whilst ending $500B in harmful subsidies), proposing that at least 30% of all degraded ecosystems were under effective restoration (summarised in Suggett et al. 2023). Consequently, reactive restoration approaches that are built on 'nature-based solutions' are being increasingly implemented on coral reefs globally to assist natural recovery, to boost or maintain genetic and biological diversity and to preserve resilience of coral reef ecosystems (Saunders et al. 2020). While application of such approaches is fast progressing (Boström-Einarsson et al. 2020; Hein et al. 2021a,2021b, Suggett et al. 2023), reported success is variable and optimisation is urgently required to deliver restoration at any meaningful scale (Boström-Einarsson et al. 2020; McAfee et al. 2021; Suggett and van Oppen, 2022).

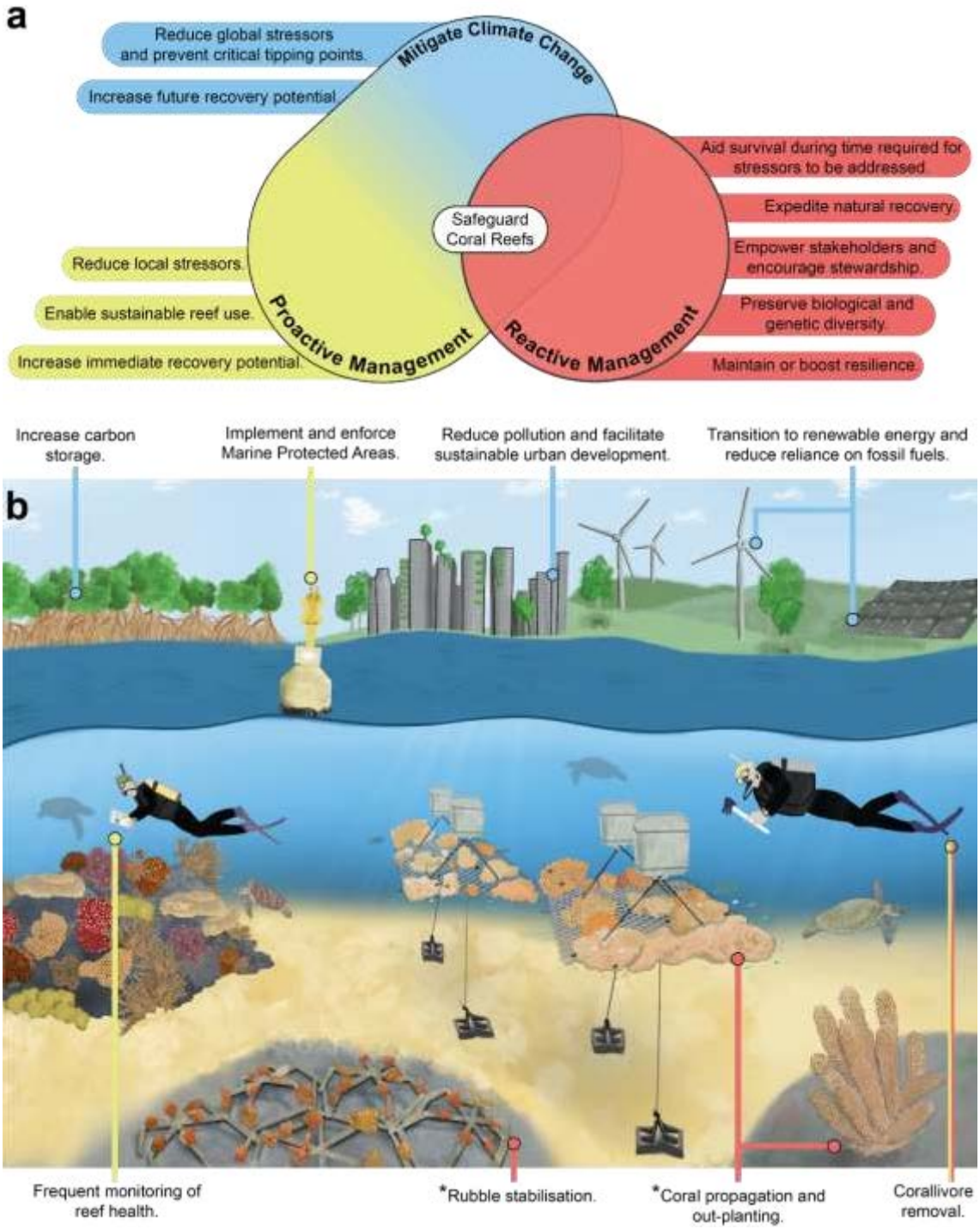


**Figure 1.1.** (**a**) The management framework required to safeguard the future of coral reef ecosystems includes reducing local threats and global climate threats, through proactive management approaches, and expediting natural recovery via reactive management (for example, reef restoration) (Knowlton et al. 2021). (**b**) A schematic of applications of this management framework and specifically *reef restoration utilising asexual reproduction of corals – during propagation with *in situ* nursery structures and on rubble stabilisation structures – investigated in this thesis.

### *1.2.1. Reactive reef management*

Reactive management-based 'restoration' approaches are being applied to tropical coral reefs in more than 55 countries through investments totalling US$1.9 billion dollars (Hein et al. 2021b). Restoration approaches are diverse and include propagation and out-planting of coral (with intermediate *in situ* nursery stages), larval enhancement and reseeding, artificial reefs, substrate enhancement, and substrate stabilisation (Boström-Einarsson et al. 2018). Although the readiness of the different approaches varies (Suggett and van Oppen, 2022), in-water coral propagation and out-planting have emerged as some of the most encouraging methods for targeted rehabilitation at a local level (Hein et al. 2020; Suggett et al. 2019; Williams et al. 2019; Fig. 1.2.), with increasing evidence for successful coral reef rehabilitation at scale (e.g., van Woesik et al. 2021, Peterson et al. 2023). On Australia's Great Barrier Reef (GBR), the first *in situ* multi-species coral propagation nurseries to grow coral material for out-planting were installed in 2018 at three sites (at one reef) on the northern GBR by the Coral Nurture Program (CNP) (Howlett et al. 2021; Suggett et al. 2019, 2020). As of 2023, there is now a large restoration footprint within the GBR Marine Park, with 124 established CNP coral nurseries and more than 100,000 corals out-planted across 30 sites (at nine reefs) through partnerships with nine tourism operators (Howlett et al. 2022; www.coralnurtureprogram.org), and a further ten propagation and out-planting projects established by other reef stakeholders (McLeod et al. 2022).

Coral propagation and out-planting can rapidly boost coral biomass and cover to help counteract the impacts of climate change and compounding anthropogenic stressors, and/or to retain disproportionately high socio-ecological and economic value at targeted sites (Howlett et al. 2021; Suggett et al. 2019, 2020; Roper et al. 2022). A standard CNP propagation and out-planting protocol used on the GBR involves sourcing coral material primarily consisting of loose, naturally fragmented Corals of

Opportunity (CoO) or harvesting fragments from 'donor' colonies and then either immediate out-planting or securing fragments to a nursery structure for a temporary growth phase prior to out-planting (Howlett et al. 2022, Fig. 1.2.). During out-planting fragments are secured to bare consolidated substrate with Coralclip®, which is a stainless-steel device that provides downward pressure (Suggett et al. 2020, Fig. 1.2.). Most coral propagation and out-planting research to date has focused on optimising processes by improving yields, where generating biomass is often the primary goal (Abelson et al. 2016; Boström-Einarsson et al. 2020; Edwards and Gomez, 2007; Montoya-Maya et al. 2016). However, the relationship between generation of biomass and preservation of ecosystem functioning or health is not linear (Suggett and van Oppen, 2022), whereby quality can be compromised by prioritising quantity; for example, by higher risks of disease in large-scale coral monostands (Moriarty et al. 2020).

Recently focus has shifted toward improving both coral restoration yield quality *and* quantity, with research investigating how practitioners can sustain inherent resilience of propagated coral and in turn reef health (e.g., genetic variance, Parkinson et al. 2020; photobiological phenotypes, Suggett et al. 2022; ecosystem service attributes, Nuñez Lendo et al. 2023, in press; coral metabolism, Gantt et al. 2023). Despite efforts to improve standard operating protocols, long-term success rates of propagated corals range from < 5% to > 90% (Boström-Einarsson et al. 2020; Suggett et al. 2019, 2020; Ware et al. 2020) and the effects of propagation and out-planting on the underlying biology of propagated coral or donor colonies and subsequent links to long-term propagated coral success under changing environmental conditions remain largely unknown (Boström-Einarsson et al. 2020). For example, communities of microorganisms closely associated with reef-building corals provide essential functions to support health and/or prevent mortality of the coral host (Montoya-Maya et al. 2016;

Putnam et al. 2017; Reshef et al. 2006; Rosenberg et al. 2007), yet their essential roles in the success of coral restoration efforts remain almost entirely unexplored.

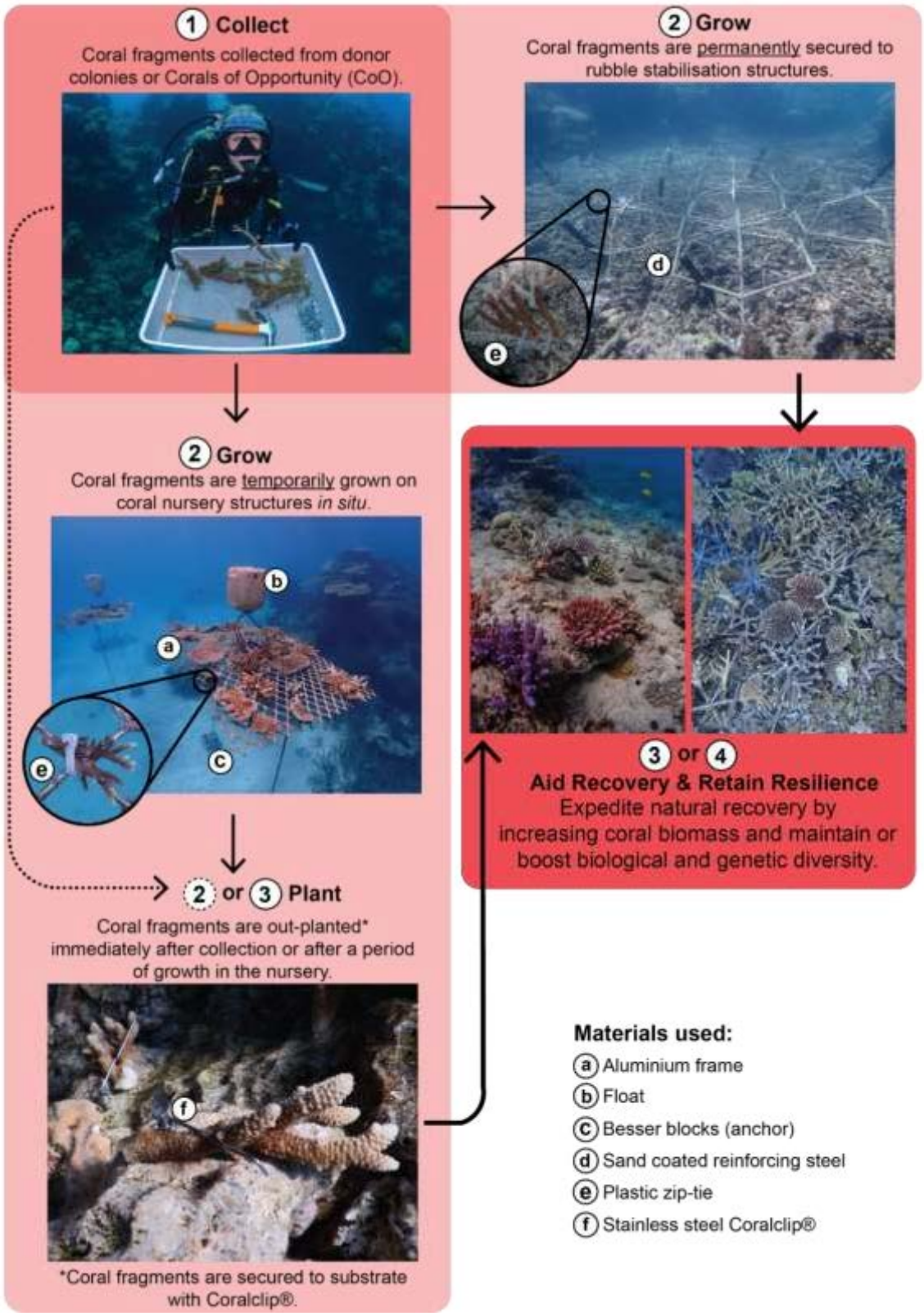


**Figure 1.2.** Two models of reef restoration (reactive management) routinely used on the Great Barrier Reef involve (i) propagation (with a temporary nursery growth phase) and/or out-planting and (ii) rubble stabilisation. The following model for propagation

and/or out-planting was established by the Coral Nurture Program. Firstly, practitioners collect coral material either directly from donor colonies or Corals of Opportunity (CoO), secondly coral fragments are either directly out-planted (secured to consolidated substrate) with Coralclip® or are temporarily attached to nursery structures for growth prior to eventual harvesting of nursery grown fragments for subsequent out-planting. Over time the out-planted coral will attach to the substrate and grow over the Coralclip®. For rubble stabilisation approaches coral fragments are collected in the same way as for (i) and are then permanently secured to pre-installed rubble stabilisation structures, Mars Assisted Reef Restoration System (MARRS) 'Reef Stars' shown here. Photographs: coral fragments (e), out-planted coral (2/3) and rubble stabilisation structures (3/4, right), credit: Paige Strudwick; coral collection, credit: Passions of Paradise; nursery frame (2) and grown out plants (3/4, left), credit: John Edmondson.

## 1.3.0 Microbial reefs

Microorganisms underpin the health of ocean ecosystems and – through nutrient cycling, primary productivity, organic matter degradation and host-microorganism relationships – are fundamental to sustaining life above and below water (Arrigo, 2004; Falkowski et al. 2008; Sharp and Ritchie, 2012; Egan and Gardiner, 2016). All animals have complex associations with microorganisms (Apprill, 2017), and in the last 20 years the mapping of host-microorganism or 'microbiome' relationships has accelerated due to increased affordability and accessibility of DNA sequencing tools (McFall-Ngai et al. 2013; Abdul-Aziz et al. 2016). Through complex associations with microorganisms within the coral microbiome (Bourne et al. 2016), corals can survive in nutrient devoid waters of tropical coral reefs (Bourne et al. 2016; Reich et al. 2022). The symbiotic consortium of microorganisms associated with corals include photosynthetic microalgae, filamentous algae, fungi, archaea, viruses, and bacteria; in totality referred to as the 'holobiont' (Rohwer et al. 2002). While the relationship between coral host and symbiotic microalgae is instrumental to holobiont survival and has been researched with increasing depth, recognition of the importance of coral-associated *bacterial* communities and related research is in its relative infancy, but is growing rapidly (van Oppen and Blackall, 2019). From early (gametes, embryos, and larvae) to later adult life stages, corals acquire diverse bacterial communities through vertical and horizontal

transmission (Ceh et al. 2013; Leite et al. 2017; Peixoto et al. 2017; van Oppen and Blackall, 2019). Distinct coral-associated bacterial communities reside in microhabitats across coral colonies within the skeleton, gastrovascular cavity, tissue, and surface mucus layers (Fig. 1.3.) and provide functions including nutrient cycling (Zilber-Rosenberg and Rosenberg, 2008), delivery of essential trace metals, vitamins and cofactors and resistance to pathogens (Bourne et al. 2016; Raina et al. 2009, 2016).

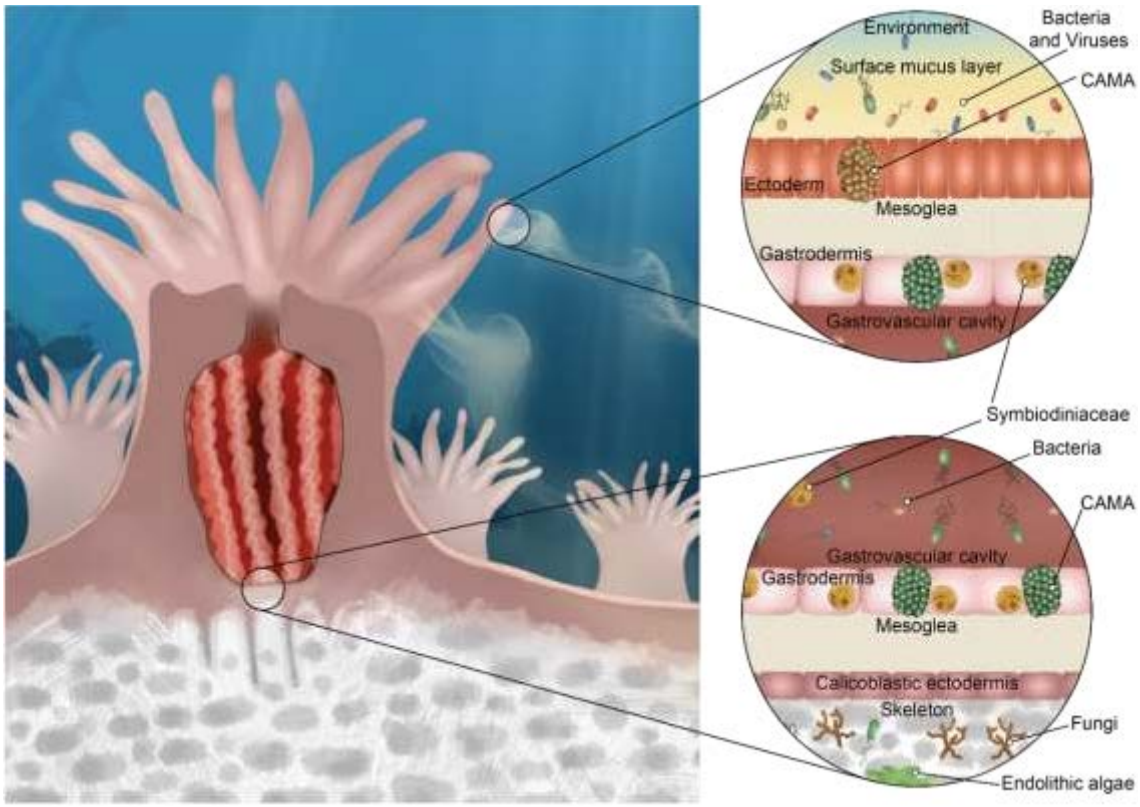


**Figure 1.3.** Microorganism communities associated with Scleractinian corals across the skeleton, gastrovascular cavity, tissue, and surface mucus layer. CAMA = Cell-Associated Microbial Aggregates (Maire et al. 2022) within the coral tissue.

### *1.3.1 Coral-associated bacterial communities in reef restoration*

Harnessing human- and plant-microbiome relationships has transformed outcomes of modern medicine and agricultural productivity (Foo et al. 2017) and 'microbiome stewardship' has been recommended to reduce global biodiversity loss (Peixoto et al. 2022). Conception of the 'Coral Probiotic Hypothesis' in 2006 postulated the potential of exploiting coral bacterial associations to fast-track holobiont adaptation

to changing environmental conditions through both the enormous genetic potential and fast generational turnover of bacteria relative to the coral host (Reshef et al. 2006). Subsequent Coral Probiotic Hypothesis investigations have catalysed development of an entire suite of approaches under 'assisted coral evolution' (van Oppen et al. 2015, 2017; Suggett and van Oppen 2022), via microbiome manipulation to protect or maintain health and resilience of corals under future environmental conditions and/or to improve success of reef restoration efforts (Peixoto et al. 2017; Rosado et al. 2018; Tang et al. 2019).

While the parallel application of microbiome manipulation or stewardship with *in situ* propagation and out-planting holds promise (Putnam and Gates, 2015; Peixoto et al. 2017; Rosado et al. 2018; Voolstra et al. 2021; Suggett and van Oppen, 2022), numerous hurdles remain in place that govern successful application (Peixoto et al. 2021; Voolstra et al. 2021). Primarily, application of such approaches *in situ* to provide broad stress tolerance to the holobiont is yet to be achieved (Peixoto et al. 2021; Voolstra et al. 2021) and the drivers of varied survival of propagated coral are unknown (Boström-Einarsson et al. 2020; Suggett et al. 2019, 2020; Ware et al. 2020). As such, 'conserving the holobiont' by investigating microbial links to varied survival and tailoring processes to consider natural microbiome dynamics is the logical first step to harnessing the microbiome in a restoration context (Carthey et al, 2019). However, the complexities of natural spatiotemporal dynamics of coral-associated bacterial communities and most pertinently how these are impacted by reef restoration methods and materials remain unresolved. For example, various components of propagation and out-planting protocols could impact coral-associated bacterial communities and subsequently influence coral survival; such as transfer of corals between locations with distinct environmental conditions (Casey et al. 2015; Egan and Gardiner, 2016; Ziegler et al. 2019; Haydon et al. 2021; Peixoto et al. 2022) and the introduction of artificial

materials e.g., plastic and steel, that harbour distinct bacterial communities in marine environments (Zettler et al. 2013; Procópio, 2019). Hence, bridging the primary knowledge gap of how coral-associated bacterial communities are impacted by reef restoration practices and the artificial materials used, presents immediate opportunities to optimise propagation and out-planting protocols in real-time. Consequently, to support conservation of the holobiont and assist preservation of coral reef ecosystem resilience through reactive management, this thesis focuses on developing the understanding of bacterial community dynamics throughout the processes of *in situ* propagation and out-planting on the Great Barrier Reef and aims to inform optimisation of approaches via systematic comparison of materials used in the process.

## 1.4 Thesis aims and hypotheses

Overall, increasing application of reactive management approaches has been recognised as one of the key pillars to ensuring the persistence of coral reef ecosystems under predicted changing ocean conditions (Knowlton et al. 2021). Further, in 2021 the announcement of the UN Decade on Ecosystem Restoration and Kunming-Montreal agreement – establishing 2030 as our last chance to halt damage to and preserve irreplaceable coral reef ecosystems – has fuelled the expansion of human interventions in the form of propagation and out-planting approaches globally (United Nations General Assembly (UN), 2019; Boström-Einarsson et al. 2020; Hein et al. 2021). Unfortunately, such interventions still have varied success (Boström-Einarsson et al. 2020). Fundamentally, identifying how propagation and out-planting techniques, and the materials used (Fig. 1.2.), impact coral-associated bacterial communities will facilitate real-time optimisation of protocols alongside their increasing application globally. Therefore, to generate essential baseline knowledge required for optimisation of scaling interventions, the overall aim of this thesis was to identify the impacts

propagation and out-planting have on the coral-associated bacterial communities and how this varies between (i) different coral species, (ii) reef sites, and (iii-iv) materials used during the process. To address these aims, I conducted *in situ* time-series propagation and/or out-planting experiments and applied amplicon (16S rRNA gene) and shotgun metagenomic sequencing to identify taxonomic and functional changes in coral-associated bacterial communities.

Four data chapters deliver and test the following aims and hypotheses, all of which were conducted within on-going restoration activities of the Coral Nurture Program (northern Great Barrier Reef) (Howlett et al. 2022; Suggett et al. 2023, Fig. 1.2.).

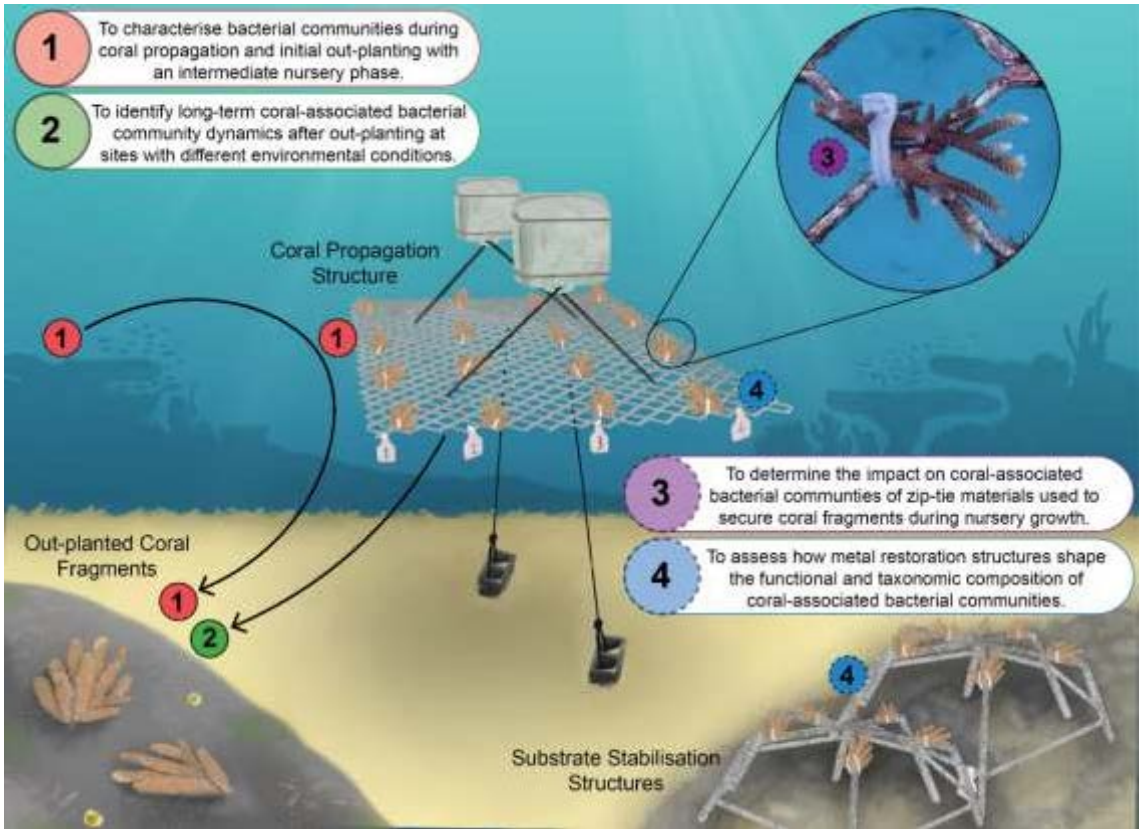


**Figure 1.4.** The four aims addressed in this thesis address either a restoration process or a component of that process (indicated by a solid or dashed circle respectively) and include (**1**) to characterise the impacts of propagation and initial out-planting on coral-associated bacterial communities, (**2**) to identify long-term bacterial community dynamics of out-planted coral fragments, (**3**) to determine the impact on coral-associated bacterial communities of different zip-tie materials and inform the viability of plastic-alternative materials to secure coral fragments during propagation, and (**4**) to

assess how metal restoration structures influence functional potential and taxonomic composition of coral-associated bacterial communities during propagation.

**Chapter 2 (Aim 1):** ***To examine temporal dynamics of coral-associated bacterial communities for two coral species routinely propagated on the Great Barrier Reef during restoration.***

Coral-associated bacterial communities are known to be vital for overall coral host health and potentially influenced by novel environmental conditions, yet the impact of nursery-based propagation and out-planting on the associated bacterial communities remained unknown. Here I tracked two coral species *Acropora millepora* and *Pocillopora verrucosa* from harvesting of initial source colonies to nursery propagation and out-planting and for the first time characterised the coral-associated bacterial communities throughout this process (**Chapter 2**). Within this chapter, the following hypothesis was tested:

(i) Coral bacterial communities will be impacted by propagation in nurseries and after out-planting.

This chapter has been published: **Strudwick, P.**, Seymour, J., Camp, E. F., Edmondson, J., Haydon, T., Howlett, L., Le Reun, N., Siboni, N., Suggett, D. J. (2022). Impacts of nursery-based propagation and out-planting on coral-associated bacterial communities. *Coral Reefs,* 41, 95-112. https://doi.org/10.1007/s00338-021-02207-6

**Chapter 3 (Aim 2):** ***To characterise the long-term coral-associated bacterial communities of Acropora millepora during out-planting at sites with contrasting environmental conditions.***

Environment plays an integral role in shaping coral-associated bacterial communities and inherently varies between reef sites. Success of out-planted corals can

also vary between reef sites. Therefore, to investigate potential reef site-specific impacts to coral-associated bacterial communities of out-planted corals, and to test if coral-associated bacterial communities are linked to differences in survivorship, I conducted an *in situ* out-planting experiment across three sites (Opal Reef, Northern Great Barrier Reef) with contrasting environmental characteristics. I measured out-plant survivorship and coral-associated bacterial communities over 12 months (**Chapter 3**). Within this chapter, the following hypotheses were tested:

(i) Bacterial communities of coral fragments will change after out-planting.

(ii) Changes in bacterial communities will be representative of different reef sites.

(iii) Shifts in out-plant bacterial communities will reflect differences in survivorship.

This chapter has been published: **Strudwick, P.,** Seymour, J., Camp, E. F., Roper, C., Edmondson, J., Howlett, L., & Suggett, D.J. (2023). Bacterial communities associated with corals out-planted on the Great Barrier Reef are inherently dynamic over space and time. *Marine Biology*, *170*(7), 1-17. https://doi.org/10.1007/s00227-023-04235-y

**Chapter 4 (Aim 3):** ***To determine the viability of plastic-alternatives in coral propagation.***

A major concern of coral restoration practices is the persistent reliance on plastic zip-ties to secure coral fragments and how this contributes to the problem of marine plastic pollution. Recently, carbohydrate-based biodegradable materials that breakdown via exposure to moisture, UV radiation and microbial metabolism have become commercially available. With the potential for coral-associated bacterial communities to be impacted by the nursery propagation process (Chapter 2) it is important to assess the

suitability of plastic alternatives by investigating whether their use further impacts the coral-associated bacterial communities (**Chapter 4**). Within this chapter, the following hypotheses were tested:

(i) Materials used to secure coral fragments during coral propagation will have different impacts on the coral-associated bacterial communities.

(ii) The associated bacterial communities of coral fragments secured with biodegradable materials will have increased relative abundance of putative coral pathogens from the *Vibrio* genus.

This chapter is in review: **Strudwick P.** [1], Camp, E. F. [1], Seymour, J. [1], Roper, C. [1], Edmondson, J. [2], Howlett, L. [1], & Suggett, D. J[1]. Impacts of Plastic-free Materials on Coral-associated Bacterial Communities During Reef Restoration, (2023).

**Chapter 5 (Aim 4):** ***To identify how metals commonly used in reef restoration for coral propagation structures potentially regulate coral health.***

Globally reef restoration efforts report variable survivorship and, as with plastic-alternatives to zip-ties, practitioners are increasingly using novel propagation structures. Both coral nurseries and rubble stabilisation structures employ metal components such as steel that could alter the propagated coral environment. Steel can corrode and release iron (Fe), which is an element essential for many physiological processes of members of the coral holobiont and, importantly, is essential for virulence in putative coral pathogens (Zughaier and Cornelius, 2018). Prior to scaled deployment of these structures, it is essential to resolve the impacts of metal structures on coral fitness during the propagation process. Consequently, we harvested fragments from known source colonies and propagated fragments on three different metal structures for six

months and subsequently characterised the taxonomic and functional profiles of associated bacterial communities through a combined amplicon (16S rRNA gene) and shotgun metagenomic approach.

Within this chapter, the following hypotheses were tested:

(i) Coral propagated on steel structures will host distinct bacterial communities.

(ii) Coral propagated on steel structures will have associated bacterial communities with different functional potential compared to those propagated on aluminium structures.

(iii) Coral propagated on steel structures will have associated bacterial communities with overrepresentation of genes related to Fe cycling or with Fe requirements (e.g., as co-factors).

This chapter is presented as a full article prepared for journal submission.

**Strudwick, P.**, Suggett, D. J. , Seymour, J, DeMeare, M. Z. , Grima, A., Edmondson, J., McCardle, A., Nicholson, F., & Camp, E. F. Assessing how metal reef restoration structures shape the functional and taxonomic profile of coral-associated bacterial communities.

Finally, the knowledge gathered through delivering these four aims is considered in **Chapter 6,** where I synthesise my findings and identify future directions for the application of this research in reef restoration via propagation and out-planting.

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# Chapter 2: Impacts of nursery-based propagation and out-planting on coral-associated bacterial communities.

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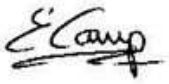
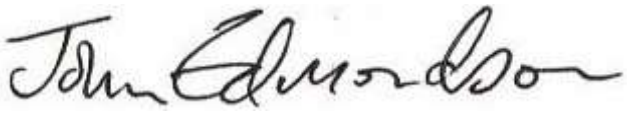
Emma Camp: 

John Edmondson: 

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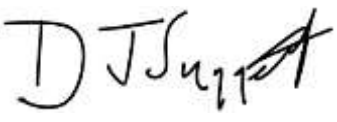
David Suggett: 

## 2.1 Abstract

Efforts to manage coral reef declines are increasingly turning towards *in situ* propagation of corals to aid reef recovery. Understanding the factors that influence 'success' throughout the propagation process is therefore critical to ensure efforts are viable and cost-effective, yet the extent to which propagation practices potentially impact the underlying coral biology remains unknown. Given growing evidence for the importance of the coral microbiome, we examined the influence of nursery-based propagation and out-planting on the bacterial communities of two coral species – *Acropora millepora* and *Pocillopora verrucosa* – increasingly propagated on the northern Great Barrier Reef (Opal Reef). Bacterial communities of coral fragments were characterised over four months of nursery propagation (sampling points: zero, seven and 125 days) and one month of subsequent out-planting (sampling points: zero, one and 30 days). Bacterial community structure differed between *A. millepora* and *P. verrucosa* throughout the experiment and species-specific temporal dynamics were observed during the transplantation of corals into nurseries and subsequent out-planting back to the reef. *P. verrucosa* bacterial community structure remained stable over time in the natural reef environment and the nursery. In contrast, *A. millepora* bacterial communities within the nursery significantly changed over time, whereas those associated with source colonies within the natural reef environment remained unchanged. However, after one month of out-planting, the composition, richness and diversity of *A. millepora* bacterial communities was not statistically different to those associated with the source colonies. We interpret the transient shift of *A. millepora* bacterial communities within the nursery as an impact of distinctive environmental conditions in nurseries compared to natural reef settings, and the greater responsiveness of *A. millepora* bacterial communities to environmental change. Our observations highlight that different coral species exhibit distinct microbial responses to coral

propagation and out-planting, and we recommend that these should be considered when designing and scaling future coral management strategies.

## 2.2 Introduction

Coral reefs worldwide are experiencing detrimental impacts from cumulative anthropogenic pressures (Hughes, 2017, 2019; Eakin et al. 2019), with climate change – through persistent ocean warming, acidification and deoxygenation – remaining the primary threat to viability of future tropical reefs. Ocean warming is driving more intense and frequent marine heat waves (Smale et al. 2019), which result in geographically widespread coral bleaching and mortality (Baker et al. 2008; França et al. 2020). For example, mass bleaching events on the Great Barrier Reef (GBR) in 2016, 2017 (Hughes et al. 2018) and again in 2020 have contributed to a loss of > 50% of all coral since the mid-1990s (Dietzel et al. 2020). Such ongoing degradation of the World Heritage listed GBR is often exacerbated by anthropogenic influences such as overfishing and pollution (Hughes et al. 2017), demonstrating the limited effectiveness of conventional marine protection and management activities alone (Selig and Bruno, 2010; Anthony et al. 2017) and the need for implementation, or expansion, of local *active* management approaches that directly target preservation of coral reef biodiversity (Anthony et al. 2017; Rinkevich, 2019).

Local active management approaches recently tested on the GBR are tailored to specific site conditions, with methods broadly falling within three categories; sexual propagation, asexual propagation, or substratum enhancement (Boström-Einarsson et al. 2020). Asexual coral propagation, via *in situ* coral nurseries and the subsequent out-planting of coral propagules, is a low-technology and low-cost active management approach (Rinkevich, 2008). *In situ* coral propagation involves growing fragments (either from ‘donor’ colonies or ‘fragments of opportunity’) to larger sizes (above a

threshold with higher post-transplantation survival; Putchim et al. 2008; Boström-Einarsson et al. 2020) on *in situ* nursery frames for later out-planting, to boost (or retain) coral cover at nearby degraded or ecologically, economically or culturally 'high-value' reef sites (Rinkevich, 2008; Suggett et al. 2019). Coral propagation and out-planting facilitate and expedite natural reef recovery (Boström-Einarsson et al. 2020) by enhancing coral growth within the nursery phase (Lirman et al. 2010; Barton et al. 2017), thereby increasing coral biomass at a faster rate than through natural recovery alone at degraded sites (Boström-Einarsson et al. 2020). Propagation efforts generally employ *in situ* coral nurseries to generate sufficient coral material for future out-planting (Boström-Einarsson et al. 2020), notably where target source material for out-planting is rare or limited (Suggett et al. 2019). However, the long-term success of coral fragments that have been propagated in coral nurseries and out-planted, including novel nursery (Suggett et al. 2019, Howlett et al. 2021) and mass out-planting efforts (Suggett et al. 2020) recently established on the GBR, is highly variable (Boström-Einarsson et al. 2020; Suggett et al. 2020). Prior assessments of coral propagation 'success' have primarily focused on coral fragment growth and survival (Forrester et al. 2012; Hein et al. 2017; Suggett et al. 2019, Howlett et al. 2021), yet other important aspects of coral biology, such as the coral microbiome, have typically been overlooked (Moriarty et al. 2020), which we suggest severely limits the scope of optimising coral propagation procedures.

Coral-associated microorganisms (including viruses, fungi, archaea, microalgae and bacteria) comprise the coral 'microbiome', which has repeatedly been shown to be fundamentally important in shaping coral growth and survival (Reshef et al. 2006; Rosenberg et al. 2007; Krediet et al. 2013; Bourne et al. 2016; Peixoto et al. 2017; Putnam et al. 2017). As such, we propose that coral propagation practitioners should operate with a sound understanding of how their procedures promote microbial

communities that support 'healthy' coral functioning (including exclusion of possible pathogens that could arise from intensive propagation) (Moriarty et al. 2020). Certain practices of the *in situ* coral propagation process, such as transplantation (Casey et al. 2015) and handling (Lirman et al. 2010) of fragments, as well as exposure to altered growth environments (Bourne et al. 2016) are known to detrimentally impact coral health (Bourne et al. 2016; Ziegler et al. 2019; Moriarty et al. 2020). Yet, despite rapid scaling of coral propagation efforts worldwide, it remains unknown to what extent such potential changes in coral fitness can be attributed to a change in coral-associated bacterial communities during propagation and out-planting. Indeed, it has recently been suggested that coral diseases may be an overlooked cause of disproportionately high mortality that can occur via the nursery-based propagation and out-planting process (Moriarty et al. 2020).

Recent evidence has highlighted that coral-associated bacterial communities can vary both naturally with space and time (Ziegler et al. 2019; Osman et al. 2020) or shift in response to environmental fluctuations (Kelly et al. 2014; McDevitt-Irwin et al. 2017; Maher et al. 2019; Camp et al. 2020), and that the extent and nature of these changes can vary according to coral species (Epstein et al. 2019; Ziegler et al. 2019). For example, the bacterial communities of *Acropora* corals are known to be relatively more variable than those of other coral genera, with natural variability sometimes amplified by intra-colony variation (Damjanovic et al. 2020; Marchioro et al. 2020). However, within a coral nursery scenario, intra-colony consistency in the bacterial communities of some species within this genus (e.g., *A. cervicornis*) has been observed (Miller et al. 2020). *Pocillopora* corals have also been recorded to exhibit stable bacterial community structure when cross-transplanted between distinct locations (Ziegler et al. 2019). However, whether such differences between reef colonies, nursery fragments and coral

species are universal across regions and over time in the context of coral propagation and out-planting process has not been explored.

Local biotic and abiotic conditions shape coral-associated bacterial communities (Bourne et al. 2013; Sweet et al. 2017; Williams et al. 2015; Hernandez-Agreda et al. 2017) and therefore would be expected to differ between coral nurseries or out-plant sites and sites where coral material is originally sourced (Boström-Einarsson et al. 2020). As such, we hypothesise that coral bacterial community dynamics will be altered by propagation and out-planting. In order to inform efforts to optimise coral propagation practices on the GBR (Suggett et al. 2019; Howlett et al. 2021) and test this hypothesis, we therefore examined the temporal dynamics of the bacterial component of the coral microbiome in propagation nurseries (as well as subsequent out-planting to the natural reef environment) for two coral species – *Acropora millepora* and *Pocillopora verrucosa* – that are commonly propagated in the Indo-Pacific (Boström-Einarsson et al. 2020) and now on the GBR (Suggett et al. 2019, Howlett et al. 2021), and how these dynamics compared to those of source colonies that remained in the natural reef environment.

## 2.3 Materials and Methods

### *2.3.1 Sampling location and experimental design*

Experiments were conducted at a coral nursery facility located at Opal Reef (16°13'37.5"S 145°53'42.0"E), which is a 24.7 $km^2$ reef situated on the northern GBR (detailed in Suggett et al. 2019, Howlett et al. 2021). The nursery site was established in 2018 and consists of multiple floating frames located at depths of 5-6 m on sand immediately adjacent to the reef. Two specific nursery frames for this study were installed and conditioned *in situ* for a period of at least two weeks prior to the beginning of the experiment. Each frame consists of 2.0 × 1.2 m aluminium diamond-mesh and is

held in place with 2 × 9 kg Besser blocks and a 20 L float approximately 1-2 m above the sand (Fig. S4). In September 2019, coral samples were harvested, propagated and out-planted at site 'Rayban' a shallow and protected area within Opal Reef, (see Suggett et al. 2019, Howlett et al. 2021) by SCUBA.

### *2.3.2 Comparison of nursery fragments and source colony associated bacterial communities*

To examine differences in coral-associated bacterial communities among corals maintained in the nurseries relative to source colonies on the reef, we performed a transplantation experiment using the coral species *A. millepora* and *P. verrucosa.* Initial reef locations of four source colonies (≥ 55 cm diameter) of each species were marked with cattle tags, with each colony representing a biological replicate. Nine fragments (≤ 5 cm) were taken from each source colony using wire clippers and subsequently transported in a sterile zip-lock bag (with seawater) by a diver to the nursery frames located 4-10 m from the source colonies. Eight fragments from each source colony were immediately attached to frames in the nursery with cable ties (the time and distance between fragmentation of source colony and attachment to nursery frame were less than 40 min and 20 m respectively) (Fig. 2.1.). To account for potential frame-effects, the four biological replicates from each species were divided equally across two nursery frames (Fig. 2.1. and Fig. S2.5.).

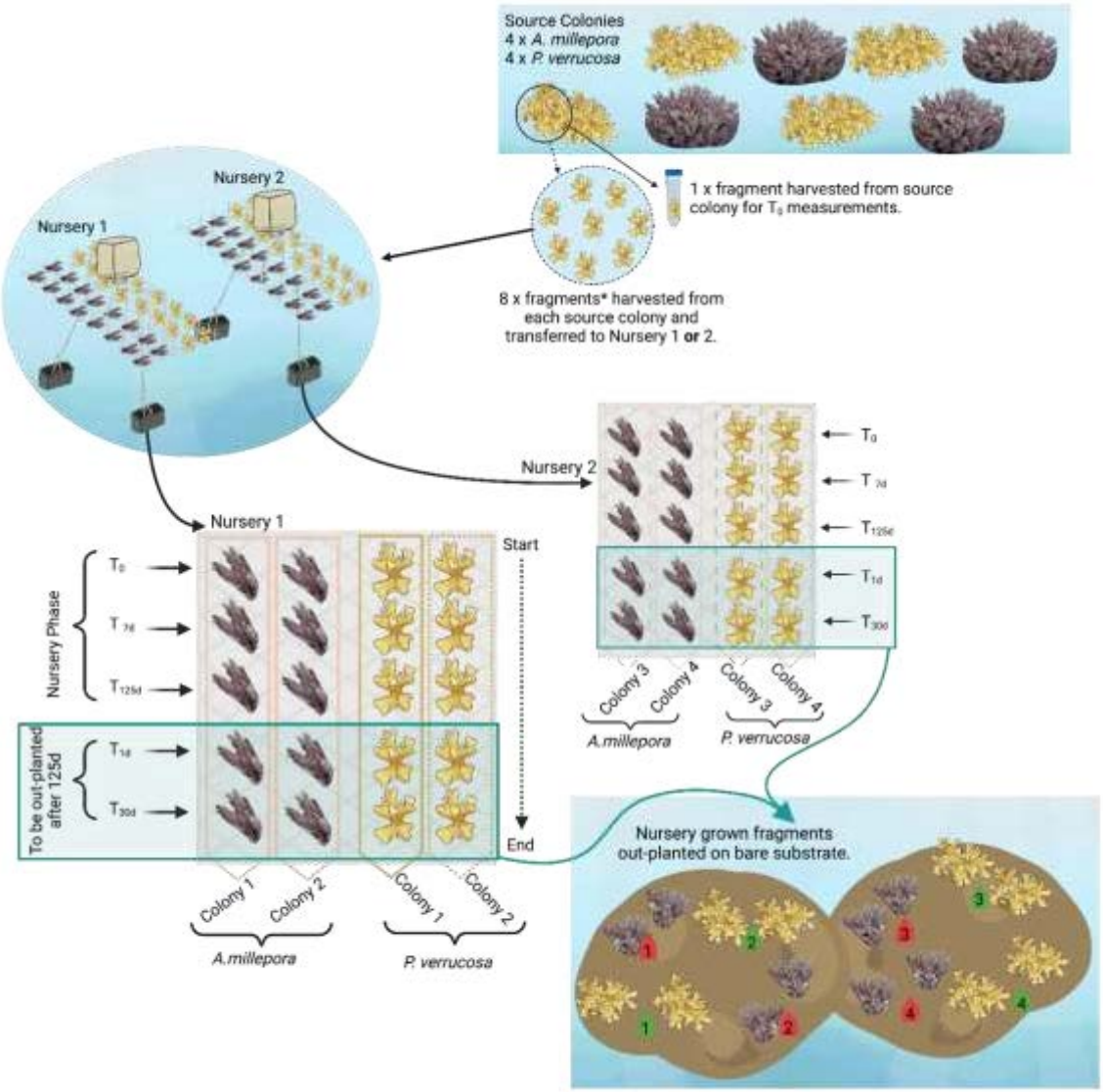



**Figure 2.1.** Experimental design for coral propagation and out-planting. Coral fragments (~5 cm) are harvested from four *Acropora millepora* source colonies and four *Pocillopora verrucosa* source colonies and attached to nursery frame one or two, one fragment per source colony was preserved immediately in RNAlater for source colony $T_0$ measurements. Fragments (n=8) were held in the nursery for 125days (three fragments from each source colony were sampled over this time period: $T_0$, $T_7$ and $T_{125d}$) and two fragments (not previously sampled) per source colony were out-planted to bare substrate on the neighbouring reef and sampled at $T_{1d}$ or $T_{30d}$ after out-planting. *n=8 were transferred to nursery frames to provide contingency for any fragment mortality throughout the experiment. Created with BioRender.com.

At the time of attaching fragments to the nursery frames, one fragment from each source colony was placed in an individual sterile zip-lock bag and immediately taken to the operations vessel (*Wavelength 5*) to be preserved in RNAlater for source reef 'time zero' ($T_0$) measurements (Fig. 2.1., S2.3. and S2.4.). One fragment from each replicate

placed onto the nursery frames was removed within 90 min to provide a separate nursery 'time zero' ($T_0$). For $T_0$ measurements, nursery and source samples were used to profile initial *in situ* bacterial communities using 16S rRNA amplicon sequencing. Following $T_0$, nursery fragments and source colonies were both re-sampled at seven days ($T_{7d}$/Nursery$T_{7d}$) and 125 days ($T_{125d}$/Nursery$T_{125d}$) (Fig. 2.1., S2.3 and S2.4.). At each time point, including $T_0$, coral fragments that had not been sampled at previous time-points were either removed from the nursery frame by detaching the cable tie (or cut using wire clippers in cases where fragments had begun to overgrow onto the nursery frame) or from the source colony in the natural reef environment using wire clippers. Overall, 48 samples were collected for bacterial community analysis: (i) nursery fragments: four replicates (two from each nursery frame) x two species x three time points ($n$ = 24) and (ii) source colonies: four replicates x two species x three time points ($n$ = 24).

### *2.3.3 Comparison of nursery grown, out-plant and natural reef coral bacterial communities*

At the end of the 125 day nursery phase, a second experiment was conducted to characterise the dynamics of bacterial communities associated with corals that were subsequently out-planted from the nursery back to the natural reef environment. For this experiment, samples that had been maintained in the nursery for 125 days ($T_{125d}$) were additionally designated as time zero for out-plants (Nursery$T_{125d}$ = Out-plant$T_0$). The remaining fragments that had been in the nursery (and had not been previously sampled) for 125 days (Nursery$T_{125d}$ = Out-plant$T_0$) were out-planted and then sampled after either one day ($T_{1d}$) or 30 days ($T_{30d}$). In total, 16 (125 day 'old') nursery fragments were out-planted within 30 m of source colonies within 40 min of being removed from the nursery frames.

Out-planting location was selected with four pre-requisites: (i) bare consolidated structurally sound substrate (i.e., not rubble or sand), (ii) absence of algal turfs, (iii) location outside of damselfish territories, (iv) presence of other coral growing within a 1-2 m radius. At chosen locations, bare substrate was brushed with a hard-bristled brush to remove algae and loose sediment. Two buddy pairs operated on SCUBA, allowing one pair to harvest propagules from the nursery and the other pair to prepare the substrate and Coralclips®, a stainless steel spring-clip that is fastened using a hammer and masonry nail (Suggett et al. 2020), for immediate out-planting to minimise propagule handling time. Coral propagules were taken off the nursery either by fragmentation (if they had attached to the nursery frames) or by cutting the cable ties with clean wire cutters, with propagules held in individual sterile zip-lock bags during underwater transportation to the out-planting site. Propagules were immediately attached to the substrate with Coralclip®. Once Coralclip® was hammered into the substrate, the spring-clip was leveraged to firmly hold the fragment in place. Out-planted fragments and source colonies were both re-sampled at one day ($T_{1d}$) and 30 days ($T_{30d}$) after out-planting (Fig. S2.3. and S2.4.). At each time point, coral fragments were either removed from the Coralclip® by leveraging the clip open (or cut using wire clippers where fragments had begun to overgrow the clip or attach to the substrate) or from the source colony as per time zero. Overall, a further 32 samples were collected for bacterial community analysis: (i) out-plants: four replicates x two species x two time points ($n$ = 16) and (ii) source colonies: four replicates x two species x two time points ($n$ = 16).

### *2.3.4 Sample preservation and DNA extraction*

Following sampling, all fragments were returned to the operations vessel (*Wavelength 5*) within 30-50 min, placed into sterile 15-mL Falcon tubes and preserved by total submersion in RNA*later*. All samples were subsequently held at 4°C for four

days during transportation from the study site to the laboratory. Once in the laboratory, RNA*later* was thoroughly removed from 15-mL Falcon tubes using an adjustable pipette with sterile tips, after which samples were preserved at –80°C for three to four weeks until DNA was extracted. Prior to DNA extraction, coral tissue was removed from the coral skeleton, using an air brushing technique. Coral fragments were thawed on ice in their respective 15-mL falcon tubes, removed from the falcon tube using sterile forceps, rinsed with autoclaved phosphate-buffered saline (PBS) (2X, pH 7.4) to remove any remaining RNA*later*, placed in sterile zip-lock bags and air brushed with sterile pipette tips into 4-mL of autoclaved PBS (2X, pH 7.4). The tissue slurry was divided across two 2-mL microcentrifuge tubes and centrifuged at 8000 g for 5 min. The supernatant was removed, and the tissue pellet was stored at –80°C for two weeks until DNA extraction. DNA was extracted from approximately 100 µL of the coral tissue pellet using a DNeasy Blood and Tissue kit (Qiagen) following the Manufacturer's protocols with a total elution volume of 40 µL. Kit negative samples were included in DNA extractions to identify any kit contaminants. Extracted DNA was quality checked and the concentration was quantified using a NanoDrop spectrophotometer.

### *2.3.5 16S rRNA amplicon sequencing*

DNA extracts were stored at –80°C for two weeks until transportation on dry ice to Ramaciotti Centre for Genomics (Sydney, NSW, Australia) for 16S rRNA amplicon sequencing to characterise the composition and diversity of coral-associated bacterial communities. The hypervariable V3 and V4 regions of the bacterial 16S rRNA gene were sequenced using primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth *et al.* 2013) on the Illumina MiSeq v3 2×300 bp platform. Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA733237.

### *2.3.6 Bioinformatics*

Raw demultiplexed sequencing data were analysed with Quantitative Insights into Microbial Ecology (QIIME 2) platform (Callahan et al. 2016). DADA2 plugin (version 2019.1.0) was used to denoise the data (Callahan et al. 2016) and taxonomy was assigned using the classify-sklearn classifier (Pedregosa et al. 2011) against the SILVA v138 database. Amplicon sequence variants (ASVs) contributing a cumulative read frequency (across all samples) of less than 0.03% (131 reads) of the entire data set total read frequency (461,848 reads across 72 samples) and/or corresponding to chloroplast or mitochondria were filtered from the data set. Fifteen ASVs that comprised > 89% of sequences in the DNA extraction negative control, and have been previously reported as common contaminants of laboratory reagents (Weyrich et al. 2019), were removed from all samples for subsequent analyses using the filter command in R. Prior to diversity analyses three *P. verrucosa* and three *A. millepora* samples were removed from the data set due to poor sequencing outputs leading to low read numbers after quality filtering and contaminant removal (< 3000 reads or < 20 ASVs), consequently six time points only had three biological replicates, whereas all other time points had four biological replicates. After initial comparison of the two coral species' alpha and diversity indices (pooled time points and treatments) all subsequent analyses were conducted on the coral species separately. The ASV table was rarefied to an even depth of 5000 reads per sample for alpha diversity analyses, whereas, for beta diversity analyses the raw reads ASV table was converted to relative abundances, scaled to 20,000 (McKnight et al. 2019) and square root transformed.

### *2.3.7 Statistical analysis*

All data analyses were performed in R version 4.0.2. (Wickham and Grolemund, 2016), PAST 4.03 statistical software (Hammer et al. 2001) and QIIME2 (Callahan et al. 2016). For multivariate statistical analysis and visualisation, the 'vegan' and 'ggplot'

R packages were used (Wickham, 2016; Oksanen et al. 2020). Alpha diversity indices (Chao1, Inverse Simpson Evenness, and Shannon Diversity) were calculated on rarefied ASV counts using the 'phyloseq' R package. After rarefying, 16 samples (7 nursery, 8 source, and 1 out-plant) were lost for *A. millepora* and 6 (2 nursery, 2 source, 2 out-plant) samples were lost for *P. verrucosa*; as such, time points were pooled, and treatment (nursery, source, or out-plant) was the fixed effect in the analysis. For the nursery phase of the experiment, differences between treatments (source and nursery) were analysed by applying Kruskal-Wallis tests to assess significance using PAST 4.03, all $p$-values < 0.05 were considered significant. Specifically, groups that were compared for the nursery phase of the experiment were source colonies and nursery fragments (within these groups Nursery$T_0$, Nursery$T_{7d}$ and Nursery$T_{125d}$ were pooled and Source$T_0$, Source$T_{7d}$ and Source$T_{125d}$ were pooled) (Fig. S2.6a.). For the out-planting phase of the experiment differences between treatments (source, out-plants and Nursery$T_{125d}$) were analysed using Kruskal-Wallis tests, if significant a Dunn's post-hoc test was applied with subsequent Bonferroni $p$-value adjustment, all $p_{adj}$ values < 0.05 were considered significant. Groups that were analysed for the out-planting phase of the experiment were out-planted fragments, nursery fragments and source colonies (within these groups Out-plant$T_{1d}$ and Out-plant$T_{30d}$ were pooled, Source$T_{125d}$, Source$T_{1d}$, Source$T_{30d}$ were pooled and Nursery$T_{125d}$ was one group) (Fig. S2.6b.). The models described (Fig. S2.6a-b.) were applied to each coral species separately.

Differences in bacterial community structure (beta diversity patterns) within treatments over time and between treatments at individual time points were analysed using the Bray-Curtis dissimilarity distance metric and these patterns were visualised using non-metric multidimensional scaling (nMDS) plots. Differences in beta diversity were tested for significance with pairwise permutational multivariate analysis of variance (PERMANOVA; perm = 999) of Bray-Curtis dissimilarities using the

*pairwise.adonis* function of the 'vegan' R package, *p*-values were adjusted by applying a Benjamini and Hochberg (a.k.a. False Discovery Rate) correction in adonis, all $p_{adj}$ values < 0.05 were considered significant. To address our overall hypotheses, fixed effects were separated to precisely address a series of systematic objectives in turn; specifically whether bacterial community structure differed for: (1) nursery fragments over time, (2) source colonies over time within the nursery phase, (3) nursery fragments versus source colonies at each time point, (4) source colonies over time within the out-planting phase, (5) out-planted fragments over time, and finally (6) out-planted fragments and source colonies at each time point. To identify changes in bacterial community structure *over time* within each treatment, treatments were separated, and time was a fixed effect (for the nursery phase: Nursery$T_0$, Nursery$T_{7d}$ and Nursery$T_{125d}$ were compared and in a separate analysis for the out-planting phase Out-plant$T_0$, Out-plant$T_{1d}$, Out-plant$T_{30d}$ were compared), *p*-values were adjusted by applying false discovery rate, all $p_{adjust}$ values < 0.05 were considered significant. To identify changes in bacterial community structure over time for source colonies the PERMANOVA was blocked by 'colony' to account for repeated sampling of the same source colonies throughout the experiment (Epstein *et al.* 2019), *p*-values were adjusted by applying false discovery rate correction, all $p_{adj}$ values < 0.05 were considered significant.

To identify differences in bacterial community structure between treatments, time points were separated, and treatment was the fixed effect in the PERMANOVA (Fig. S2.6g. and S2.6h.), all *p*-values < 0.05 were considered significant. When significant differences in the bacterial community structure were identified over time within treatments or between treatments at individual time points, similarity percentage analysis (SIMPER) was used in PAST 4.03 to identify and calculate the percentage contribution of each ASV to dissimilarity between groups. To identify core bacterial community members (present in > 75% samples with relative abundance > 0.1%) for

nursery fragments and source colonies of each species throughout the nursery phase, the panbiom.py script was used as detailed in Kahlke (2017).

## 2.4 Results

### *2.4.1 Bacterial associations with P. verrucosa and A. millepora are species-specific*

Diversity, richness and evenness of bacterial communities associated with the two coral species *P. verrucosa* and *A. millepora* (Fig. S2.1.) did not statistically differ ($p > 0.05$) between species across the entire 155 day experimental period (pooled time points, Fig. S2.3. and Table S2.3.). However, the structure of the bacterial communities associated with the two coral species (*A. millepora* and *P. verrucosa*) were significantly different from each other throughout the experiment (pooled treatments and time points) (PERMANOVA; $F = 8.1197$, $p = 0.0001$, $df = 1$, Fig. S2.2.). During the experimental period, bacterial communities were more variable for *A. millepora* (with significant shifts observed in bacterial communities associated with nursery and out-plant fragments (Fig. 2.4b-e. and Fig. 2.8b.)) than for *P. verrucosa* colonies which by comparison did not exhibit any significant bacterial community changes across treatments (nursery, out-plants and source) or over time (Fig. 2.2a-e. and 2.7a-e.).

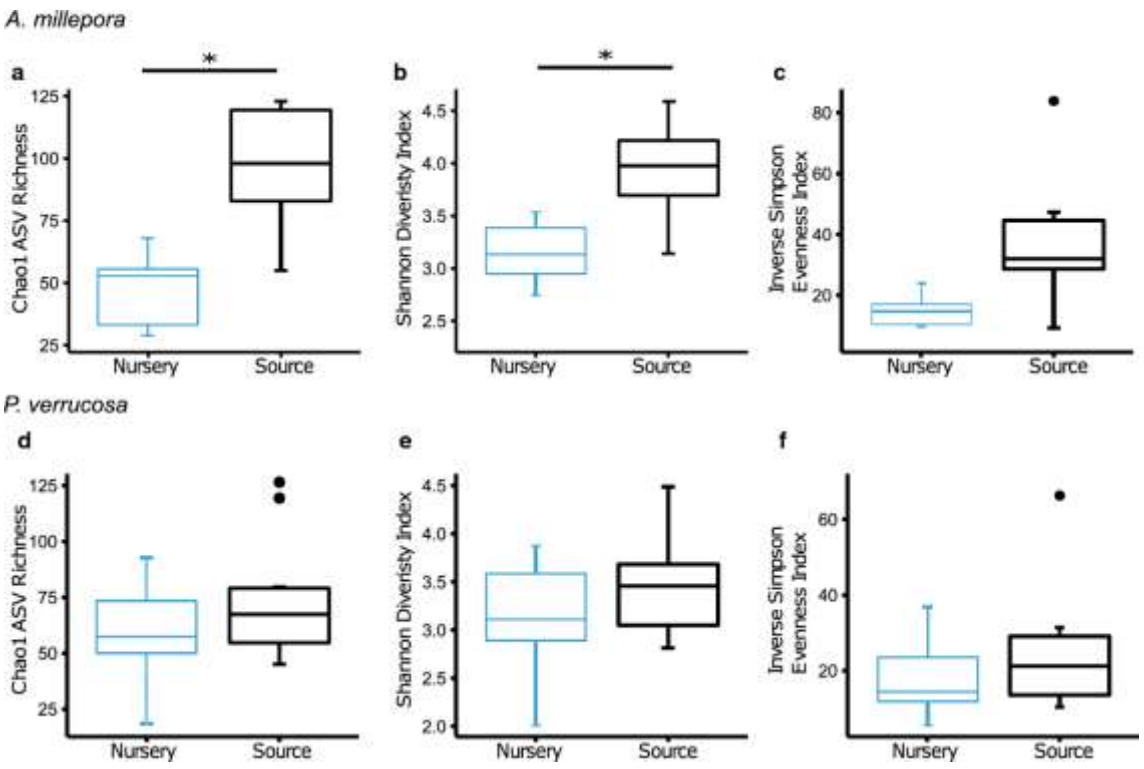



**Figure 2.2.** Bacterial community richness, diversity and evenness of *Acropora millepora* nursery fragments and source colonies (**a**, **b**, **c** respectively) and bacterial community richness, diversity and evenness *Pocillopora verrucosa* nursery fragments and source colonies (**d**, **e**, **f** respectively). Boxes represent the 25th to 75th percentile, centre lines show medians. The whiskers mark 1.5-times the inter-quartile range and the values beyond these upper and lower bounds are considered outliers, marked with dots. Kruskal-Wallis test significance levels: $p < 0.05$ = *. Source data are provided as a Source data file.

### *2.4.2 Initial core bacterial community of colonies of P. verrucosa and A. millepora*

Five 'core' ASVs were present across *P. verrucosa* reef colonies at $T_0$, with three ASVs classified as members of the *Rhodobacteraceae* family (mean relative abundance (RA) = 1.23%) and two from the *Sphingomonadaceae* family (*Erythrobacter* spp.) (mean RA = 0.46%). Six core ASVs were present across *A. millepora* reef colonies at $T_0$, with five of them classified as members of the *Pseudomonadaceae* family (mean RA = 2.68%), and one ASV from the *Rhodobacteraceae* family (mean RA = 1.05%).

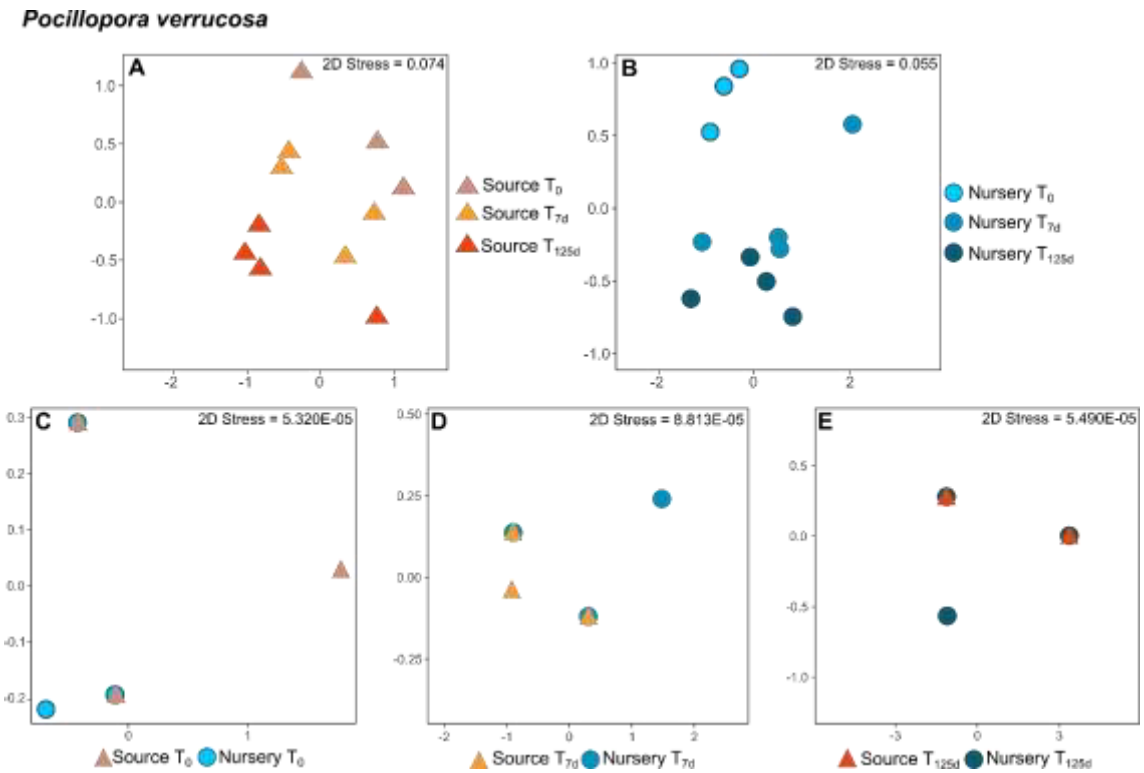



**Figure 2.3**. Bacterial community structure and relative dispersion of the microbial communities of the coral species *Pocillopora verrucosa* source colonies over time, nursery fragments over time (**a** & **b**) and source colonies and nursery fragments at each time point $T_0$, $T_{7d}$ and $T_{125d}$ (**c**, **d** & **e**). Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. Source data are provided as a Source data file.

### *2.4.3 Bacterial community dynamics of P. verrucosa during the coral nursery phase*

Richness, diversity, and evenness of associated bacterial communities for *P. verrucosa* did not significantly differ between source colonies and nursery fragments (Fig. 2.2d., 2.2e., 2.2f. respectively and Table S2.2.). Bacterial community structure of *P. verrucosa* source colonies did not exhibit significant changes over time (Fig. 2.3a. and Fig. 2.5d.). *P. verrucosa* fragments within the nursery did not significantly change over time (Fig. 2.3a. and Fig. 2.5b.). Furthermore, the bacterial community structure of *P. verrucosa* nursery fragments and source colonies were not statistically different at all time point comparisons over the course of the nursery phase (Fig. 2.3c., 2.3d., 2.3e. and Table S2.1.). Despite no significant changes in the overall structure of the *P. verrucosa*

bacterial community in nursery fragments or source colonies, no members of the *P. verrucosa* $T_0$ core bacterial community were retained within nursery fragments or source colonies over the 125 day period. After 125 days, all five ASVs from the initial core bacterial community of *P. verrucosa* source colonies were absent. Similarly, nursery fragments of *P. verrucosa* lost all five ASVs from the initial core bacterial community. Nursery fragments lost core bacterial community members faster (after 7 days) than source colonies (125 days), yet the overall core bacterial community dynamics of nursery fragments were similar to source colonies.

### *2.4.4 Bacterial community dynamics of A. millepora during the coral nursery phase*

*A. millepora* nursery fragments were characterised by significantly lower bacterial community richness and diversity compared to source colonies (Kruskal-Wallis$_{Chao1}$, Chi square = 6.1027, $p = 0.021$, $df = 1$, Kruskal-Wallis$_{Shannon}$ Chi square = 4.8, $p = 0.047$, $df = 1$) (Fig. 2.2a-b.), and bacterial community evenness for *A. millepora* was not significantly different between nursery fragments and source colonies (Fig. 2.2c. and Table S2.2.). Bacterial community structure of *A. millepora* source colonies did not change significantly over time (Fig. 2.4a., Fig. 2.5c. and Table S2.1.). In contrast, while bacterial communities of *A. millepora* fragments held in the nursery did not change significantly from $T_0$ to $T_{7d}$, nursery fragments after 125 days hosted bacterial communities that were significantly different to those at $T_0$ (PERMANOVA, $F = 1.8894$, $p_{adj} = 0.0464$, $df = 1$) and $T_{7d}$ (PERMANOVA, $F = 2.4607$, $p_{adj} = 0.0464$, $df = 1$) (Fig. 2.4b. and Fig. 2.5a). The difference between $T_{125d}$ nursery fragments and $T_0$ /$T_{7d}$ nursery fragments was primarily driven by increases in RA of six *Pseudomonas* ASVs, an increase of one *Sphingomonas* ASV, the loss of one *Endozoicomonas* ASV, the decrease in RA of another *Endozoicomonas* ASV and an increase in another *Endozoicomonas* (SIMPER) (Fig. S2.8a.). Fragments of *A. millepora* from the nursery

also exhibited significantly different bacterial communities to source colonies after 125 days within the nursery (PERMANOVA, $F = 2.2111$, $p = 0.0259$, $df = 1$). Pairwise comparisons of the *A. millepora* bacterial communities in the nursery relative to the source colonies at individual time-points indicated nursery fragments and source colonies were not statistically different at $T_0$ and $T_{7d}$ (Fig. 2.4c., 2.4d. and Table S2.1.), but were significantly different at $T_{125d}$ (PERMANOVA, $F = 2.2111$, $p = 0.0259$, $df = 1$) (Fig. 2.4e.). The significant difference between nursery fragments and source colonies at $T_{125d}$ was primarily explained by the shifts observed within the nursery over time (above) — a higher relative abundance of six *Pseudomonas* ASVs, a *Sphingomonas* ASV, and an *Endozoicomonas* ASV in nursery fragments compared to source colonies (SIMPER analysis) (Fig. S2.8b.). Despite the significant bacterial community shift in *A. millepora* nursery fragments, there were no notable increases in putatively pathogenic taxa. All six ASVs classified as core members of the *A. millepora* source colony bacterial communities at the onset of the experiment were lost after 7 days. In contrast, and despite significant changes to bacterial community structure, the core bacterial community of *A. millepora* nursery fragments was stable over time (from $T_0$ to $T_{125d}$) and *A. millepora* nursery fragments retained a consistent constituent of four *Pseudomonas* ASVs (out of the five *Pseudomonas* ASVs in the initial core) throughout 125 days in the nursery (mean $T_{7d}$RA = 2.34%, mean $T_{125d}$RA = 8.37%).

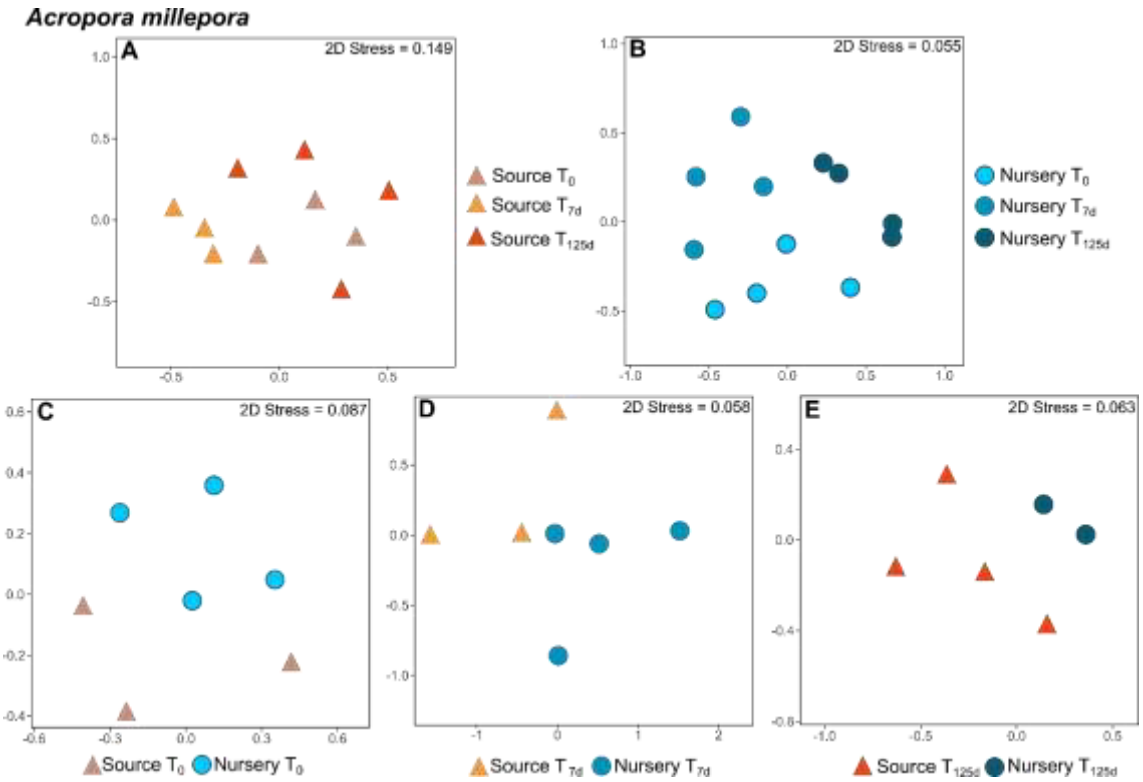



**Figure 2.4.** Bacterial community structure and relative dispersion of the microbial communities of the coral species *Acropora millepora* source colonies over time, nursery fragments over time (**a** & **b**) and source colonies and nursery fragments at each time point T0, T7d and T125d (**c**, **d** & **e**). Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. Ellipses are for illustrative purposes only to highlight significantly different groups. Source data are provided as a Source data file.

### *2.4.5 Bacterial community dynamics of P. verrucosa during the out-planting phase*

Richness and evenness of the bacterial communities of *P. verrucosa* source colonies and out-planted fragments (moved from the nursery back to the neighbouring reef) were not significantly different (Fig. 2.6d., 2.5f. and Table S2.2.). Out-planted fragments had significantly higher bacterial community diversity than ($T_{125d}$ nursery) fragments at time of out-planting (Dunn's post-hoc$_{Shannon}$ $p_{adj} = 0.016$) (Fig. 2.6e.). Bacterial community structure of *P. verrucosa* source colonies did not significantly differ over time (Fig. 2.7a., Fig. 2.9d. and Table S2.1.). The structure of bacterial communities associated with *P. verrucosa* fragments did not change throughout the out-planting process (Fig. 2.7b., Fig. 2.9b. and Table S2.1.). Further, bacterial communities

of out-planted *P. verrucosa* fragments were not significantly different to source colonies when compared at each time point (Fig. 2.7c., 2.6d., 2.6e. and Table S2.1.).

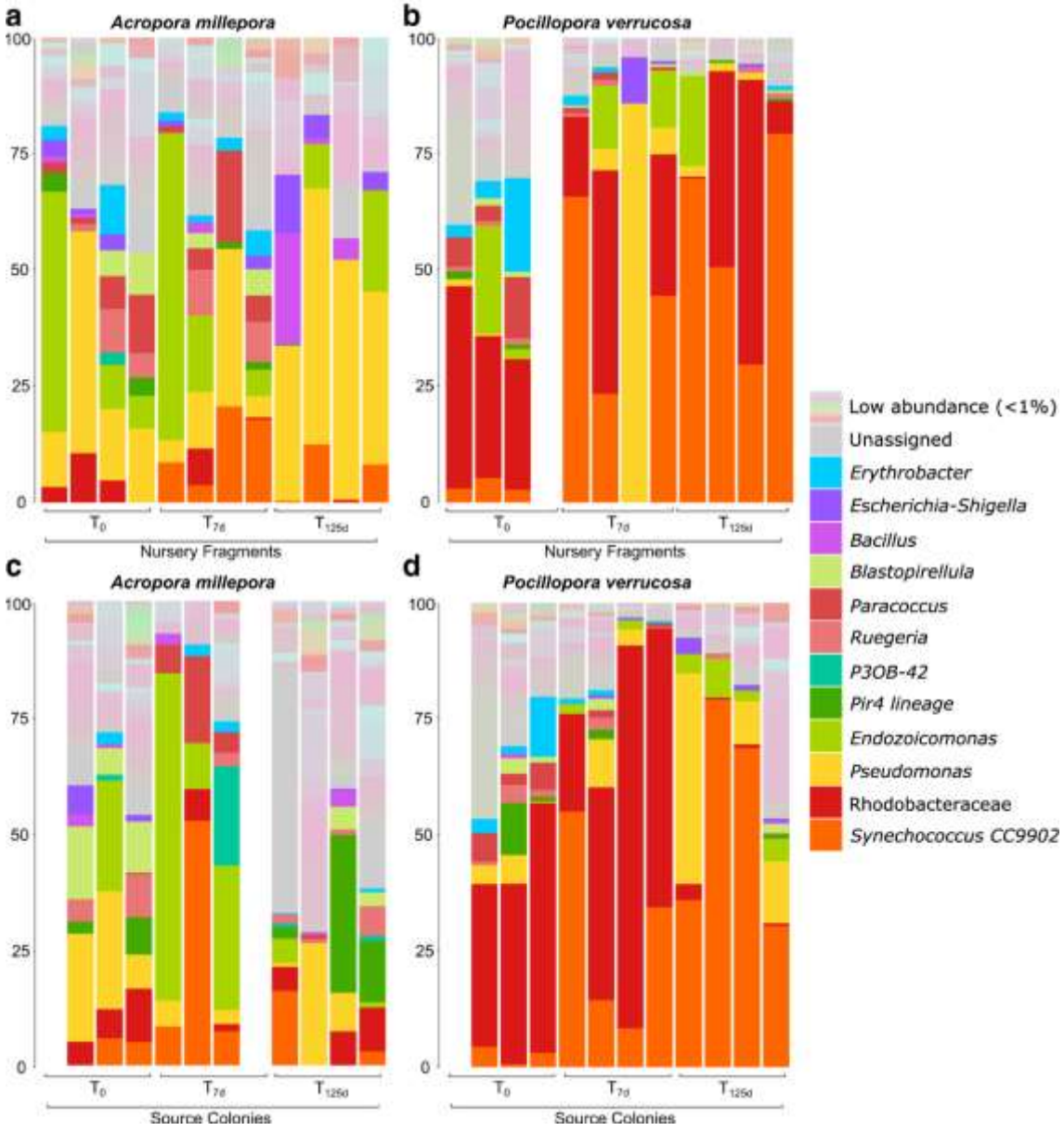



**Figure 2.5.** Bacterial community composition (relative abundances) by genus of (**a**) *Acropora millepora* nursery fragments at T0, T7d and T125d, (**b**) *Pocillopora verrucosa* nursery fragments at T0, T7d and T125d, (**c**) *A. millepora* source colonies at T0, T7d and T125d and (**d**) *P. verrucosa* source colonies at T0, T7d and T125d. Pastel colours represent genera with average of <1% relative abundance in all samples, full legend provided as supplemental data (Supplementary Data Sheet 6.). Non-italic text corresponds to Family taxonomy level which was the finest taxonomic resolution available for those specific ASVs.

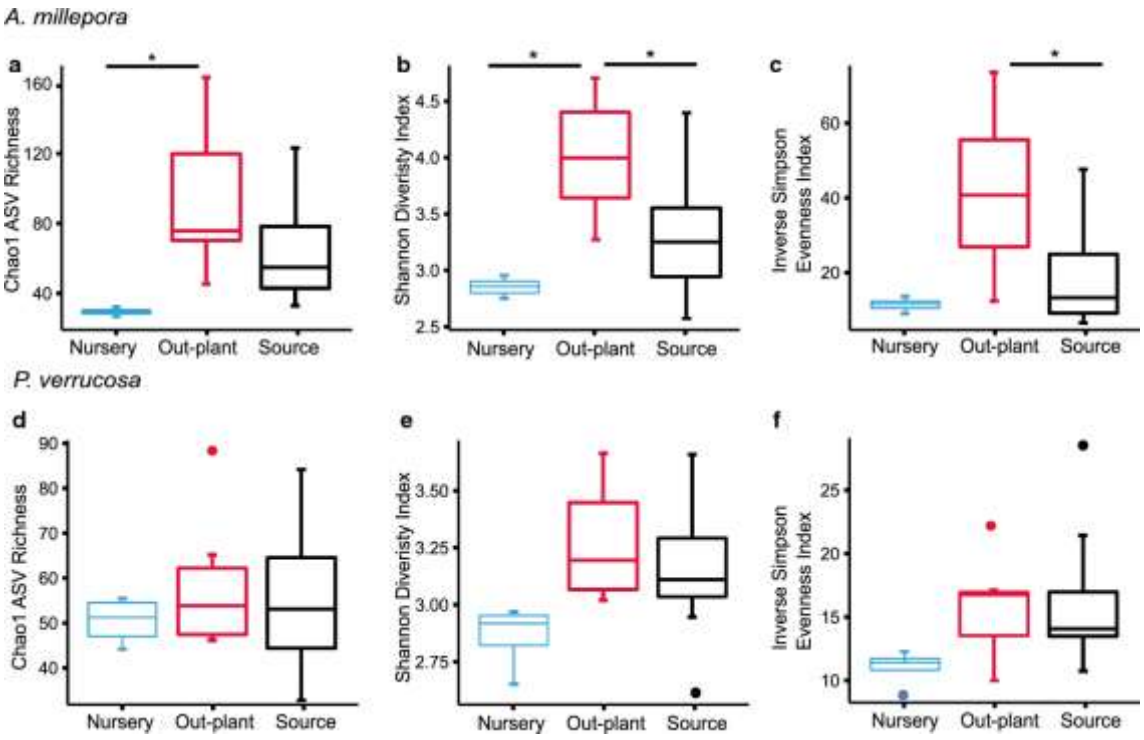



**Figure 2.6.** Bacterial community richness, diversity, and evenness of Acropor*a millepora* T125d nursery fragments out-planted fragments and source colonies (a, b, c respectively) and bacterial community richness, diversity, and evenness *Pocillopora verrucosa* T125d nursery fragments, out-planted fragments, and source colonies (d, e, f respectively). Boxes represent the 25th to 75th percentile, centre lines show medians. The whiskers mark 1.5-times the inter-quartile range and the values beyond these upper and lower bounds are considered outliers, marked with dots. Kruskal Wallis test significance levels: $p < 0.05$ = *. Source data are provided as a source data file.

### *2.4.6 Bacterial community dynamics of A. millepora during the out-planting phase*

Out-planting of *A. millepora* fragments from the nursery back into the reef environment led to significant increases in bacterial community diversity compared to the 125 day nursery (Out-plant$T_0$) fragments (Dunn's post-hoc$_{Shannon}$ $p_{adj} = 0.031$) (Fig. 2.6b.) and source colonies (Dunn's post-hoc$_{Shannon}$ $p_{adj} = 0.007$) (Fig. 2.6b.). Out-planted *A. millepora* fragments also exhibited significantly greater bacterial community evenness compared to source colonies (Dunn's post-hoc$_{InvSimpson}$ $p_{adj} = 0.009$) (Fig. 2.6c.) and greater richness compared to 125 day nursery fragments (Dunn's post-hoc$_{Chao1}$ $p_{adj} = 0.019$) (Fig. 2.6a). Bacterial community structure of *A. millepora* source colonies did not change over the course of the out-planting phase (Fig. 2.8a., Fig. 2.9c.

and Table S2.1.). Out-planting of *A. millepora* fragments (grown for 125 days) from the nursery back to the natural reef environment led to a significant change in the associated bacterial community structure of out-planted fragments, whereby after 1 day and 30 days the out-planted fragments had significantly different bacterial community structure compared to the fragments at $T_0$ of out-planting ($PERMANOVA_{Bray\text{-}Curtis}$; $F = 2.6923$, $p_{adj} = 0.0467$, $df = 1$; $F = 2.4274$, $p_{adj} = 0.0467$, $df = 1$ respectively) (Fig. 2.8b. and Fig. 2.9a.). After the initial shift in bacterial community structure between $T_0$ to $T_{1d}$, out-planted *A. millepora* fragments did not display any significant changes in bacterial community structure over time from $T_{1d}$ to $T_{30d}$ (Fig. 2.8b. and Table S2.1.). At both $T_{1d}$ and $T_{30d}$ the bacterial community structure of out-planted fragments was not statistically different from the source colonies (Fig. 2.8d., 2.7e. and Table S2.1.). The difference between the nursery fragments at $T_0$ of out-planting ($NurseryT_{125d}$) and $T_{1d}$/$T_{30d}$ out-plants (after 1 day and 30 days) was primarily explained by an immediate and then continued decrease in the relative abundance of four *Pseudomonas* ASVs, an immediate loss of two further *Pseudomonas* ASVs and a *Sphingomonas* ASV, an immediate increase in the relative abundance of one *Synechococcus* ASV, an immediate decrease in the relative abundance of one *Endozoicomonas* ASV and after 30 days the appearance of two Myxococcus ASVs (SIMPER analysis, Fig. S2.10.).

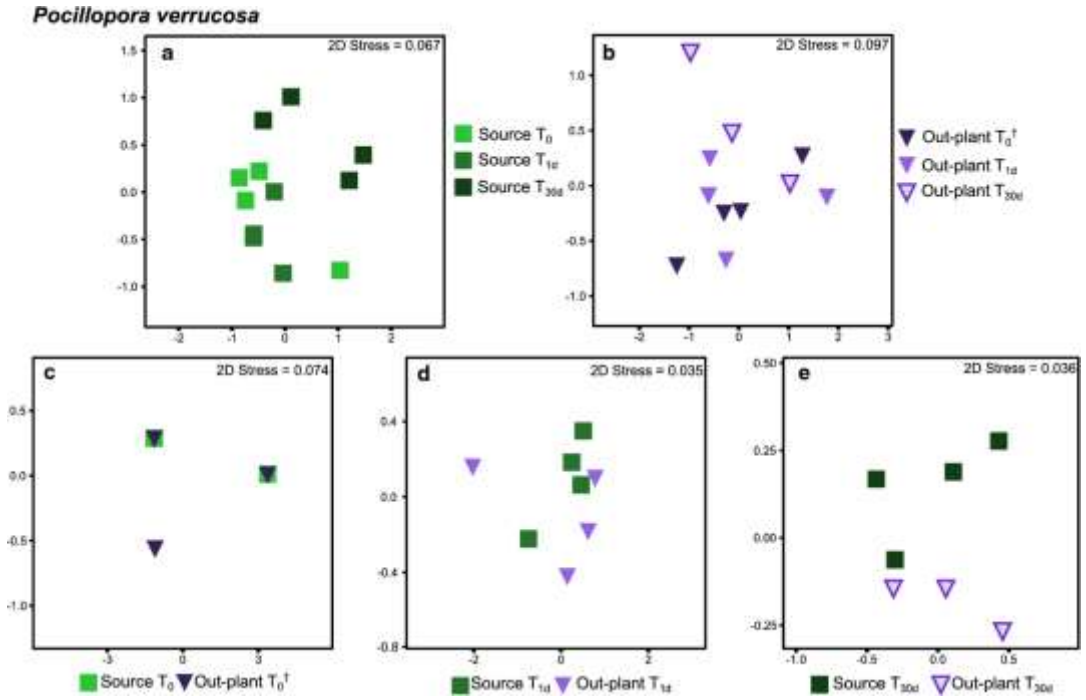



**Figure 2.7.** Bacterial community structure and relative dispersion of the microbial communities of the coral species *Pocillopora verrucosa* source colonies over time, out-planted fragments over time (**a** & **b**) and source colonies and out-planted fragments (**c**, **d** & **e**) at each time point $T_0^{\dagger}$, $T_{1d}$ and $T_{30d}$. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. †The source colony sample at $T_{125d}$ (of the nursery phase of the experiment) was considered $T_0$ in the out-planting phase, and the $T_{125d}$ nursery fragment was considered $T_0$ of out-planting phase. Source data are provided as a Source data file.

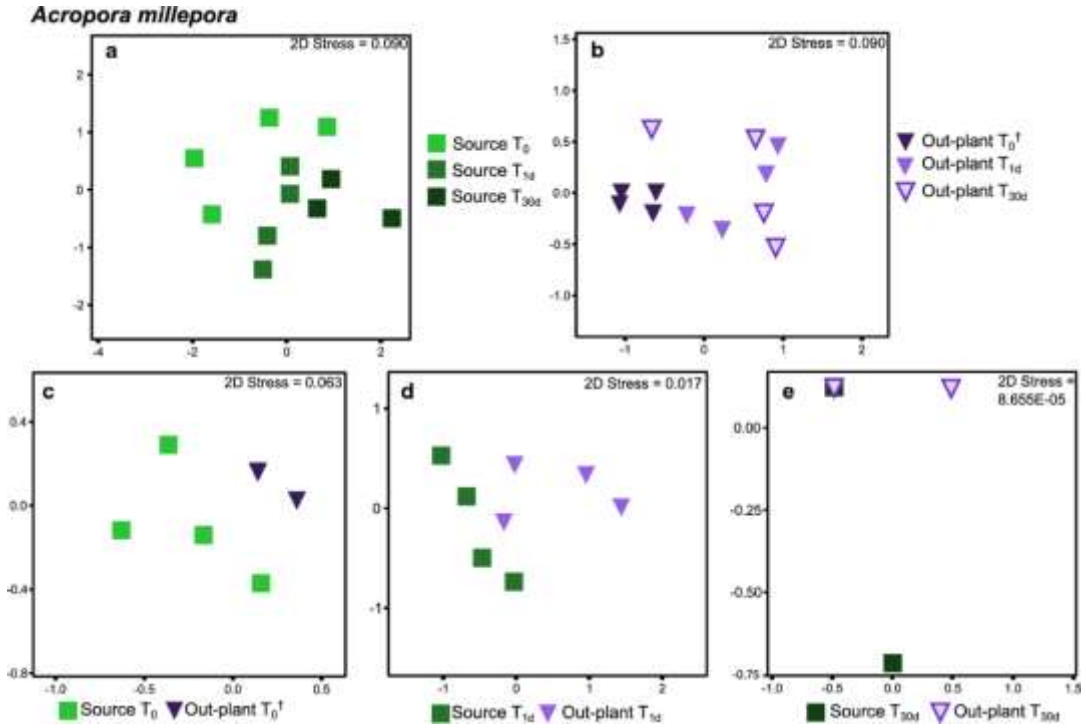



**Figure 2.8.** Bacterial community structure and relative dispersion of the microbial communities of the coral species *Acropora millepora* source colonies over time, out-

planted fragments over time (**a** & **b**) and source colonies and out-planted fragments (**c**, **d** & **e**) at each time point T0†, T1d and T30d. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. †The source colony sample at T125d (of the nursery phase of the experiment) was considered T0 in the out-planting phase, and the T125d nursery fragment was considered T0 of out-planting phase. Source data are provided as a Source data file.

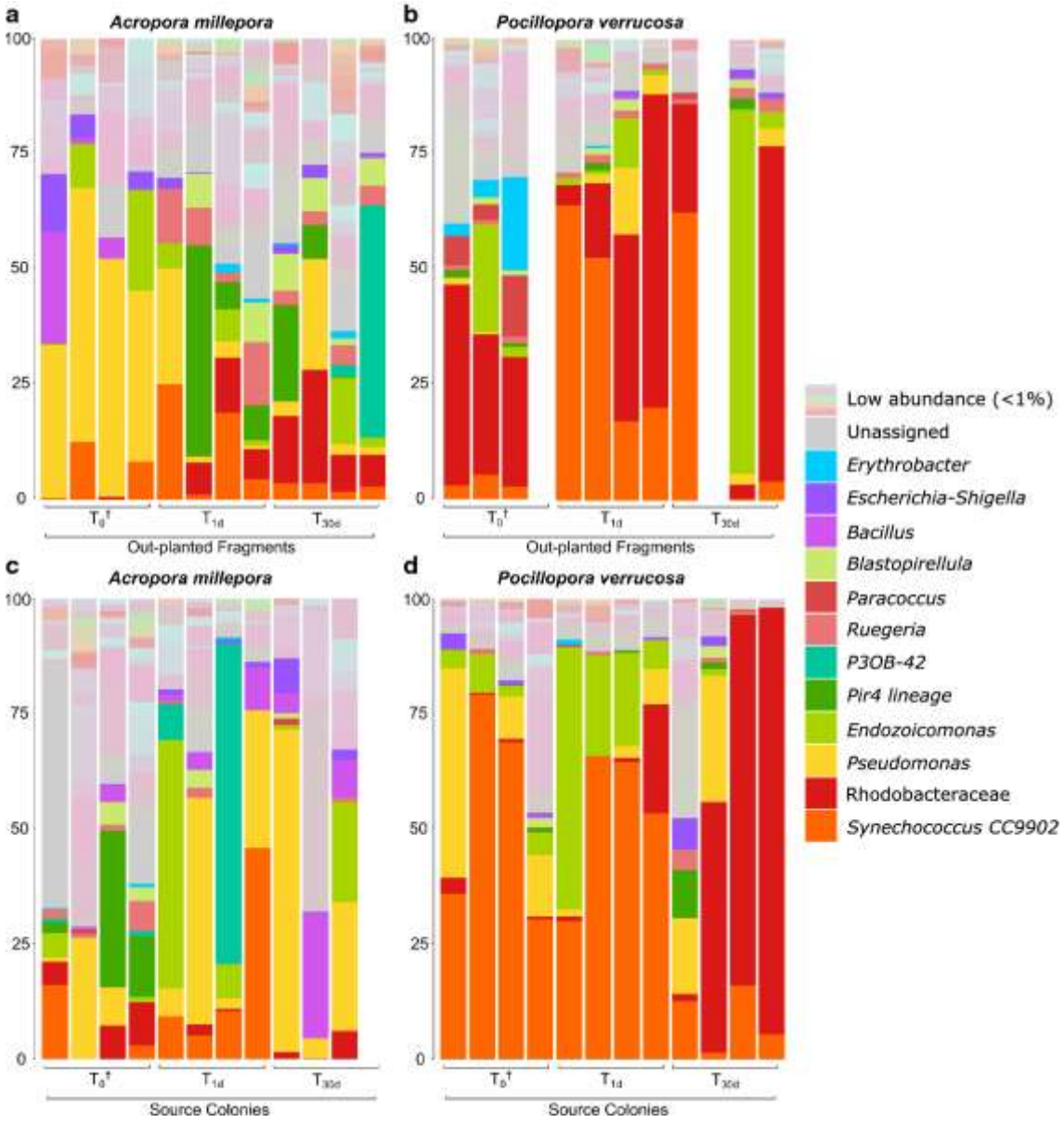



**Figure 2.9.** Bacterial community composition (relative abundances) by genus of (**a**) *Acropora millepora* out-planted fragments at T0†, T1d and T30d, (**b**) *Pocillopora verrucosa* out-planted fragments at T0†, T1d and T30d, (**c**) *A. millepora* source colonies at T0†, T1d and T30d and (**d**) *P. verrucosa* source colonies at T0†, T1d and T30d. Pastel colours represent genera with average of <1% relative abundance in all samples, full legend provided as supplemental data (Supplementary Data S6.). Non-italic text corresponds to Family taxonomy level which was the finest taxonomic resolution available for those specific ASVs. †The source colony sample at T125d (of the nursery phase of the experiment) was considered T0 in the out-planting phase, and the T125d nursery fragment was considered T0 of out-planting phase.

## 2.5 Discussion

Coral propagation and out-planting efforts are growing worldwide (Boström-Einarsson et al. 2020), including on the Great Barrier Reef (GBR; Suggett et al. 2019, 2020, Howlett et al. 2021), yet how these practices potentially drive alterations in coral-associated bacterial communities – that could regulate coral health, including disease susceptibility (Moriarty et al. 2020) – remains unknown. As part of a nursery-based propagation and out-planting program at Opal Reef (GBR), we have shown that two coral species exhibit different bacterial community responses. Specifically, bacterial communities of *A. millepora* fragments were altered during nursery propagation but changed to more closely resemble source colonies within 30 days of out-planting. In contrast, *P. verrucosa* bacterial communities were unaltered during nursery propagation or after out-planting, retaining similar bacterial community dynamics compared to source colonies on the reef throughout the whole process. Our results thus highlight that propagation and out-planting can impact microbiomes for some commonly used coral species, thereby highlighting potential for modification of microbial consortia that underpin healthy functioning. We discuss how these changes may be either beneficial or detrimental (depending on the environmental context) and therefore the need for future coral restoration practices to 'conserve the holobiont' to further improve effectiveness.

Distinct bacterial communities associated with the two coral species examined here, *A. millepora* and *P. verrucosa,* are consistent with previous observations that coral-associated bacterial communities can exhibit substantial species-specificity (Dunphy et al. 2019; Osman et al. 2020). Furthermore, the different bacterial community dynamics observed between the two coral species are also reflective of previous observations that *P. verrucosa* bacterial communities are often well conserved over time and between locations (Pogoreutz et al. 2018; Wang et al. 2018; Epstein et al. 2019; Ziegler et al. 2019), whereas *Acropora* spp. bacterial communities have been

shown to display higher levels of variability across time and location (Pogoreutz et al. 2018; Dunphy et al. 2019; Ziegler et al. 2019). The stability of coral-associated bacterial communities has been suggested to be linked to the persistent presence of dominant bacterial taxa, which may be indicative of tight ecological relationships between the host and specific microbes, while in contrast microbiome flexibility has been suggested to be linked to high community evenness and/or functional redundancy within the bacterial community that can facilitate retention of essential functions under stress, even if the composition of the bacterial community changes (Voolstra and Ziegler, 2020).

Propagation of *P. verrucosa* colonies within *in situ* coral nurseries had no significant impacts to the coral-associated bacterial communities (and compared to the natural community dynamics on the reef). While some, relatively low abundance, core ASVs were lost from the *P. verrucosa* core bacterial community, the overall structure of the bacterial community associated with *P. verrucosa* fragments remained stable. In contrast, propagation of *A. millepora* using *in situ* coral nurseries led to differences between the bacterial communities in nursery fragments and source colonies remaining within the reef environment, a response previously observed for experimentally 'stressed' corals (e.g., Grottoli et al. 2018). While changes in the relative abundance of the bacterial taxa (*Pseudomonas*, *Endozoicomonas* and *Sphingomonas*) driving differences between nursery and source colonies observed in our study have been linked to stressed corals as an immune-like response (Reschef et al. 2006), they have also been suggested to provide putatively beneficial functions (Raina et al. 2009; Peixoto et al. 2017; Grottoli et al. 2018). Given *A. millepora* nursery fragments did not display any other signs of stress (bleaching or tissue loss) or increases in putatively pathogenic bacteria, we propose that significant shifts in bacterial community structure, richness and diversity of nursery fragments over time distinct to source colonies, and loss of a core bacterial community in source colonies but retention in nursery fragments, is likely

a result of differences in biotic and abiotic conditions (Dunphy et al. 2019) between the reef benthos and the floating nursery frames (Zaneveld et al. 2017). Specific biotic and abiotic factors known to drive bacterial community differences (Wang et al. 2018; Ezzat et al. 2020) that are likely to vary from nursery frames to the source reef (Boström-Einarsson et al. 2018) can include: more complex benthic assemblage on the reef (other corals, fleshy/turf macroalgae, CCA) (Casey et al. 2015), novel fish communities on nursery frames (Xin et al. 2016; Taira et al. 2017) and differences in nutrient availability (Kelly et al. 2014; Wang et al. 2018). Environmental differences between natural reef and nursery frames are potentially compounded by the fragmentation of a reef colony, which modifies the architectural complexity (Ainsworth et al. 2020) of the coral and will potentially alter the diversity of coral microniches and hence contribute toward the establishment of novel bacterial communities over time (Putnam et al. 2017).

Transfer of *P. verrucosa* from the reef to the nursery and back to the reef during out-planting had no significant effects on the associated bacterial communities over time or compared to the source colonies. However, out-planting nursery grown *A. millepora* led to an immediate shift in the associated bacterial community, with the bacterial community returning to a state that was consistent with source corals that had remained within the reef environment. Such an immediate shift observed in the *A. millepora* associated bacterial community after out-planting and reversion of key bacterial taxa (driving differences between nursery fragments and source colonies) to relative abundances more similar to source colonies is consistent with previously observed bacterial community flexibility in *Acroporid* corals (Epstein et al. 2019; van Oppen and Blackall, 2019; Ziegler et al. 2019; Damjanovic et al. 2020; Marchioro et al. 2020). The role of the bacterial taxa from the Myxococcales order that increased in abundance after out-planting (and had a 4.5% contribution to differences between 30 day out-plants and 125 day nursery fragments) has not been thoroughly investigated but

has been suggested to be linked to pathogen control (Rosales et al. 2019). Importantly, these patterns observed in the bacterial communities of *A. millepora* (when transferred from source reef to nursery and then back to the source reef) provide evidence that the external environment primarily governs the bacterial component of the *A. millepora* microbiome and suggest the shift observed in the nursery fragments was a response to, and a temporary artefact of, the environmental conditions (Casey et al. 2015; Nicolet et al. 2018; Ezzat et al. 2020; Koval et al. 2020; Moriarty et al. 2020) within the coral nursery resulting from the innate flexibility of *A. millepora* bacterial communities (Voolstra and Ziegler, 2020).

While the study of coral microbiomes throughout the coral propagation process remains in its relative infancy, numerous previous studies have highlighted how captive breeding of other animals, from mammals to fish, can impact host-microbiome dynamics (Amato et al. 2013, Bennett et al. 2016 , Borbon-Garcia et al. 2017; Carthey et al. 2019; Cheng et al. 2015; Chong et al. 2019; Delport et al. 2016; Dhanasiri et al. 2011; McKenzie et al. 2017; Nelson et al. 2021; Wasimuddin et al. 2017). These previous studies have emphasised the importance of focussing efforts on 'conserving the holobiont' (Carthey et al. 2019). In the majority of documented cases, (alpha) diversity measures of mammalian associated gut microbiota are reduced in captivity, which has been suggested to be an outcome of reduced interactions with variable environmental substrates that act as sources for bacterial diversity (McKenzie et al. 2017). However, it has been noted in mammals that variability in bacterial community diversity observed in captivity can also be influenced by host traits (McKenzie et al. 2017). Interestingly, we observed decreased bacterial diversity in *A. millepora* coral nursery fragments compared to source colonies remaining on the neighbouring reef, while the bacterial diversity of nursery grown fragments increased after out-planting back onto the natural reef substrate. The bacterial diversity dynamics observed in *A.*

*millepora* are therefore similar to cases of decreased (alpha) diversity measures in bacterial communities of some mammal species held in captivity compared to wild populations (McKenzie et al. 2017) and those held in captivity then released into the wild (Chong et al. 2019). However, these patterns were not observed in *P. verrucosa* throughout the same process, where bacterial diversity did not change significantly.

Aside from changes in overall microbiome (alpha) diversity, differences in the specific bacterial community composition have also been recorded between captive and wild animals. For example, the gut-microbiota of captive bred mammals have been observed to undergo significant restructuring after time in captivity, whereby the bacterial community subsequently reverts to a 'wild-type' constituent after release into the wild (Chong et al. 2019; Cheng et al. 2015). Microbiome shifts observed for mammals in captivity appear driven by a range of factors that differ between wild environments and captivity (Carthey et al. 2019), notably altered group size and environmental conditions. In the latter case, conditions include the introduction of microorganisms from nonendemic sources (Delport et al. 2016), and the degree of habitat degradation, which can result in compromised diet (Amato et al. 2013), according to food type or availability (Dhanasiri et al. 2011, Borbon-Garcia et al. 2017, Carthey et al. 2019). The implications of these microbiome perturbations on host health remain largely unexplored, however microbiome shifts in captive mammals have been characterised by loss or depletion of beneficial microbes and related microbial services, specifically for digestive health (butyrate production crucial for colonic epithelial tissue health), have led to reduced health status of the holobiont (Amato et al. 2013, Borbon-Garcia et al. 2017). In our study, the process of out-planting nursery grown coral fragments did not promote putative coral pathogens or clearly indicate loss of putatively beneficial bacteria; however, the observed bacterial community shift in *A. millepora* out-plants and hypothesised link to environmental conditions suggests that there is

potential for transition to a pathogenic state if fragments are out-planted at sites with high inherent pathogen presence within the reef benthos, or indeed if nurseries are maintained in a manner that may promote pathogens to proliferate (e.g. Moriarty et al. 2020). The species-specific differences in bacterial community dynamics observed throughout the propagation and out-planting processes may also have implications for holobiont success in the future with climate change altering environmental conditions, where the innate flexibility or stability of coral-associated bacterial communities are posited as individual ecological adaptation strategies (Voolstra and Ziegler, 2020). Consequently, clear understanding of the extent or scope of shifts in coral-associated bacterial communities within the context of coral propagation and out-planting, especially where coral fragments are temporarily held in nurseries that may expose coral to atypical environmental conditions (compared to the natural reef environment), requires more detailed attention.

We have provided the first description of how coral propagation within a coral nursery and subsequent out-planting into the natural reef environment affects coral-associated bacterial community dynamics. Our data suggest that shifts observed in bacterial communities of commonly propagated *A. millepora* appear to be driven by transplantation of corals between distinct environments, rather than handling during the propagation or out-planting process – this notion therefore warrants more targeted investigation given the heterogeneous nature of reef environmental conditions. Notably, the impacts of coral propagation and out-planting on coral-associated bacterial communities are species-specific, with the bacterial communities of *P. verrucosa* not significantly altered during the nursery or out-planting phases. While we found no evidence for proliferation of putative pathogenic bacterial associates, it is important to note that coral propagation practices are diverse and variable (Rinkevich, 2019; Boström-Einarsson et al. 2020), therefore it is critical to consider how the responses

observed in our current study hold across to other practices and coral species. The responses observed for *A. millepora* provide clear evidence supporting the notion that the external environment significantly shapes coral-associated bacterial communities for some coral species. As such, nursery and out-planting practices may require more fine-tuned, species-specific, protocols that can account for the potential plasticity of coral-associated bacterial communities. Importantly, this is the first study to examine these questions, to further address the impact of propagation and out-planting on coral-associated bacterial communities, future studies with larger sample sizes and more coral species across different practices – along with characterisation of environmental differences between coral nurseries and source reefs – will be beneficial. Coral reef ecosystems are experiencing a broad array of accelerating threats compounded by climate change, and so researchers and local active management strategies are proactively initiating and scaling *in situ* propagation and out-planting efforts to rapidly increase coral cover and maintain diversity. Our findings suggest that such efforts will benefit by expanding focus to include coral-associated microbial changes in understanding – and therefore optimising – effectiveness of practices, in particular as they continue to scale across increasingly diverse reef (and nursery) environments.

## 2.6 Acknowledgements

The authors wish to express thanks to the Great Barrier Reef Marine Park Authority, whose support established the permit for the coral nurseries at Opal Reef (G18/40023.1 and G19/42553 to EFC, DJS and J Edmondson), as well as staff from Wavelength Reef Cruises, who have continuously supported the project. This research was supported by an Australian Government Research Training Program (RTP) Fee Offset Scholarship to P Strudwick. Operations at Opal Reef – including site access – were supported by funding to the Coral Nurture Program from the Australian & Queensland Governments

("Solving the bottleneck of reef rehabilitation through boosting coral abundance: Miniaturising and mechanising coral out-planting" to DJ Suggett, EF Camp, J Edmondson). Contribution of EF Camp was through the University of Technology Sydney Chancellor's Postdoctoral Research Fellowship and ARC Discovery Early Career Research Award (DE190100142).

## 2.7 Data Availability

Raw data files used for coral and environmental bacterial community analysis in this study have been deposited in FASTQ format in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA733237. All other data is provided as Electronic Supplementary Materials online at doi.org/10.1007/s00338-021-02207-6.

## 2.8 Supplementary Materials

### *2.8.1 Supplementary Figures*

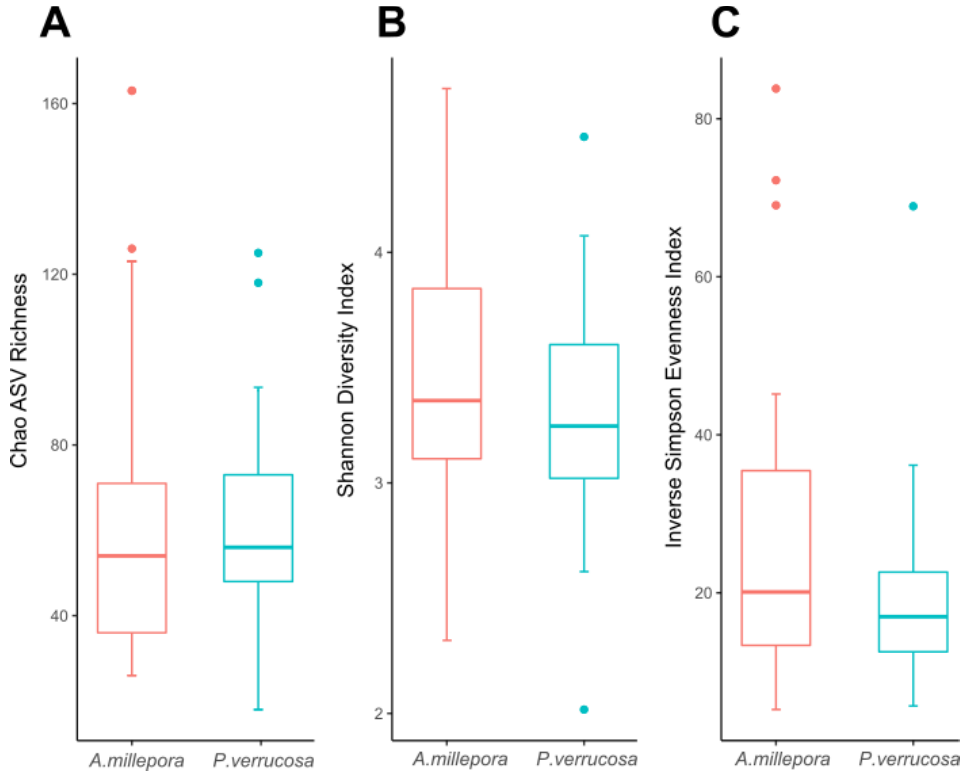



**Figure S2.1.** (**a**) Bacterial richness, (**b**) diversity and (**c**) evenness *of Acropora millepora* and *Pocillopora verrucosa* over the course of the 155 day experiment (time points and treatments pooled). Boxes represent the 25$^{th}$ to 75$^{th}$ percentile, centre lines

show medians. The whiskers mark 1.5-times the inter-quartile range and the values beyond these upper and lower bounds are considered outliers, marked with dots.

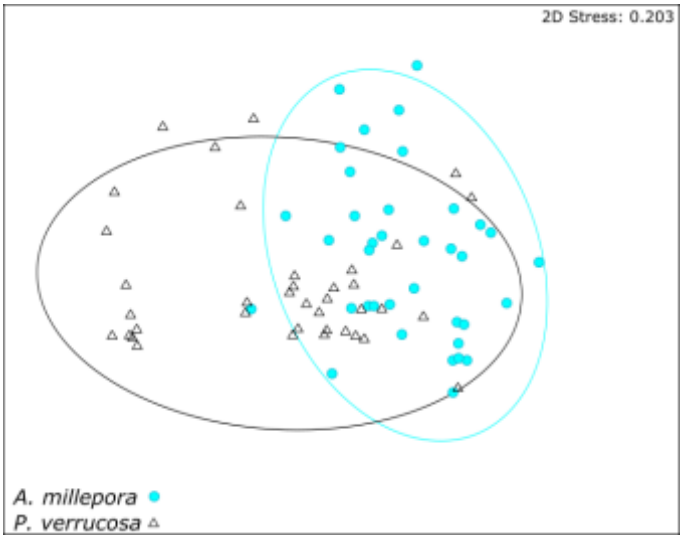



**Figure S2.2.** Bacterial community structure and relative dispersion of the microbial communities of the coral species *Acropora millepora* and *Pocillopora verrucosa* (all time points and treatments pooled). Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community compositions associated with coral samples. Ellipses denote 90% confidence intervals.

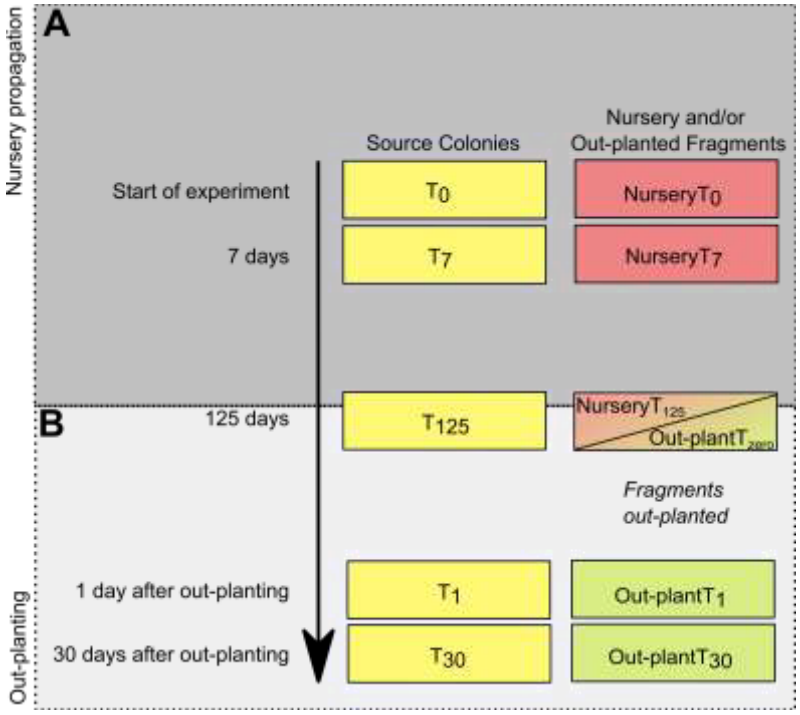



**Figure S2.3.** Experimental design and sampling timeline. (a) Part 1 "Nursery Phase" of the experiment: Nursery fragments and source colonies were both sampled at the start of the experiment ($T_0$ and Nursery$T_0$) at 7 days ($T_{7d}$ and Nursery$T_{7d}$) and at 125 days ($T_{125d}$ and Nursery$T_{125d}$) after the start of the experiment. (b) Part 2 "Out-planting Phase" of the experiment: Out-planting occurred 125 days after the start of the experiment. Fragments that were held within the nursery for 125 days (but had not previously been sampled) were out-planted and as such samples collected to quantify the bacterial

community of nursery fragments at 125 days (Nursery$T_{125d}$) were used in analysis as time zero for the out-planting phase and are referred to as Nursery$T_{125d}$/Out-plant$T_0$. Out-plants and source colonies were then both sampled at 1 day ($T_{1d}$/Out-plant$T_{1d}$) and 30 days ($T_{30d}$/Out-plant$T_{30d}$) after out-planting.

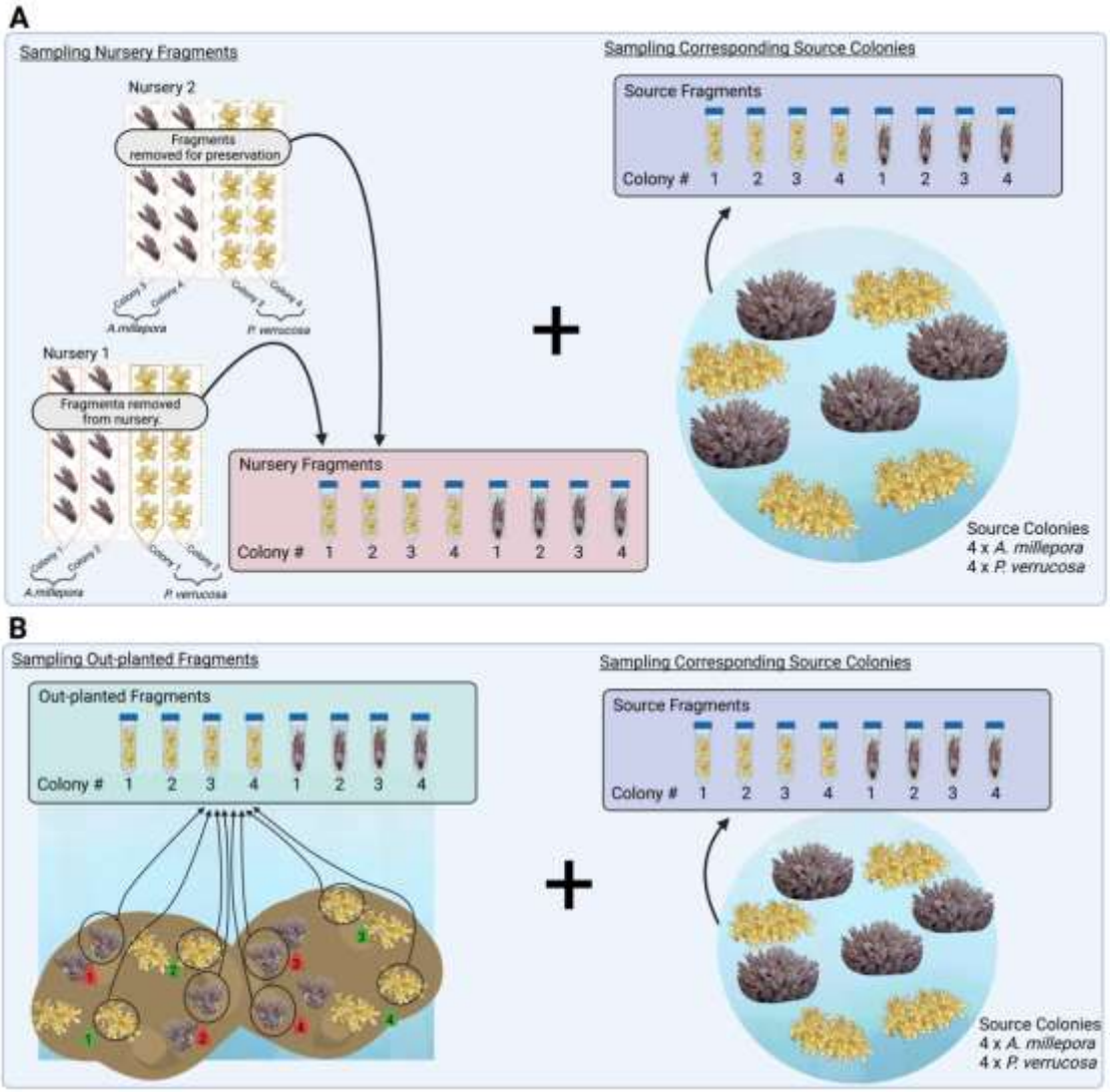



**Figure S2.4.** Diagram of nursery or out-plant and corresponding source colony sampling at an individual time-point during either the nursery phase or out-planting phase of the experiment. For a sampling event during (**a**) the nursery phase of the experiment two fragments of each species – that had not been sampled previously - were removed from the two nursery frames (eight fragments in total) and the four corresponding source colonies of each species were sampled (eight fragments in total). For a sampling event during (**b**) the out-planting phase of the experiment four fragments of each species were removed from the out-plant plot (eight fragments in total) and the four corresponding source colonies of each species were sampled (eight fragments in total). Created with BioRender.com.

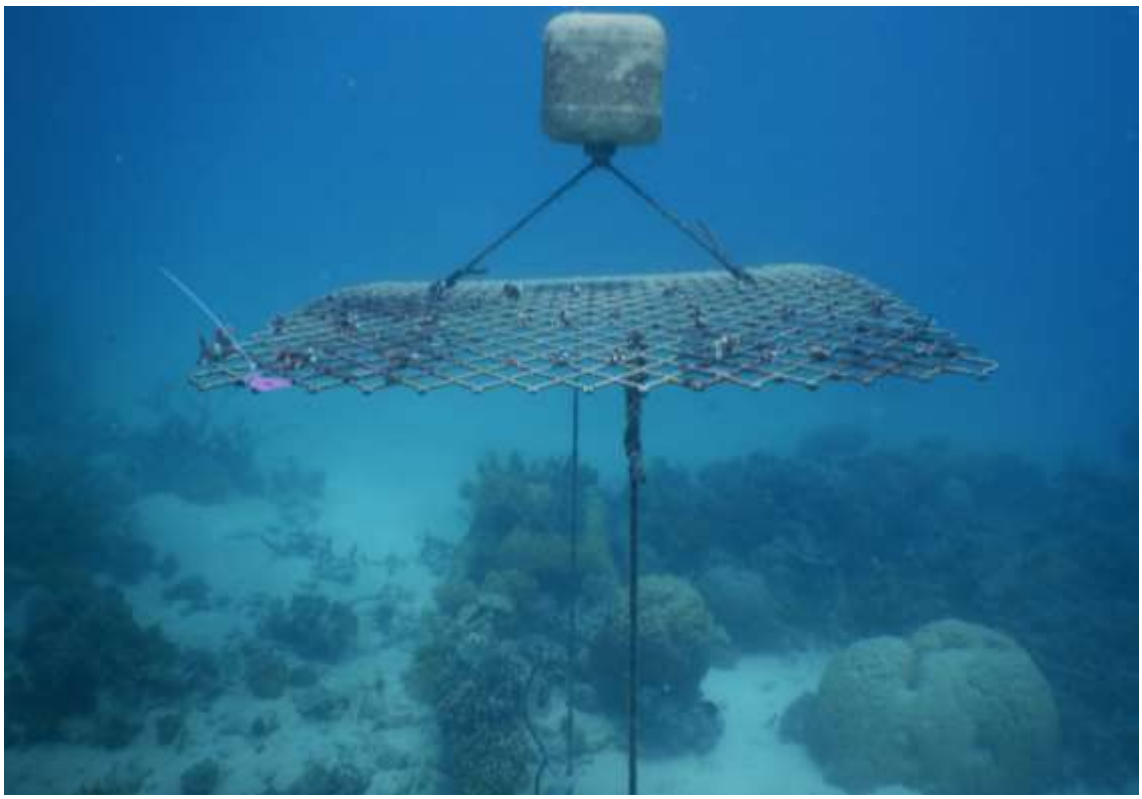


**Figure S2.5.** In situ floating nursery frame. Nursery frame consisting of 2.0 × 1.2 m aluminium diamond-mesh, held in place with 2 × 9 kg Besser blocks and a 20 L float approximately 2 m above the sand at study site ‘Rayban’ on the Northern Great Barrier Reef.

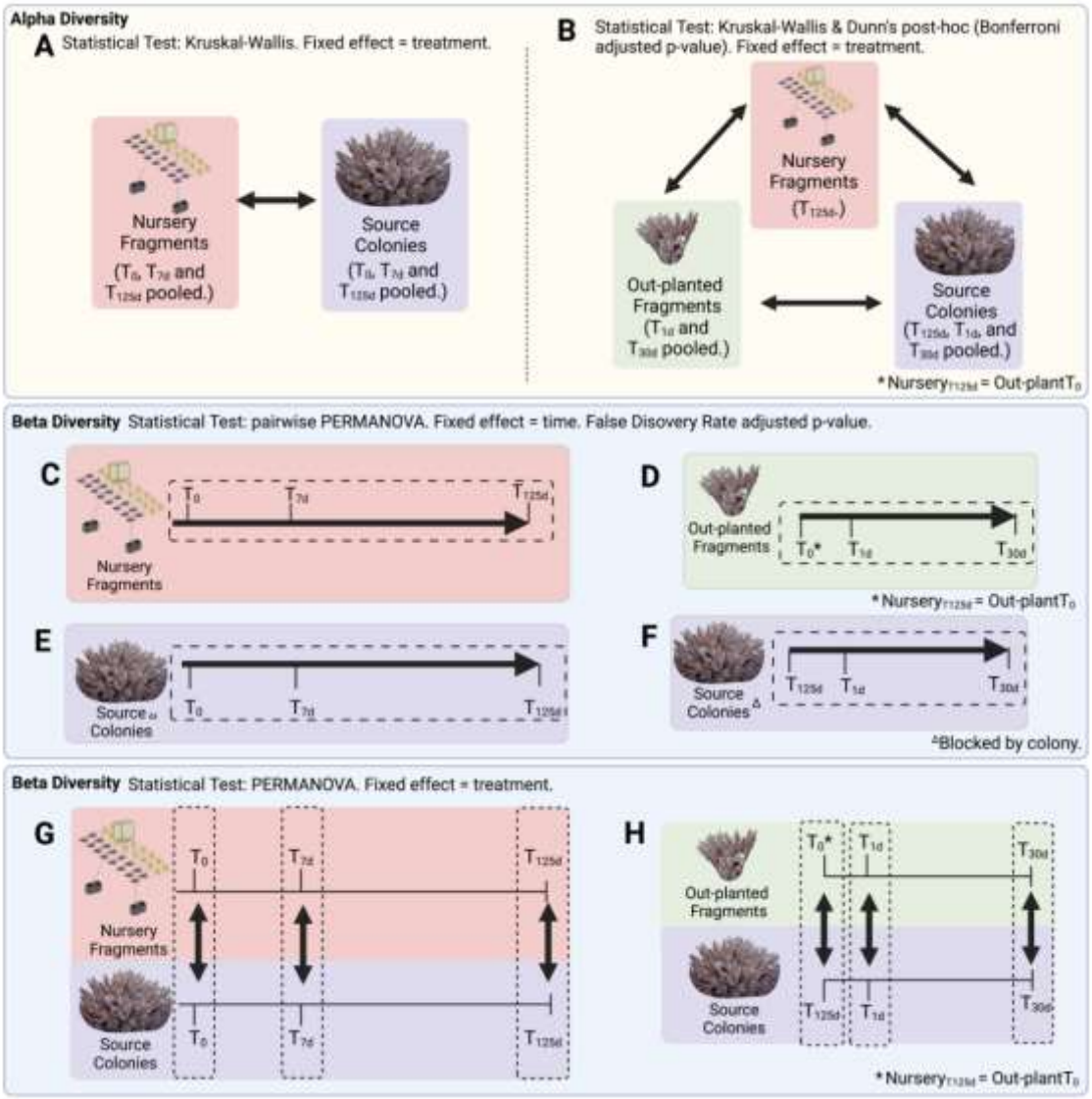



**Figure S2.6.** Diagram of statistical analyses. For alpha diversity indices time points were pooled and treatments were compared. Differences between nursery and source colonies were assessed using Kruskal-Wallis tests (**a**), and differences between out-planted fragments, source colonies and $T_{125d}$ nursery fragments (**b**) were assessed using Kruskal-Wallis tests and if significant subsequent Dunn's post-hoc tests with sequential Bonferroni *p*-value corrections were applied. Bacterial community structure (beta diversity) of nursery fragments over time (**c**), source colonies over time during the nursery phase (**d**), out-planted fragments over time (**e**) and source colonies over time during the out-planting phase (**f**) were analysed with pairwise permutational multivariate analysis of variance (PERMANOVA) and *p*-values were adjusted using a False Discovery Rate (FDR) correction. In separate analysis bacterial community structure of nursery fragments and source colonies were compared at individual time points (**g**) and bacterial community structure of out-planted fragments and source colonies were compared at individual time points (**h**) with PERMANOVA. Created with BioRender.com.

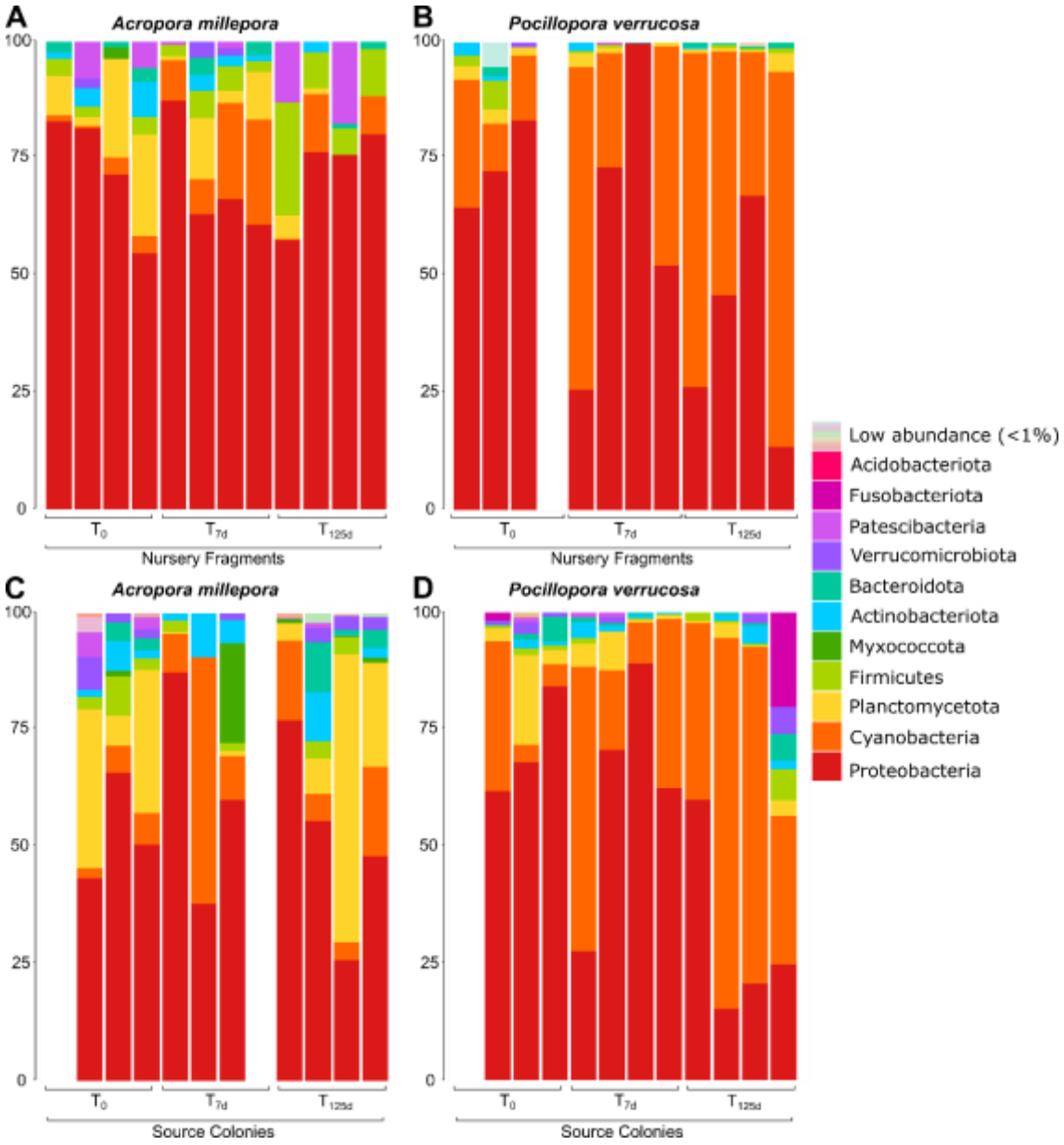



**Figure S2.7.** Bacterial community composition (relative abundances) by phylum of (**a**) *Acropora millepora* nursery fragments at $T_0$, $T_{7d}$ and $T_{125d}$, (**b**) *Pocillopora verrucosa* nursery fragments at $T_0$, $T_{7d}$ and $T_{125d}$, (**c**) *A. millepora* source colonies at $T_0$, $T_{7d}$ and $T_{125d}$ and (d) *P. verrucosa* source colonies at $T_0$, $T_{7d}$ and $T_{125d}$. Pastel colours represent phyla with average of <1% relative abundance in all samples, full legend provided as supplemental data (Supplementary Data S7.).

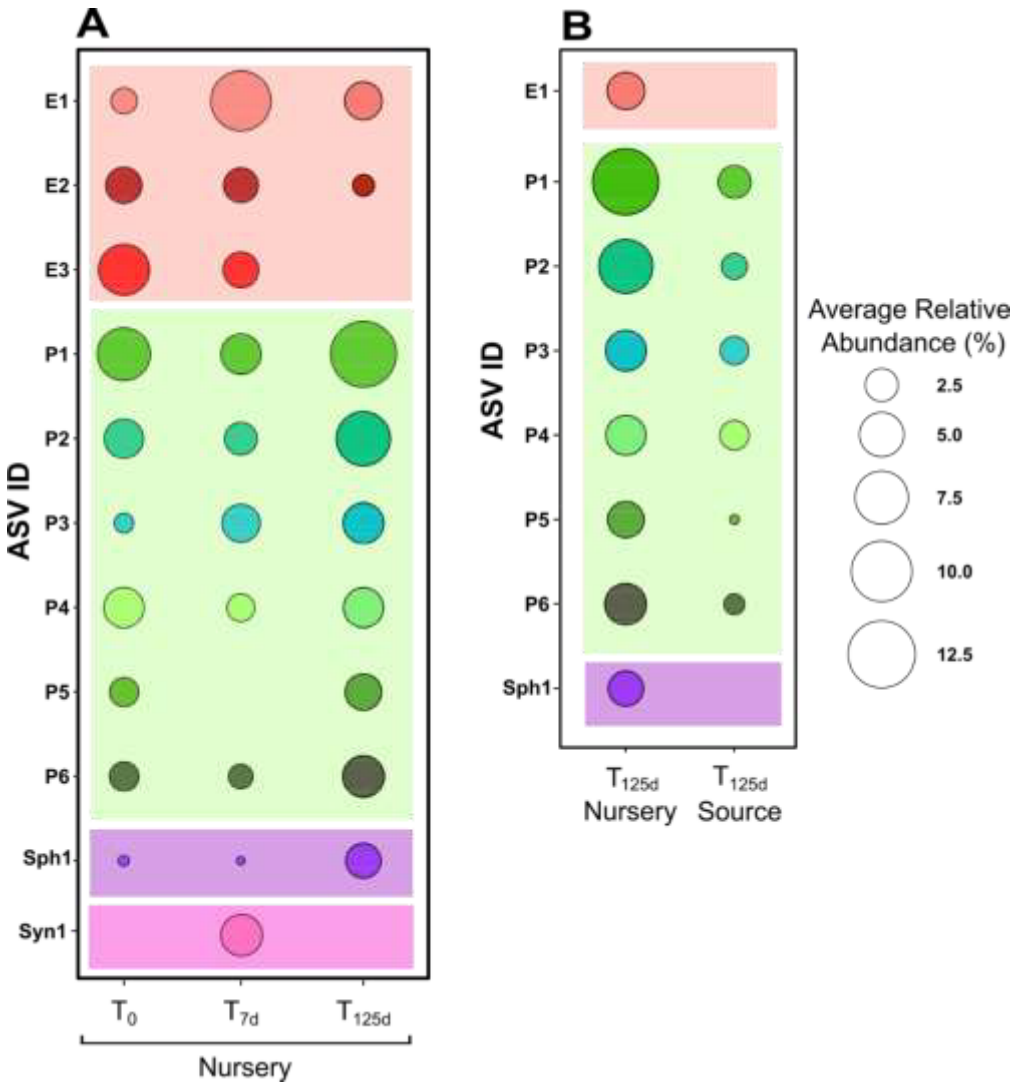



**Figure S2.8.** SIMPER outputs from analysis of significantly different groups identified in PERMANOVA of Bray-Curtis dissimilarities. Showing average relative abundance of ASVs that cumulatively explain ≥25% of the dissimilarity between *Acropora millepora* groups, (**a**) $T_0$ and $T_{7d}$ nursery fragments to $T_{125d}$ nursery fragments and (**b**) $T_{125d}$ nursery fragments to $T_{125d}$ source colonies. E = *Endozoicomonas*, P = *Pseudomonas*, Syn = *Synechococcus*, Sph = *Sphingomonas*.

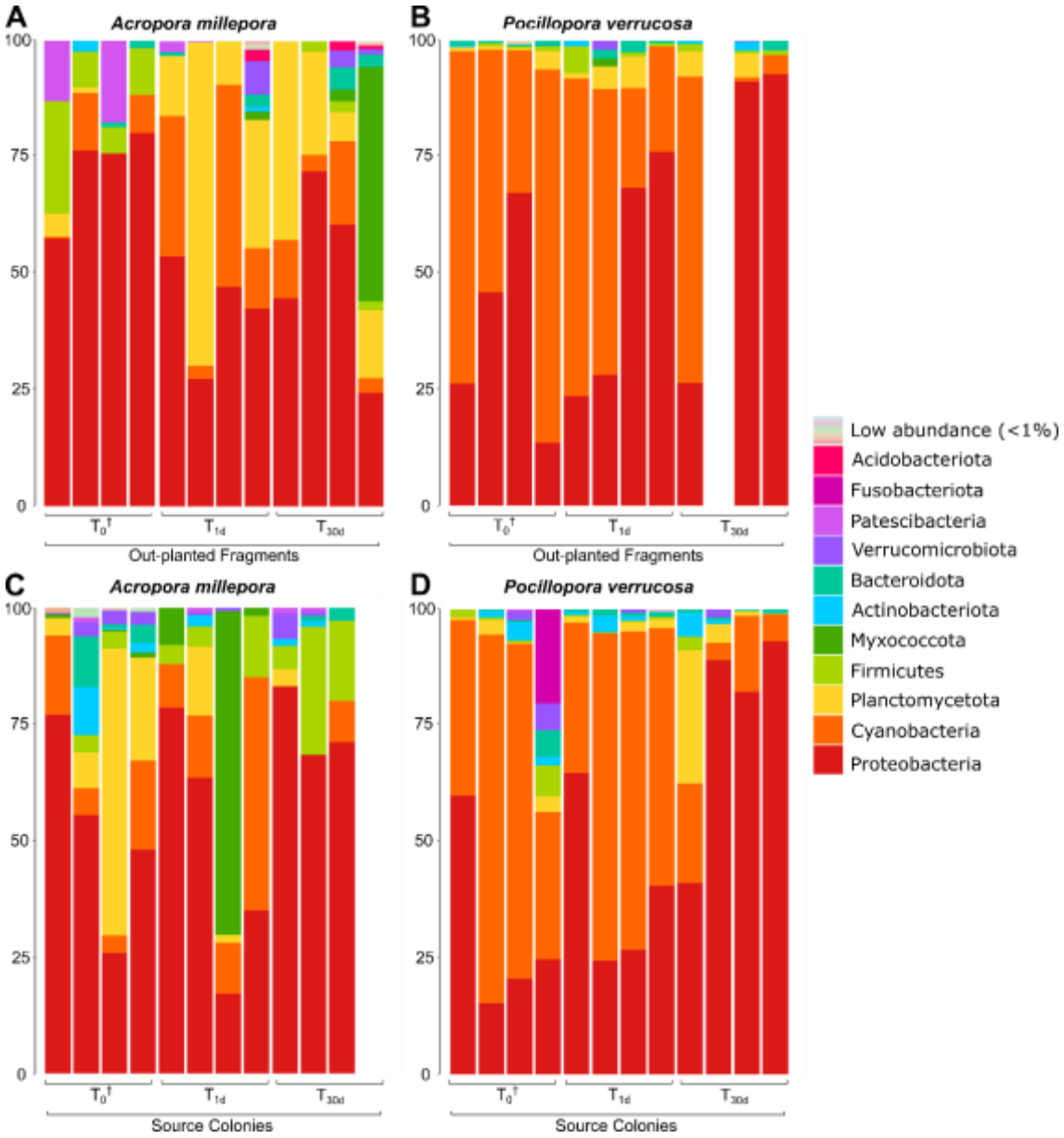



**Figure S2.9.** Bacterial community composition (relative abundances) by phylum of (**a**) *Acropora millepora* out-planted fragments at $T_0^{\dagger}$, $T_{1d}$ and $T_{30d}$, (**b**) *Pocillopora verrucosa* out-planted fragments at $T_0^{\dagger}$, $T_{1d}$ and $T_{30d}$, (**c**) *A. millepora* source colonies at $T_0^{\dagger}$, $T_{1d}$ and $T_{30d}$ and (**d**) *P. verrucosa* source colonies at $T_0^{\dagger}$, $T_{1d}$ and $T_{30d}$. Pastel colours represent phyla with average of <1% relative abundance in all samples, full legend provided as supplemental data (Supplementary Data S7.). †The source colony sample at $T_{125d}$ (of the nursery phase of the experiment) was considered $T_0$ in the out-planting phase, and the $T_{125d}$ nursery fragment was considered $T_0$ of out-planting phase.

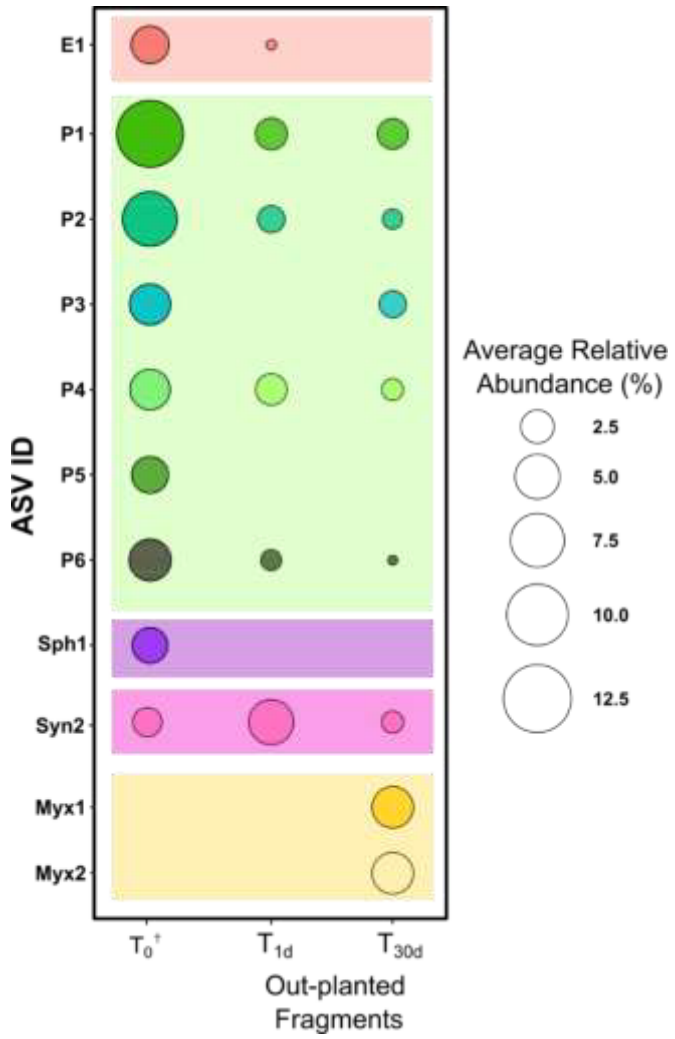



**Figure S2.10.** SIMPER outputs from analysis of significantly different groups identified in PERMANOVA of Bray-Curtis dissimilarities. Showing average relative abundance of ASVs that cumulatively explain ≥25% of the dissimilarity between *Acropora millepora* groups $T_0^{\dagger}$ out-planted fragments to $T_{1d}$ and $T_{30d}$ out-planted fragments. †The $T_{125d}$ nursery fragments were considered $T_0$ of out-planting phase. E = *Endozoicomonas*, P = *Pseudomonas*, Syn = *Synechococcus*, Sph = *Sphingomonas*, Myx = *Myxococcus*.

### *2.8.2 Supplementary Tables*

| **Pairwise comparisons over time.** | | | | | | |
|---|---|---|---|---|---|---|
| **pairs** | **df** | **Sum of Squares** | **F** | **$R^2$** | ***p-value*** | ***p*adj value** |
| *A.m* Reef $T_{125d}$ vs *A.m* Reef $T_{7d}$ | 1 | 0.60781 | 1.687 | 0.3 | 0.125 | 0.375 |
| *A.m* Reef $T_{7d}$ vs *A.m* Reef $T_{0d}$ | 1 | 0.50555 | 1.575 | 0.3 | 0.25 | 0.375 |
| *A.m* Nurs $T_{0d}$ vs *A.m* Nurs $T_{125d}$ | 1 | 0.50608 | 1.889 | 0.2 | 0.027 | 0.0442 5* |
| *A.m* Nurs $T_{0d}$ vs *A.m* Nurs $T_{7d}$ | 1 | 0.41671 | 1.387 | 0.2 | 0.083 | 0.0834 |
| *A.m* Nurs $T_{125d}$ vs *A.m* Nurs $T_{7d}$ | 1 | 0.63032 | 2.461 | 0.3 | 0.03 | 0.0442 5* |
| *A.m* Reef $T_{125d}$ vs *A.m* Reef $T_{1d}$ | 1 | 0.49381 | 0.193 | 1.4 | 0.125 | 0.375 |
| *A.m* Reef $T_{125d}$ vs *A.m* Reef $T_{30d}$ | 1 | 0.57415 | 0.234 | 1.5 | 0.25 | 0.375 |
| *A.m* Reef $T_{1d}$ vs *A.m* Reef $T_{30d}$ | 1 | 0.42411 | 0.215 | 1.4 | 0.375 | 0.375 |
| *A.m* O-P $T_{0d}$ vs *A.m* O-P $T_{1d}$ | 1 | 0.74497 | 2.692 | 0.3 | 0.031 | 0.0466 5* |
| *A.m* O-P $T_{0d}$ vs *A.m* O-P $T_{30d}$ | 1 | 0.73546 | 2.427 | 0.3 | 0.03 | 0.0466 5* |
| *A.m* O-P $T_{1d}$ vs *A.m* O-P $T_{30d}$ | 1 | 0.34162 | 0.959 | 0.1 | 0.488 | 0.4881 |
| *P.v* Reef $T_{7d}$ vs *P.v* Reef $T_{125d}$ | 1 | 0.44221 | 0.198 | 1.5 | 0.375 | 0.5 |
| *P.v* Reef $T_{125d}$ vs *P.v* Reef $T_{0d}$ | 1 | 0.74307 | 0.317 | 2.3 | 0.125 | 0.375 |
| *P.v* Reef $T_{7d}$ vs *P.v* Reef $T_{0d}$ | 1 | 0.31817 | 0.171 | 1 | 0.5 | 0.5 |
| *P.v* Nurs $T_{0d}$ vs *P.v* Nurs $T_{125d}$ | 1 | 0.65208 | 2.39 | 0.3 | 0.057 | 0.0857 1 |
| *P.v* Nurs $T_{0d}$ vs *P.v* Nurs $T_{7d}$ | 1 | 0.62263 | 2.075 | 0.3 | 0.057 | 0.0857 1 |
| *P.v* Nurs $T_{125d}$ vs *P.v* Nurs $T_{7d}$ | 1 | 0.26435 | 0.805 | 0.1 | 0.626 | 0.6365 |
| *P.v* Reef $T_{0d}$ (a.k.a $T_{125d}$) vs *P.v* Reef $T_{1d}$ | 1 | 0.23554 | 0.134 | 0.9 | 0.125 | 0.1875 |
| *P.v* Reef $T_{0d}$ (a.k.a $T_{125d}$) vs *P.v* Reef $T_{30d}$ | 1 | 0.52463 | 0.21 | 1.6 | 0.375 | 0.375 |
| *P.v* Reef $T_{30d}$ vs *P.v* Reef $T_{1d}$ | 1 | 0.75129 | 0.312 | 2.7 | 0.125 | 0.1875 |
| *P.v* O-P $T_{0d}$ vs *P.v* O-P $T_{1d}$ | 1 | 0.14035 | 0.45 | 0.1 | 0.973 | 0.9727 |
| *P.v* O-P $T_{0d}$ vs *P.v* O-P $T_{30d}$ | 1 | 0.31582 | 0.99 | 0.2 | 0.486 | 0.5934 1 |
| *P.v* O-P $T_{1d}$ vs *P.v* O-P $T_{30d}$ | 1 | 0.2729 | 0.835 | 0.1 | 0.514 | 0.5934 1 |

| **Comparisons of individual time points.** | | | | | |
|---|---|---|---|---|---|
| **pairs** | **df** | **Sums of Squares** | **F Model** | **$R^2$** | ***p-value*** |
| *A.m* Reef $T_{0d}$ vs *A.m* Nurs $T_{0d}$ | 1 | 0.32597 | 1.007 | 0.2 | 0.514 |
| *A.m* Reef $T_{7d}$ vs *A.m* Nurs $T_{7d}$ | 1 | 0.40523 | 1.38 | 0.2 | 0.057 |
| *A.m* Reef $T_{125d}$ vs *A.m* Nurs $T_{125d}$ | 1 | 0.68923 | 2.211 | 0.3 | 0.028* |
| *A.m* Reef $T_{0d}$ (a.k.a $_{125d}$) vs *A.m* O-P $T_{0d}$ | 1 | 0.68923 | 2.211 | 0.3 | 0.028 * |
| *A.m* Reef $T_{1d}$ vs *A.m* O-P $T_{1d}$ | 1 | 0.45872 | 1.48 | 0.2 | 0.171 |
| *A.m* Reef $T_{30d}$ vs *A.m* O-P $T_{30d}$ | 1 | 0.54052 | 1.478 | 0.2 | 0.086 |

| | | | | | |
|---|---|---|---|---|---|
| *P.v* Nurs $T_{0d}$ vs *P.v* Reef $T_{0d}$ | 1 | 0.22325 | 0.792 | 0.2 | 1 |
| *P.v* Nurs $T_{125d}$ vs *P.v* Reef $T_{125d}$ | 1 | 0.28762 | 0.939 | 0.1 | 0.486 |
| *P.v* Nurs $T_{7d}$ vs *P.v* Reef $T_{7d}$ | 1 | 0.18331 | 0.573 | 0.1 | 0.913 |
| *P.v* Nurs $T_{0d}$ vs *P.v* Reef $T_{0d}$ | 1 | 0.28762 | 0.939 | 0.1 | 0.488 |
| *P.v* O-P $T_{1d}$ vs *P.v* Reef $T_{1d}$ | 1 | 0.2857 | 1.098 | 0.2 | 0.34 |
| *P.v* O-P $T_{30d}$ vs *P.v* Reef $T_{30d}$ | 1 | 0.27787 | 0.804 | 0.1 | 0.6 |

**Table S2.1.** Results for pairwise permutational multivariate analysis of variance (PERMANOVA) of beta diversity indices (Bray-Curtis) for *Acropora millepora* (*A.m*) and *Pocillopora verrucosa* (*P.v*) coral-associated bacterial communities of nursery fragments (Nurs) over time, source colonies (Reef) over time, out-planted fragments (O-P) over time with false discovery rate (FDR) *p*-value adjustment for multiple comparisons, and PERMANOVA of Bray-Curtis indices of coral-associated bacterial communities of nursery fragments and source colonies compared at individual time points and source colonies and out-planted fragments at individual time points. * Indicates significant *p*-value or adjusted *p*-value < 0.05.

| **Kruskal-Wallis** | | | | |
|---|---|---|---|---|
| **Comparisons** | **Chao1** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| *A. m* nursery vs source | 1 | 5.28 | 5.345 | 0.02078* |
| *P. v* nursery vs source | 1 | 1.116 | 1.116 | 0.2908 |
| | **Inverse Simpson** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| *A.m* nursery vs source | 1 | 2.455 | 2.455 | 0.1172 |
| *P.v* nursery vs source | 1 | 1.79 | 1.79 | 0.1809 |
| | **Shannon** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| *A.m* nursery vs source | 1 | 3.938 | 3.938 | 0.0472* |
| *P.v* nursery vs source | 1 | 1.269 | 1.269 | 0.2599 |
| **Inverse Simpson** | | | | |
| *A.m* out-plant vs 125 day nursery vs source | **Kruskal-Wallis** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| | 2 | 7.714 | 7.714 | 0.02113* |
| | **Dunn's post-hoc *p*-value** | | | |
| | source | source - | nursery 1 | out-plant 0.009773* |
| | nursery | 1 | - | 0.073 |
| | out-plant | 0.009773* | 0.073 | - |
| | **Kruskal-Wallis** | | | |

| | | | | |
|---|---|---|---|---|
| *P.v* out-plant vs 125 day nursery vs source | df | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| | 2 | 0.3238 | 0.3253 | 0.8499 |
| | **Dunn's post-hoc *p*-value** | | | |
| | | source | nursery | out-plant |
| | source | - | 0.7941 | 0.6872 |
| | nursery | 0.7941 | - | 0.5805 |
| | out-plant | 0.6872 | 0.5805 | - |
| **Shannon** | | | | |
| *A.m* out-plant vs 125 day nursery vs source | **Kruskal-Wallis** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| | 2 | 9.129 | 9.129 | 0.01042* |
| | **Dunn's post-hoc *p*-value** | | | |
| | | source | nursery | out-plant |
| | source | - | 0.7842 | 0.006963 |
| | nursery | 0.7842 | - | 0.03144* |
| | out-plant | 0.006963* | 0.03144* | - |
| *P.v* out-plant vs 125 day nursery vs source | **Kruskal-Wallis** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| | 2 | 6.539 | 6.539 | 0.03802* |
| | **Dunn's post-hoc *p*-value** | | | |
| | | source | nursery | out-plant |
| | source | - | 0.02553* | 0.6168 |
| | nursery | 0.02553* | - | 0.0158* |
| | out-plant | 0.6168 | 0.0158* | - |
| **Chao1** | | | | |
| *A.m* out-plant vs 125 day nursery vs source | **Kruskal-Wallis** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| | 2 | 8.27 | 8.278 | 0.01594* |
| | **Dunn's post-hoc *p*-value** | | | |
| | | source | out-plant | nursery |
| | source | - | 0.2182 | 0.2959 |
| | out-plant | 0.2182 | - | 0.01853* |
| | nursery | 0.2959 | 0.01853* | - |
| *P.v* out-plant vs 125 day nursery vs source | **Kruskal-Wallis** | | | |
| | **df** | **H (chi-squared)** | **Hc (tie corrected)** | ***p*-value** |
| | 2 | 5.849 | 5.849 | 0.05368 |
| | **Dunn's post-hoc *p*-value** | | | |
| | | source | nursery | out-plant |
| | source | - | 0.06186 | 1 |
| | nursery | 0.06186 | - | 0.1182 |
| | out-plant | 1 | 0.1182 | - |

**Table S2.2.** Results for Kruskal-Wallis (and Dunn's post-hoc with Bonferroni p-value adjustment for multiple comparisons) analysis of alpha diversity indices for coral-associated bacterial communities of *Acropora millepora* (*A.m*) and *Pocillopora verrucosa* (*P.v*) nursery fragments compared to source colonies and out-planted fragments compared to source colonies and 125day nursery fragments.

| Kruskal-Wallis | | | | |
|---|---|---|---|---|
| **Alpha Diversity** | **df** | **chi-squared** | ***p*-value** | |
| Chao1 | 1 | 1 | 0.5484 | |
| Inverse Simpson | 1 | 1 | 0.3682 | |
| Shannon | 1 | 2.297 | 0.1296 | |
| **PERMANOVA** | | | | |
| **df** | **Sums of Squares** | **F Model** | **$R^2$** | ***p*-value** |
| 1 | 2.813271 | 8.119702 | 0.10134 | 0.0004* |

**Table S2.3.** Results from Kruskal-Wallis analysis of alpha diversity indices of coral-associated bacterial communities between species *Acropora millepora* and *Pocillopora verrucosa* (with pooled time points and treatments) and permutational multivariate analysis of variance of beta diversity (Bray-Curtis) indices of coral-associated bacterial communities between species (with pooled time points and treatments).

### *2.8.3 Supplementary Data*

The supplementary data files from the publication have, where possible, been supplied as supplementary tables however following supplementary data files are within *Supplementary file1* online at doi.org/10.1007/s00338-021-02207-6

**Data S6** Full legend for relative abundance plot of Figure 2.5 and 2.9.

**Data S7** Full legend for relative abundance plot of Figure S2.7 and S2.9.

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# Chapter 3: Bacterial communities associated with corals out-planted on the Great Barrier Reef are inherently dynamic over space and time.

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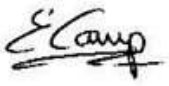
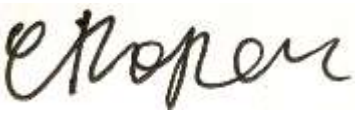
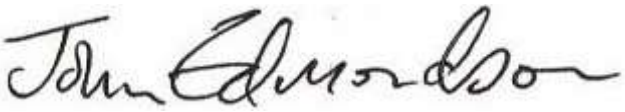
Emma Camp: 

Christine Roper: 

John Edmondson: 

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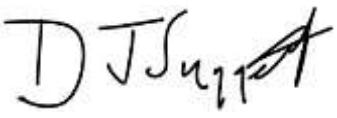
David Suggett: 

## 3.1 Abstract

Coral propagation and out-planting are becoming commonly adopted as part of reef stewardship strategies aimed at improving reef resilience through enhanced natural recovery and rehabilitation. The coral microbiome has a crucial role in the success of the coral holobiont and can be impacted shortly after out-planting. However, long-term characterisation of the out-plant microbiome in relation to out-plant survival, and how these properties vary across reef sites is unexplored. Therefore, at three reef sites on Opal Reef, Great Barrier Reef (Mojo, Sandbox and Rayban, 16°12'18" S 145°53'54" E) we examined bacterial communities associated with out-planted *Acropora millepora* coral and monitored coral survival over 12 months (February 2021-22). Bacterial communities of out-planted corals exhibited significant changes from donor colonies 7 days to 1.5 months after out-planting. Further, bacterial community composition differed for sites Sandbox and Rayban with low overall survival (0-43 %) versus Mojo with higher overall survival (47-75 %). After initial dissimilarity in bacterial communities of out-plants across sites at 1.5 months, and despite changes within sites over time, out-plants exhibited similar microbial communities across sites at 7 days and 6, 9 and 12 months. We hypothesise these trends reflect how bacterial communities are shaped by rapid changes in local environmental characteristics (e.g., from source to out-planting site), where out-plant bacterial communities 'conform' to out-planting site conditions. After initial changes, out-plant bacterial communities may then be under the influence of global environmental conditions – such as annual trends in temperature across seasons. Such outcomes indicate the importance of site-selection in shaping initial coral bacterial communities and subsequent out-plant success. Importantly, continued differences in out-plant survival trajectory but similar bacterial communities across sites after 1.5 months indicate that other factors – apart from bacterial community changes – likely govern out-plant success in the longer term. Our research highlights the

need to resolve drivers of small-scale site differences alongside higher resolution spatiotemporal monitoring of environmental conditions to distinguish key drivers of (i) microbial change during out-planting and (ii) out-plant survival to subsequently inform out-plant site selection to optimise future restoration efforts.

## 3.2 Introduction

Increasingly frequent and intense anthropogenic disturbances threaten the persistence of tropical coral reefs and hence the wealth of ecological, economic, social and heritage values they provide (Hughes et al. 2017; Eakin et al. 2019; Eddy et al. 2021; Sully et al. 2022). Future survival of coral reef ecosystems is primarily reliant on mitigation of climate change to reduce global stress from ocean warming, acidification, and deoxygenation (Kleypas et al. 2021; Shaver et al. 2022). However, parallel application of strategic, active management approaches will be pivotal for sustaining resilience of coral reef ecosystems and safeguarding against future climate conditions (Duarte et al. 2020; Kleypas et al. 2021; Shaver et al. 2022). Increasing application of active management approaches has accelerated over the last two decades in almost all tropical coral-reef-associated regions, whereby coral propagation and out-planting techniques have been utilised to rapidly increase coral biomass and cover at degraded or high-value reef sites (Boström-Einarsson et al. 2020; Hein et al. 2021), including on the Great Barrier Reef (Howlett et al. 2022; McLeod et al. 2022).

The ultimate success of coral propagation and out-planting rests on the capacity to rapidly up-scale and optimise approaches (Boström-Einarsson et al. 2020, McAfee et al. 2021, Suggett and van Oppen 2022). Whilst the readiness of successful implementation of different active management approaches is variable (Suggett and van Oppen, 2022), in-water coral propagation and out-planting has become one of the most promising for local and targeted recovery or rehabilitation (Williams et al. 2019, Hein et

al. 2020, Suggett et al. 2019). Even so, the long-term survival of corals during the propagation and out-planting process is highly variable, ranging from < 5% to > 90% (Boström-Einarsson et al. 2020; Ware et al. 2020, Suggett et al. 2019, 2020). Many factors can contribute to success in coral out-planting such as out-planting technique (method of attachment), coral species, fragment size, reef area (back-reef, fore-reef vs reef crest) and geographical subregion (Bayraktarov et al. 2016; van Woesik et al. 2021; Boström-Einarsson et al. 2018; Suggett et al. 2019), but these are often site and context specific. Consequently, investigating how environmental factors and biological factors of the holobiont interact to influence out-plant survival is critical to long-term success of active management approaches that are increasingly necessary to ensure the long-term viability of reef ecosystems (Hein et al. 2020).

Coral microbiomes are an essential component of the coral holobiont (Bourne et al. 2016; Peixoto et al. 2017; Voolstra et al. 2021). However, only recently has the coral microbiome been considered as an important element in the success of active management approaches (van Oppen and Blackall, 2019; Moriarty et al. 2020; Peixoto et al. 2021; Voolstra et al. 2021; Strudwick et al. 2022). For example, coral species can display community restructuring or stability during long-term (6 months) nursery propagation and subsequent early out-planting (1 day-1 month) (Strudwick et al. 2022). However, the longer-term dynamics of the bacterial communities associated with out-planted coral after relocation to the reef environment – and hence the role of bacterial communities influencing the success of newly out-planted coral – remains unexplored. Addressing such gaps may be critical where coral diseases have potential to impact survival during propagation and out-planting efforts (Moriarty et al. 2020). In fact, how microbes impact the survival of coral may be particularly important at degraded reef sites that are often targeted for out-planting, where there is potential for copiotrophic microorganisms, many of which are known coral pathogens, to be present in higher

abundances (Dinsdale et al. 2008; Kelly et al. 2014; Haas et al. 2016; Silveira et al. 2017).

Opal Reef, on the northern Great Barrier Reef (GBR), has been a site of intensive propagation and out-planting activity since the 2018 initiation of the Coral Nurture Program, a research-led reef stewardship approach (Howlett et al. 2022). Relatively high survivorship (typically > 80 %) has been recorded for *Acropora* spp., however survivorship remains highly variable across sites ranging from 79.8 - 100 % (Suggett et al. 2020; Howlett et al. 2022). Considering that environmental conditions are known to drive bacterial community shifts (Kelly et al. 2014; McDevitt-Irwin et al. 2017; Maher et al. 2019; Camp et al. 2020) we hypothesised that (i) out-planted fragments will undergo changes in the bacterial community composition after out-planting, and that (ii) these changes will differ between reef sites. Furthermore, given that associated bacterial communities are essential for coral host health (Bourne et al. 2016; Peixoto et al. 2017; Voolstra et al. 2021), we hypothesise that (iii) shifts in coral-associated bacterial communities are likely to reflect differences in survivorship. To test these hypotheses and inform future out-planting efforts, over 12 months we examined temporal dynamics of coral-associated bacterial communities and survivorship of *Acropora millepora* fragments out-planted across three sites on Opal Reef (northern GBR) characterised by different defining features and environmental conditions.

## 3.3 Materials and Methods

### *3.3.1 Sampling location and experimental design*

To examine differences in coral-associated bacterial communities amongst coral fragments out-planted at each reef site with contrasting characteristics, we performed a transplantation experiment using the coral species *A. millepora*. This species is routinely grown in coral nurseries and used for out-planting on the GBR (e.g., Howlett et al.

2022) and exhibits microbiome variability over time and space during propagation in coral nurseries at Opal Reef (Strudwick et al. 2022). Experiments were conducted at three sites across Opal Reef (16°12'18"S 145°53'54"E), which is a 24.7 $km^2$ reef situated on the northern GBR (detailed in Suggett et al. 2019, Howlett et al. 2021) (Fig. 3.1.). Each site had contrasting characteristics: (i) "Mojo", subject to strong tidal currents due to its close proximity to a deep-water channel leading to the coral sea at the north of Opal Reef; (ii) "Rayban", is not subject to strong currents due to its central location at Opal Reef within a sheltered sandy lagoon area (see Suggett et al. 2019; Howlett et al. 2021), and (iii) "Sand Box", is adjacent to a channel on the southern edge of Opal reef and consequently has elements of both sites with some sheltered sandy lagoons and mild currents (Edmondson personal obs.).

In February 2021, coral fragments (~ 5-10 cm) were harvested from five established *A. millepora* nursery colonies at Rayban for out-planting across five separate 'plots' within each of the three sites (Fig. 3.2a. and S3.2.). Five nursery colonies (~ 40 cm diameter) were selected from two adjacent established (2 years) nursery frames. From each donor colony, ~ 60 fragments (standardised to sizes between 5-10 cm) were harvested. Two fragments (≤ 5 cm) were randomly selected after the donor colony was fragmented and were retained to capture any potential heterogeneity in bacterial communities across the colony (Marchioro et al. 2020; Damjanovic et al. 2020) – in a sterile zip-lock bag, returned to the operations vessel and preserved in RNAlater for microbiome characterisation (defined as '$T_F$' – time of fragmentation). The other 58 fragments were held in wire trays on the nurseries at Rayban for 24-48 h – to enable identification of any fragments that exhibited mortality from fragmentation and also because of sporadic access to the three sites prior to out-planting; specifically, corals out-planted at Rayban were held for 24 h, whilst those out-planted at Sandbox and Mojo were held for 48 h prior to out-planting. At each reef site, 11-23 fragments

from each donor were out-planted within five separate plots (Fig. 3.2a-b. and Table S3.1.). Out-planting plots were approximately 4-5 m apart and within an area of ≥ 2 $m^2$ (Fig. S3.2.) and marked with cattle tags to identify the donor colony they were planted from. Each plot satisfied four pre-requisites: (i) bare consolidated structurally sound substrate (i.e., not rubble or sand), (ii) absence of algal turfs, (iii) located outside of damselfish territories, (iv) presence of other coral growing within a 1-2 m radius. Coralclip® (a stainless-steel spring-clip that is fastened using a hammer and masonry nail) was used to fasten each fragment to the substrate (as per Suggett et al. 2020). At the time of initial out-planting (24 h: $T_{O\text{-}1}$ and 48 h: $T_{O\text{-}2}$) two fragments (≤ 5 cm) from each donor colony were retained and preserved in the same manner as for $T_F$ (above) for microbiome characterisation (Fig. 3.2a.). This was to ensure adequate representation of the fragments at time of out-planting, and to assess whether the bacterial communities changed from time of fragmenting ($T_F$) to time of out-planting ($T_{O\text{-}1}$ and $T_{O\text{-}2}$). Once all fragments were out-planted, photographs were taken of the entire plot and the out-plants were counted. Fragment survivorship and coral-associated bacterial communities of out-planted fragments were then tracked over 12 months.

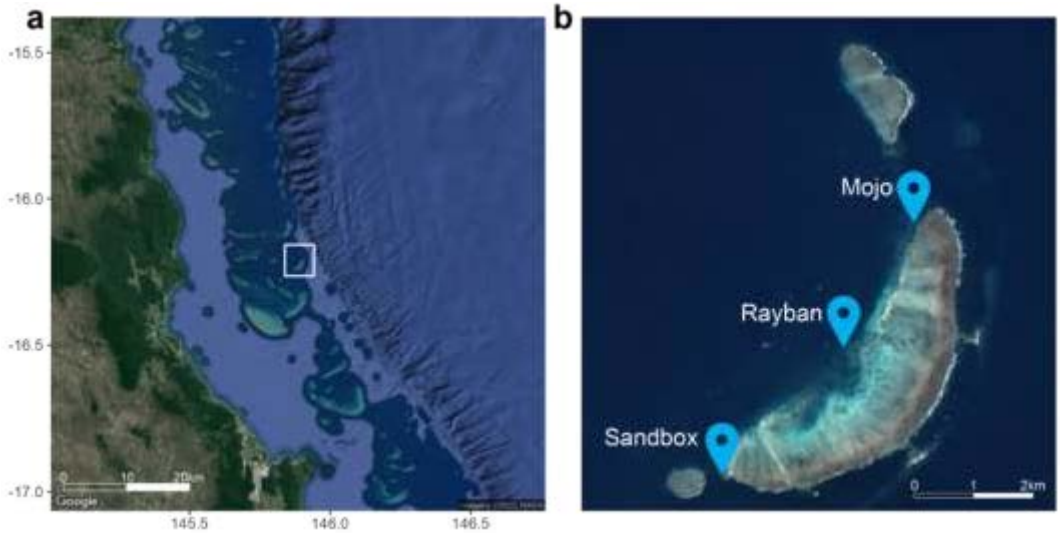



**Figure 3.1.** (**a**) Satellite image of Opal Reef, northern Great Barrier Reef, Australia, (**b**) Opal Reef with relative locations of the three reef sites where fragments were out-planted during the study. Satellite image sourced from GoogleEarth and

allencoralatlas.org respectively.

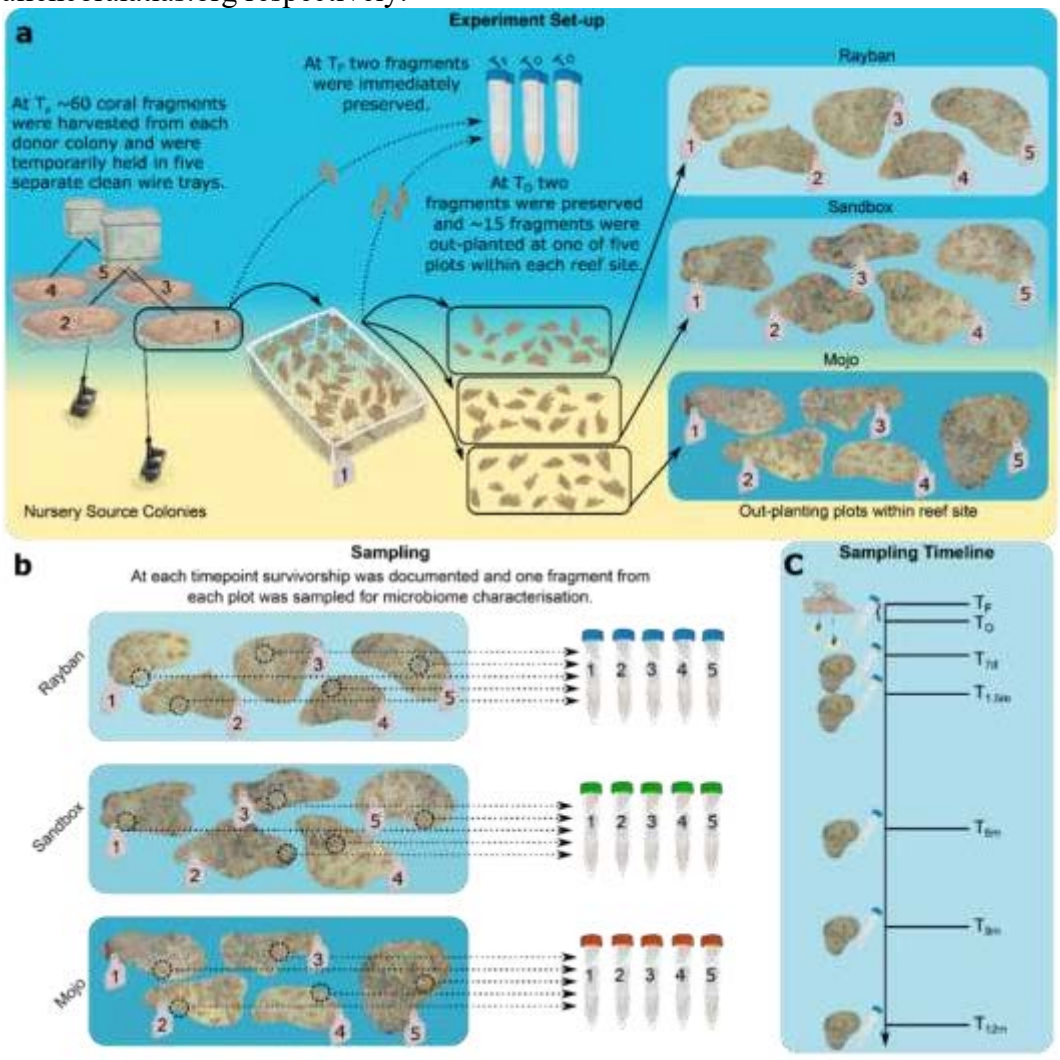



**Figure 3.2.** (**a**) Experimental set up: illustrating the process of fragmentation (TF) for one donor colony from the nursery through to out-planting ($T_{O\text{-}1}$ at 24hrs and $T_{O\text{-}2}$ at 48hrs) at one plot within the three reef sites. This was repeated for the remaining four donor colonies. (**b**) Sampling: illustrating the process of one sampling time point, at each reef site the five out-plant plots were sampled five times* over a 12-month period. (**c**) Timeline: illustrating the points at which sampling occurred, at $T_{F/O}$ only the donor colonies from the nursery frames were sampled and at subsequent sampling points only out-plants were sampled $T_{7d\text{-}12m}$. *Unless there was total mortality at the plot prior to completion of the experiment.

### *3.3.2 Coral sample preservation and DNA Extraction*

Following $T_F$, $T_{O\text{-}1}$ and $T_{O\text{-}2}$, one out-plant was sampled from each plot and site (5 plots x 3 sites = 15 fragments total) at seven days ($T_{7d}$), 1.5 months ($T_{1.5m}$), six months ($T_{6m}$), nine months ($T_{9m}$) and 12 months ($T_{12m}$) (Fig. 4b-c.). At each time point,

fragments (≤ 5 cm) were subsampled from coral out-plants using wire clippers. After sampling, all fragments were returned to the operations vessel (*Wavelength IV*) in sterile zip-lock bags within 30-50 min, placed into sterile 15 mL falcon tubes and preserved by total submersion in RNA*later*. All samples were subsequently held at ambient temperature for 6 days during transportation from the study site to the laboratory for processing (as per Strudwick et al. 2022). One donor colony sample was compromised during transit and consequently one replicate for donor colony five at $T_F$ proceeded to subsequent DNA extraction and sequencing. Once in the laboratory, RNA*later* was thoroughly removed from 15 mL falcon tubes using an adjustable pipette with sterile tips, after which samples were preserved at –80 °C for 1-11 months and DNA extractions were all conducted at the same time. Prior to DNA extraction, coral tissue was removed from the coral skeleton, using an air brushing technique. Coral fragments were thawed on ice in their respective 15 mL falcon tubes, removed from the falcon tube using sterile forceps, rinsed with autoclaved phosphate-buffered saline (PBS) (3X, pH 7.4) to remove RNA*later* residue, placed in sterile zip-lock bags and air brushed with sterile pipette tips into 4 mL of autoclaved PBS (3X, pH 7.4). The tissue slurry was divided across two 2 mL micro centrifuge tubes and centrifuged at 8000 rpm for 5 min. The supernatant was removed, and the pellet was stored at –80 °C for 4-6 weeks until DNA extraction. DNA was extracted from approximately 100 µL of the pellet using a DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol (July 2020 version) with a total elution volume of 40 µL. Extractions were conducted in randomised batches of 23 with one kit negative sample included. Extracted DNA was quality checked and the concentration was quantified using a NanoDrop spectrophotometer.

### *3.3.3 Environmental logging*

Upon initiation of the experiment, temperature, light attenuation and flow at each reef site were measured using HOBO™ loggers for a 14-day period to identify potential site-specific differences in key environmental factors that can influence coral growth and survivorship. Due to site access, sensors could only be deployed for a 14-day period. Whilst this is a relatively short timeframe, it allowed a spring-neap cycle to be captured to identify relative differences across the sites. Light attenuation was measured by securing two temperature/light data loggers (HOBO Pendant® UA-002-64), positioned 1 m apart onto a PVC pipe with metal brackets and vertically suspended the structure in the water column. Each logger was secured in a horizontal orientation so that the light sensors were parallel to the surface and did not shade one another (Fig. S3.1a-b.). Light sensors were routinely cleaned (approximately every three days) to prevent biofouling. Light and temperature data were recorded hourly and temperature readings were taken from only the bottom data logger on the PVC pipe set-up. All temperature/light data loggers were inter-calibrated with data from a 4 h period of logging at 10-min intervals in the same position and conditions. Average temperature and light intensity of the three loggers was calculated at each 10-min interval. Relative error was calculated for individual loggers at every 10-min interval during the calibration period. Light and temperature readings recorded at each site during the 14-day monitoring period were then adjusted by their logger's average relative error calculated during the calibration. Light attenuation was calculated using the following equation (Kirk, 1994):

$$k = Ln(l_a) - Ln(l_b)$$

$l_a$ is the PAR recorded by the deepest logger and $l_b$ is the PAR recorded by the shallower logger (separated by a 1 m interval), and $k$ is the attenuation coefficient ($m^{-1}$).

Flow was recorded using a G data logger (HOBO Pendant® UA-004-64) that was assembled into a tilting current metre (based on Crookshanks, 2008, see Supplementary Appendix 3.1. for detailed description): The tilting current meters were cross-calibrated and flow readings ranged from low (0) to high (1), as calibration to a known flow rate ($m/s^{-1}$) was not possible (Fig. S3.1f.). To inter-calibrate tilting current metres, for a 24 h period all tilting current metres were secured to an *in situ* nursery frame within 30 cm of each other (Fig. S3.1c.) with G data loggers recording at 20-min intervals. Average flow was calculated at each 20-min interval. Relative error was calculated for individual tilting current metres at every 20-min interval during the calibration period. Recordings from each site during the 14-day monitoring period were then adjusted by their logger's average relative error calculated during the calibration (calibrated flow, temperature and light attenuation data provided as Supplementary Data S1.).

### *3.3.4 Environmental sampling and DNA extraction*

Water and substrate samples were collected to characterise environmental bacterial communities prior to introduction of coral material and were only collected at time of initial out-planting, as such it was not possible to characterise any potential variability in substrate and water communities over time. Water samples for microbiome characterisation were collected from the surface at each reef site using 10 L pre-sterilised plastic containers and filtered in triplicate through 47 mm, 0.22 µm pore-size membrane filters (Millipore, DURAPORE PVDF 0.22 µm WH PL) using a peristaltic pump (100 rpm), within 10 min of sample collection. Before each sample was filtered, 250 mL 10% bleach was run through the pump, followed by 500 mL Milli-Q water, and then 3 L of sample. Filters were stored in cryovials, snap frozen and stored in a dewar for transport back to the laboratory where they were stored at –80 °C. DNA was extracted from the membrane filters using the PowerWater DNA isolation Kit (QIAGEN) as per the manufacturer's protocol (June 2016 version). DNA extractions

were performed alongside three kit blanks, which were subsequently included in all sequencing analysis to exclude kit contaminants.

Substrate samples for microbiome characterisation were also collected using a hammer and chisel to leverage a 2 cm x 2 cm x 2 mm piece of substrate off each plot into a sterile zip lock bag. Following sampling, all substrate samples were preserved, stored and transported to the laboratory in the same manner as coral samples. Once in the laboratory samples were preserved at 4 °C for 11 months until RNA*later* was thoroughly removed from 15 mL falcon tubes using an adjustable pipette with sterile tips, after which DNA was extracted. DNA was extracted from approximately 100 µg of the substrate sample using a DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol (July 2020 version) with a total elution volume of 40 µL. Extractions were performed alongside one kit negative sample and the kit negative sample was included in subsequent sequencing analysis to exclude kit contaminants. All extracted DNA was quality checked and the concentration was quantified using a NanoDrop spectrophotometer.

Overall, 126 samples were collected for bacterial community analysis: (i) out-plants: three sites x three-five plots (some plots experienced total mortality) x five time points ($n$ = 64), (ii) donor colonies: two fragments x five donor colonies x $T_F$ *plus* two fragments x five donor colonies x two out-planting times $T_O$ ($n$ = 30), (iii) environmental: three sites x three replicate water samples (n = 9) and three sites x five plots x one substrate sample (n = 15) *plus* eight x DNA extraction blank samples.

### *3.3.5 Quantification of coral out-plant survival*

Coral survival was recorded at each sampling time point ($T_{7d}$, $T_{1.5m}$, $T_{6m}$, $T_{9m}$, $T_{12m}$) and through additional opportunistic observations at 35, 40, 167, 344, and 346 days after out-planting. Survival was defined as the number of fragments still alive in the out-plant plot at each time interval as a percentage of the number out-planted. Corals

in the out-plant plot observed with < 5 % live tissue were counted as 'dead', whilst fragments either missing or dislodged from the Coralclip® were defined as 'detached'. Corals sampled for preservation at time of survival counts were included in 'live' counts. Both dead and detached coral fragments were considered as 'lost' and excluded from survival counts.

### *3.3.6 16S rRNA amplicon sequencing*

Extracted DNA was stored at –80 °C for two weeks prior to 16S rRNA amplicon sequencing, which was used to characterise the composition and diversity of coral, sediment, water and kit negative bacterial communities. The hypervariable V3 and V4 regions of the bacterial 16S rRNA gene were amplified using the primers 341F (5'–CCTAYGGGRBG-CASCAG-3') and 805R (5'–GACTACHVGGGTATC-TAATCC-3') (Klindworth et al. 2013), prior to sequencing on the Illumina MiSeq platform (Ramaciotti Centre for Genomics (Sydney, NSW, Australia)). Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA929655.

### *3.3.7 Bioinformatics*

Raw demultiplexed sequencing data were analysed with Quantitative Insights into Microbial Ecology (QIIME 2, version 2020.6) platform (Callahan et al. 2016). The DADA2 plugin was used to denoise the data (Callahan et al. 2016) and taxonomy was assigned using the classify-sklearn classifier (Pedregosa et al. 2011) against the SILVA v138 database. In total 9,156,785 reads were generated (after denoising) from 126 samples. Amplicon sequence variants (ASVs) corresponding to chloroplast or mitochondria were filtered from the data set. 18 ASVs that comprised 51 % of sequences in the DNA extraction negative controls and have been previously reported as contaminants of laboratory reagents (Weyrich et al. 2019), were removed for subsequent analyses using the filter command in R (version 4.2.2). Prior to diversity

analyses, four samples were removed from the data set due to poor sequencing outputs leading to low read numbers after quality filtering and contaminant removal (< 900 reads or < 58 ASVs). As a result of filtering and complete mortality at some plots, six time points had three biological replicates and one time point had four biological replicates, all other time points had five biological replicates. For beta diversity analyses, the raw read ASV table was converted to relative abundances, scaled to 20,000 (McKnight et al. 2019) and square root transformed.

### *3.3.8 Statistical analysis*

Differences in bacterial community structure and dispersion (beta diversity patterns) of out-planted corals between reef sites at each time point (reef site = fixed effect), between donor colonies at time of fragmenting ($T_F$) and out-planted corals over the 12-month time period within a site and over time – from 7 days to 1.5 months ($T_{7d}$-$T_{1.5m}$), 1.5 months to 6 months ($T_{1.5m}$-$T_{6m}$), 6 months to 9 months ($T_{6m}$-$T_{9m}$) and 9 months to 12 months ($T_{9m}$-$T_{12m}$) – were analysed using the Bray–Curtis dissimilarity distance metric. Differences in bacterial community structure of water and substrate between reef sites were also analysed using the Bray Curtis dissimilarity metric. Permutation tests for homogeneity in multivariate dispersion (PERMDISP) of coral-associated bacterial community were calculated using the *betadisper* function of the '*vegan*' R package. Patterns in bacterial community structure were visualised using non-metric multidimensional scaling (nMDS) plots. Differences in community structure were tested for significance with pairwise permutational multivariate analysis of variance (PERMANOVA; perm = 999) of Bray–Curtis dissimilarities using the *pairwise.adonis* function of *vegan* and differences in community dispersion were tested for significance – with site as a single factor and with pairwise comparisons between sites at each time point – using the *permutest.betadisper* function of *vegan* (perm = 999), *p*-values were adjusted by applying a Benjamini and Hochberg (a.k.a. False

Discovery Rate) correction, all $p_{adj}$ values < 0.05 were considered significant. When significant differences in the bacterial community structure were identified similarity percentage analysis (SIMPER) was used in PAST 4.03 to identify and calculate the percentage contribution of each ASV to dissimilarity between groups, ASVs contributing > 1.5 % dissimilarity were reported. The *core_members* function of the '*microbiome*' R package was used to identify core bacterial community members (present in > 80 % samples with relative abundance > 0.1 %) for out-planted fragments over time and at time of fragmenting ($T_F$).

Kaplan-Meier survival curves were estimated for out-plant survival at each site. To test for differences in survival between sites we conducted a pairwise log-rank test, *p*-values were adjusted by applying Bonferroni adjustment. To assess differences between sites in flow, attenuation and temperature during the 14-day monitoring period each variable was analysed separately, and sites were compared via a Kruskal-Wallis tests to assess significance, when significant ($p < 0.05$) a Dunn's post hoc test was applied with subsequent Bonferroni adjustment, all $p_{adj}$ values < 0.05 were considered significant. To qualitatively describe overall similarity of sites (from the recorded parameters) a dissimilarity matrix of the mean temperature, light attenuation and flow was used (Table S3.2.).

## 3.4 Results

### *3.4.1 Site-specific environmental characteristics and microbial communities*

Flow rates, light attenuation and temperature differed between the three reef sites during the 14-day monitoring period (Kruskal-Wallis test, $p < 0.001$, Table S3.3.). All sites exhibited different variance for flow, light attenuation and temperature (Dunn's post hoc, $p_{adj} < 0.001$, Table S3.3.). The largest range in light attenuation was at Mojo (1.54 $m^{-1}$), followed by Rayban and Sandbox (1.52 $m^{-1}$ and 1.11 $m^{-1}$ respectively, Fig.

S3.3b.), and highest mean light attenuation was at Mojo, followed by Rayban and Sandbox (0.415, 0.317 and 0.200 $m^{-1}$ respectively, Fig. S3.3b.). The largest range in flow was also observed at Mojo (1.20), followed by Sandbox and Rayban (1.13 and 0.58 respectively, Fig. S3.3a.) and similarly highest mean flow was observed for Mojo, followed by Sandbox and Rayban (0.455, 0.335 and 0.240 respectively, Fig. S3.3a.). The largest range in temperature was observed at Rayban (2.91 ˚C) followed by Mojo and Sandbox (2.41 ˚C and 1.91 ˚C respectively, Fig. S3.3c.). The highest mean temperature was observed at Sandbox (31.61 ± 0.42 ˚C) followed by Rayban (30.36 ± 0.54 ˚C) and Mojo (29.80 ± 0.44 ˚C) (Fig. S3.3c.). In summary, Mojo was characterised by the highest mean flow, the lowest water clarity (the highest mean light attenuation), the lowest mean temperature, and the largest range in temperature and flow. Sandbox had intermediate mean flow and the highest temperature, but the highest water clarity (lowest light attenuation), and the smallest range in light attenuation and temperature. Rayban had intermediate mean temperature, the lowest mean flow, intermediate water clarity and the highest range in temperature. Overall, Rayban and Mojo were more similar than Rayban and Sandbox based on a qualitative comparison of the recorded parameters (Table S3.2.).

Bacterial community composition differed between water and substrate within every site ($PERMANOVA_{Bray\text{-}Curtis}$ $p_{adj} < 0.05$, Fig. S3.4-5. and Table S3.4.*)*, reflecting predominantly different environmental bacterial communities. However, water samples were generally the same between sites ($PERMANOVA_{Bray\text{-}Curtis,}$ $p_{adj} > 0.05$, Table S3.4.*)* whereas substrate samples associated with Sandbox differed with those from Rayban ($PERMANOVA_{Bray\text{-}Curtis,}$ $F = 1.403$, $p_{adj} = 0.021$, Fig. S4-5., and Table S3.4.*)*. These differences in substrate bacterial communities between Rayban and Sandbox occurred in parallel with differences between the two sites in measured mean flow, temperature and light attenuation. However, there were no further differences in substrate bacterial

communities between sites despite flow, temperature and light attenuation also differing between Rayban and Mojo, and Sandbox and Mojo.

### *3.4.2 Coral-associated bacterial communities changed after out-planting across three reef sites*

Bacterial community composition associated with the donor colonies at time of fragmenting ($T_F$) was first compared to that at initial out-planting ($T_{O\text{-}1}$ and $T_{O\text{-}2}$) to account for any possible responses induced by the different periods of time with which fragmented material was held prior to out-planting ($T_{O\text{-}1}$: 24 h Rayban vs $T_{O\text{-}2}$: 48 h Sandbox, Mojo). No changes were observed from $T_F$ vs $T_O$ for any site (both $T_{O\text{-}1}$ and $T_{O\text{-}2}$) ($PERMANOVA_{Bray\text{-}Curtis}$, $p_{adj} < 0.05$, Table S3.7.), and hence subsequent comparisons between donor colonies and out-planted fragments were conducted against samples from time of fragmenting ($T_F$) to ensure consistency between sites. After out-planting bacterial communities associated with the corals at all sites became significantly different to those at time of fragmenting ($T_F$) this occurred 1.5 months after out-planting at Mojo and Rayban ($PERMANOVA_{Bray\text{-}Curtis}$, $F = 2.250$, $p_{adj} = 0.009$, $df = 1$; $F = 2.571$, $p_{adj} = 0.002$, $df = 1$ respectively), but after only 7 days at Sandbox ($PERMANOVA_{Bray\text{-}Curtis}$, $F = 2.501$, $p_{adj} = 0.017$, $df = 1$, Fig. 3.3a-c., Fig. 3.4. and Fig. S3.6a-c.). As such, shifts in bacterial community composition were more rapid at Sandbox compared to both Rayban and Mojo.

One 'core' ASV was present at time of fragmenting ($T_F$) and classified as a member of the *Endozoicomonas* genus (mean RA = 50.3 %). Following significant changes in the bacterial community of out-plants at 7 days and 1.5 months after out-planting (Sandbox and Mojo/Rayban respectively), the core ASV from the *Endozoicomonas* genus (identified at $T_F$) was only retained as a core ASV at Sandbox and Rayban and was completely lost from three of five replicates at Mojo and no longer classified as a core member in out-plants. In fact, while still classified as a core ASV the

*Endozoicomonas* ASV declined in relative abundance from time of fragmenting ($T_F$ mean RA = 50.3 %) after 7 days at Sandbox ($T_{7d}$ mean RA = 22.6 %) and 1.5 months at Rayban ($T_{1.5m}$ mean RA = 8.4 %) (Fig. S3.7.).

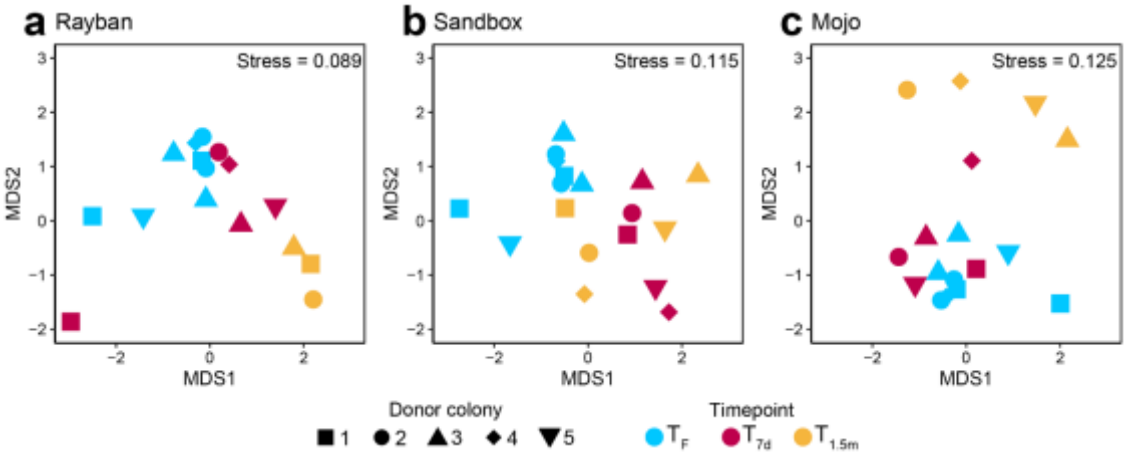



**Figure 3.3.** Bacterial community structure and relative dispersion of the microbial communities at time of fragmenting ($T_F$) and out-planted fragments at (**a**) Rayban, (**b**) Sandbox and (**c**) Mojo over time. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. Source data are provided as a Source data file.

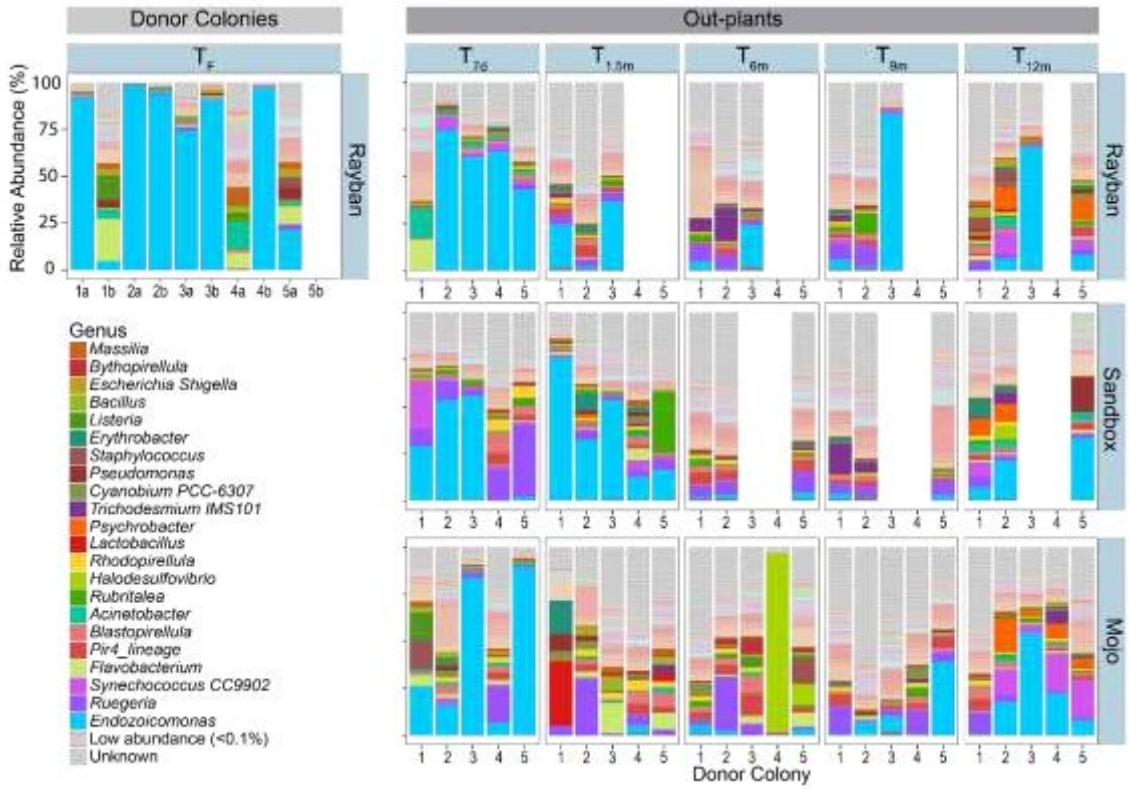



**Figure 3.4.** Bacterial community composition (relative abundances) by genus at time of fragmenting ($T_F$) and of out-planted fragments over the 12-month monitoring period across the three sites. Pastel colours represent genera with an average relative abundance of < 0.1% in all samples, full legend provided as supplemental data (Supplementary Data S4.).

### *3.4.3 Temporal dynamics of out-plant associated bacterial communities varied across sites*

At Mojo there were significant changes in the bacterial community structure from 7 days ($T_{7d}$) to 1.5 months ($T_{1.5m}$) post-out-planting after which the bacterial communities remained stable until a change in the out-plant bacterial community structure from 9 to 12 months ($T_{9m\text{-}12m}$) (PERMANOVA$_{Bray\text{-}Curtis}$, $p_{adj} = 0.031$, $F = 1.497$, $df = 1$, and $p_{adj} = 0.037$, $F = 1.432$, $df = 1$ respectively). Differences in bacterial communities of out-planted fragments at Mojo from 7 days to 1.5 months ($T_{7d}$-$T_{1.5m}$) were primarily explained by (> 1.5 % dissimilarity contribution) decreased relative abundance (RA) the ($T_F$) core *Endozoicomonas* ASV, increased abundance of 5 ASVs from the *Lactobacillus fermentum*, *Ruegeria*, *PseudoVibrio*, and *Erthryobacter* genera

and loss of an ASV from the *Tenacibaculum* genus (SIMPER, Table S3.8.). Differences in bacterial communities of out-planted fragments at Mojo from 9 to 12 months ($T_{9m}$-$T_{12m}$) were explained by increased RA of the ($T_F$) core *Endozoicomonas* ASV (Fig. S3.7.) and four ASVs from the *Endozoicomonas, Ruegeria*, *Psychrobacter*, and *Synechococcus_CC9902* genera and increased RA of an ASV from the *Hungateiclostridiaceae* family (SIMPER, Table S3.8.). Bacterial communities of out-planted fragments also exhibited restructuring over time at Rayban. At Rayban the coral-associated bacterial community structure of out-plants changed from 7 days ($T_{7d}$) to 1.5 months ($T_{1.5m}$), and from 1.5 months ($T_{1.5m}$) to 6 months ($T_{6m}$) after out-planting ($PERMANOVA_{Bray\text{-}Curtis}$, $p_{adj} = 0.032$, $F = 1.610$, $df = 1$, and $p_{adj} = 0.048$, $F = 1.247$, $df = 1$ respectively). There were no further changes in coral out-plant bacterial community structure after 6 months ($PERMANOVA_{Bray\text{-}Curtis}$, $p_{adj} > 0.05$). Similar to Mojo, differences in bacterial communities of out-planted fragments at Rayban from 7 days to 1.5 months ($T_{7d}$-$T_{1.5m}$) were also primarily explained by decreased relative abundance (RA) of the ($T_F$) core *Endozoicomonas* ASV and one other ASV from the *Endozoicomonas* genus (SIMPER, Table S3.8.). Differences in bacterial communities of out-planted fragments from 1.5 months to 6 months ($T_{1.5m}$-$T_{6m}$) at Rayban were explained by further decreases in RA of the two *Endozoicomonas* ASVs (Fig. S3.7.) and increases in RA of ASVs from the *Limnothrix, and Thrichodesmium_IMS101* genera (SIMPER, Table S3.8.). After the initial change in bacterial community structure from time of fragmenting ($T_F$) to 7 days after out-planting ($T_{7d}$) at Sandbox there were no further changes in the bacterial communities of out-planted coral fragments ($PERMANOVA_{Bray\text{-}Curtis}$, $p_{adj} > 0.05$, Fig. S3.6. and Table S3.7.). There were no changes in the dispersion of coral-associated bacterial communities for out-plants over the 12 months at any site (PERMUTEST, $p_{adj} > 0.05$, Fig. S3.8. and Table S3.9.).

### *3.4.4 Out-plant survival and associated bacterial communities varied across sites*

Mean survival of out-plants at the final time point of sampling (12 months after out-planting) was higher at Mojo (mean 58.96 % ± 1.72, n = 5) than Rayban and Sandbox (31.75 % ± 6.25, n = 4 and 32.66 % ± 7.31, n = 3). Throughout the experiment, mean survival remained 3.85-32.59 % higher at Mojo compared to both other sites. Kaplan-Meier survival curves were significantly different between Mojo and Rayban (Pairwise log-rank, $p_{adj} < 0.001$) and Mojo and Sandbox (Pairwise log-rank test, $p_{adj} < 0.0001$, Fig. 3.5a.). Bacterial community composition of out-planted corals only significantly differed for Mojo vs Rayban and Mojo vs Sandbox at 1.5 months ($T_{1.5m}$) ($PERMANOVA_{Bray\text{-}Curtis}$, $F = 1.346$, $p_{adj} = 0.048$, $df = 1$; $F = 1.232$, $p_{adj} = 0.048$, $df = 1$ respectively) at all other time points there were no differences across sites in the structure of the coral-associated bacterial communities ($PERMANOVA_{Bray\text{-}Curtis}$ $p_{adj} > 0.05$, Fig. 3.5b. and Table S3.7.). Differences in bacterial communities of out-planted fragments between sites were primarily explained by higher RA at Sandbox and Rayban compared to Mojo of two ASVs from the genus *Endozoicomonas* (one of which was from the 'core' microbiome characterised at $T_F$), as well as a lower relative abundance at Sandbox and Rayban of ASVs from the *Lactobacillus*, *Ruegeria*, *Erythrobacter* and *PseudoVibrio* genera compared to Mojo (SIMPER, and Table S3.8.). The out-plant associated bacterial communities were more heterogenous at Mojo compared to out-plants at Rayban at 1.5 months and 6 months (PERMUTEST, $p_{adj} < 0.05$, Fig. S3.8. and Table S3.9.) and compared to out-plants at Sandbox at 6 months and 9 months (PERMUTEST, $p_{adj} < 0.05$, Fig. S3.8. and Table S3.9.).

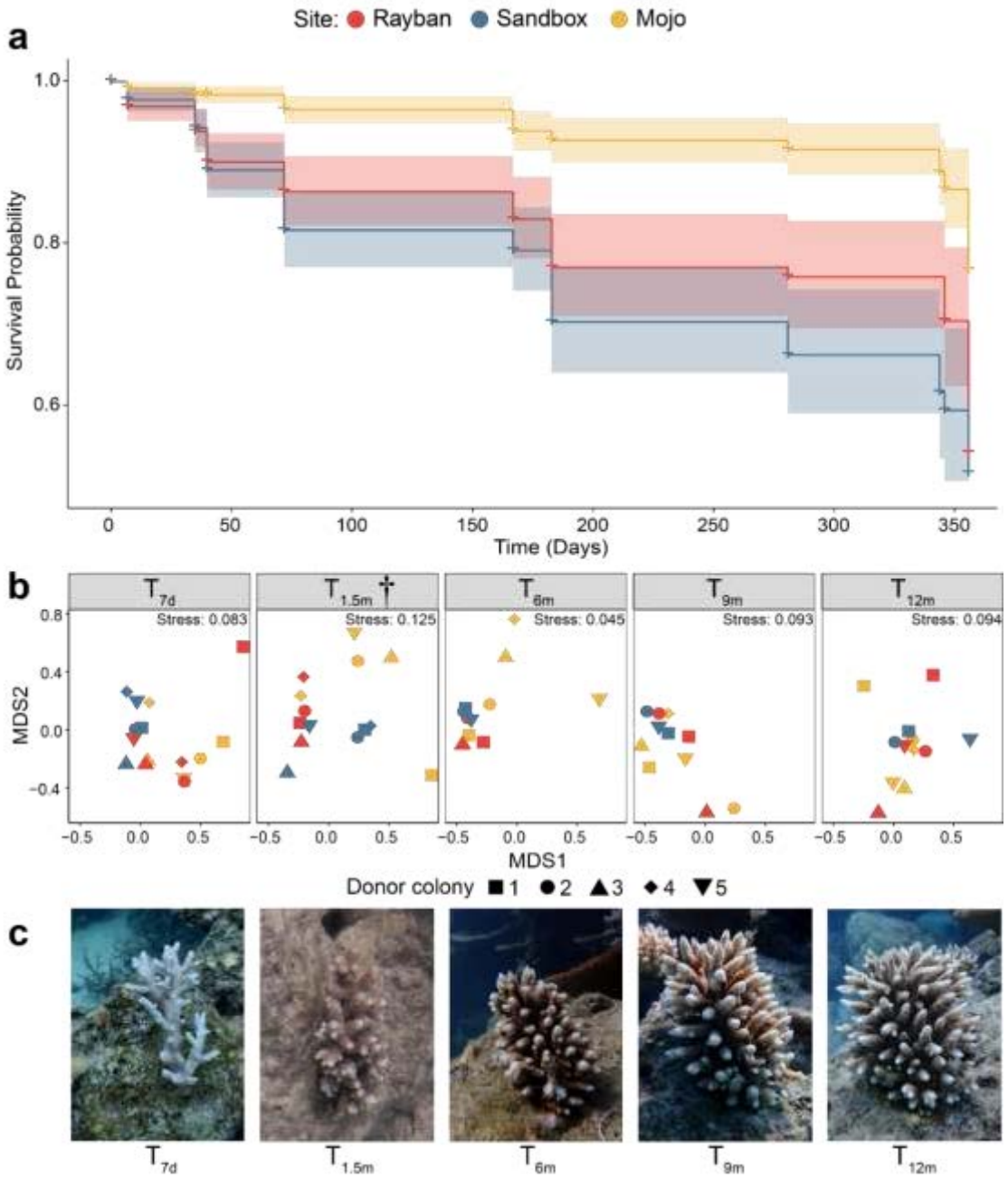



**Figure 3.5. (a)** Kaplan-Meier survival curve for out-plants at Mojo, Sandbox and Rayban, **(b)** bacterial community structure and relative dispersion of the microbial communities of out-planted fragments at Mojo, Sandbox and Rayban at individual time points and **(c)** photographs showing growth of a singular tracked fragment over time. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. Source data are provided as Supplementary Data S5. † Indicates a significant difference in microbial community structure between Mojo and Rayban, and Mojo and Sandbox ($PERMANOVA_{Bray\text{-}Curtis}$, $p_{adj} < 0.05$).

## 3.5 Discussion

Active reef management approaches that include the translocation of corals within reef systems to enhance natural recovery are expanding on the Great Barrier Reef (McLeod et al. 2022; Howlett et al. 2022) and worldwide (Boström-Einarsson et al. 2020). However, some evidence indicates that coral-associated bacterial communities are impacted by transplantation (Casey et al. 2015; Ziegler et al. 2019; Haydon et al. 2021), as well as nursery-based propagation and out-planting (Strudwick et al. 2022) and it has been proposed that these changes are driven by exposure to novel environmental conditions between propagation sites (Strudwick et al. 2022). Even so, to date there has been little quantification and comparison of long-term out-plant survival and associated bacterial community composition across reef sites with contrasting environmental conditions on the GBR. Most studies have only quantified survival of out-planted *Acroporid spp* (van Woesik et al. 2018, 2021; Howlett et al. 2022) or monitored bacterial communities of out-planted corals for short periods of time (1-30 days, Strudwick et al. 2022). Here we show that on the northern GBR reef environmental conditions, out-plant survival, and coral-associated bacterial communities all exhibit inter-site variability. Specifically, *A. millepora* fragments from known donor colonies out-planted across three diverse reef sites, exhibited different survival trajectories and different rates of change of associated bacterial communities to site-specific compositions. We discuss the variability and potential interaction of environmental conditions, survival and coral-associated bacterial communities, and hence the importance of integrating these factors into future planning, initiation, and monitoring of active reef management approaches to optimise success.

### *3.5.1 Site differences in out-plant survival*

Reef rehabilitation practitioners face the challenge of highly variable survivorship of coral propagules with limited understanding of which factors primarily

influence survival (e.g., Caribbean, Young et al. 2012; Lirman et al. 2014, and Great Barrier Reef, Suggett et al. 2020; Howlett et al. 2022). Such a core gap in knowledge represents a fundamental road-block to successfully scaling propagation and out-planting protocols (Ware et al. 2020; Hein et al. 2020; Boström-Einarsson et al. 2020). Differences in the survival of corals transplanted between environments during management interventions potentially stem from factors including site selection (e.g., prevailing environmental conditions and corallivore presence) (Pausch et al. 2018; Ware et al. 2020), coral-associated bacterial community composition (Moriarty et al. 2020), as well as coral species, coral size, and attachment type (Yap, 2004; Goergen et al. 2018; Munasik et al. 2020). In our current study, we standardised coral species and attachment type, and fragment size to explore the potential role of coral-associated bacterial communities and environmental conditions between sites. At the start of the experiment ($T_F$), we observed differences in flow, light attenuation, and temperature across the study sites. Overall coral survival was generally higher at the site with historically the lowest recent impact ("Mojo", Edmondson pers. obs.; see also Roper et al. 2022) that was initially characterised by higher flow rates, light attenuation, and lower temperatures. Such factors have previously been described to promote resilience of corals at other sites (low temperature, Mediterranean - Rubio-Portillo et al. 2014; high flow, Palmyra Atoll - Rogers et al. 2016; low temperature and increased light attenuation/heterotrophy, Red Sea - Tremblay et al. 2016; increased light attenuation/heterotrophy, Caribbean, Indian and Pacific Ocean - Fox et al. 2018); however, it is unknown if initial environmental differences between sites were consistent throughout the experiment. Importantly, out-planting of *Acropora* spp. at Rayban has previously yielded 70-100 % survival (mixed species assemblages after 3-7 months, Suggett et al. 2020; *A. gemnifera*, *A. intermedia*, *A. spathulata* after 11 months; Howlett et al. 2022) in contrast to the 0-35 % survival after 12 months observed across

plots within the same site in our current study. Further, similar bacterial community composition and low overall survival were observed at sites Rayban and Sandbox (0-43 %) despite different initial environmental conditions (flow, temperature, and light attenuation), indicating the need for finer resolution spatiotemporal monitoring of environmental conditions (e.g., flow, temperature, light, pH, dissolved oxygen, substrate composition, fish communities, and corallivore communities) and associated bacterial communities within-sites as well as between sites with differential out-plant survival. Resolving the relationships between environmental conditions and bacterial community change in out-plant plots (1-30 $m^2$) and out-planted corals respectively and identifying differentiating factors between sites with low versus high out-plant survival will be instrumental in improving the success of restoration approaches through informed site-selection.

### *3.5.2 Spatiotemporal dynamics of out-plant bacterial communities*

Environmental heterogeneity is a suggested driver of host-microbiome composition (Dunphy et al. 2019) with nutrient gradients (Kelly et al. 2014), temperature variability (Littman et al. 2009; Santos et al. 2014; van Oppen and Blackall, 2019), fish communities, (McDevitt-Irwin et al. 2017), pH and oxygen variability (Camp et al. 2020; Haydon et al. 2021) all contributing and sometimes interacting synergistically towards reshaping coral-associated microbial communities (Maher et al. 2019). In our current study we observed variability in the bacterial community composition of *A. millepora* after out-planting at three reef sites. This is consistent with previous observations for this genus, where transplanting corals between impacted sites and control sites (Ziegler et al. 2019), between mangrove and reef areas (Haydon et al. 2021) and specifically at Opal Reef previously during propagation and out-planting over 125 and 30 days (respectively) led to bacterial community changes (Maher et al. 2019; Strudwick et al. 2022). Interestingly, in our study we observed bacterial community

changes in out-planted coral at different times across sites. Whilst it remains unknown what specifically influences the rate of bacterial community change in corals over time, in other host-microbiome relationships, such as human-gut microbiome, responses to extreme changes in extrinsic factors (e.g., diet and exposure to foreign substances) can occur within days, whereas responses to mild changes in extrinsic factors occur within weeks to months (Uhr et al. 2019; Schlomann and Parthasarathy, 2019). In the case of our study the rate of bacterial community changes was variable between reef sites – 7 days versus 1.5 months – suggesting the site where changes occurred more rapidly likely had the most different environmental conditions compared to the donor site. Interestingly, in relation to the environmental variables measured in this study, the site where bacterial communities changed within 7 days was more dissimilar to the donor site compared to the site where bacterial communities changed within 1.5 months. It is important to note there are likely variations in other environmental conditions (that we did not measure) such as pH (Zhang et al. 2015), algal exudates (Smith et al. 2006; Barott et al. 2012) and fish communities (Ezzat et al. 2019) between the donor and out-plant sites contributing to the site-specific rate of change and subsequent composition of coral-associated bacterial communities. It is integral to identify these contributing factors so restoration practitioners can estimate the likelihood of potential impacts to coral biology when transplanting coral fragments between sites with contrasting environmental conditions.

### *3.5.3 Potential links between microbial communities and out-plant survival*

Associated bacterial communities are well known to play a role in resilience of the coral holobiont to environmental change (Reshef et al. 2006; Ainsworth and Gates, 2016; Bourne et al. 2016; Glasl et al. 2016) – and hence the importance of considering the microbiome in reef management is integral (e.g., Voolstra et al. 2021). Certain bacteria have the capacity to play relevant roles in the coral holobiont functioning – and

these may confer resilience, resistance or susceptibility to specific biotic and abiotic stressors – however the mechanisms remain unresolved (Ben-Haim et al. 2003; Alagely et al. 2011; Bourne et al. 2016; Santos et al. 2016; Peixoto et al. 2017; Welsh et al. 2017; Rosado et al. 2019). Some species of *Acropora* thrive across a range of environmental conditions including those predicted to exist under future climate change scenarios found in mangrove lagoons (Camp et al. 2020) and can persist in these conditions in part due to specific bacterial community composition and/or changes in relative abundance of bacterial taxa in response to prevailing environmental conditions (Ziegler et al 2017, 2019). During our current study distinct bacterial community restructuring was observed in out-plants that was consistent with our previous study where out-plants were monitored for only one month at Rayban (Strudwick et al. 2022). However, we observed site-specific post-out-planting microbiome changes accompanied by variable survival across sites with contrasting site topography and environmental conditions, suggesting the bacterial community changes observed were not necessarily a beneficial response to the novel environmental conditions of the out-planting site; rather, such changes potentially reflect loss of microbial taxa providing essential functions or dysbiosis (microbiome imbalance), thereby contributing to mortality (Egan and Gardiner, 2016). Interestingly, increased heterogeneity of bacterial communities – previously suggested to indicate dysbiosis (Maher et al. 2019) – was paradoxically recorded at the site with the highest survival and no signs of dysbiosis were recorded at the sites with the lowest survival. We therefore suggest, that observed initial differences in out-plant bacterial communities across sites may reflect a response to the novel local-scale environmental conditions at the out-planting site, with subsequent similarities across sites (in the bacterial communities) representing a successive period in which bacterial communities are primarily shaped by larger-scale environmental conditions across sites – such as global trends in temperature (Sharp et

al. 2017; Cai et al. 2018) – and mortality is driven by other factors not measured in this study (e.g. corallivore presence) (Pausch et al. 2018).

Differences in bacterial community composition between the sites were explained by higher relative abundance of bacterial taxa (*Ruegeria, Lactobacillus, PseudoVibrio* and *Erythrobacter*) at the site with the highest survival (Mojo); these taxa have previously been linked to pathogen resistance (Karthikeyan and Santosh, 2008 - *Lactobacillus*; Kitamura et al. 2021; Miura et al. 2019; Rosado et al. 2019 – *Ruegeria*; Raina et al. 2016; - *PseudoVibrio;* Pereira et al. 2017 - *Erythrobacter*). However, differences in bacterial taxa between sites were also explained by higher relative abundance of *Endozoicomonas* at the sites with lower survival (Sandbox and Rayban). Such an outcome is perhaps counter-intuitive where *Endozoicomonas* has also been linked to putatively beneficial functions in coral (Neave et al. 2016; Tandon et al. 2020) and are usually observed to decrease in abundance during stress (van Oppen and Blackall, 2019), and hence contradictory to the higher mortality rates at these sites. Changes in the bacterial community structure of out-plants (at Mojo and Rayban) over time were also explained by fluctuations in the relative abundance of the core ASV from the *Endozoicomonas* genus and were consistent with previous observations of increased abundance of *Endozoicomonas* in February on the GBR (Epstein et al. 2019). We suggest similar changes in abundance of *Endozoicomonas* in out-plant bacterial communities across sites could indicate out-plant bacterial communities are not only influenced by local environmental conditions but also global trends in temperature and/or other environmental conditions. Putatively pathogenic bacteria were not recorded to proliferate in coral-associated bacterial communities at sites with lower out-plant survival. However, sampling dying coral where pathogens were likely to be present was not possible, as such we cannot confirm if a driver of out-plant mortality was microbial disease. Considering bacteria underpin the health and resilience of reef ecosystems

(Reshef et al. 2006; Rosenberg et al. 2007; Ainsworth and Gates, 2016; Bourne et al. 2016**;** van Oppen et al. 2019; van Oppen and Blackall, 2019) and that bacterial communities associated with out-plants at sites of high mortality differed from out-plants at sites with low mortality, our study reinforces the importance of considering bacterial communities in the suite of factors influencing survival of out-planted corals. Future investigations into the role *and* source of specific bacterial taxa associated with out-planted corals at sites with high survival versus low survival will improve understanding of site-selection for increased success.

### *3.5.4 Importance of integrating microbiome in planning, initiation, and monitoring of future active reef management*

For the first time we have shown long-term site-specific changes in coral-associated bacterial communities of out-planted fragments *alongside* differences in out-plant survival and environmental conditions within a reef. Our findings suggest that contrasting environmental conditions between sites of the same reef could have an important role in survival of out-planted corals and likely influence speed of change and composition of coral-associated bacterial communities. These findings emphasise that improved understanding of the mechanisms through which environmental conditions impact coral-associated bacterial communities, and how this in turn effects coral host health, is essential to guide optimisation of restoration activities through improved site selection; in particular, in terms of out-planting site relative to nursery propagation site. Although we did not observe proliferation of putative pathogens at sites with lower coral out-plant survival, it is critical to note that increased abundance of microbes with the potential to incite pathogenesis have been observed at degraded sites (Haas et al. 2016; Silveira et al. 2017) and depending on the mechanisms of bacterial community change or the inherent microbiome variability of the host coral species, corals out-planted at these sites could remain vulnerable to pathogenesis (Thurber et al. 2009; Sato

et al. 2016; Ziegler et al. 2019; Moriarty et al. 2020). On the contrary, coral species that have variable bacterial communities – such as *Acropora* spp. – may exhibit higher survival through bacterial community 'conforming' to the out-planting environment, compared to coral species that maintain stable bacterial communities potentially poorly suited to novel environments (Ziegler et al. 2019). Recent enthusiasm to use environmental DNA to improve reef monitoring methods (West et al. 2020; Richards et al. 2022) and probiotics during reef restoration (Peixoto et al. 2021) will have limited scope without thorough understanding of how microbial communities are influencing the health of rehabilitated reef ecosystems and *how* bacterial communities are influenced by active management processes. Therefore, it is critical to resolve the mechanisms of bacterial community change in out-planted corals, including through experiments involving systematic moderation of environmental conditions, to (i) clarify their role in out-plant mortality, (ii) identify whether findings hold across coral species and (iii) how the responses observed in this study translate across sites with varying degrees of degradation prior to expansion of activities into more degraded areas. Our results indicate that careful selection of appropriate out-planting sites has the potential to improve success of interventions, but it will only be possible to inform site selection by first thoroughly investigating the *role* of microorganisms in the survival/mortality of out-planted corals.

## 3.6 Declarations

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### *3.6.3 Data Availability*

Raw data files used for coral and environmental bacterial community analysis in this study have been deposited in FASTQ format in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA929655. All other data is provided as Electronic Supplementary Materials online at doi.org/10.1007/s00227-023-04235-y.

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## 3.8 Supplementary Materials

### *3.8.1 Supplementary Figures*

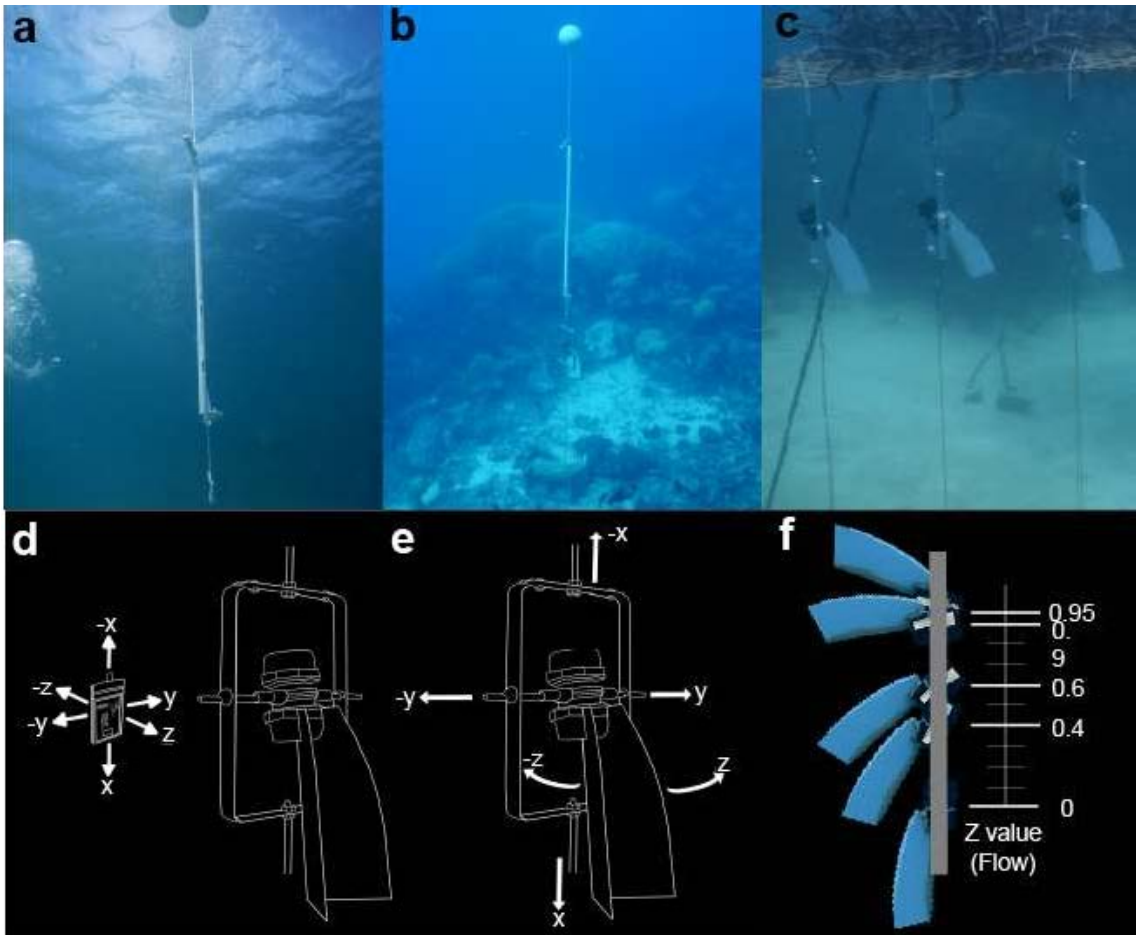


**Figure S3.1.** (**a**) Light attenuation measurement system in situ, (**b**) tilting current meter in situ (with light attenuation measurement system) (**c**) three tilting current meters in situ during inter-calibration (**d**) Illustration of the tilting current meter and orientation of the internal HOBO Pendant G logger with (**e**) associated planes of measurement (**f**) orientation of tilting current meter at neutral (0) with corresponding Z value ≤ 1 as flow increases.

**Figure S3.2.** Example of out-planting plot at Mojo.

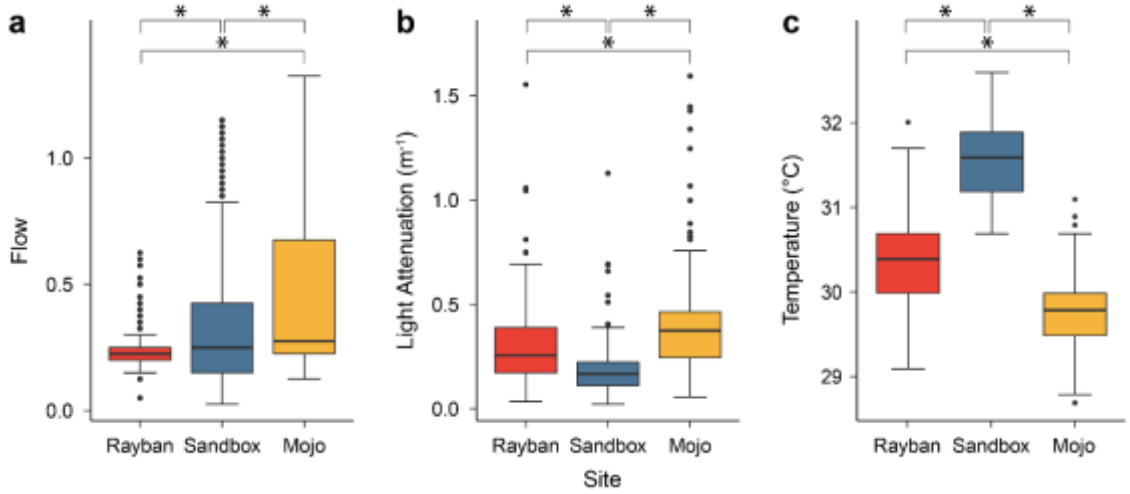


**Figure S3.3.** Flow (**a**), light attenuation (**b**) and temperature (**c**) at each out-planting site during initial two-week site profiling). Boxes represent the 25$^{th}$ and 75$^{th}$ percentile, centre lines show medians. The whiskers mark 1.5 times the inter-quartile range and the values beyond these upper and lower bounds are considered outliers, marked with dots. * = Wilcoxon rank sum test significance levels: $p_{adj} < 0.05$. Source data are provided in Supplementary Data S2.

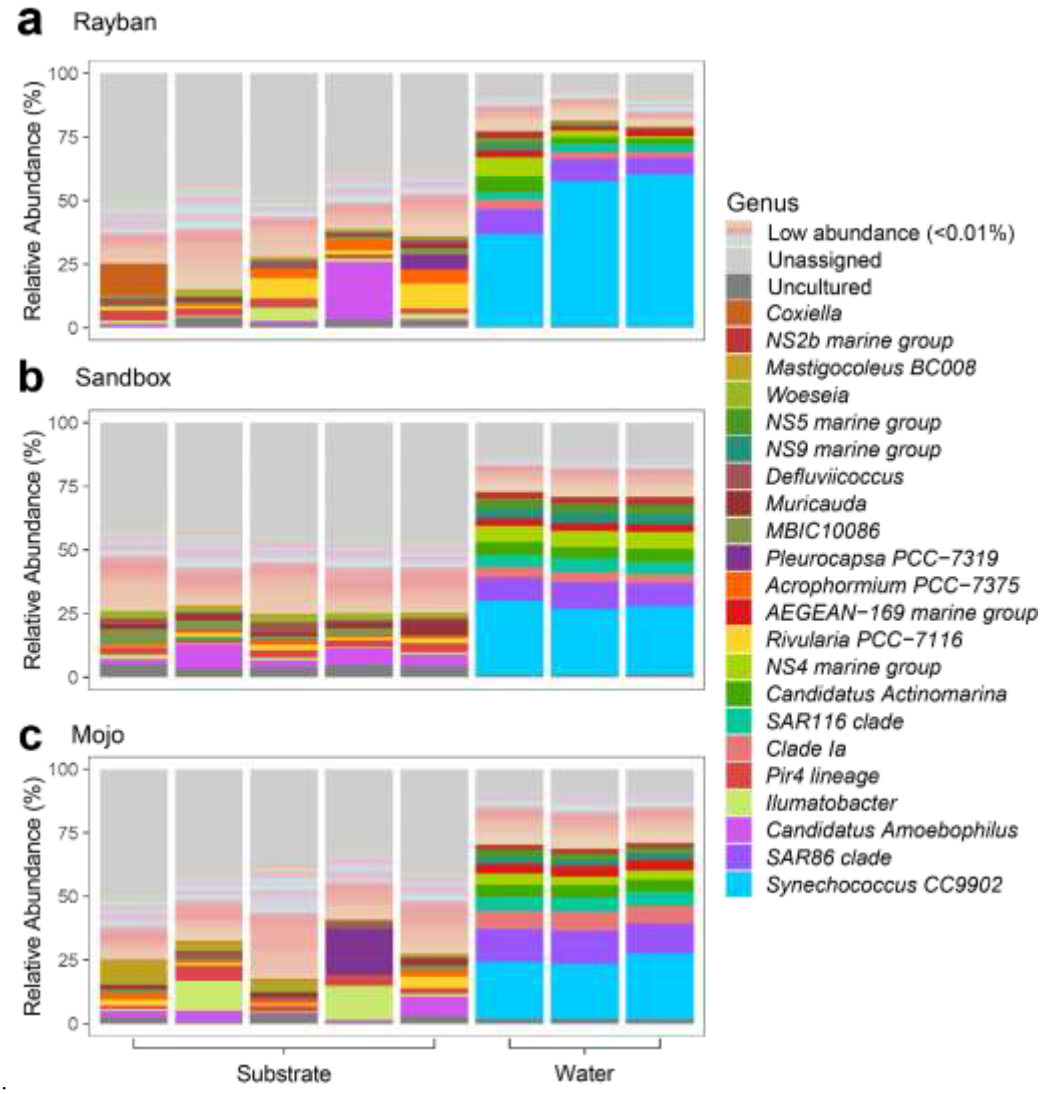


**Figure S3.4.** Bacterial community composition (relative abundances) by genus of water and substrate at the start of the experiment at (**a**) Rayban, (**b**) Sandbox and (**c**) Mojo. Pastel colours represent genera with an average relative abundance of < 0.1% in all samples, full legend provided as supplemental data (Supplementary Data S8.).

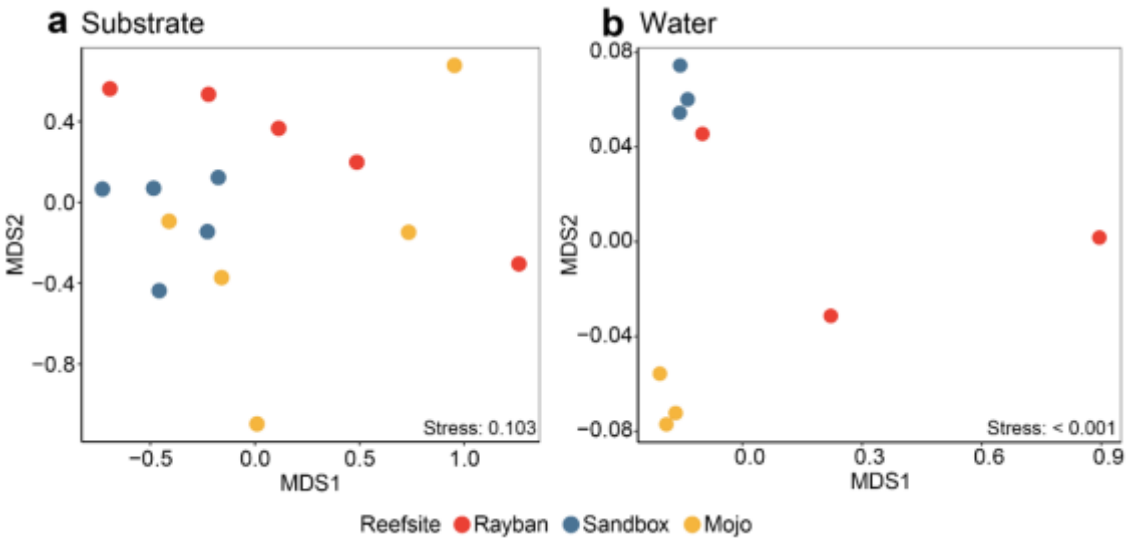


**Figure S3.5.** Bacterial community structure and relative dispersion of the microbial communities of (**a**) substrate and (**b**) water at the start of the experiment at Rayban, Sandbox and Mojo. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. Source data are provided as a Source data file.

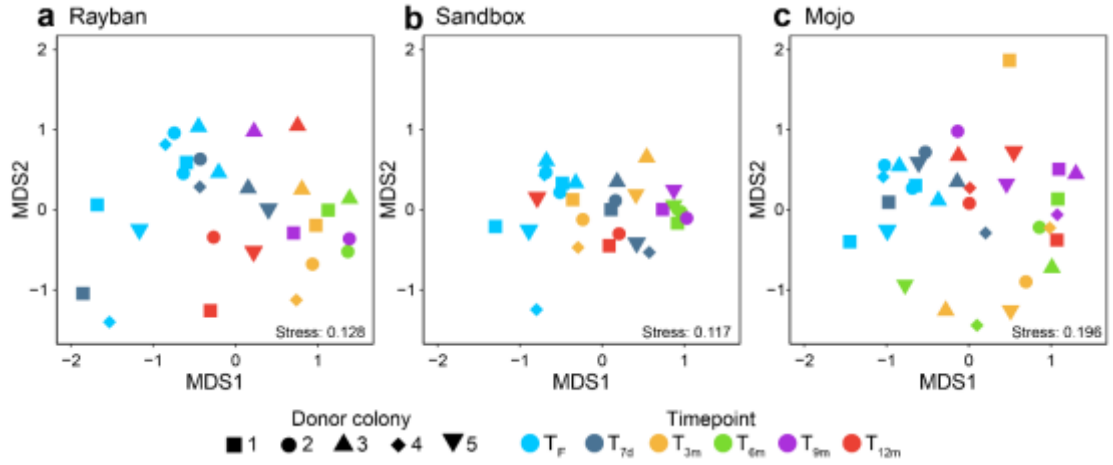


**Figure S3.6.** Bacterial community structure and relative dispersion of the microbial communities of the donor colonies ($T_{fragmenting}$) and out-planted fragments at (**a**) Mojo, (**b**) Sandbox and (**c**) Rayban over time. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure. Source data are provided as a Source data file.

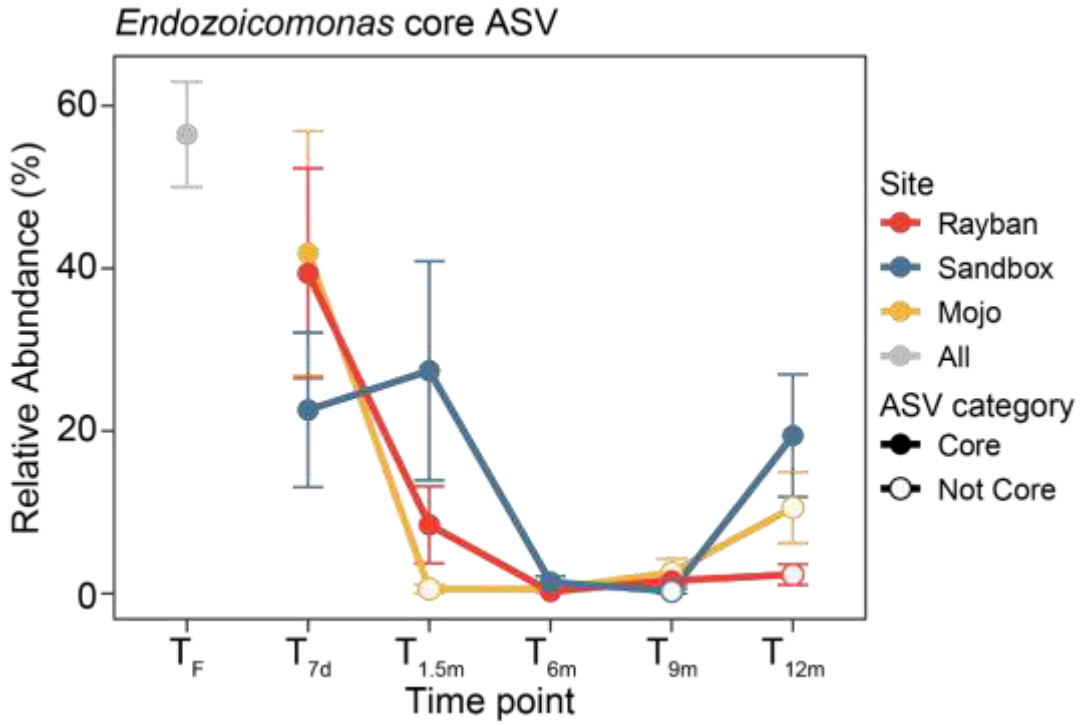


**Figure S3.7.** Relative abundance of the core ASV from the *Endozoicomonas* genus for *Acropora millepora* at time of fragmenting ($T_F$) and coral fragments out-planted at Rayban, Sandbox or Mojo over 12 months with filled circles indicating the ASV was classified as a core member of the bacterial community and empty circles indicating when the ASV was no longer classified as a core member.

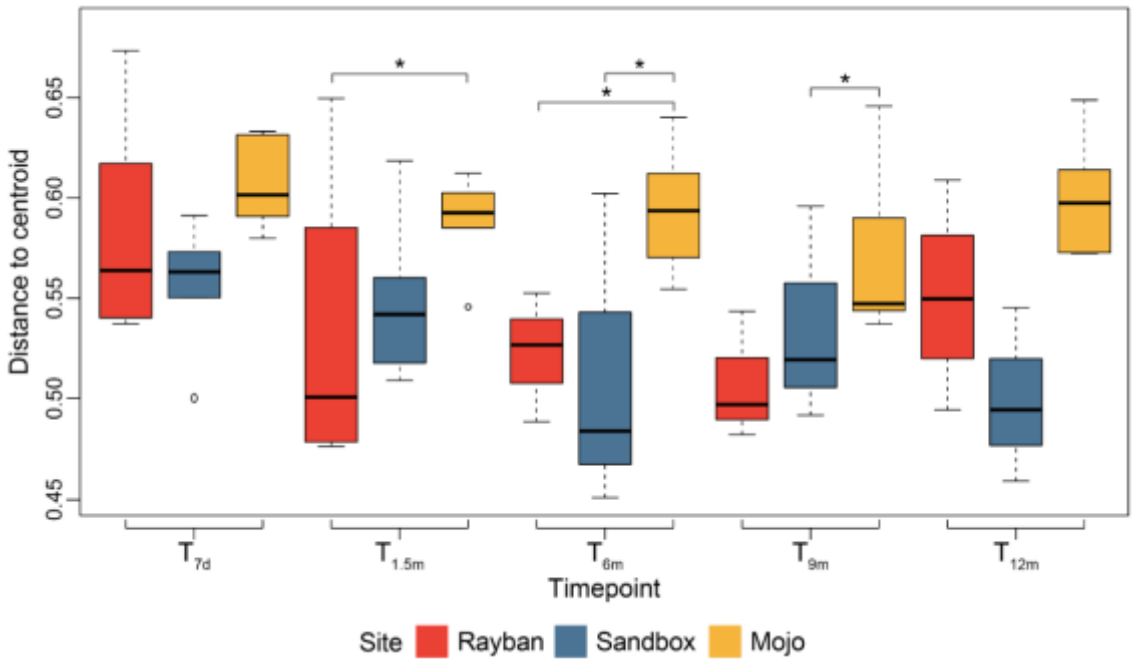


**Figure S3.8.** Bray-Curtis mean distance-to-centroid or heterogeneity of bacterial communities of out-plant *Acropora millepora* fragments by site over time. * denotes permutation test for homogeneity of multivariate dispersions post-hoc $p_{adj} < 0.05$.

### *3.8.2 Supplementary Tables*

| | Mojo | | | | | Rayban | | | | | Sandbox | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Plot/Donor colony number** | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** | ***1*** | ***2*** | ***3*** | ***4*** | ***5*** |
| **Number of out-plants** | 19 | 17 | 12 | 14 | 18 | 17 | 17 | 15 | 11 | 13 | 19 | 17 | 17 | 23 | 23 |

**Table S3.1.** Number of out-plants at each plot in each plot within the three reef sites.

| *Category of variable low-high.* | **Sandbox** | **Rayban** | **Mojo** |
|---|---|---|---|
| **Temperature** | High (31.61˚C) | Mid (30.36˚C) | Low (29.80˚C) |
| **Flow** | Mid (0.335) | Low (0.240) | High (0.455) |
| **Light Attenuation** | Low ($0.200m^{-1}$) | Mid ($0.317m^{-1}$) | High ($0.414m^{-1}$) |
| *Total difference to Rayban (source).* | *1.362* | - | *0.872* |

**Table S3.2.** Qualitative dissimilarity matrix of environmental parameters across reef sites organised according to mean value relative to Rayban (source site).

| | Variable | Min | Max | Range | Average | Coefficient of Variance | Standard Deviation |
|---|---|---|---|---|---|---|---|
| **Rayban** | **Temperature (°C)** | 29.132 | 32.046 | 2.914 | 30.364 | 0.018 | 0.539 |
| | **Light ($m^{-1}$)** | 0.0339 | 1.553 | 1.519 | 0.317 | 1.690 | 0.536 |
| | **Flow** | 0.05 | 0.625 | 0.575 | 0.240 | 2.236 | 0.536 |
| **Sandbox** | **Temperature (°C)** | 30.729 | 32.634 | 1.905 | 31.614 | 0.013 | 0.419 |
| | **Light ($m^{-1}$)** | 0.0219 | 1.128 | 1.107 | 0.200 | 2.082 | 0.416 |
| | **Flow** | 0.025 | 1.15 | 1.125 | 0.335 | 1.244 | 0.416 |
| **Mojo** | **Temperature (°C)** | 28.728 | 31.134 | 2.406 | 29.796 | 0.0148 | 0.440 |
| | **Light ($m^{-1}$)** | 0.0572 | 1.594 | 1.537 | 0.414 | 1.167 | 0.481 |
| | **Flow** | 0.125 | 1.325 | 1.2 | 0.455 | 1.125 | 0.512 |

| **Light Attenuation** | | | |
|---|---|---|---|
| **Kruskal-Wallis rank sum test** | | | |
| | **Chi squared** | **df** | ***p*-value** |
| Light Attenuation x Site | 73.198 | 2 | < 2.2e-16 |
| **Dunn's post-hoc (Bonferroni adjustment)** | | | |
| **comparison** | **z** | ***p*-value** | $p_{adj}$ **value** |
| Mojo-Rayban | 3.44177 | 2.89E-04 | 8.67E-04 |
| Mojo-Sandbox | 8.55404 | 5.94E-18 | 1.78E-17 |

| | | | |
|---|---|---|---|
| Rayban-Sandbox | 4.6218 | 1.90E-06 | 5.71E-06 |
| **Flow** | | | |
| **Kruskal-Wallis rank sum test** | | | |
| | **Chi squared** | **df** | ***p*-value** |
| Flow x Site | 191.31 | 2 | < 2.2e-16 |
| **Dunn's post-hoc (bonferroni adjustment)** | | | |
| **comparison** | **z** | ***p*-value** | $p_{adj}$ **value** |
| Mojo-Rayban | 13.4692 | 1.19E-41 | 3.56E-41 |
| Mojo-Sandbox | 9.45863 | 1.56E-21 | 4.68E-21 |
| Rayban-Sandbox | -4.01059 | 3.03E-05 | 9.09E-05 |
| **Temperature** | | | |
| **Kruskal-Wallis rank sum test** | | | |
| | **Chi squared** | **df** | ***p*-value** |
| Temperature x Site | 653.25 | 2 | < 2.2e-16 |
| **Dunn's post-hoc (Bonferroni adjustment)** | | | |
| **comparison** | **z** | ***p*-value** | $p_{adj}$ **value** |
| Mojo-Rayban | -9.23202 | 1.33E-20 | 3.98E-20 |
| Mojo-Sandbox | -25.2562 | 4.84E-141 | 1.45E-140 |
| Rayban-Sandbox | -16.0242 | 4.33E-58 | 1.30E-57 |

**Table S3.3.** Summary of environmental conditions at each out-planting sites and Kruskal-Wallis and Dunn's post-hoc test for statistical comparison of environmental conditions (flow, light attenuation and temperature) between sites.

| **Water** | | | | | | |
|---|---|---|---|---|---|---|
| **pairs** | **df** | **Sums of Squares** | **F Model** | **$R^2$** | ***p*-value** | **$p_{adj}$ value** |
| Mojo vs Rayban | 1 | 0.2072065 | 2.915205 | 0.42156 | 0.1 | 0.1 |
| Mojo vs Sandbox | 1 | 0.1031328 | 5.656184 | 0.58576 | 0.1 | 0.1 |
| Rayban vs Sandbox | 1 | 0.156822 | 2.306424 | 0.36573 | 0.1 | 0.1 |
| **Substrate** | | | | | | |
| **pairs** | **df** | **Sums of Squares** | **F Model** | **$R^2$** | ***p*-value** | **$p_{adj}$ value** |
| Mojo vs Rayban | 1 | 0.3832989 | 0.9900336 | 0.11013 | 0.4604 | 0.4604 |
| Mojo vs Sandbox | 1 | 0.4375223 | 1.2608048 | 0.13614 | 0.0598 | 0.0897 |
| Rayban vs Sandbox | 1 | 0.4843931 | 1.4025576 | 0.14917 | 0.007 | 0.0210 |
| **Water vs Substrate** | | | | | | |
| **pairs** | **df** | **Sums of Squares** | **F Model** | **$R^2$** | ***p*-value** | **$p_{adj}$ value** |
| Water_Mojo vs Substrate_Mojo | 1 | 1.2233556 | 4.5936634 | 0.43362 | 0.0199 | 0.03045 |
| Water_Rayban vs Substrate_Rayban | 1 | 1.122469 | 3.7757783 | 0.38624 | 0.0178 | 0.03045 |
| Water_Sandbox vs Substrate_Sandbox | 1 | 1.3614968 | 6.5289257 | 0.52111 | 0.0153 | 0.03045 |

**Table S3.4.** Permutational multivariate analysis of variance (PERMANOVA) pairwise test results comparing structure of water and substrate bacterial communities between and within sites. Adjusted *p*-value = FDR correction.

| Site | Time (Days) | n.risk | n.event | survival | std.err | lower 95% CI | upper 95% CI |
|---|---|---|---|---|---|---|---|
| Mojo | 1 | 595 | 0 | 1 | 0 | 1 | 1 |
| | 30 | 515 | 5 | 0.992 | 0.00374 | 0.984 | 0.999 |
| | 60 | 369 | 4 | 0.984 | 0.00534 | 0.973 | 0.994 |
| | 90 | 294 | 7 | 0.965 | 0.00873 | 0.948 | 0.982 |
| | 180 | 242 | 8 | 0.939 | 0.01249 | 0.915 | 0.964 |
| | 270 | 167 | 3 | 0.927 | 0.01403 | 0.9 | 0.955 |
| Rayban | 1 | 357 | 0 | 1 | 0 | 1 | 1 |
| | 30 | 285 | 11 | 0.969 | 0.00915 | 0.951 | 0.987 |
| | 60 | 172 | 18 | 0.901 | 0.0178 | 0.867 | 0.936 |
| | 90 | 129 | 7 | 0.864 | 0.02181 | 0.822 | 0.908 |
| | 180 | 98 | 5 | 0.831 | 0.0256 | 0.782 | 0.882 |
| | 270 | 65 | 7 | 0.771 | 0.03213 | 0.711 | 0.837 |
| Sandbox | 1 | 442 | 0 | 1 | 0 | 1 | 1 |
| | 30 | 343 | 10 | 0.977 | 0.00707 | 0.964 | 0.991 |
| | 60 | 193 | 27 | 0.891 | 0.01727 | 0.858 | 0.925 |
| | 90 | 131 | 16 | 0.817 | 0.02374 | 0.772 | 0.865 |
| | 180 | 108 | 4 | 0.792 | 0.02608 | 0.742 | 0.845 |
| | 270 | 52 | 12 | 0.704 | 0.03333 | 0.642 | 0.772 |
| | **Pairwise comparisons of survival trajectories using Log-Rank test (Bonferroni correction)** | | | | | | |

| | Mojo | Rayban |
|---|---|---|
| Rayban | 6.80E-08 | - |
| Sandbox | 2.80E-13 | 0.65 |

**Table S3.5.** Results of Kaplan-Meier survival analysis of out-plants at three reef sites: Mojo, Rayban and Sandbox.

| Reefsite_Time | Average percentage of out-plants from $T_0$ surviving (%) | Standard Deviation | SEM |
|---|---|---|---|
| Mojo_$T_{7d}$ | 94.05474 | 4.220884 | 1.887637 |
| Mojo_$T_{1.5m}$ | 86.2519 | 3.838977 | 1.716843 |
| Mojo_$T_{6m}$ | 73.56086 | 0 | 0 |
| Mojo_$T_{9m}$ | 69.23067 | 4.004142 | 1.790707 |
| Mojo_$T_{12m}$ | 58.96481 | 3.851056 | 1.722244 |
| Rayban_$T_{7d}$ | 82.54984 | 20.4754 | 9.156876 |
| Rayban_$T_{1.5m}$ | 46.60469 | 18.16603 | 8.124095 |
| Rayban_$T_{6m}$ | 40.96531 | 9.129766 | 4.564883 |
| Rayban_$T_{9m}$ | 44.70588 | 8.247861 | 4.761905 |
| Rayban_$T_{12m}$ | 31.74962 | 12.5 | 6.25 |
| Sandbox_$T_{7d}$ | 90.20057 | 7.28114 | 3.256225 |
| Sandbox_$T_{1.5m}$ | 51.70043 | 14.76459 | 6.602925 |
| Sandbox_$T_{6m}$ | 53.72639 | 5.925256 | 3.420948 |
| Sandbox_$T_{9m}$ | 44.94548 | 16.72326 | 9.655179 |
| Sandbox_$T_{12m}$ | 32.65581 | 12.65401 | 7.305796 |

**Table S3.6.** Survival metrics for coral out-plants at three reef sites: Mojo, Rayban and Sandbox.

| Site | pairs | df | Sums Of Squares | F Model | $R^2$ | *p*-value | $p_{adj}$ value |
|---|---|---|---|---|---|---|---|
| Donor Colonies | $T_F$ vs $T_{O-1}$ | 1 | 0.31188 | 0.9311 | 0.058 | 0.486 | 0.486 |
| | $T_F$ vs $T_{O-2}$ | 1 | 0.37976 | 1.1015 | 0.061 | 0.234 | 0.486 |
| | $T_{O-1}$ vs $T_{O-2}$ | 1 | 0.32585 | 0.9941 | 0.058 | 0.392 | 0.486 |
| Rayban | donor vs out-plant | 1 | 1.58391 | 4.3178 | 0.089 | 0.0001 | 0.0001 |
| | $T_F$ vs out-plant_$T_{12m}$ | 1 | 0.72998 | 1.9753 | 0.152 | 0.004 | 0.0295 |
| | $T_F$ vs out-plant_$T_{1.5m}$ | 1 | 0.91556 | 2.5711 | 0.189 | 0.002 | 0.0295 |
| | $T_F$ vs out-plant_$T_{6m}$ | 1 | 0.93539 | 2.6904 | 0.212 | 0.006 | 0.0295 |
| | $T_F$ vs out-plant_$T_{9m}$ | 1 | 0.68844 | 1.8811 | 0.158 | 0.015 | 0.0435 |
| | $T_F$ vs out-plant_$T_{7d}$ | 1 | 0.4912 | 1.345 | 0.101 | 0.087 | 0.1299 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{7d}$ | 1 | 0.61147 | 1.6102 | 0.187 | 0.009 | 0.031875 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{6m}$ | 1 | 0.43699 | 1.2467 | 0.2 | 0.029 | 0.047833 |
| | out-plant_$T_{6m}$ vs out-plant_$T_{9m}$ | 1 | 0.36524 | 0.977 | 0.196 | 0.5 | 0.514286 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{9m}$ | 1 | 0.38604 | 0.9264 | 0.156 | 0.514 | 0.514286 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{7d}$ | 1 | 0.48165 | 1.2014 | 0.146 | 0.098 | 0.133091 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{9m}$ | 1 | 0.39692 | 1.0253 | 0.17 | 0.371 | 0.428571 |
| | out-plant_$T_{9m}$ vs out-plant_$T_{7d}$ | 1 | 0.46241 | 1.1556 | 0.161 | 0.178 | 0.22225 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{1.5m}$ | 1 | 0.56139 | 1.4389 | 0.193 | 0.029 | 0.047833 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{6m}$ | 1 | 0.57675 | 1.5172 | 0.233 | 0.029 | 0.047833 |
| | out-plant_$T_{6m}$ vs out-plant_$T_{7d}$ | 1 | 0.64281 | 1.7389 | 0.225 | 0.02 | 0.047833 |
| Sandbox | donor vs out-plant | 1 | 1.753 | 4.885 | 0.100 | 0.0001 | 0.0001 |
| | $T_F$ vs out-plant_$T_{12m}$ | 1 | 0.58138 | 1.6162 | 0.139 | 0.048 | 0.0714 |
| | $T_F$ vs out-plant_$T_{1.5m}$ | 1 | 0.65009 | 1.7683 | 0.128 | 0.013 | 0.042 |
| | $T_F$ vs out-plant_$T_{6m}$ | 1 | 1.02311 | 3.0155 | 0.232 | 0.005 | 0.0245 |
| | $T_F$ vs out-plant_$T_{9m}$ | 1 | 0.9434 | 2.7144 | 0.213 | 0.005 | 0.0245 |
| | $T_F$ vs out-plant_$T_{7d}$ | 1 | 0.87016 | 2.5008 | 0.172 | 0.001 | 0.0165 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{7d}$ | 1 | 0.45742 | 1.2422 | 0.134 | 0.074 | 0.101182 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{6m}$ | 1 | 0.62605 | 1.7365 | 0.224 | 0.034 | 0.056 |
| | out-plant_$T_{6m}$ vs out-plant_$T_{9m}$ | 1 | 0.35797 | 1.1668 | 0.226 | 0.2 | 0.214286 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | out-plant_$T_{12m}$ vs out-plant_$T_{9m}$ | 1 | 0.58444 | 1.6329 | 0.29 | 0.1 | 0.115385 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{1.5m}$ | 1 | 0.41988 | 1.064 | 0.151 | 0.283 | 0.2827 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{6m}$ | 1 | 0.62171 | 1.8435 | 0.315 | 0.1 | 0.115385 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{9m}$ | 1 | 0.5692 | 1.5207 | 0.202 | 0.032 | 0.056 |
| | out-plant_$T_{6m}$ vs out-plant_$T_{7d}$ | 1 | 0.5857 | 1.8236 | 0.233 | 0.02 | 0.042 |
| | out-plant_$T_{9m}$ vs out-plant_$T_{7d}$ | 1 | 0.52901 | 1.5793 | 0.208 | 0.018 | 0.042 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{7d}$ | 1 | 0.49105 | 1.3822 | 0.187 | 0.017 | 0.042 |
| **Site** | **pairs** | **df** | **Sums Of Squares** | **F Model** | **$R^2$** | ***p-value*** | ***p*adj value** |
| **Mojo** | donor vs out-plant | 1 | 1.01682 | 2.4618 | 0.071 | 0.0001 | 0.0001 |
| | $T_F$ vs out-plant_$T_{12m}$ | 1 | 0.84783 | 2.3645 | 0.165 | 0.002 | 0.009 |
| | $T_F$ vs out-plant_$T_{1.5m}$ | 1 | 0.86225 | 2.25 | 0.158 | 0.002 | 0.009 |
| | $T_F$ vs out-plant_$T_{6m}$ | 1 | 0.87365 | 2.3037 | 0.161 | 0.001 | 0.009 |
| | $T_F$ vs out-plant_$T_{9m}$ | 1 | 0.90463 | 2.4531 | 0.17 | 0.002 | 0.009 |
| | $T_F$ vs out-plant_$T_{7d}$ | 1 | 0.44606 | 1.2325 | 0.093 | 0.13 | 0.1295 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{7d}$ | 1 | 0.58961 | 1.4291 | 0.152 | 0.016 | 0.0305 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{6m}$ | 1 | 0.49319 | 1.1246 | 0.123 | 0.099 | 0.106393 |
| | out-plant_$T_{6m}$ vs out-plant_$T_{9m}$ | 1 | 0.48554 | 1.1647 | 0.127 | 0.089 | 0.102346 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{9m}$ | 1 | 0.55247 | 1.4318 | 0.152 | 0.027 | 0.037091 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{1.5m}$ | 1 | 0.60998 | 1.4967 | 0.158 | 0.031 | 0.038625 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{6m}$ | 1 | 0.58592 | 1.4591 | 0.154 | 0.024 | 0.0354 |
| | out-plant_$T_{1.5m}$ vs out-plant_$T_{9m}$ | 1 | 0.58517 | 1.3839 | 0.147 | 0.015 | 0.0305 |
| | out-plant_$T_{12m}$ vs out-plant_$T_{7d}$ | 1 | 0.53742 | 1.431 | 0.152 | 0.016 | 0.0305 |
| | out-plant_$T_{6m}$ vs out-plant_$T_{7d}$ | 1 | 0.62334 | 1.5331 | 0.161 | 0.016 | 0.0305 |
| | out-plant_$T_{9m}$ vs out-plant_$T_{7d}$ | 1 | 0.63459 | 1.6235 | 0.169 | 0.018 | 0.0305 |
| **$T_{7d}$** | Mojo_$T_{7d}$ vs Rayban_$T_{7d}$ | 1 | 0.34163 | 0.8862 | 0.1 | 0.774 | 0.7741 |
| | Mojo_$T_{7d}$ vs Sandbox_$T_{7d}$ | 1 | 0.51701 | 1.4375 | 0.152 | 0.042 | 0.0636 |
| | Rayban_$T_{7d}$ vs Sandbox_$T_{7d}$ | 1 | 0.51067 | 1.4007 | 0.149 | 0.032 | 0.0636 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Mojo_$T_{1.5m}$ vs Rayban_$T_{1.5m}$ | 1 | 0.55266 | 1.3458 | 0.161 | 0.033 | 0.04875 |
| | Mojo_$T_{1.5m}$ vs Sandbox_$T_{1.5m}$ | 1 | 0.51891 | 1.2322 | 0.133 | 0.025 | 0.04875 |
| **$T_{1.5m}$** | Rayban_$T_{1.5m}$ vs Sandbox_$T_{1.5m}$ | 1 | 0.46116 | 1.2012 | 0.146 | 0.058 | 0.0584 |
| | Mojo_$T_{6m}$ vs Rayban_$T_{6m}$ | 1 | 0.52954 | 1.3313 | 0.182 | 0.037 | 0.0549 |
| | Mojo_$T_{6m}$ vs Sandbox_$T_{6m}$ | 1 | 0.54846 | 1.4292 | 0.192 | 0.036 | 0.0549 |
| **$T_{6m}$** | Rayban_$T_{6m}$ vs Sandbox_$T_{6m}$ | 1 | 0.3648 | 1.1879 | 0.229 | 0.1 | 0.1 |
| | Mojo_$T_{9m}$ vs Rayban_$T_{9m}$ | 1 | 0.33191 | 0.8149 | 0.12 | 0.943 | 0.9432 |
| | Mojo_$T_{9m}$ vs Sandbox_$T_{9m}$ | 1 | 0.38939 | 1.034 | 0.147 | 0.275 | 0.45 |
| **$T_{9m}$** | Rayban_$T_{9m}$ vs Sandbox_$T_{9m}$ | 1 | 0.38366 | 1.0271 | 0.204 | 0.3 | 0.45 |
| | Mojo_$T_{12m}$ vs Rayban_$T_{12m}$ | 1 | 0.35501 | 0.9114 | 0.115 | 0.714 | 0.7144 |
| | Mojo_$T_{12m}$ vs Sandbox_$T_{12m}$ | 1 | 0.41434 | 1.1006 | 0.155 | 0.133 | 0.3993 |
| **$T_{12m}$** | Rayban_$T_{12m}$ vs Sandbox_$T_{12m}$ | 1 | 0.40436 | 1.0003 | 0.167 | 0.486 | 0.7144 |

**Table S3.7.** Permutational multivariate analysis of variance pairwise test results comparing structure of coral-associated bacterial communities between and within sites/variables and over time. Adjusted *p*-value = FDR correction.

| Comparison | Taxon | Average dissim. | Contr. (%) | Mean RA (%) Mojo | Mean RA (%) Rayban |
|---|---|---|---|---|---|
| Between Sites at $T_{1.5m}$ | *g__Endozoicomonas 1* | 4.082 | 4.375 | 0.542 | 8.45 |
| | *g__Lactobacillus fermentum* | 3.766 | 4.036 | 7.49 | 0.0769 |
| | *g__Endozoicomonas 2* | 3.24 | 3.473 | 0.0399 | 6.5 |
| | *g__Ruegeria 1* | 2.803 | 3.004 | 5.67 | 1.42 |
| | *g__Erythrobacter* | 2.185 | 2.342 | 3.85 | 1.2 |
| | *g__PseudoVibrio* | 1.581 | 1.694 | 3.16 | 0 |
| | **Taxon** | **Average dissim.** | **Contr. (%)** | **Mean RA (%) Mojo** | **Mean RA (%) Sandbox** |
| | *g__Endozoicomonas 1* | 13.47 | 14.21 | 0.542 | 27.4 |
| | *g__Lactobacillus fermentum* | 3.743 | 3.947 | 7.49 | 0 |
| | *g__Endozoicomonas 2* | 5.191 | 5.474 | 0.0399 | 10.4 |
| | *g__Ruegeria 1* | 2.852 | 3.007 | 5.67 | 1.59 |
| | *g__Erythrobacter* | 2.405 | 2.535 | 3.85 | 1.79 |
| | *g__PseudoVibrio* | 1.587 | 1.674 | 3.16 | 0.0211 |
| | **Taxon** | **Average dissim.** | **Contr. (%)** | **Mean RA (%) 7d** | **Mean RA (%) 1.5m** |
| At Mojo between $T_{7d}$ and $T_{1.5m}$ | *g__Endozoicomonas 1* | 24.24 | 25.22 | 49 | 0.542 |
| | *g__Lactobacillus fermentum* | 3.743 | 3.895 | 0 | 7.49 |
| | *g__Ruegeria 1* | 2.833 | 2.948 | 0.6 | 5.67 |
| | *g__Ruegeria 2* | 2.086 | 2.171 | 2.42 | 3.23 |
| | *g__Erythrobacter* | 2.02 | 2.102 | 0.54 | 3.85 |
| | *g__Tenacibaculum* | 1.7 | 1.769 | 3.4 | 0 |
| | *g__PseudoVibrio* | 1.581 | 1.645 | 0 | 3.16 |
| | **Taxon** | **Average dissim.** | **Contr. (%)** | **Mean RA (%) 9m** | **Mean RA (%) 12m** |
| At Mojo between $T_{9m}$ and $T_{12m}$ | g__Synechococcus_CC9902 | 5.173 | 5.686 | 0.959 | 11 |
| | *g__Endozoicomonas 1* | 5.005 | 5.501 | 2.57 | 10.6 |
| | f__Hungateiclostridiaceae | 4.668 | 5.131 | 9.34 | 0 |
| | *g__Endozoicomonas 3* | 3.96 | 4.352 | 4.59 | 5.61 |
| | *g__Ruegeria 2* | 2.035 | 2.237 | 4.43 | 1.05 |
| | *g__Psychrobacter* | 1.486 | 1.633 | 0 | 2.97 |

| | Taxon | Average dissim. | Contr. (%) | Mean RA (%) 7d | Mean RA (%) 1.5m |
|---|---|---|---|---|---|
| **At Rayban between T7d and T1.5m** | *g__Endozoicomonas 1* | 17.13 | 19.5 | 39.4 | 8.45 |
| | *g__Endozoicomonas 2* | 5.511 | 6.272 | 7.2 | 6.5 |
| | **Taxon** | **Average dissim.** | **Contr. (%)** | **Mean RA (%) 1.5m** | **Mean RA (%) 6m** |
| **At Rayban between T1.5m and T6m** | *g__Endozoicomonas 2* | 4.557 | 5.234 | 6.5 | 5.6 |
| | *g__Endozoicomonas 1* | 4.102 | 4.711 | 8.45 | 0.242 |
| | *g__Limnothrix* | 3.715 | 4.267 | 0 | 7.43 |
| | *g__Trichodesmium_IMS101* | 1.368 | 1.571 | 0 | 2.74 |

**Table S3.8.** Relative abundance of bacterial taxa identified (with SIMPER analysis) to be driving differences between sites and over time in out-planted coral-associated bacterial communities. Average dissim. = Average dissimilarity, Contr. = Contribution.

| Variable | | df | Sum of Squares | Mean of Squares | F | N.Perm | Pr (>F) |
|---|---|---|---|---|---|---|---|
| $T_{7d}$ | Groups | 2 | 0.00351 | 0.00175 | 0.3921 | 999 | 0.723 |
| | Residuals | 12 | 0.05364 | 0.00447 | | | |
| $T_{1.5m}$ | Groups | 2 | 0.01178 | 0.00589 | 6.373 | 999 | 0.013 |
| | Residuals | 11 | 0.01016 | 0.00092 | | | |
| $T_{6m}$ | Groups | 2 | 0.05179 | 0.02589 | 38.465 | 999 | 0.001 |
| | Residuals | 8 | 0.00539 | 0.00067 | | | |
| $T_{9m}$ | Groups | 2 | 0.01831 | 0.00916 | 5.7112 | 999 | 0.023 |
| | Residuals | 8 | 0.01283 | 0.0016 | | | |
| $T_{12m}$ | Groups | 2 | 0.00448 | 0.00224 | 0.4828 | 999 | 0.622 |
| | Residuals | 9 | 0.04178 | 0.00464 | | | |
| $T_{7d}$ pairwise comparisons | | Mojo | | Rayban | | Sandbox | |
| | Mojo | - | | 0.975 | | 0.31 | |
| | Rayban | 0.94894 | | - | | 0.557 | |
| | Sandbox | 0.27713 | | 0.49052 | | - | |
| $T_{1.5m}$ pairwise comparisons | | Mojo | | Rayban | | Sandbox | |
| | Mojo | - | | 0.01 | | 0.142 | |
| | Rayban | 0.00839 | | - | | 0.103 | |
| | Sandbox | 0.12768 | | 0.09481 | | - | |
| $T_{6m}$ pairwise comparisons | | Mojo | | Rayban | | Sandbox | |
| | Mojo | - | | 0.006 | | 0.004 | |
| | Rayban | 0.00079 | | - | | 0.172 | |
| | Sandbox | 0.00028 | | 0.1699 | | - | |
| $T_{9m}$ pairwise comparisons | | Mojo | | Rayban | | Sandbox | |
| | Mojo | - | | 0.26 | | 0.009 | |
| | Rayban | 0.2686 | | - | | 0.137 | |
| | Sandbox | 0.01147 | | 0.13595 | | - | |
| $T_{12m}$ pairwise comparisons | | Mojo | | Rayban | | Sandbox | |
| | Mojo | - | | 0.73 | | 0.526 | |
| | Rayban | 0.7346 | | - | | 0.372 | |
| | Sandbox | 0.53176 | | 0.39291 | | - | |

**Table S3.9.** Permutation test for homogeneity of multivariate dispersions (PERMUTEST) and post-hoc analysis of microbial community distribution/variance of out-planted corals.

### *3.8.3 Appendix 3.1*

## *Construction of tilting current meter*

Flow was recorded using a G data logger (HOBO Pendant® UA-004-64) that was assembled into a tilting current meter (based on Crookshanks et al. 2008); the G data logger was secured inside a PVC pipe casing, that was suspended in the water column with fins that allowed the device to orient to the direction of flow. Caps were attached to either end and fastened with plumbers' tape. Hollow aluminium rods were secured to the logger casing using marine epoxy and a cable tie. Two eye bolts were fastened to the top and bottom of a rectangular aluminium frame. Two holes were drilled on either side of the aluminium frame, lined with rubber grommets/O-rings and aluminium rods were fed through to sit within the hollow aluminium rods attached to the logger casing. Two plastic fins were attached to the logger casing with marine grade adhesive and the logger casing was able to tilt freely and orient to the flow (Fig. S1b – e.). HOBO Pendant G loggers record tilt in 3 planes, however only the Z plane was recorded as this aligned to the direction of tilt induced by water flow (Fig. S1e). The tilting flow meter was suspended in the water column with tethers from each eye bolt attached to a weight or float (where convenient an established nursery was used as the float (Fig. S1b-c.)) and tilt was recorded every 20 min.

**Reference**

Crookshanks, S. (2008). High-Energy Sedimentary Processes in Kluane Lake, Yukon Territory. Doctoral dissertation, Queen's University, Kingston, Ontario, Canada. https://www.collectionscanada.gc.ca/obj/s4/f2/dsk3/OKQ/TC-OKQ-1219.pdf

### *3.8.4 Supplementary Data*

The supplementary data files from the publication have, where possible, been supplied as supplementary tables however following supplementary data files are within *Supplementary file2* online at doi.org/10.1007/s00227-023-04235-y

**Data S1** Calibrated flow, temperature and light attenuation data.

**Data S2** Results of analysis of environmental conditions across sites.

**Data S4** Full legend for Fig 3.4.

**Data S5** Results of Kaplan-Meier survival analysis for out-planted corals across sites.

**Data S8** Full legend for Fig S3.4.

# Chapter 4: Assessing efficacy of plastic-free alternative ties for coral propagation in reef restoration.

**In review at *Environmental Microbiology Reports* as:**

**Strudwick P.** [1], Camp, E. F. [1], Seymour, J. [1], Roper, C. [1], Edmondson, J. [2], Howlett, L. [1], & Suggett, D. J[1]. Impacts of Plastic-free Materials on Coral-associated Bacterial Communities During Reef Restoration, 05 September 2023.

[1] University of Technology Sydney, Climate Change Cluster, Faculty of Science, Ultimo, NSW, 2007, Australia

[2] Wavelength Reef Cruises, 6/43 Macrossan St., Port Douglas, QLD, 4877, Australia

**Author Contributions**

Paige Strudwick, David J. Suggett, Emma F. Camp and Justin Seymour designed the experiments. Paige Strudwick, Lorna Howlett and John Edmondson initiated field experiments. Paige Strudwick, Emma F. Camp, Christine Roper, Lorna Howlett and John Edmondson collected and preserved samples. Paige Strudwick conducted all laboratory processing and data analysis. Paige Strudwick wrote the original manuscript. David J. Suggett, Emma F. Camp and Justin Seymour provided editing and all co-authors read and approved the final manuscript.

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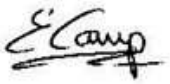
Emma Camp: 

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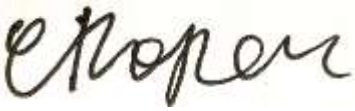
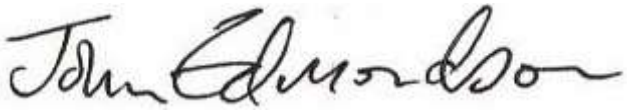
Christine Roper: 

John Edmondson: 

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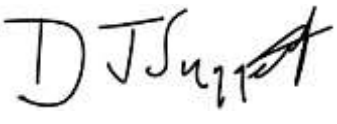
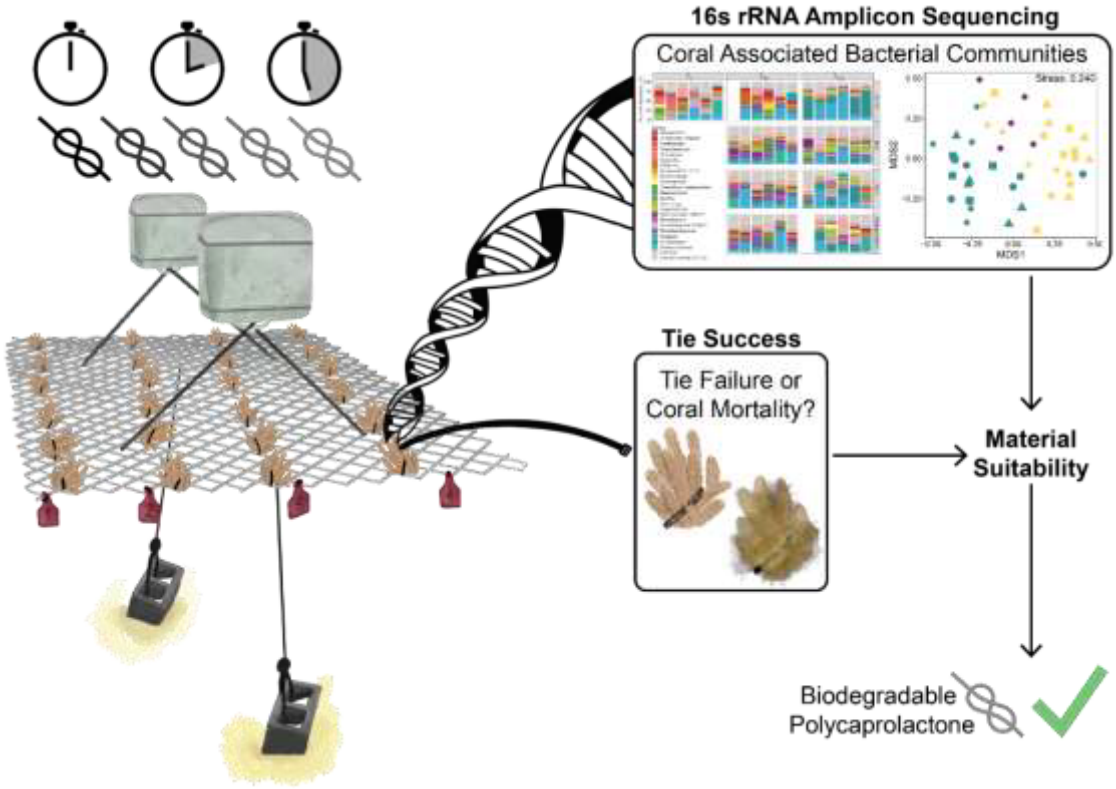
David Suggett: 

## 4.1 Abstract

Coral propagation and out-planting based restoration approaches are increasingly being applied as tools to assist natural recovery and preserve resilience of coral reefs. However, many out-planting and propagation methods rely on plastic zip-ties to fasten corals to structures in coral nurseries, which is potentially problematic and unsustainable for the marine environment. Plastic-free biodegradable alternatives are becoming available but may pose unique risks by impacting coral-associated bacterial communities that are integral to coral health. We therefore examined the bacterial communities of *Acropora millepora* coral fragments propagated in coral nurseries in two experiments on the northern Great Barrier Reef to identify whether biodegradable materials differentially impact coral-associated bacterial communities. In each study coral fragments were secured to nursery frames with conventional plastic, metal, and biodegradable (polyester and polycaprolactone) ties, and both tie failure and coral-associated bacterial communities were characterised during a six month period. Minimal coral mortality was observed (3.6-8 %) and all ties tested had low failure rates (0-4.2 %) with the exception of the polyester biodegradable material (29.2 % failure). No differences were observed between coral-associated bacterial communities of fragments secured in the coral nursery with different tie types, and no proliferation of putatively pathogenic bacteria was recorded for fragments secured with biodegradable ties. Overall, our findings suggest that reducing reliance on conventional plastic can be achieved through transitions to biodegradable materials, without any notable impacts on coral-associated bacterial communities, but we caution the need to examine wider coral taxa of different morphologies and growth dynamics, and any new plastic-free materials prior to application.




Graphical Abstract.

## 4.2 Introduction

Coral propagation and out-planting based restoration practices are accelerating globally to aid conventional reef management approaches (Boström-Einarsson et al. 2020; Kleypas et al. 2021; Suggett and van Oppen 2022). Such practices commonly use an *in situ* 'nursery' phase to increase coral biomass prior to out-planting material onto bare reef substrate to increase coral cover at rates faster than from natural recovery alone (e.g., Rinkevich, 2019; Ware et al. 2020; Boström-Einarsson et al. 2020; Howlett et al. 2022). Plastics are relied upon by restoration practitioners during coral nursery set-up (e.g., zip-ties and fishing line), for identification purposes (e.g., cattle tags), and as vessels during collection or movement of samples (Boström-Einarsson et al. 2020). Plastic zip-ties have particularly become the 'staple' for restoration practitioners – primarily for attaching corals to nurseries, but also anchoring corals back to the reef during out-planting (Boström-Einarsson et al. 2020; Goergen and Gilliam, 2018) –

given their low cost, widespread availability, ease and speed of deployment, and overall lowest chance of fragment dislodgement (Bruckner et al. 2008; Goergen and Gilliam, 2018). However, these benefits of plastic zip-ties are fast becoming outweighed by the high cost of potential generation of micro- and macro-plastics (Caron et al. 2018; Huang et al. 2021; Reichert et al. 2022), which pose risks to coral reefs and endemic marine organisms (Bidegain et al. 2018; Lamb et al. 2018; Manfra et al. 2021). Specifically, microbial communities colonising marine plastic debris (within the 'plastisphere') could impact coral microbiomes through direct contact or ingestion and subsequent transfer of foreign microbial communities including pathogens (Rotjan et al. 2019; Hchaichi et al. 2020; Lartaud et al. 2021).

Recently we have shown that coral-associated microbial communities essential to coral holobiont health can change during propagation and out-planting practices (Strudwick et al. 2022, 2023), likely from environmental conditions that differ for propagation or out-planting areas compared to the native reef. Variations in environmental conditions are known to influence coral-associated bacterial communities (Kelly et al. 2014; McDevitt-Irwin et al. 2017; Maher et al. 2019; Camp et al. 2020; Ziegler et al 2017, 2019); however, how this applies to environmental changes induced by the materials used during the propagation process (e.g., metal structures and/or fixing devices, such as plastic zip-ties) remains untested. Consequently, while transitioning to plastic-alternatives in reef construction and engineering (Nauta et al. 2022; Manfra et al. 2021; Kenyon et al. 2023 in press) – including intervention practices – is a matter of urgency (Boström-Einarsson et al. 2020, Ceccarelli et al. 2020), plastic alternatives such as metal or biodegradable plastics may present microbial risks to coral reefs. Biodegradable materials can have enhanced biofouling (Dussud et al. 2018; Peng et al. 2022) through high microbial affinity (Peng et al. 2022) and microbial driven break-down (Gan and Zhang, 2019; Manfra et al. 2021) and could result in proliferation of

putative pathogens within the coral microbiome (Zettler et al. 2013; Dussud et al. 2018; Hchaichi et al. 2020; Ceccarelli et al. 2020). Indeed, increased transfer of putatively pathogenic *Vibrio* spp. and trace metals – linked to increased pathogenicity (Rubio-Portillo et al. 2020) – from plastic-free biodegradable materials to marine organisms can occur (catfish, Jang et al. 2022). As such, use of plastic alternatives could arguably negatively impact coral bacterial communities despite best intentions for a more environmentally positive attachment solution. To incentivise the use of plastic-free alternatives (that may be more expensive or pose unique risks to coral fragments) and ensure investment in more environmentally positive products in reef restoration – a field typically operating on limited funding opportunities – it is essential to determine the nature of any potential impacts to the coral holobiont.

Whilst stainless-steel metal ties have long been available, biodegradable zip-ties have only more recently become commercially available (Haider et al. 2018). Biodegradable zip-ties are fabricated from polymers that degrade under prolonged exposure to UV light, heat, moisture, and microbial metabolic activity through several stages including biodegradation, bio-fragmentation, assimilation and mineralisation (Lucas et al. 2008; Delacuvellerie et al. 2021). Surface bacterial communities of both degradable and non-degradable marine plastics show similarities after short time frames (~ 80 days) (Delacuvellerie et al. 2021); however, how these plastisphere microbial communities evolve over longer periods remains unexplored (Delacuvellerie et al. 2023). It is known that biodegradable materials have high microbial affinity and biomass (Peng et al. 2022) and can transfer putative pathogenic bacteria (e.g., *Vibrio* spp.) to marine organisms (Jang et al. 2022). As such, it is plausible to expect evolution of microbial communities in the biodegradable plastisphere to differ from that of conventional plastic (Dussud et al. 2018; Delacuvellerie et al. 2021) and potentially include proliferation of putatively pathogenic bacteria of the *Vibrio* genus. Therefore,

biodegradable material zip-ties may impact coral-associated bacterial communities differentially to conventional plastic, especially during coral propagation in nursery environments that already promote shifts in coral-associated bacterial communities for some coral species (Strudwick et al. 2022).

Here we compared performance of zip-ties fabricated from plastic and plastic-free materials for coral propagation processes testing the hypotheses that: (i) differences in zip-tie material will shape coral-associated bacterial communities and (ii) coral fragments attached with biodegradable zip-ties will have increased relative abundance of bacteria that are putative coral pathogens from the *Vibrio* genus. To test these hypotheses, and to inform coral reef restoration practitioners of the suitability of plastic-alternatives, we tracked the microbiome of coral fragments attached to *in situ* nurseries with five different zip-tie materials, via two consecutive experiments each lasting six months.

## 4.3 Materials and Methods

### *4.3.1 Sampling location and experimental design*

Experiments were conducted at coral nursery sites located at Opal Reef (16°12'18"S 145°53'54"E), which is a 24.7 $km^2$ reef situated on the northern Great Barrier Reef (GBR) (detailed in Suggett et al. 2019; Howlett et al. 2021). All nursery sites at Opal Reef consist of multiple floating frames located at depths of 5-6 m on sand immediately adjacent to the reef (detailed in Howlett et al. 2021) (Fig. S4.1.). For each experiment two dedicated nursery frames were installed and conditioned *in situ* for a period of at least two weeks prior to beginning the experiment.

Coral fragments were harvested from donor colonies of *Acropora millepora* and secured to nursery frames via three different attachment materials (Fig. S4.2.). The first experiment was conducted from August 2020-February 2021 at site “Blue Lagoon”,

which is subject to tidal currents due to proximity to a deep-water channel (see Suggett et al. 2019; Howlett et al. 2021), where corals have previously been demonstrated to achieve good recovery from bleaching and storm damage (Edmondson personal obs.). For this experiment, the three ties compared were plastic (black Nylon-66), biodegradable A (Poly-1,4-butanediol Succinate, supplier gocableties.co.uk) and metal (316 grade stainless steel) (full specifications; Table S4.1.). After fastening corals, excess tie was cut off, apart from for the metal tie where this was not possible.

A second experiment was conducted from February-August 2022 at site "Mojo", which is 150 m from Blue Lagoon and shares topographical characteristics and similar natural recovery to past disturbance events. A different combination of ties were used for this experiment, including plastic ('natural' colour Nylon-66), biodegradable material B (Polycaprolactone, supplier rapstrap.com) and Rapstrap soft plastic (Polyurethane Elastomer, supplier rapstrap.com) (full specifications; Table S4.1). Excess tie was cut after fastening corals (Fig. S4.2a-b.). Although the timing for the two experiments differed, the same Nylon-66 plastic ties were used and hence provided a control group.

The sampling design for both experiments consisted of three (start, intermediate and end) sampling events; however, the respective timing of intermediate and end sampling events differed due to logistical and reef access constraints. At Blue Lagoon, six donor colonies (≥ 55 cm diameter) were identified on the reef adjacent to nurseries and marked with cattle tags, with each colony representing a biological replicate. In total 13 fragments (≤ 5 cm) were harvested from each donor colony using wire cutters and transported in a sterile zip-lock bag with seawater by a diver to nursery frames located 10-20 m away. Harvested fragments from three of six donor colonies were taken to frame one and the remainder were taken to frame two (3 m apart) to account for potential frame effects. Fragments from an individual donor colony were divided into

three groups of four and immediately attached to nursery frames using one of the three tie types (Fig. 4.1a. and S4.3.). At Mojo, five *A. millepora* donor colonies were identified and marked with cattle tags, in total 19 (≤ 5 cm) fragments were harvested from each donor colony divided into three groups of six and immediately attached to nursery frames using one of the three tie types (again spread over two nursery frames to account for frame effect). At the time of harvesting fragments from donor colonies, one fragment from each donor colony was placed in an individual sterile zip-lock bag and taken to the operations vessel (*Wavelength 4*) and preserved in RNA*later* for donor colony 'time zero' ($T_0$) bacterial community characterisation (Fig. S4.3a-b.). In the first experiment (Blue Lagoon, Aug 2020 – Feb 2021), nursery fragments and donor colonies were re-sampled at 56 days ($T_{56d}$) and 189 days ($T_{189d}$) (Fig. S4.3a-b.). In the second experiment (Mojo, Feb 2022 – Aug 2022), nursery fragments and donor colonies were re-sampled at 32 days ($T_{32d}$) and 147 days ($T_{147d}$) (Fig. S4.3a-b.).

At each time point, coral fragments were removed from the nursery frame by detaching the cable tie (or fragmented using wire clippers where fragments had self-attached to the nursery frame) and from the original marked donor colony on the reef using wire clippers and preserved by submersion in RNA*later* in sterile falcon tubes. Fragments were sub-sampled where a ~5cm fragment was selected for coral-associated bacterial community analysis as close to the coral-material interface as possible depending on colony structure and growth over the tie. In the first experiment, 54 samples were collected for bacterial community analysis: (i) nursery fragments: six replicates (three from each nursery frame) x three attachment materials x two time points ($n$ = 36) plus (ii) donor colonies: six replicates x three time points ($n$ = 18). In the second experiment, 45 samples were collected for bacterial community analysis: (i) nursery fragments: five replicates (two or three from each nursery frame) x

three attachment materials x two time points ($n$ = 30) plus (ii) donor colonies: five replicates x three time points ($n$ = 15).

### *4.3.2 Quantification of failure and coral mortality*

At each sampling time point, counts were conducted to record the number of (i) live coral fragments present with tie, (ii) live coral fragment present without tie, (iii) tie present with coral missing, (iv) tie and coral missing and (v) dead coral. Tie failure was considered to have occurred when either live coral fragments were present without tie, when a tie was present without coral fragment, or when both tie and coral fragment were missing. At the end of the study, tie failure rate percentage (%) was calculated by dividing the total number of failed ties by the total number of coral fragments attached at $T_0$ and multiplying by 100.

### *4.3.3 DNA extraction, 16S rRNA amplicon sequencing and bioinformatics*

DNA was extracted from coral tissue isolated using an airbrushing technique (see Supplementary Methods) as per Strudwick et al. (2022) using DNeasy Blood and Tissue kit (Qiagen) following the Manufacturer's protocol (July 2020 version) with a total elution volume of 40 µL. Extracted DNA was stored at –30 °C for one week prior to 16S rRNA amplicon sequencing. The hypervariable V3 and V4 regions of the bacterial 16S rRNA gene were amplified using the primers 341F (5'–CCTAYGGGRBG-CASCAG-3') and 805R (5'–GACTACHVGGGTATC-TAATCC-3') (Klindworth et al. 2013), prior to sequencing on the Illumina MiSeq platform (Ramaciotti Centre for Genomics, Sydney, NSW, Australia). Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA945487.

For both experiments raw demultiplexed sequencing data were analysed using the Quantitative Insights into Microbial Ecology (QIIME 2, version 2020.6) platform (Callahan et al. 2016). The DADA2 plugin was used to denoise the data (Callahan et al.

2016) and taxonomy was assigned using the classify-sklearn classifier (Pedregosa et al. 2011) against the SILVA v138 database. Amplicon sequence variants (ASVs) corresponding to chloroplast or mitochondria were removed from the data set. To remove contaminants identified in 'kit blank' extractions, ASVs were removed with (i) > 5 % relative abundance in kit blank samples or (ii) greater relative abundance than total coral samples and (iii) that have been previously reported as contaminants of laboratory reagents (Weyrich et al. 2019). Overall, 11 and 13 ASVs were removed from the August 2020-February 2021 and February 2022-August 2022 data, respectively. Prior to diversity analyses, one sample was removed from the August 2020-February 2021 data set due to poor sequencing output providing low read numbers after quality filtering and contaminant removal. For beta diversity analyses, the raw read ASV table was converted to relative abundances, scaled to 20,000 (McKnight et al. 2019) and square root transformed.

### *4.3.4 Statistical analysis*

To identify whether bacterial community structure differed after corals were placed in the nursery, beta diversity patterns of nursery corals at both intermediate ($T_{32d/56d}$) and end ($T_{147d/189d}$) time points and donor colonies at time of harvesting fragments ($T_0$) were analysed using the Bray–Curtis dissimilarity distance metric and patterns were visualised using non-metric multidimensional scaling (nMDS) plots. Further analysis was conducted to identify differences in associated bacterial community structure between nursery fragments attached with different materials (fixed effect = tie type). Differences in beta diversity were tested for significance with pairwise permutational multivariate analysis of variance (PERMANOVA; perm = 999) of Bray–Curtis dissimilarities using the pairwise.adonis function of the 'vegan' R package, *p*-values were subsequently adjusted by applying a Benjamini and Hochberg (a.k.a. False Discovery Rate) correction to account for multiple comparisons, all $p_{adj}$

values < 0.05 were considered significant. To test whether coral fragments secured with biodegradable materials (A or B) harboured bacterial communities with higher relative abundance of putative coral pathogens from the *Vibrio* genus, *Vibrio* genus ASVs were grouped due limited resolution to species level of ASVs within this genus, average relative abundance for each tie type and time point was calculated and compared with a Kruskal-Wallis test to assess significance.

## 4.4 Results and Discussion

### *4.4.1 Impact of tie-material on propagated coral survival*

Survival of propagated coral was high across all tie types (including conventional plastic). Specifically, only two fragments (that were attached with plastic) out of 162 total fragments exhibited mortality (Table S4.2, S4.3.). Almost all fragments had begun to overgrow their respective ties after 32-56 days and no signs of disease were recorded (Fig. 4.1a-b.). Although failure of some kind (e.g., loss of coral or loss of tie) was observed for most ties, low failure was observed across both experiments for all tie materials (0-4.17 %), except for biodegradable material A that had the highest failure rate (29.17 %) (Table S4.3.). The high failure rate observed in biodegradable material A ties potentially resulted from early compromise – presumably via degradation – indicating that using this material for other coral species (with slower growth) would likely have even higher failure. As such our results suggest that biodegradable material B (polycaprolactone) zip-ties appeared more suited for securing coral in nursery propagation; however, wider species-specific investigation is required to ensure tie time-to-failure exceeds coral time-to-attachment prior to widespread use.

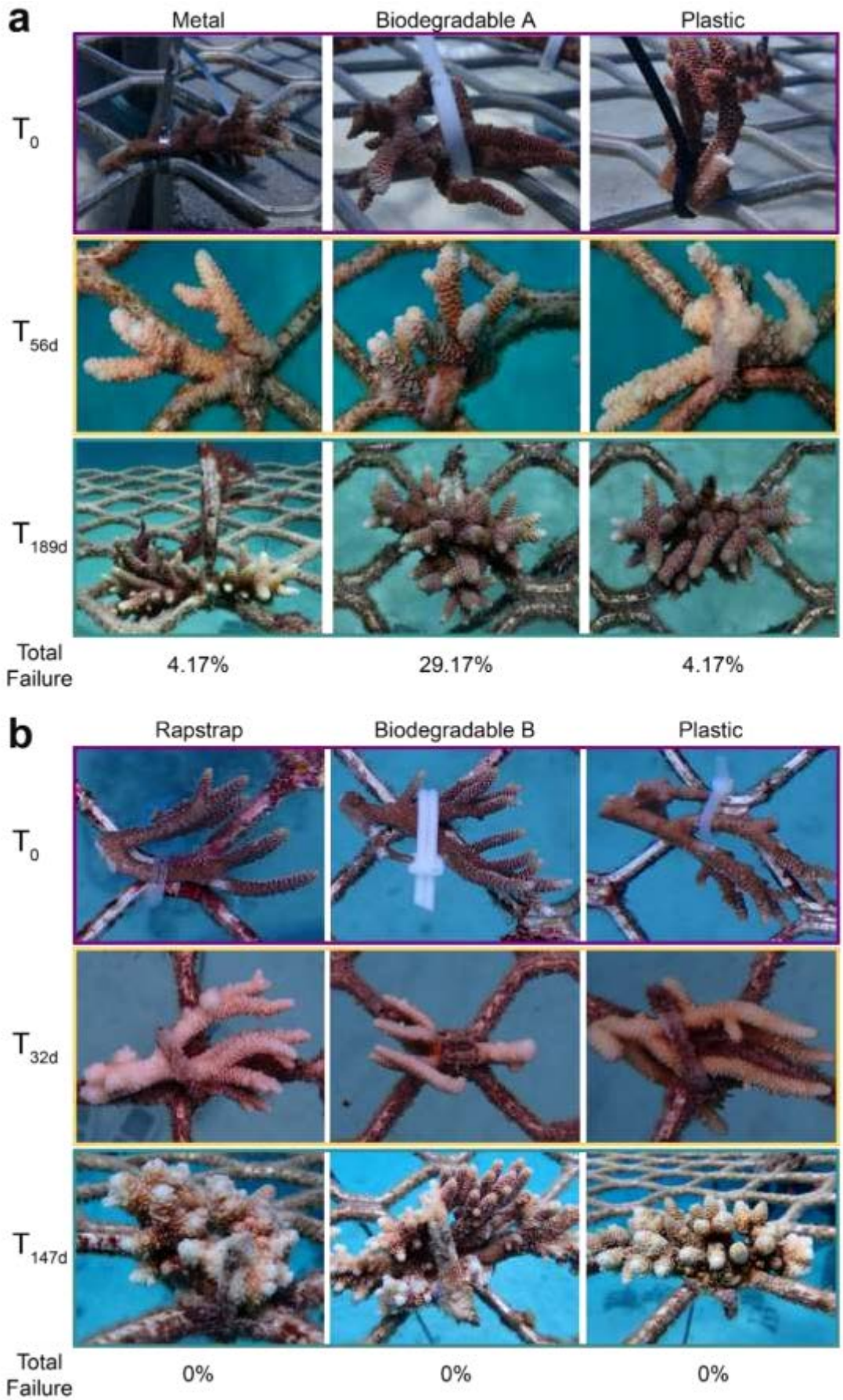



**Figure 4.1.** Coral fragments of *Acropora millepora* secured in nurseries at with three different attachment materials at (**a**) Blue Lagoon over six months from August 2020 to

February 2021 and (**b**) at Mojo over six months from February 2022 to August 2022 at Opal Reef, northern Great Barrier Reef. Note different fragments are shown at each time point for any given tie type.

### *4.4.2 Impact of tie-material on coral-associated bacterial communities*

For the first experiment assessing biodegradable material A, the structure of coral-associated bacterial communities of all propagated corals after 56 days in the nursery – regardless of attachment type – significantly changed from that at time of harvesting fragments ($T_0$) (PERMANOVA$_{Bray\text{-}Curtis}$, $p_{adj} < 0.05$, Fig 4.2a., S4.5. and Supplementary Data S1.). For the second experiment assessing biodegradable material B, 'Rapstrap' and plastic ties, the structure of coral-associated bacterial communities of propagated corals attached with plastic and Rapstrap ties changed after 32 days ($T_{32d}$) compared to time of harvesting fragments ($T_0$) (PERMANOVA$_{Bray\text{-}Curtis}$, $p_{adj} < 0.05$, Fig. 4.2d., S4.6. and Supplementary Data S1.). In contrast, bacterial community structure of coral fragments attached to nurseries with biodegradable (B) ties differed from time of harvesting fragments ($T_0$) after a longer period in the nursery (147 days; $T_{147d}$) (PERMANOVA$_{Bray\text{-}Curtis}$, $F = 1.283$, $p_{adj} = 0.022$, Fig. 4.2d., S4.6. and Supplementary Data S1.). Changes in coral-associated bacterial communities after transfer from native reef to coral nurseries observed in this study are consistent with previous observations for *A. millepora* during nursery propagation on the GBR (Strudwick et al. 2022) and are typical of this coral genus when transported between distinct environments (Ziegler et al. 2019; Haydon et al. 2021; Strudwick et al. 2023). In line with previous studies highlighting variability of *Acropora* spp. bacterial communities over time and space (Ziegler et al. 2019; Haydon et al. 2021; Strudwick et al. 2023), coral fragments growing in nurseries over the course of both experiments exhibited temporal changes in associated bacterial community structure; specifically, for experiment one from 56 days ($T_{56d}$) to 189d ($T_{189d}$) (PERMANOVA$_{Bray\text{-}Curtis}$ $p_{adj} < 0.05$, Fig. 2a., S4.5. and Supplementary Data S1.), and for experiment two from 32 days (T32d) to 147 days

(T147d) (PERMANOVA$_{\text{Bray-Curtis}}$ $p_{adj} < 0.05$, Fig. 4.2d., S4.6. and Supplementary Data S1.). However, in both experiments there were no differences between the structure of bacterial communities of fragments in nurseries attached with different ties at any time; specifically, in experiment one for plastic, biodegradable (A) and metal ties at 56 days ($T_{56d}$) and 189 days ($T_{189d}$) (PERMANOVA$_{\text{Bray-Curtis}}$ $p_{adj} > 0.05$, Fig. 4.2b-c., S4.5. and Supplementary Data S1.) and in experiment two for plastic, biodegradable (B) or Rapstrap ties at 32 days ($T_{32d}$) and 147 days ($T_{147d}$) (PERMANOVA$_{\text{Bray-Curtis}}$ $p_{adj} > 0.05$, Fig. 4.2e-f., S4.6. and Supplementary Data S1.). While different materials are suggested to be colonized by distinct microbial communities (Caruso, 2020) there were no differential impacts to coral bacterial communities between tie types. Biodegradable materials A and B did not differentially impact coral bacterial communities suggesting high suitability for use in nursery-based reef restoration activities.

### *4.4.3 Abundance of putatively pathogenic Vibrio spp.*

Enriched populations of putatively pathogenic *Vibrio* spp. have been observed on plastic and biodegradable plastic materials in marine environments (Zettler et al. 2013; Dussud et al. 2018). However, in our study we observed no differences in the relative abundance (RA) of *Vibrio* spp. between coral fragments secured with biodegradable (A) or plastic ties (mean RA = 0.88 %, mean RA = 1.21 %, respectively) and donor colonies (mean RA = 0.26 %) or other nursery fragments secured with metal ties (RA range = 0.57 %) (Kruskal-Wallis, $p > 0.05$, and Fig. S4.4a.). Similarly, in the second experiment RA of *Vibrio* spp. did not differ between coral fragments secured with biodegradable (B), plastic or Rapstrap ties (mean RA = 0.28 %, mean RA = 0.16 %, mean RA = 0.54 %, respectively) and donor colonies (mean RA = 0.12 %) (Wilcoxon rank sum, $p < 0.05$, and Fig. S4.4b.). Consequently, there was no evidence to suggest that using the biodegradable materials tested in this study would increase abundance of putatively pathogenic taxa in propagated coral microbiomes.

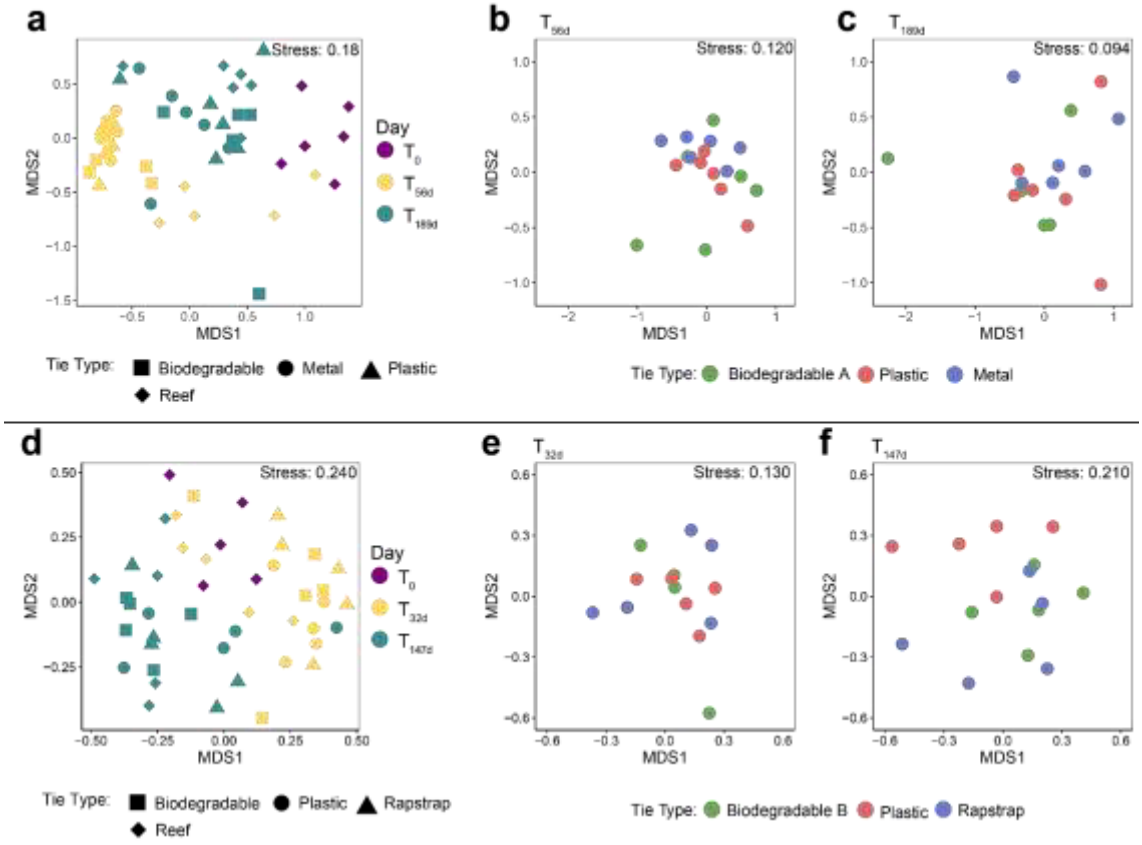



**Figure 4.2.** Bacterial community structure and relative dispersion of the microbial communities of (**a**) donor colonies at time of harvesting ($T_0$) and nursery fragments and donors at $T_{56d}$ and $T_{189d}$, and just nursery fragments at (**b**) $T_{56d}$ and (**c**) $T_{189d}$ for the first experiment. Bacterial community structure and relative dispersion of (**d**) donor colonies at time of harvesting ($T_0$) and nursery fragments and donor colonies at $T_{32d}$ and $T_{147d}$ and just nursery fragments at (**e**) $T_{32d}$ and (**f**) $T_{147d}$ in the second experiment. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure.

### *4.4.4 Conclusion*

Reducing reliance on plastic materials and optimising protocols is key to advancing coral propagation and out-planting approaches. However, little is known about the impact of changing the materials used to fasten corals to artificial propagation structures. Here we examined whether biodegradable materials used to secure coral to nurseries differentially impact the coral-associated bacterial communities compared to conventional plastics. In contrast to our hypotheses that tie material would influence coral-associated bacterial communities (and that biodegradable ties would cause higher putative pathogen loads), we found no significant change in coral bacteria communities

that could be explained by the tie material. Generally plastic-free alternatives had low failure rates and high coral survival similar to conventional plastic, except for biodegradable material A. However, how well these materials perform for other coral taxa with particularly different growth rates and morphologies needs to be tested. Further, as highlighted here – not all biodegradable materials have equal failure – and as more biodegradable options become available it will be essential to quantify their respective 'life spans' in marine environments to ensure degradation does not occur prior to coral self-attachment. In conclusion, in this study we show that biodegradable materials do not differentially impact associated bacterial communities of fragments grown in coral nurseries and suggest that transitions from conventional plastic to particular biodegradable alternatives while avoiding impacts to coral-associated bacterial communities is possible.

## 4.5 Acknowledgements

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rehabilitation through boosting coral abundance: Miniaturising and mechanising coral out-planting" to David J. Suggett, Emma F. Camp and John Edmondson). Emma F. Camp was supported through the University of Technology Sydney Chancellor's Postdoctoral Research Fellowship and ARC Discovery Early Career Research Award (DE190100142). Justin Seymour was supported by the Australian Research Council Grant (DP210101610).

## 4.7 Data Availability

The datasets generated and/or analysed during the current study are available in the NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA945487 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA945487) or within the Supplementary Data sheets provided online at doi.org/10.21203/rs.3.rs-2729419/v1.

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## 4.9 Supplementary Materials

### *4.9.1 Supplementary Methods*

All samples were held at 4 °C for four to six days during transportation from the study site to the laboratory. Once in the laboratory, RNA*later* was thoroughly removed, and samples were preserved at –80 °C for up to six months until DNA was extracted. Prior to DNA extraction, samples were thawed and rinsed with autoclaved phosphate-buffered saline (PBS) (3X, pH 7.4), and coral tissue was removed from the skeleton via air brushing into 4 mL of PBS. The tissue slurry was divided across two 2-mL micro centrifuge tubes and centrifuged at 8000 rpm for 5 min. The supernatant was removed, and the tissue pellet was stored at –80°C for one to two weeks until DNA extraction. DNA was extracted from approximately 200 µl of the coral tissue pellet using a DNeasy Blood and Tissue kit (Qiagen) following the Manufacturer's protocol (July 2020 version) with a total elution volume of 40 µL. Kit 'blank' samples were included in DNA extractions to identify any laboratory contaminants. Extracted DNA was quality checked and the concentration was quantified using a NanoDrop spectrophotometer.

### *4.9.2 Supplementary Figures*

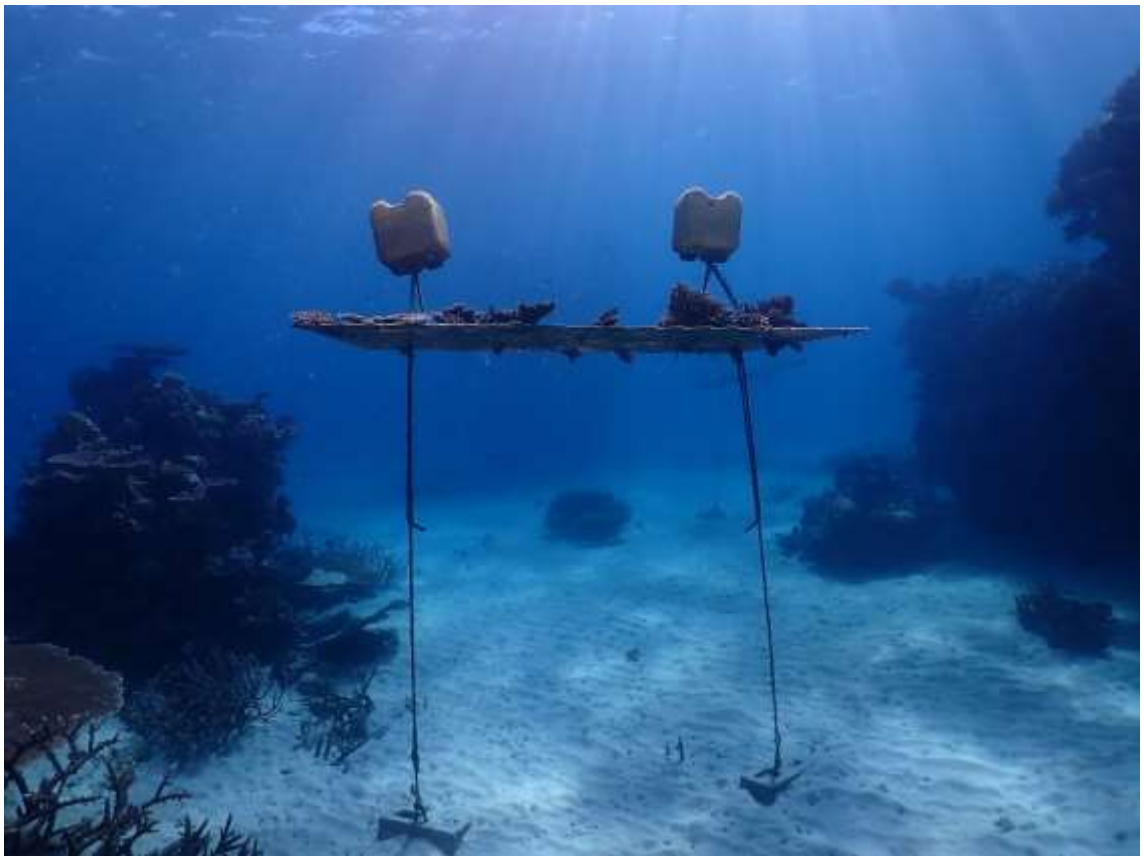


**Figure S4.1.** Example of coral nursery at Opal Reef, photo: John Edmondson.

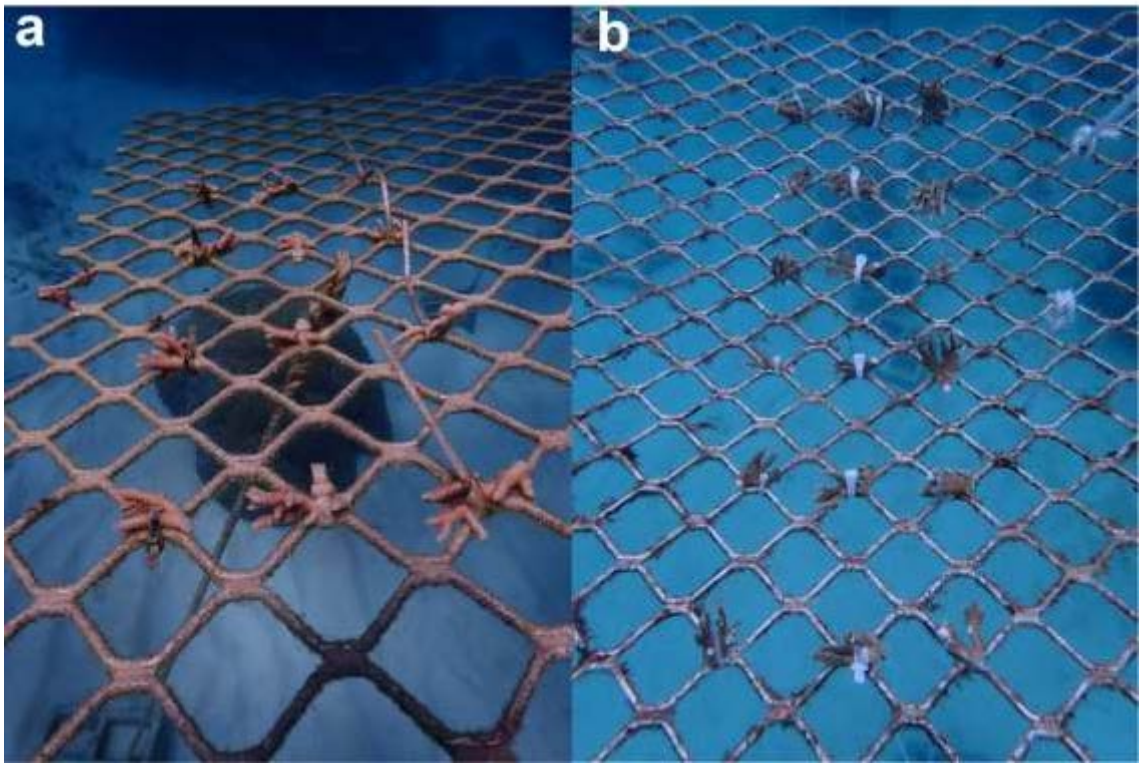



**Figure S4.2.** Fragments of *Acropora millepora* in coral nurseries at Opal Reef, northern GBR. Coral fragments are secured with (from left to right) (**a**) plastic, biodegradable material A and metal ties in September 2020; and (**b**) plastic, biodegradable material B and Rapstrap ties in February 2022.

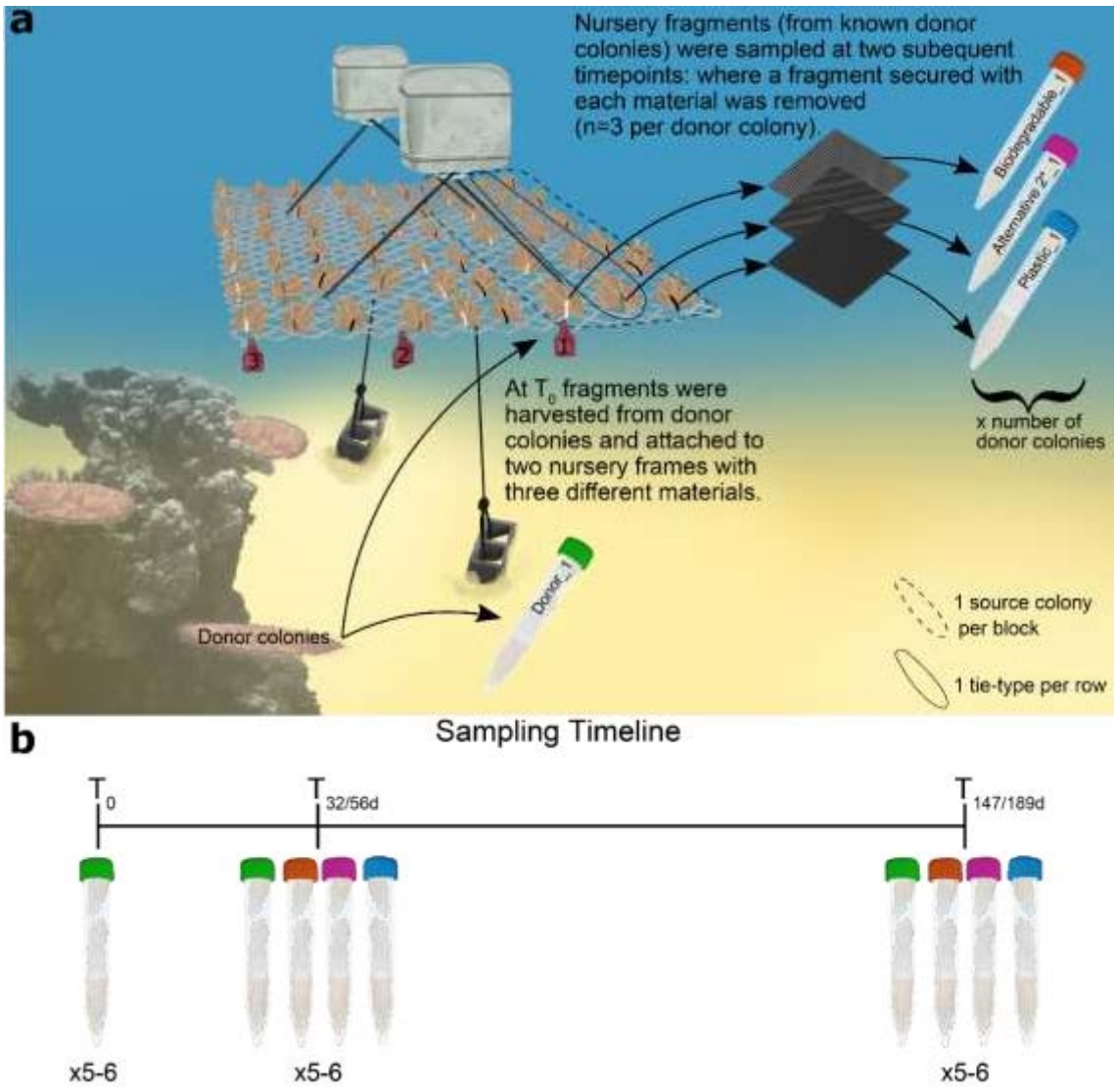



**Figure S4.3.** Illustration of (**a**) the experimental design and nursery set up and (**b**) sampling timeline of the two experiments.

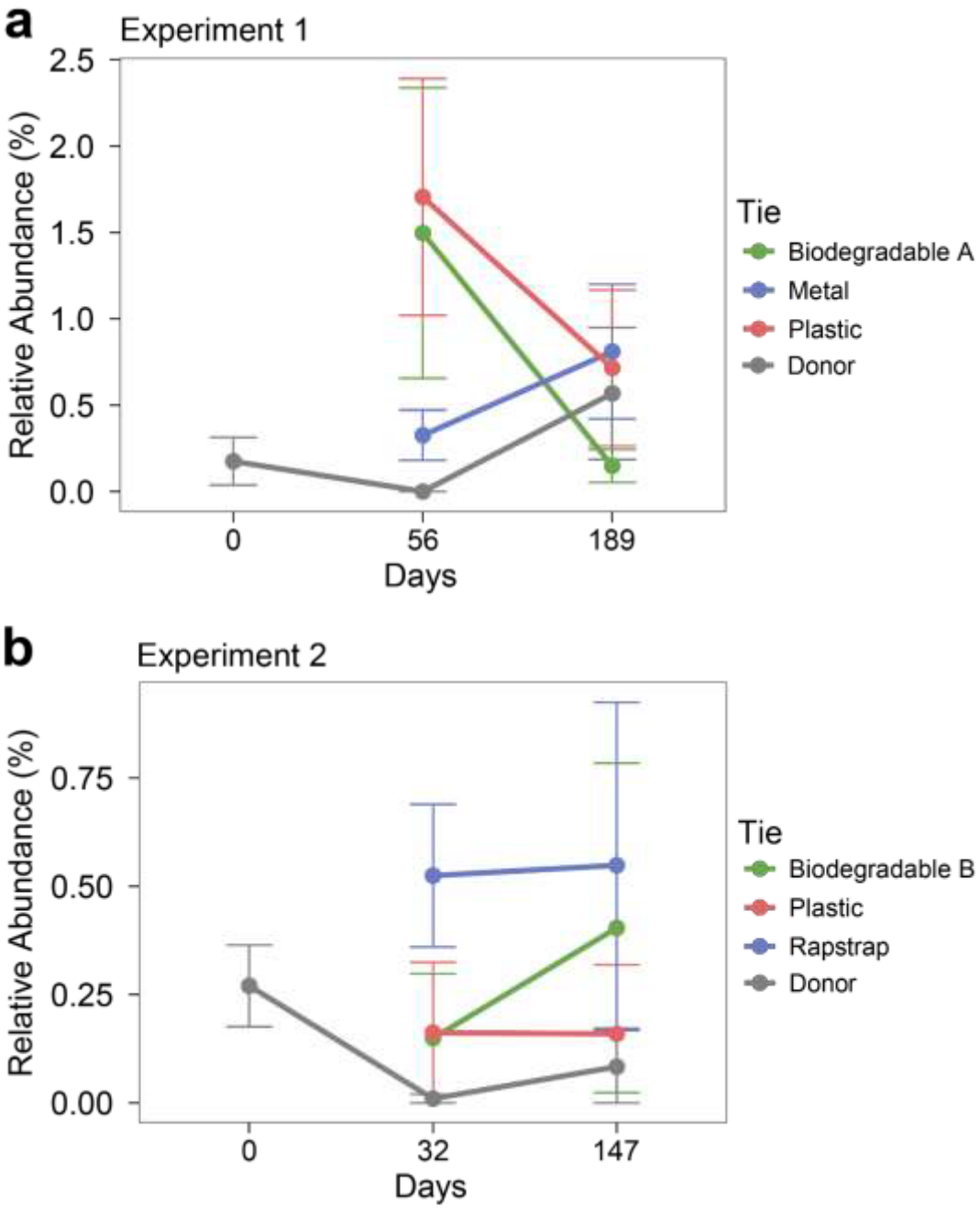



**Figure S4.4.** Relative abundance of bacteria within the *Vibrio* genus for *Acropora millepora* donor colonies and fragments within a coral nursery over time; in two studies assessing (**a**) biodegradable material A, plastic, and metal zip-ties and (**b**) biodegradable material B, plastic and Rapstrap zip-ties to secure coral fragments (Source Data provided as Supplementary Data S2.).

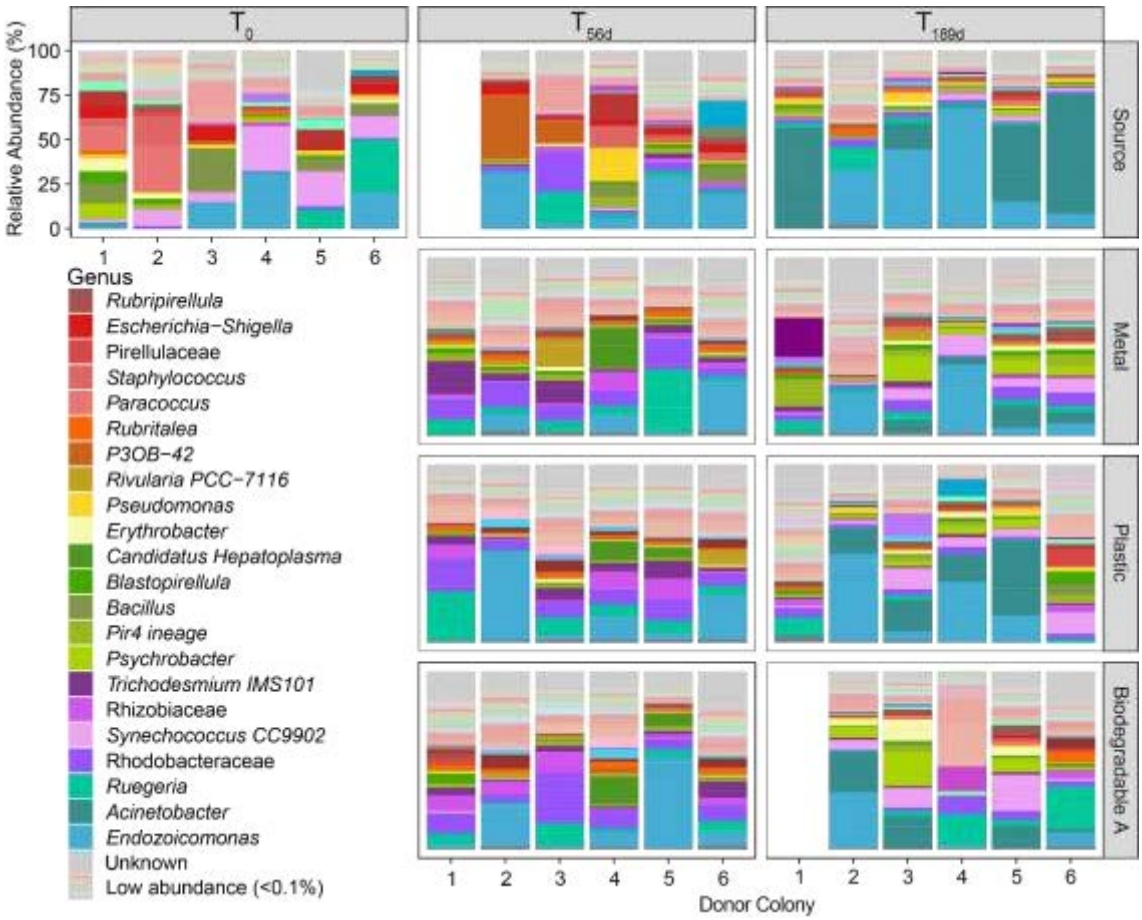



**Figure S4.5.** Bacterial community composition (relative abundances) by genus* of donor colonies at the start of the experiment ($T_0$) and fragments after 56 and 189 days (from August 2020 to February 2021) within a nursery secured with different materials; metal, plastic and biodegradable material A. Pastel colours represent genera with an average relative abundance of < 0.1 % in all samples, full legend provided as supplemental data (Supplementary Data S3a.) *non-italic text indicates family where – taxonomic resolution to genus level was unavailable.

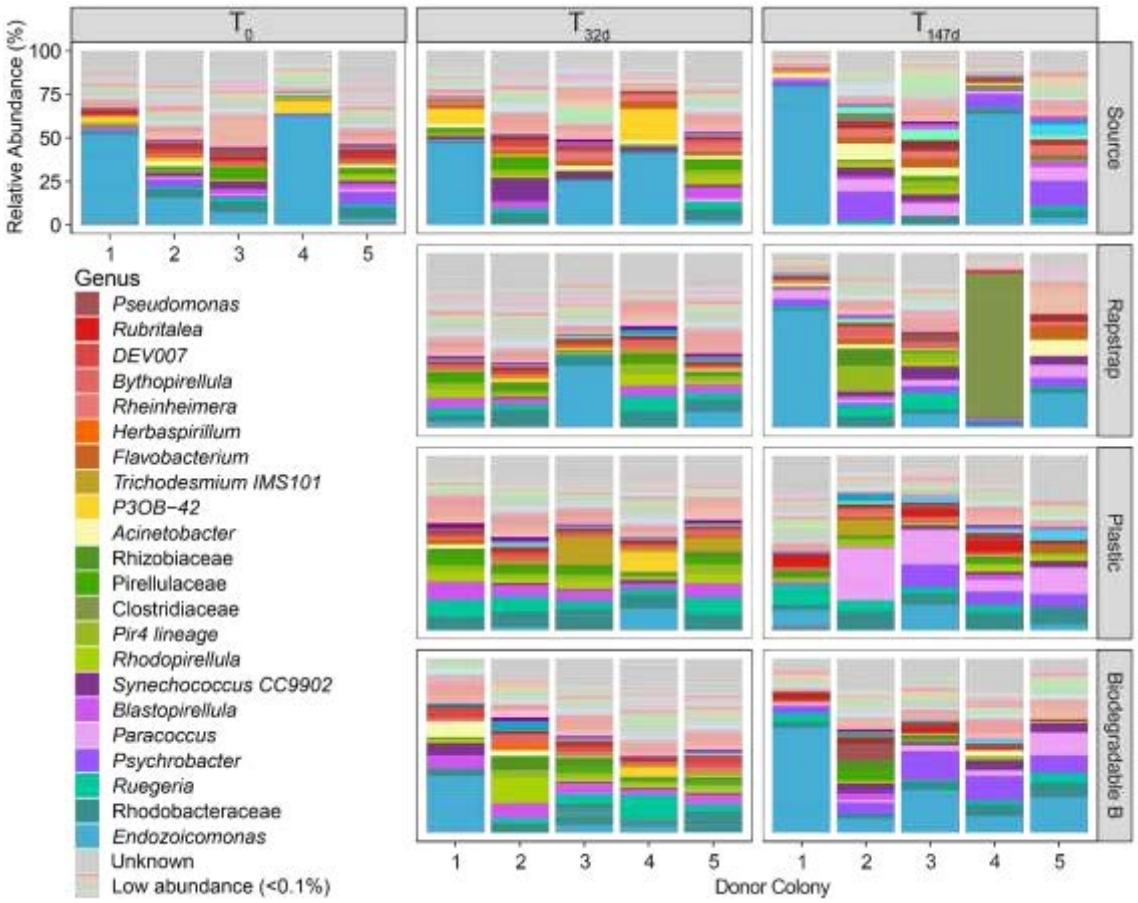



**Figure S4.6.** Bacterial community composition (relative abundances) by genus* of donor colonies at the start of the experiment ($T_0$) and fragments after 32 and 147 days (from February to August 2022) within a nursery secured with different materials; Rapstrap, plastic and biodegradable material B. Pastel colours represent genera with an average relative abundance of < 0.1 % in all samples, full legend provided as supplemental data (Supplementary Data S3b.) *non-italic text indicates family – where taxonomic resolution to genus level was unavailable.

### *4.9.3 Supplementary Tables*

| **Experiment 1** | | |
|---|---|---|
| **Material Details** | | |
| **Biodegradable A (gocableties.co.uk)** | **Metal** | **Plastic** |
| SES-QUICK BIO biodegradable polyester (partially biobased of wheat or sugar cane). Poly 1,4-butanediol Succinate – PBS. 300 mm x 7.6 mm | 316 Grade stainless steel. 200 mm x 4.6 mm | Nylon 66 – polyamide. Black colour. 300 mm x 4.8 mm |
|  |  |  |
| **Experiment 2** | | |
| **Material Details** | | |
| **Biodegradable B (rapstrap.com)** | **Rapstrap** (i-Tie: 052-MPU) **(rapstrap.com)** | **Plastic** |
| Polycaprolactone 300 mm x 8 mm | Polyurethane Elastomer. 300 mm x 4.8 mm | Nylon 66 – polyamide. 'Natural' colour. 300 mm x 4.8 mm |
|  |  |  |

**Table S4.1.** Details of the attachment materials used in each experiment with photographs of corals attached to nursery frames.

| Failure | Photograph, Experiment Number and Tie Details | |
|---|---|---|
| Missing coral fragment and tie. |  Experiment 1; Biodegradable (A). | |
| Missing tie and coral fragment still present. |  Experiment 1; Biodegradable (A). |  Experiment 1; Biodegradable (A). |
| Dead corals |  Experiment 2; Plastic tie. |  Experiment 2; Rapstrap tie. |

**Table S4.2.** Examples of tie failure and coral mortality.

| Experiment 1 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Time** | **$T_0$** | | | **$T_{56d}$** | | | **$T_{189d}$** | | |
| | **Metal** | **Bio A** | **Plastic** | **Metal** | **Bio A** | **Plastic** | **Metal** | **Bio A** | **Plastic** |
| attached with tie | 24 | 24 | 24 | 24 | 21 | 24 | 22 | 11 | 22 |
| missing entirely | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 5 | 0 |
| coral missing tie present | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| coral present tie missing | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 |
| dead coral | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *expected coral fragments* | *24* | *24* | *24* | *24* | *24* | *24* | *23* | *18* | *23* |
| **Tie type** | Metal | | | Bio A | | | Plastic | | |
| **Total failure %** | 4.167 | | | 29.167 | | | 4.167 | | |
| **Experiment 2** | | | | | | | | | |
| **Time** | **$T_0$** | | | **$T_{32d}$** | | | **$T_{147d}$** | | |
| | **Rap-strap** | **Bio B** | **Plastic** | **Rap-strap** | **Bio B** | **Plastic** | **Rap-strap** | **Bio B** | **Plastic** |
| attached with tie | 28 | 29 | 29 | 26 | 29 | 29 | 23 | 28 | 27 |
| missing entirely | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| coral missing tie present | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| coral present tie missing | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| dead coral | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 1 |
| *expected coral fragments* | *28* | *29* | *29* | *28* | *29* | *29* | *25* | *28* | *28* |
| **Tie type** | Rapstrap | | | Bio B | | | Plastic | | |
| **Total failure %** | 0 | | | 0 | | | 0 | | |

**Table S4.3.** Survivorship and failure counts for experiment 1 and 2 at sampling time points.

### *4.9.4 Supplementary Data*

The following supplementary data files are within the *Supplementary Data* file online at doi.org/10.21203/rs.3.rs-2729419/v1

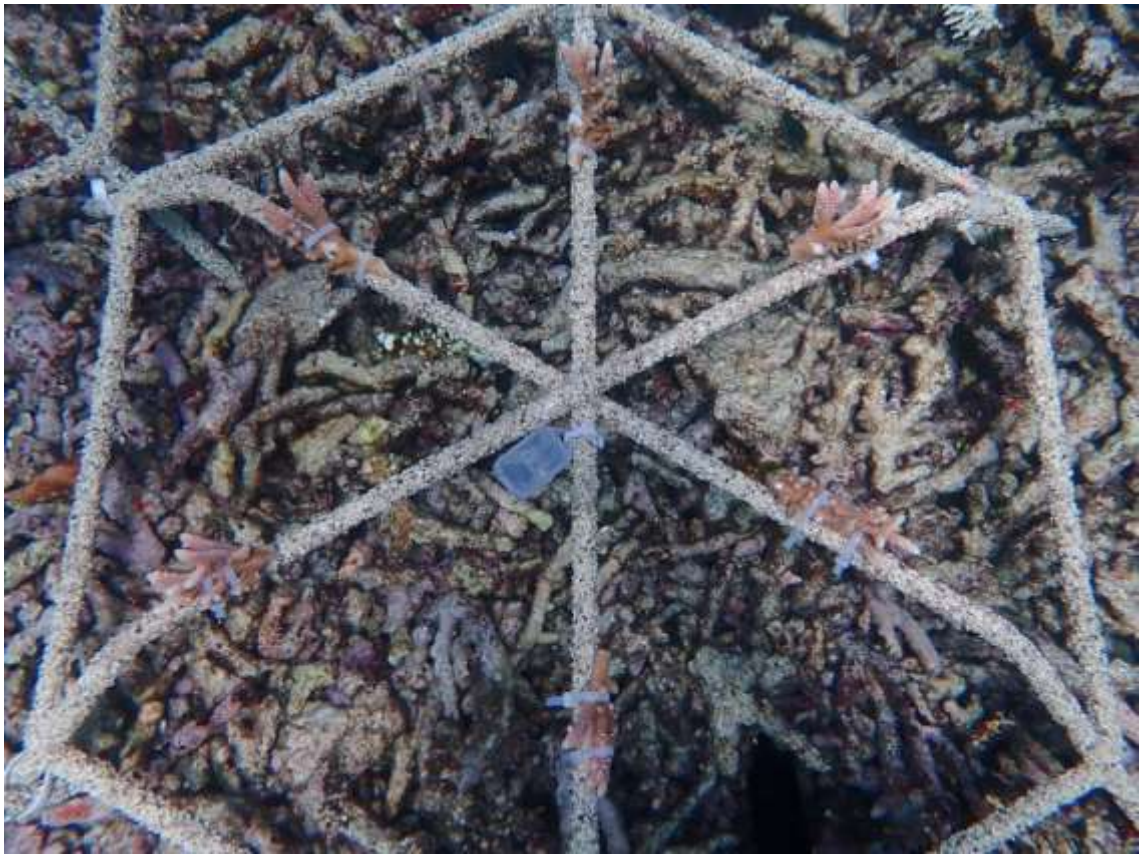
**Data S1** PERMANOVA analysis of microbial community structure of nursery corals secured with different material ties and donor colonies.

**Data S2** Analysis of relative abundance of *Vibrio* spp. bacteria in associated bacterial communities of corals secured with different material ties and donor colonies.

**Data S3a** Full legend for Fig S4.5.

**Data S3b** Full legend for Fig S4.6.

# Chapter 5: Assessing how metal reef restoration structures shape the functional and taxonomic profile of coral-associated bacterial communities.



**In preparation as:**

**Strudwick, P.**[1], Suggett, D. J.[3] , Seymour, J[1], DeMeare, M. Z.[4] , Grima, A.[1], Edmondson, J.[2], McCardle, A.[5], Nicholson, F.[5], & Camp, E. F.[1]. Assessing how metal reef restoration structures shape the functional and taxonomic profile of coral-associated bacterial communities.

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Paige Strudwick, David J. Suggett, Emma F. Camp and Justin Seymour designed the experiments. Paige Strudwick, David Suggett, Emma F. Camp, Amanda Grima, Freda Nicholson, Alicia McCardle, and John Edmondson initiated field experiments. Paige Strudwick, Emma F. Camp, Amanda Grima and David Suggett collected and preserved samples. Paige Strudwick conducted all laboratory processing. Matthew DeMeare provided advice and processing of shotgun metagenomic output. Paige Strudwick processed amplicon sequencing output and conducted all data analysis. Paige Strudwick wrote the original manuscript. David J. Suggett, Emma F. Camp and Justin Seymour provided core editing and all co-authors read and approved the final manuscript.

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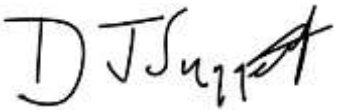
David Suggett: 

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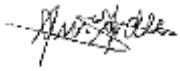
Alicia McCardle: 

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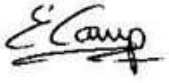
Emma Camp: 

## 5.1 Abstract

Significant threats to the long-term persistence of coral reefs have accelerated the adoption of coral propagation and out-planting restoration approaches. However, how materials commonly used for propagation structures potentially affect essential coral-associated bacterial communities remains untested. Here we examined the impact of metal propagation structures on coral-associated bacterial communities on the Great Barrier Reef. Fragments of the commonly propagated coral species *Acropora millepora* were grown on aluminium frames, sand/epoxy coated steel modular structures (Reef Stars), and uncoated steel (rebar) stakes. After six months the functional and taxonomic profiles of coral-associated bacterial communities of propagated corals and source reef colonies were characterised using amplicon (16S rRNA gene) and shotgun metagenomic sequencing. No differences in the phylogenetic structure or functional profile of coral-associated bacterial communities were observed between propagated corals and source reef colonies. However, specific genes and pathways were overrepresented in associated bacterial communities of corals grown on different structure materials and different taxa were indicative of the structures. Several metabolic pathways were elevated in rebar and (to a lesser extent) Reef Star corals, including lipid, nucleotide, and carbohydrate metabolism pathways, suggesting that propagation on steel structures may alter the functional potential of coral-associated bacterial communities. Whether changes are directly a result of altered iron availability from steel structures remains to be tested. These findings indicate that propagation of coral on different structure materials can lead to differences in individual bacterial taxa and functional potential of coral-associated bacterial communities, but how these contribute to changed holobiont fitness presents a key question to be addressed.

## 5.2 Introduction

Reef restoration approaches that involve propagation and out-planting and/or substrate stabilisation are commonly being applied to assist natural recovery and retain reef resilience in the face of mounting local and global stressors (Boström-Einarsson et al. 2018; GBRMPA, 2019; Hein et al. 2021). Coral propagation and out-planting approaches facilitate increases in coral biomass and cover at target sites (Suggett et al. 2020; Hein et al. 2020; Roper et al. 2022; Howlett et al. 2023), whereas reef stabilisation techniques aid substrate consolidation, but similarly enhance coral biomass when corals are attached to the structures (Williams et al. 2019; Fox et al. 2019; Ceccarelli et al. 2020; Kenyon et al. 2023). There is a strong motivation to boost abundance of resilient corals during propagation to improve long-term survival under future climate scenarios (Caruso et al. 2021; Camp et al. 2022). However, this is often conducted empirically, by selecting coral stock that has survived previous stress events (Caruso et al. 2021). Arguably, the efficacy of such a proactive approach would be increased if bolstered by knowledge of underlying coral biology dynamics during restoration (Voolstra et al. 2021; Shaver et al. 2022). However, whilst such approaches continue to be adopted with accelerating enthusiasm (Boström-Einarsson et al. 2020; McAfee et al. 2021; Suggett and van Oppen, 2022), how the processes and materials used impact coral biology (and related resilience) has only recently been considered (Morikawa and Palumbi, 2019; Strudwick et al. 2022, 2023a, 2023b in press; Nuñez Lendo et al. 2023 in press).

Multifaceted communities of microorganisms – the coral ‘microbiome’ – are central to the health of the coral host, and in totality, the host and associated microorganisms are referred to as the coral ‘holobiont’ (Rohwer et al. 2002; Reshef et al. 2006; Rosenberg et al. 2007). Recently, the focus of restoration research has expanded to consider approaches to ‘conserve the holobiont’ to improve restoration

success (Carthey et al. 2019; Voolstra et al. 2021; Peixoto et al. 2022). Such an approach is important for coral reefs as associated microorganisms can impact reef resilience (Putnam and Gates, 2015; Rosado et al. 2018; Peixoto et al. 2022), and thus present an opportunity (e.g., engineering of an optimal microbial consortium; Peixoto et al. 2017) or risk (e.g., disease outbreaks; Rosenberg et al. 2007; Moriarty et al. 2020) in restoration activities. Coral-associated bacterial communities are known to be impacted by transfer between distinct environments during nursery propagation and out-planting (Strudwick et al. 2022, 2023a). However, the extent to which materials used during propagation influence the coral environment and subsequently contribute – negatively or positively – to bacterial community changes is relatively unexplored.

A range of materials are used in reef restoration, from concrete to chemical adhesives, metals, plastics, ropes, and natural fibres (Nedimyer et al. 2011; Meesters et al. 2015; Williams et al. 2019; Suggett et al. 2020; Ceccarelli et al. 2020; Boström-Einarsson et al. 2020; Denhert et al. 2022). Materials are often selected due to their low-cost, structural integrity, scalability, and ease of deployment (Ceccarelli et al. 2020), rather than considering how they may detrimentally impact, or benefit propagated corals and the other members of their holobiont. Use of artificial materials may create a unique biogeochemical interface for corals that could act as a potential source of essential resources, such as trace elements (Ray et al. 2010; Reich et al. 2022), or harbour distinct bacterial communities and consequently impact coral-associated bacterial communities (Ceccarelli et al. 2020). Recent work has revealed that the type of plastic material used to secure corals to substrates does not differentially impact coral-associated bacterial communities, yet some zip-tie materials have greater coral retention rates (Strudwick et al. 2023b, in press). However, whether metal structures influence environmental conditions for propagated corals and subsequently influence coral-associated bacterial communities remains untested.

Across multiple studies we have recorded changes in the bacterial communities of *Acropora millepora* when transferred from native reef to aluminium nursery structures during restoration (Strudwick et al. 2022, 2023b in press). Aluminium is generally considered to be inert (although alloys can also corrode, Ezuber et al. 2008), whereas steel corrodes and releases iron oxides (Ray et al. 2010) in marine environments. Fe plays essential roles in physiological processes of both the coral host and associated microorganisms (Duckworth et al. 2009; Patel et al. 2020; Rubio-Portillo et al. 2020) and has potential to leach into the environment at the coral-material interface as steel structures interact with the marine environment (Procópio, 2019). Thus, compared to aluminium, it is plausible to expect that steel structures will differentially impact the coral holobiont during propagation. We therefore hypothesise that coral propagated on steel structures will (i) host distinct bacterial communities, (ii) with different functional potential compared to those propagated on aluminium structures, (iii) specifically involving overrepresentation of genes related to Fe cycling or with Fe requirements (e.g., as co-factors) and/or higher abundance of putatively pathogenic bacterial taxa. To test these hypotheses, we compared the functional and taxonomic characteristics of associated bacterial communities of the coral species *A. millepora* propagated on aluminium frames, sand/epoxy coated steel modular structures (Reef Stars; Williams et al. 2019) and uncoated steel stakes.

## 5.3 Materials and Methods

The study period spanned six months from February to August 2022 and was conducted on the northern Great Barrier Reef (GBR), at site “Long Bommie” on Opal Reef (16°22'17.2"S 145°87'60.6"E, Fig 5.1a-b.). Long Bommie was impacted by Cyclone Ita in 2014, which caused high structural degradation over most of the site and substantial areas of unconsolidated rubble. Despite widespread coral bleaching on the

GBR in 2016/17, 2020 and 2022 good recovery has been observed over the last two years on the reef flat and crest (Fig 5.1c-d.). Coral cover has particularly increased in the shallow (< 3 m depth) areas of the site (Fig 5.1c-d.). However, recovery has been relatively slow within the rubble, and subsequently areas of unconsolidated rubble remain at the southern side of the bommie (Fig 5.1e.). Two 2.0 × 1.2 m aluminium diamond-mesh frames were installed approximately 30-40 cm above the sand secured to and held in place with 4 × 9 kg Besser blocks (Fig 5.2a.). Six hexagonal Reef Stars (coated steel) 54 cm diameter x 33 cm height, constructed from rebar coated with fibreglass and sand (Fig 5.2b.), were installed directly over coral rubble and secured with steel rods (as per Williams et al. 2019, Fig 5.2b.). Six uncoated 60 cm rebar stakes were hammered ~ 15 cm into rubble (Fig 5.2c.). All structures were installed at the start of the experiment without pre-conditioning.

### *5.3.1 Coral harvesting and experimental set-up*

Coral sampling methods were designed to minimise any trace metal contamination (as per Grima et al. 2022). In brief, clear polypropylene plastics were prepared via a series of wash steps (as per Rodriguez et al. 2016) and used to mark and transfer the corals, while wooden chisels (or new bone cutters wrapped in parafilm) were used to sample coral. The location of six *A. millepora* source colonies (≥ 55 cm diameter) – representing biological replicates – on the native reef were marked with transparent polypropylene plastic tags. Sixteen fragments (≤ 5 cm) were taken from each source colony. One fragment was retained in a sterile Whirl-Pak® bag for $T_0$ microbial community characterisation, while the remaining 15 fragments were then divided into three plastic baskets. Fragments were then transported to the three different structures by SCUBA: five fragments from each of the six source colonies were taken to each of the aluminium frames, rebar stakes or the Reef Stars (Fig 5.3.). Coral fragments were evenly divided and attached to the two aluminium frames (to account for potential

frame effect), six Reef Stars and six rebar stakes with clear cable ties (Fig 5.3.). On the aluminium frames each row of corals corresponded to one source colony, fragments from only one source colony were attached to each structure for rebar stakes and Reef Stars. Clear tags were attached to the individual structures (or rows on the aluminium frames) to identify respective source colonies (Fig 5.2a-c. and 5.3.).

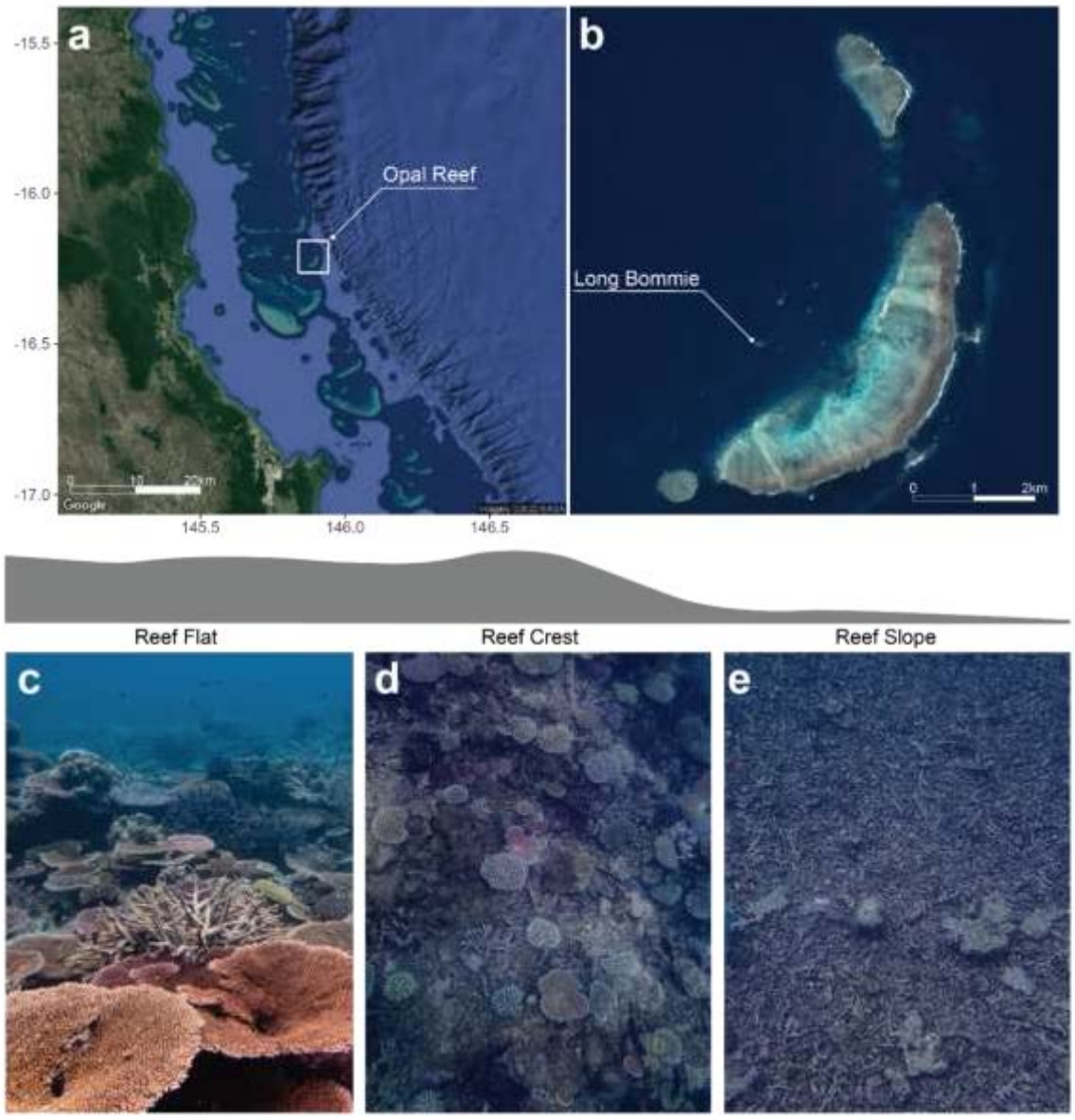



**Figure 5.1.** (**a**) Satellite image of Opal Reef, Northern Great Barrier Reef, Australia, (**b**) Opal Reef with Long Bommie the field site in this study. Coral cover has increased on the reef flat (0.5-1.5 m depth) (**c**) and crest (1-2 m depth) (**d**) since 2014 Cyclone Ita damaged the site, yet unconsolidated rubble fields remain on the reef slopes (3.5-4.5 m depth) (**e**). Satellite image sourced from GoogleEarth and allencoralatlas.org respectively. Photograph credit: (**c**) Christine Roper, *February 2023*; (**d-e**) Dr Emma Camp, *February 2022*.

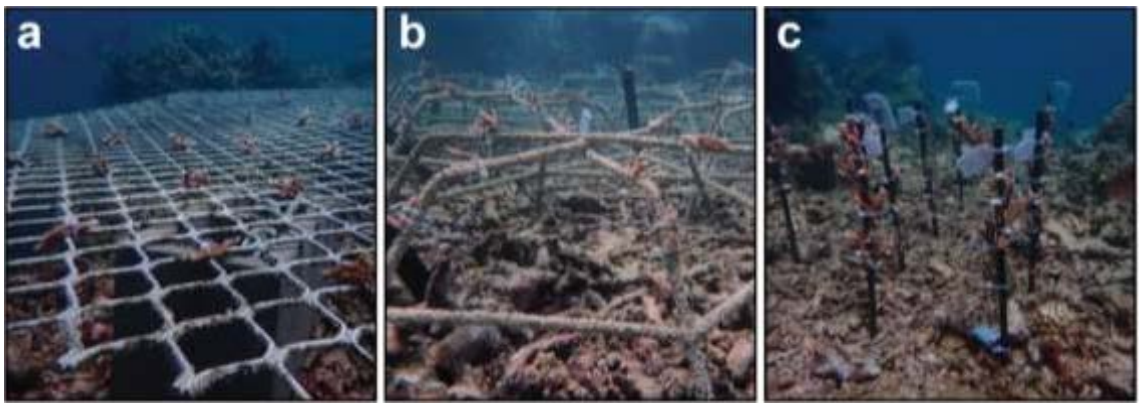



**Figure 5.2.** Coral fragments at Long Bommie secured to (**a**) an aluminium frame, (**b**) Reef Stars and (**c**) rebar stakes.

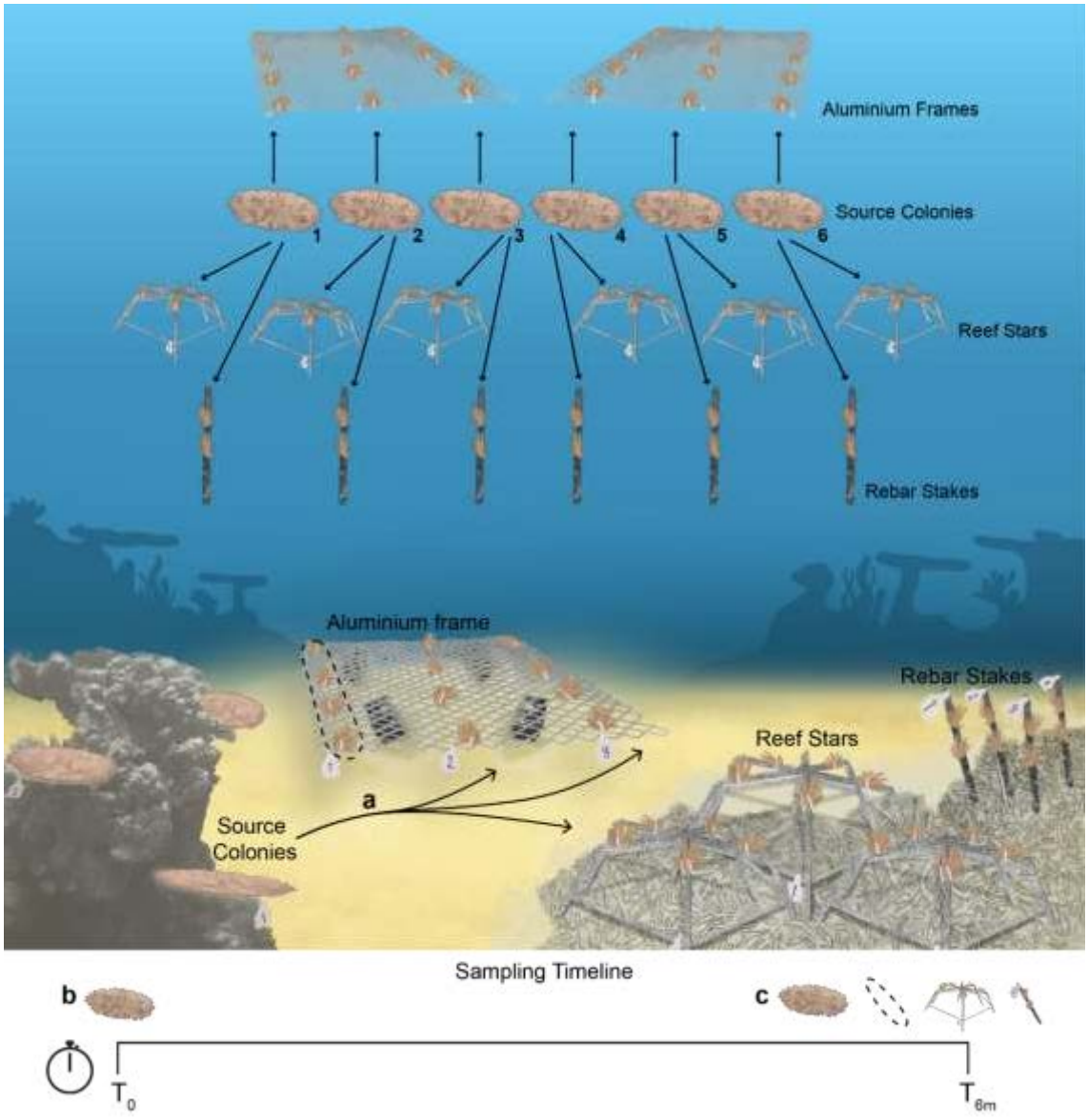



**Figure 5.3**. Coral fragments were harvested from source colonies at $T_0$ and (**a**) transferred to either aluminium frames, coated steel Reef Stars or uncoated steel rebar stakes or (**b**) retained for initial bacterial community characterisation. Fragments from individual source colonies (n = 6) were secured in rows on two aluminium frames (see

dashed line) or to one (of six) individual Reef Star or rebar stake and marked with clear plastic tags. Corals on propagation structures and source colonies were then sampled (**c**) after six months ($T_{6m}$) for bacterial community analysis.

### *5.3.2 Sampling regime*

At the start of the experiment, one (5-10 cm) fragment from each source colony was placed in an individual sterile Whirl-Pak® bag and immediately taken to the operations vessel to be snap frozen in liquid nitrogen to characterise the bacterial community of source colonies at 'time zero' ($T_0$) (Fig 5.3b.). Following $T_0$, corals on the propagation structures (aluminium frames, Reef Stars, rebar stakes) and source colonies were re-sampled at six months ($T_{6m}$) (Fig 5.3c.). After six months, coral fragments (5-10 cm) were either removed from the propagation structures by detaching the cable tie (or cut using bone cutters wrapped in parafilm in cases where fragments had begun to overgrow the structure) or from the source colony in the natural reef environment using parafilm wrapped bone cutters. Samples were placed into sterile Whirl-Pak® bags and snap frozen in liquid nitrogen as per sampling at $T_0$.

In total, 30 coral samples were collected, and up to two DNA extractions were conducted from each sample to provide DNA for shotgun metagenomics and amplicon (16S rRNA gene) sequencing. To increase the yield of coral-associated bacterial DNA a phenol-chloroform extraction with an endonuclease step to remove coral host and Symbiodiniaceae DNA was used for shotgun metagenomic applications (detailed in Appendix 5.1). This approach is not required for amplicon sequencing where the target region is amplified via PCR prior to sequencing, hence, we proceeded with a previously successful DNA extraction kit for amplicon sequencing.

In some cases, there was not adequate material for two DNA extractions and replication of n = 3 for each treatment across consistent source colonies (e.g., colonies 1, 2 and 3 sampled at both $T_0$ and $T_{6m}$ for source reef colonies) for metagenomic sequencing was prioritised (Fig S5.1.). DNA was extracted from 15 samples for shotgun

metagenomic analyses: three replicates x one time point for each of the propagation structures (n = 9) and three replicates x two time points for the source reef colonies (n = 6). DNA was extracted from 25 samples for amplicon (16S rRNA gene) sequencing: five to six replicates x two time points for source reef colonies ($T_0$ n = 5 and $T_{6m}$ n = 5), four to six replicates x one time point for propagated corals ($T_{6m}$: aluminium frames n = 4, Reef Stars n = 4 and rebar stakes n = 6) (Fig S5.1.), and one blank DNA extraction to identify laboratory contaminants.

### *5.3.3 DNA extractions*

All samples were transported from the field site to the laboratory in a dry shipper and then stored at –80 °C for 3-9 months, so that all DNA extractions could be conducted simultaneously. To minimise sample bias based on fragment size, preliminary analysis of the coral elementome (specifically Fe content) was performed (as per Grima et al. 2022) to identify a location on the coral branch (tip, middle or base) for standardised metagenomics sampling. Results from this testing did not indicate any areas of host tissue or symbiotic algae with significant Fe enrichment relative to other fragment locations and thus, sampling proceeded in the following manner. A 3-4 cm fragment was sub-sampled 1 cm from the base (structural contact point) of the propagated coral (Fig S5.2. a-b.) and a 3-4 cm fragment of the source colony sample were placed in sterile zip-lock bags and coral tissue was removed from the coral skeleton via air brushing with sterile pipette tips into 2 mL of autoclaved PBS (3X, pH 7.4). The tissue slurry was transferred to a 2-mL microcentrifuge tube and centrifuged at 8000 x G for 5 min. The supernatant was removed, and the pellet was stored at –80 °C for two days until DNA extraction. Coral tissue pellets were resuspended in 3X PBS prior to a series of homogenisation steps (as per Voolstra et al. 2023 in press, detailed in Appendix 5.1.). Following all homogenisation steps, a 250 µl aliquot of tissue slurry was used for phenol-chloroform DNA extraction that included an incubation with

Benzonase to remove free host and Symbiodiniaceae DNA (as per Voolstra et al. 2023 in press, detailed in Appendix 5.1.).

Prior to DNA extraction for amplicon (16S rRNA gene) sequencing, coral tissue was removed from the coral skeleton, using an air brushing technique. For each sample a 3-4 cm fragment was sub-sampled from the same area as before and placed in a sterile zip-lock bag to be air brushed with sterile pipette tips into 4 mL of autoclaved PBS (3X, pH 7.4). The tissue slurry was divided across two 2-mL micro centrifuge tubes and centrifuged at room temperature for 5 min at 8000 x G. The supernatant was removed, and DNA was extracted from approximately 100 µL of the coral tissue pellet using a DNeasy Blood and Tissue kit (Qiagen – July 2020 version) following the manufacturer's protocols with a total elution volume of 40 µL. A kit negative (no sample material added) was included in the DNA extraction to identify any kit contaminants. For both the shotgun metagenomic and 16S amplicon DNA extraction methods, extracted DNA was quality checked and the concentration was quantified using a NanoDrop spectrophotometer prior to sequencing.

### *5.3.4 Sequencing*

DNA samples were stored at –80 °C for 2-4 days until transportation on dry ice to the Ramaciotti Centre for Genomics (Sydney, NSW, Australia) for (16S rRNA gene) amplicon and shotgun metagenomic sequencing to characterise (taxonomic and functional profiles of) the coral-associated bacterial communities. To taxonomically characterise coral-associated bacterial communities the hypervariable V3 to V4 region of the bacterial 16S rRNA gene was amplified using the primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al. 2013) and the amplicons were sequenced using the Illumina MiSeq v3 2 × 300 bp platform. To characterise the functional profile of the coral-associated bacterial communities, shotgun metagenomic sequencing was performed using the

Illumina NovaSeq 6000 SP 2 x 150 bp Flowcell platform (Ramaciotti Centre for Genomics). Raw reads from 16S rRNA amplicon sequencing and shotgun metagenomics were deposited in NCBI Sequence Read Archive (SRA) in FASTQ format under Bioproject number PRJNA988823 and will be released upon publication or by request.

### *5.3.5 Bioinformatics*

**16S rRNA Amplicon Sequencing**

Raw demultiplexed sequencing data were analysed with Quantitative Insights into Microbial Ecology (QIIME 2, version 2020.6) platform (Callahan et al. 2016). The data was denoised (with the DADA2 plugin) prior to taxonomic assignment against the SILVA v138 database using the classify-sklearn classifier (Pedregosa et al. 2011). After denoising, 3,666,205 reads were generated from 25 samples. Mitochondrial or chloroplast amplicon sequence variants (ASVs) were filtered from the data set. Nine ASVs that comprised 100% of sequences in the kit negative DNA extraction were removed for subsequent analyses using the filter command in R (version 4.2.2). Two samples were removed before diversity analyses due to poor sequencing outputs, resulting in low read numbers after quality filtering and contaminant removal (< 55 ASVs) (Fig S5.1.). For beta diversity analyses, the raw read ASV table was converted to relative abundances, scaled to 20,000 (McKnight et al. 2019) and square root transformed.

**Shotgun Metagenomic Sequencing**

Filtering of low-quality reads, trimming of adapter and low-quality sequences, and deduplication was performed on raw reads using fastp (v0.23.2) (Chen et al. 2018). Removal of contaminating host *A. millepora* (acc:GCF_013753865.1) and human (acc:GCF_013753865.1) DNA was performed using HoCoRT (v1.0.0) (Rumbavicius et al 2023, in press). Read-based taxonomic profiles were generated from cleaned read sets

using Sourmash (v4.8.2) (Brown and Irber, 2016) against the Genome Taxonomy Database (GTDB) (release 207) (Parks et al. 2021). Overlapping pairs were first merged for each read-set using fastp, and a (pooled) co-assembly was then constructed using MEGAHIT (v1.2.9), where merged reads were passed as single-ended (Li et al. 2015). Co-assembled contigs were passed as a user-supplied assembly to the SqueezeMeta (v1.6.1) pipeline (analysis mode: coassembly) and read-sets for each sample were aligned to the co-assembly to predict genes, annotate gene functions and estimate the abundance of individual genes per sample (Tamames and Puente-Sánchez, 2019).

### *5.3.6 Statistical analysis*

**Analysis of taxonomic structure of coral-associated bacterial communities (16S rRNA amplicon sequencing)**

Differences in bacterial community structure and dispersion (beta diversity patterns) of source reef corals over time ($T_0$ to $T_{6m}$), and between source colonies and propagated corals after 6 months ($T_{6m}$), were analysed using the Bray–Curtis dissimilarity distance metric. Patterns in bacterial community structure were visualised using non-metric multidimensional scaling (nMDS) plots. Differences in community structure were tested for significance with permutational multivariate analysis of variance (PERMANOVA; perm = 999) of Bray–Curtis dissimilarities using the *adonis* function of the '*vegan'* R package and subsequent pairwise comparisons (if significant) with *pairwise.adonis*, *p*-values were adjusted by applying a Benjamini and Hochberg correction, all $p_{adj}$ values < 0.05 were considered significant. Permutation tests for homogeneity of multivariate dispersion (PERMDISP) of coral-associated bacterial community was calculated using the *betadisper* function of the '*vegan*' R package (perm = 999), *p*-values were adjusted by applying a Benjamini and Hochberg correction, all $p_{adj}$ values < 0.05 were considered significant. The *core_members* function of the '*microbiome*' R package was used to identify core bacterial community

members (present in > 75 % samples with relative abundance (RA) > 0.1 %) for source reef corals and propagated fragments at $T_0$ and $T_{6m}$. To identify bacterial taxa significantly associated with different propagation structures we applied an Indicator Species Analysis with the *multipatt* function of the R '*indicspecies'* package. Results were cross-referenced against the relative abundance (RA) of each ASV and retained if present in > 75% of replicates.

**Analysis of the functional profile of coral-associated bacterial communities (shotgun metagenomics)**

Metagenome reads were assigned gene functions and pathways based on the database structure of the Kyoto Encyclopedia of Genes and Genomes (KEGG). To quantify the abundance of KEGG Orthologs (KOs) transcripts per million (tpm) values were calculated. Functional analysis at KO level was conducted using a Bray–Curtis dissimilarity distance metric on the KO tpm matrix. Patterns in the diversity of functions at the community level were visualised using nMDS plots and differences between treatments were tested for significance with PERMANOVA of Bray–Curtis dissimilarities using the *adonis* function of *vegan*.

To identify functions unique to each treatment a presence/absence analysis was conducted. KOs were isolated based on presence in source colonies at $T_{6m}$ (in all replicates) and simultaneous absence (0% RA in) in a given propagated coral (e.g., in all replicates and for each structure separately) or for their absence in source colonies at $T_{6m}$ (in all replicates) and simultaneous presence in a given propagated coral (in all replicates) at $T_{6m}$. Functional analysis was also conducted at the Path level for pathways that were related to KOs identified in the absence/presence analysis. All KOs were grouped by KEGG classified pathway and differences between treatments were visualised with bar plots. Differences between treatments for each pathway were tested for significance using a Kruskal-Wallis test, where significant Dunn's post-hoc test was

applied with Benjamini and Hochberg *p*-value adjustment, all $p_{adj}$ values < 0.05 were considered significant.

## 5.4 Results

### *5.4.1 Associated bacterial communities of source reef colonies remain the same over time (16S taxonomy).*

Taxonomic profiles of coral-associated bacterial communities generated from shotgun metagenomic sequencing (Fig S5.4.) did not match those acquired from the (more directed) amplicon sequencing of the 16S rRNA gene (Fig S5.3a.). Such contrasting outcomes are potentially from the two different databases used for the taxonomic classification of reads and/or two different extraction methods used to prepare the DNA for sequencing. Considering the taxonomic profiles acquired from the amplicon sequencing were more comprehensive (2899 ASVs vs 96 unique taxa) – and to capture any differences in rare taxa that were not quantified in the shotgun sequencing output – all subsequent analysis of the phylogenetic characteristics of coral-associated bacterial communities was conducted using the amplicon (16S rRNA gene) dataset.

No significant changes were observed for the taxonomic structure of bacterial communities associated with *A. millepora* source colonies over the course of the experiment ($T_0$ to $T_{6m}$: $PERMANOVA_{Bray\text{-}Curtis,}$ $F = 1.156$, $p_{adj} = 0.182$, Fig S5.3a-b.). Consequently, all comparisons between the source colony and propagated corals were conducted only at the six month sampling point ($T_{6m}$). Two ASVs (*Synechococcus* genus and *Endozoicomonas acroporae,* mean relative abundance (RA) = 5.13% and 14.92% respectively) were identified as 'core' members of the bacterial communities of source colonies at the start of the experiment ($T_0$) and were retained throughout the six

month study period. After six months, an additional ASV (*Psychrobacter pacificensis*, mean RA = 6.61%) was identified as core for source reef colonies (Table S5.2.).

### *5.4.2 Propagated corals exhibit similar bacterial community structure and core taxa to source reef colonies but have distinct indicator species (16S taxonomy).*

After six months, no significant differences were observed between the taxonomic structure of coral-associated bacterial community of propagated corals across all propagation materials or versus source reef colonies ($T_{6m}$) (PERMANOVA$_{Bray\text{-}Curtis,}$ $p_{adj} > 0.05$, Fig 5.4a., 5.4c., and Table S5.1.). No differences were found for the core bacterial taxa of source reef colonies ($T_{6m}$) versus all propagated coral (regardless of propagation structure material). The core included one *P. pacificensis* species ASV, one *Synechococcus* genus ASV and one *E. acroporae* species ASV (Table S5.2.). Mean RA of the *E. acroporae* ASV was higher in aluminium and rebar corals (mean RA = 33.33% and 34.79% respectively) than source reef colonies ($T_{6m}$ mean RA = 24.88%) but lower in Reef Star corals (mean RA = 2.38%). Highlighting minor differences in core bacterial community members across propagation structures. Reef Star corals formed a discrete cluster on the nMDS plot (Fig 5.4b.) and exhibited significantly less multivariate dispersion compared to source reef colonies (at $T_{6m}$) and rebar corals (pairwise PERMUTEST, $p_{adj} = 0.041$ and $p_{adj} = 0.048$ respectively), but no significant differences in taxonomic structure between Reef Star coral and source reef colonies or other propagated corals was observed (PERMANOVA$_{Bray\text{-}Curtis,}$ $p_{adj} > 0.05$). Thus, differences in coral-associated bacterial communities between propagation structures may have been too small to detect. Consequently, we further assessed bacterial communities to identify any discriminating factors at the ASV level using indicator species analysis.

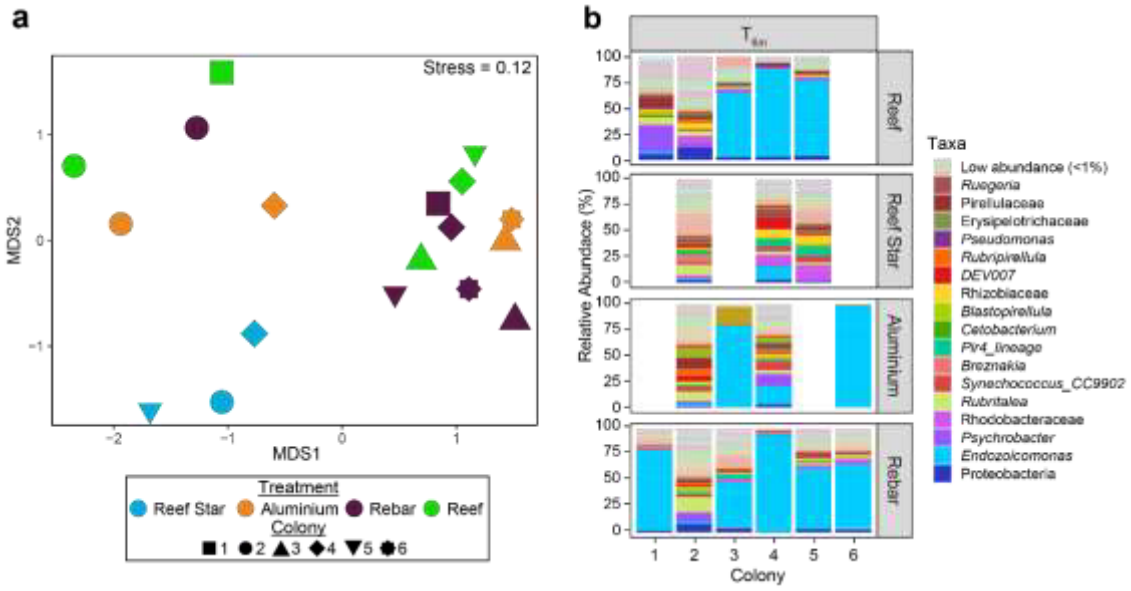



**Figure 5.4.** (**a**) Bacterial community structure and relative dispersion of the microbial communities of the source reef colonies and corals propagated on different metal structures (Reef Stars, rebar stakes and aluminium frames) after six months ($T_{6m}$). Plot is based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure from 16S rRNA gene taxonomy profiles. (**b**) Bacterial community composition (relative abundances) by genus* of source reef colonies and of corals grown on three different propagation structures after six months ($T_{6m}$). Pastel colours represent genera with an average relative abundance of < 0.1% in all samples, full legend provided as supplemental data (Supplementary Data S1.). * family/class classification were used when the genus was unknown.

Although no community-level (beta diversity) differences were observed between propagated corals, seven different ASVs were significant 'indicators' for bacterial communities associated with propagated corals across the different metal structures and source reef colonies at $T_{6m}$. Three ASVs from the *Ruegeria* and *Trichodesmium_IMS101* genera (mean RA = 1.91% and 1.10% respectively) and from the family Rhizobiaceae (mean RA = 1.45 %) were indicative of Reef Star coral-associated bacterial communities. Three ASVs of the *E. acroporae* species were indicators of rebar coral-associated bacterial communities (mean RA = 1.19%, 2.41% and 0.33%). One ASV of the *Prosthecochloris Vibrioformis* species was indicative of aluminium frame coral-associated bacterial communities (mean RA = 4.47%). One ASV of the *Flavobacteriaceae* family was indicative of source reef coral bacterial communities (mean RA = 0.91%).

### *5.4.3 Functional profiles of coral-associated bacterial communities were consistent for source reef colonies and propagated corals (shotgun metagenomics)*

No differences in the functional profiles of coral-associated bacterial communities were observed in source colonies over time (from $T_0$ to $T_{6m}$) ($PERMANOVA_{Bray\text{-}Curtis,}$ $F = 0.915$, $p_{adj} = 0.5$, Fig S5.5a.). Consequently, (as with the taxonomic data) all analysis of bacterial community function between the source colony and propagated corals was conducted at the six month sampling point ($T_{6m}$). Overall, there were no differences in the functional profiles of coral-associated bacterial communities between corals grown on different metal propagation structures and source reef colonies at $T_{6m}$, nor were there any differences between propagation structures ($PERMANOVA_{Bray\text{-}Curtis,}$ $p_{adj} > 0.05$, Fig S5.5b.).

### *5.4.4 Presence and absence of KOs in coral metagenomes differed across propagation structures (shotgun metagenomics)*

To resolve any high-resolution differences in coral-associated bacterial community functioning potential e.g., from a more nuanced response to the propagation materials (that might not be detected when comparing the whole profiles) and that could be linked to differences in indicator taxa, we analysed the abundance of individual KOs. At the six month time point ($T_{6m}$), a presence/absence analysis highlighted 22 KEGG Orthologs (KO) (groups of genes performing the same functions) of the 5125 assigned KOs were present in propagated corals and completely absent in source reef colonies. One KO was present in source reef colonies, but was absent from the aluminium frame coral and no KOs were present in source reef colonies and absent in rebar and Reef Star coral. Hence, we focused on the KOs present in coral-associated bacterial communities across different metal propagation structures (Fig 5.5a.). Four KOs were present in the metagenomes of corals grown on aluminium propagation structures (and absent in

source reef colonies) assigned to genetic information processing (KEGG classification 2) and environmental information processing pathways (KEGG classification 3.2, Table S5.3. and Appendix 5.2.). Three KOs were present in Reef Star coral metagenomes (and absent in source reef colonies) relating to environmental information processing (KEGG classification 3.1), metabolic pathways (KEGG classification 1.2) and one unknown pathway (Table S5.3. and Appendix 5.2). Fifteen KOs were present in rebar coral-associated bacterial communities (and absent in source reef colonies), four were related to genetic information processing pathways (KEGG classification 2.1, 2.3, and 2.4), two KOs were related to environmental information processing pathways (KEGG classification 3.1), four KOs were related to metabolic pathways (KEGG classification 1.1, 1.4), one KO was related to a signalling and cellular processes pathway (KEGG classification 4.1) and three KOs were from poorly described pathways (Table S5.3. and Appendix 5.2.).

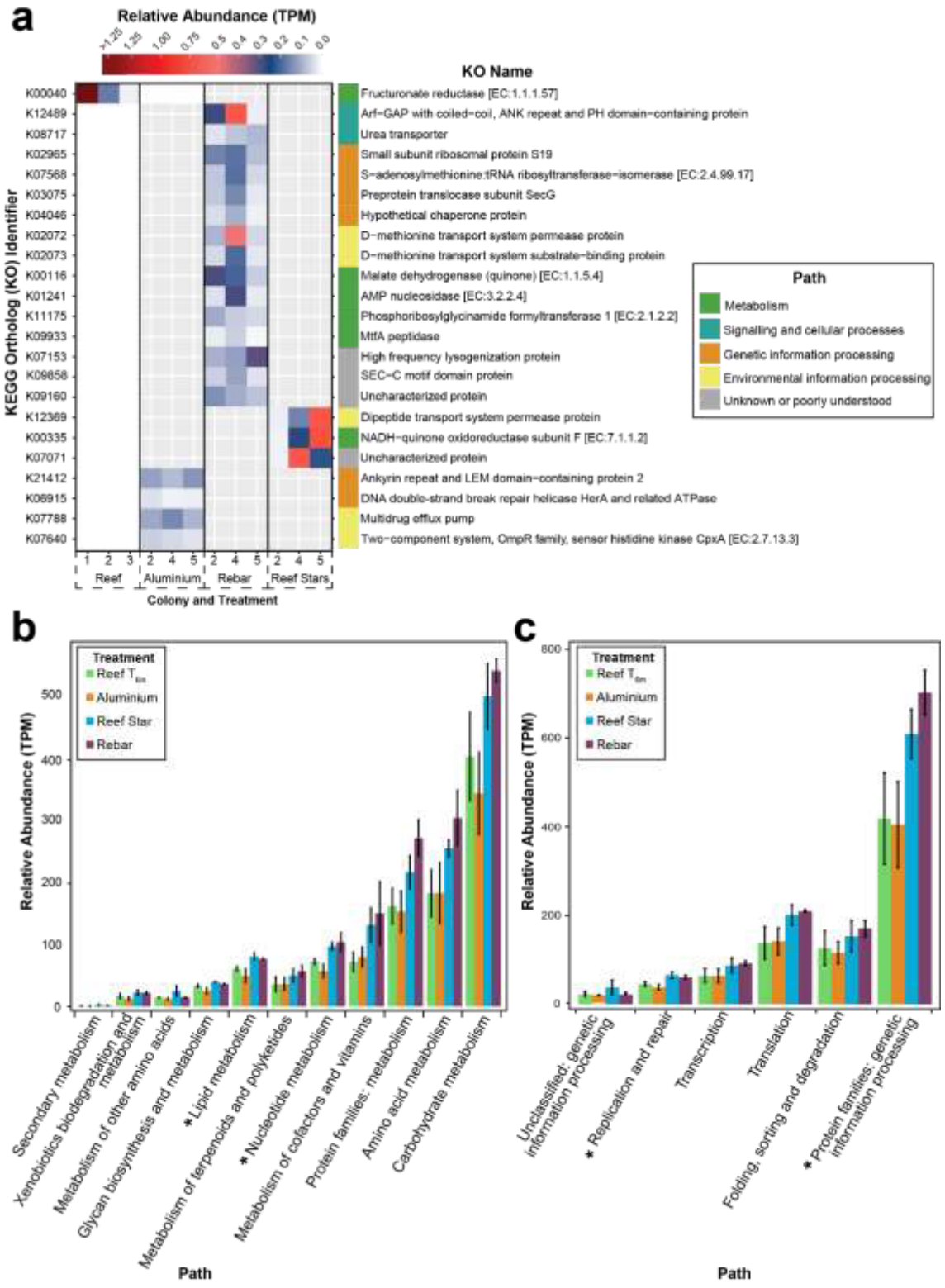



**Figure 5.5.** (**a**) Presence/absence analysis used to identify propagation material-related functions in coral-associated bacterial communities of propagated corals. The heatmap shows KEGG orthologs (KO) in the metagenome of corals propagated on different materials (coated steel: Reef Stars, uncoated steel: Rebar and aluminium frames) and source reef colonies after six months ($T_{6m}$). The colour gradient corresponds to the transcripts per million (tpm) (higher tpm = higher abundance). KO identifier is provided on the left of the heatmap, and the respective name is on the right of the heat plot for each KO. KO names are colour by their related functional path and entries are organised by these functions. KOs are only shown if present in 100% of replicates for each

treatment. (**b**) KEGG metabolism and (**c**) genetic information processing pathways in the metagenomes of source reef colonies or corals propagated on different material propagation structures after six months. KOs were grouped by path and a mean of the total tpm values is plotted with standard error bars. Asterisk indicates where Kruskal-Wallis test on each pathway by treatment showed $p < 0.05$.

### *5.4.5 Different pathways were overrepresented in propagated corals and source reef colonies*

Across restoration structures, the KOs present in propagated corals but absent in source reef colonies were components of carbohydrate and nucleotide metabolic pathways, translation, membrane transport and folding, sorting and degradation pathways and signalling pathways (Fig 5.5a.). Therefore, to determine whether entire functional pathways were elevated in propagated corals, we analysed the functional profiles at the pathway level. All metabolic and genetic information processing pathways (KEGG classification 1 and 2) were overrepresented in the metagenomes of corals propagated on Reef Stars and rebar stakes compared to source reef colonies and aluminium corals (Fig 5.5b-c.) apart from energy metabolism (KEGG classification 1.2), which was underrepresented in rebar coral compared to coral propagated on other metal structures and source reef colonies (Fig S5.6.). Differences across propagated corals and source reef colonies were significant for only two categories of metabolic pathways (KEGG classification 1.3 - Lipid metabolism, Kruskal-Wallis, $\chi^2 = 8.5385$, $df = 3$, $p = 0.0361$ and KEGG classification 1.4 - Nucleotide metabolism, Kruskal-Wallis, $\chi^2 = 8.641$, $df = 3$, $p = 0.0345$, Fig 5.5b.) and two genetic information processing pathways (KEGG classification 2.4 - Replication and repair, Kruskal-Wallis, $\chi^2 = 8.641$, $df = 3$, $p = 0.03446$; Genetic information processing protein families, Kruskal-Wallis, $\chi^2 = 9.0513$, $df = 3$, $p = 0.02862$, Fig 5.5c.). No significant differences were observed in the post-hoc tests (Dunn's post-hoc, $p_{adj} > 0.05$).

## 5.5 Discussion

Various materials are being used in ever-scaling reef restoration approaches (Nedimyer et al. 2011; Meesters et al. 2015; Williams et al. 2019; Suggett et al. 2020; Ceccarelli et al. 2020; Boström-Einarsson et al. 2020; Denhert et al. 2022), that have the potential to alter coral holobiont fitness by influencing the composition and functioning of coral-associated bacterial communities (Ceccarelli et al. 2020; Reich et al. 2022). Yet, it remains unclear if the use of different metals positively or negatively affects coral-associated bacterial communities during propagation, and in turn how this regulates reef restoration success (van Oppen and Blackall, 2019; Voolstra et al. 2021; Peixoto et al. 2022; Strudwick et al. 2022,2023a). Here we show, across different propagation structures after a six month period, minor differences in individual bacterial taxa and functional potential of coral-associated bacterial communities can emerge. Specifically, propagated corals exhibited several KOs that were absent in bacterial communities of source reef colonies, and coral propagated on steel structures exhibit overrepresentation of various metabolic and genetic information processing pathways. Further, distinct bacterial taxa were indicative of coral-associated bacterial communities across the different metal propagation structures. Together, the minor differences in functional potential and individual members of coral-associated bacterial communities demonstrate that propagated coral may have been under distinct environmental conditions (Maher et al. 2019; Camp et al. 2020; Ziegler et al. 2017, 2019). Consequently, further testing is required to disentangle the confounding findings of differences in functional potential and lack of differences in community composition of coral-associated bacterial communities, to determine whether Fe from steel structures plays a role in these changes, and to identify the implications of such differences in coral-associated bacterial communities on the fitness and resilience of the holobiont.

### *5.5.1 Coral-associated bacterial communities did not differ between propagation structures*

We compared three propagation structures to identify whether the type of metal used differentially impacts coral-associated bacterial communities. No differences in the structure of coral-associated bacterial communities were recorded between *A. millepora* source reef colonies and propagated corals propagated across structures. Restructuring of coral-associated bacterial communities of *Acropora* spp. are typically seen during transplantation between distinct environments (Ziegler et al. 2019; Haydon et al. 2021), and for *A. millepora* particularly during reef restoration on the GBR (Strudwick et al. 2022, 2023b in press). Previously coral-associated bacterial community differences have been recorded after four to six months of propagation on aluminium structures at other sites at Opal Reef (Strudwick et al. 2022, 2023b in press). Therefore, it is perhaps surprising that no differences in coral-associated bacterial communities were observed between source reef colonies and propagated corals on aluminium structures (or any other structure) after six months in our current study. Such a discrepancy of difference in coral-associated bacterial communities between source reef colonies and propagated coral may be linked to environmental site differences (Haydon et al. 2021). High flow, for example, has previously been linked to more stable coral-associated bacterial communities (Lee et al. 2017) and the location of our current study (Long Bommie, Opal Reef) experiences greater flow and wave action than Rayban and Mojo of Opal Reef in previous studies (Edmondson personal obs.; Strudwick et al. 2022, 2023b in press). At Long Bommie, corals were also grown on structures positioned 30-40 cm above the substrate, whereas mid-water aluminium frames suspended 2-3 m above the benthos were used at Rayban and Mojo (Strudwick et al. 2022, 2023b in press). The different positioning of propagation structures in relation to the substrate likely resulted in exposure to lower water flow (Perkol-Finkel et al. 2008) for propagated corals, or

more importantly, similar environmental conditions to source reef colonies at Long Bommie. Whilst this notion remains to be verified, it is consistent with the growing evidence of site-specific environmental conditions as drivers of coral-associated bacterial communities (Maher et al. 2019; Ziegler et al. 2019; Camp et al. 2020; Osman et al. 2020). It also highlights the importance of further characterising coral-associated bacterial communities during restoration across diverse sites, to combine knowledge of species-specific and site-specific bacterial community trends necessary to tailor local protocols as approaches scale geographically.

### *5.5.2 Propagated corals exhibited distinct indicator taxa*

Whilst propagation on different materials did not yield changes in the structure of the coral-associated bacterial communities, distinct taxa were identified at the individual ASV level as representative of the different coral environments. Three ASVs of the putative coral symbiont *Endozoicomonas acroporae* (Tandon et al. 2022) were identified as indicator taxa for rebar coral. Three bacterial taxa that were putative symbionts and/or had nitrogen fixing and iron-binding abilities were identified as indicator taxa for Reef Star coral (*Ruegeria*, Kitamura et al. 2021; *Trichodesmium_IMS101*, Capone et al. 1997, Held et al. 2022; Rhizobiaceae, Rincón-Rosales et al. 2010). Finally, one photosynthetic green sulfur bacterium *Prosthecochloris Vibrioformis* (Nie et al. 2023) ASV was indicative of coral grown on aluminium frames. We hypothesised pathogenic bacterial taxa may proliferate on steel structures, due to the role of Fe in pathogenesis and virulence (Kelly et al. 2012; Rubio-Portillo et al. 2020; Gnanagobal and Santader, 2022), but importantly no putative pathogens were identified as indicator species across any of the propagation structures. However, differences in indicator taxa suggest propagated coral may be under distinct environmental conditions. Thus, our results indicate that the potential unique biogeochemical niche surrounding each propagation structure had a subtle influence on

propagated coral and effected individuals within coral-associated bacterial communities for the period of growth in this study.

### *5.5.3 Coral bacterial communities gained several distinct KOs across propagation structures*

No differences were observed in the overall functional profile of coral-associated bacterial communities across propagation structures and source reef colonies. However, several KOs were elevated in propagated corals that were absent from source reef colonies, and various pathways were differentially represented between all corals (propagated and source reef colonies). Given our hypothesis that coral propagated on steel structures would display enrichment of genes associated with Fe within associated bacterial communities, we specifically investigated whether overrepresented KOs were related to Fe. Coral propagated on rebar exhibited the most overrepresented KOs and these were components of nucleotide (purine and adenosine monophosphate) and carbohydrate (pyruvate and glucose) metabolism pathways, and genetic information processing (translation, membrane transport and folding, sorting and degradation) pathways. Though only components of these pathways were enriched, the overrepresentation of carbohydrate metabolism KOs (malate dehydrogenase and MtfA peptidase) and nucleotide metabolism KOs (AMP nucleosidase and phosphoribosylglycinamide formyltransferase 1) in rebar coral compared to source reef colonies suggests that rebar coral-associated bacterial communities could have altered or increased metabolic potential (Zhang et al. 2015). In fact, both rebar and (to a lesser extent) Reef Star coral-associated bacterial communities exhibited overrepresentation of several KEGG metabolic pathways (carbohydrate, lipid, amino acid, and nucleotide metabolism) compared to both aluminium coral and source reef colonies. Metabolic potential of coral-associated bacterial communities has been shown to reflect local oceanographic conditions (Kelly et al. 2014; Zhang et al. 2015) and metabolic demands

can increase in response to Fe enrichment (Rädecker et al. 2017). Consequently, the possible increase in metabolic potential is likely linked to different environmental conditions (e.g., nutrient availability, Kelly et al. 2014; Rädecker et al 2017) between propagation structures and source reef colonies. Further, Fe leaching rates possibly differ between steel structures due to the rust preventative on Reef Stars (Williams et al. 2019) and lack thereof on rebar stakes, which could explain the different extent of metabolic pathway overrepresentation between Reef Star and rebar corals. However, whether steel structures (coated or not) alter Fe availability in the coral micro-environment and whether Fe availability is responsible for the altered functional potential is yet to be resolved.

Only three KOs were present in Reef Star coral and absent in source reef colonies, yet two of these had putative links to Fe transport or utilisation (a periplasmic transport protein that binds iron containing molecules (hemes), Hogle et al. 2014; and an oxidative phosphorylation protein that shuttles electrons from NADH via iron-sulfur centers in the respiratory chain, Leif et al. 1995). Presence of these KOs in Reef Star coral indicates potential contribution of Fe to the coral micro-environment from the steel propagation structures. Our results suggest that depending on propagation structure material there are implications for the functional potential of coral-associated bacterial communities, but whether this directly relates to Fe and the extent to which this impacts holobiont fitness requires further testing.

### *5.5.4 Conclusions and implications for coral propagation*

Successful preservation of beneficial coral-microorganism associations will likely be essential for maintaining coral health and resilience during reef restoration activities (Peixoto et al. 2022). The materials used to manufacture propagation structures and to secure corals during propagation are diverse (Boström-Einarsson et al. 2018) yet their impacts on coral-associated bacterial communities have until now been

untested. In this study, we demonstrate that propagation on different materials can lead to subtle differences in individual bacteria and functional potential of coral-associated bacterial communities, however the implications of these differences on holobiont fitness are unknown. KOs that were present in Reef Star coral and pathways overrepresented in both Reef Star and rebar corals point to differing conditions in coral local environment between steel structures and source reef colonies or aluminium structures, but further testing is required to determine the role of Fe. Considering low-level nutrient (e.g., Fe) enrichment can enhance coral thermal performance (Becker et al. 2021) we suggest future research should assess Fe accumulation within the coral elementome during propagation on steel structures and any related changes in stress tolerance (Reich et al. 2020; Becker et al. 2021). In conclusion, our findings highlight that propagation structure material has minimal impacts on the taxonomic composition of coral-associated bacterial communities, but may have implications for the functional potential of these communities that deserves further attention.

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## 5.7 Data Availability Statement

The data generated and/or analysed during the current study will be available in the NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA988823 (https://www.ncbi.nlm.nih.gov/bioproject/ PRJNA988823) upon publication or by request.

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## 5.9 Supplementary Materials

### *5.9.1 Supplementary Figures*

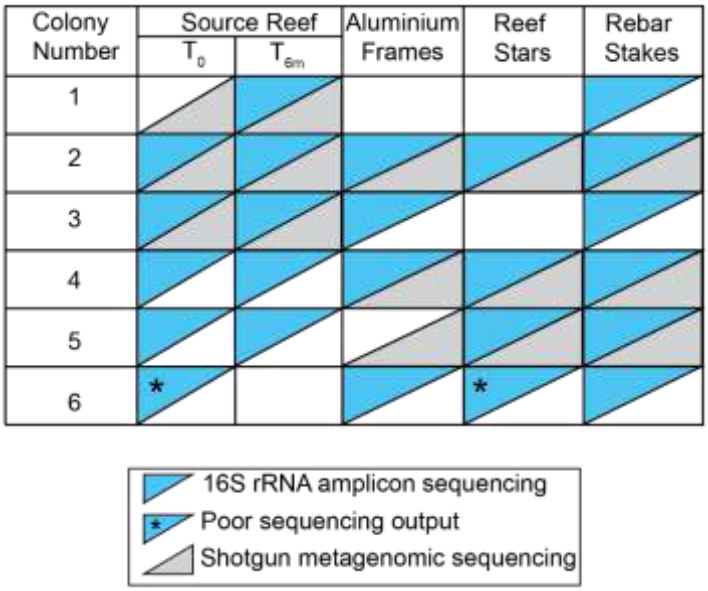


**Figure S5.1.** Graphic of replication and respective source colonies for the two sequencing types across the six source colonies (numbered from 1-6).

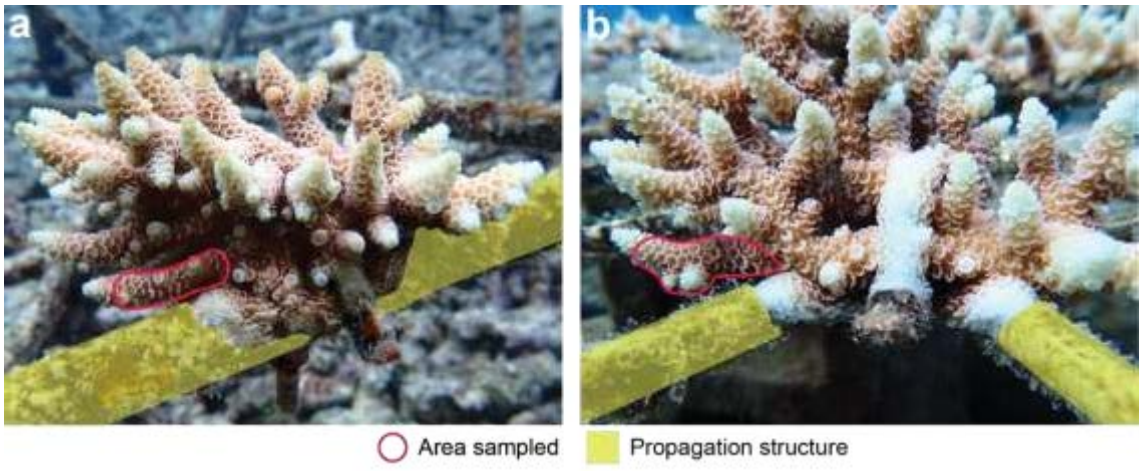


**Figure S5.2**. The area sampled for DNA extractions from propagate coral fragments for example on (**a**) Reef Stars or (**b**) aluminium frames.

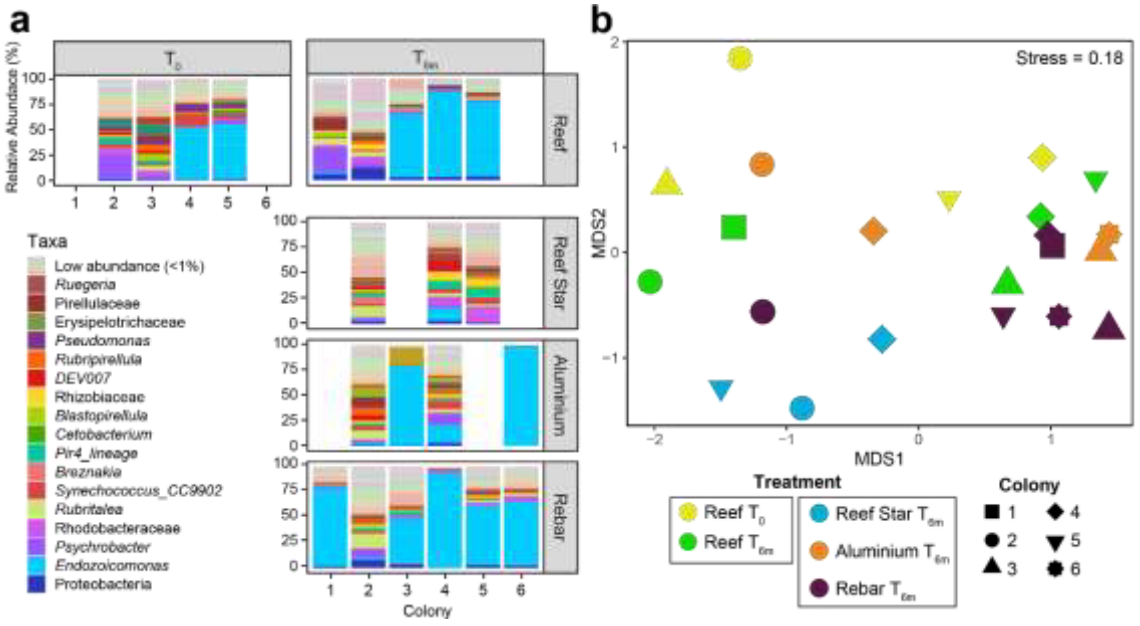


**Figure S5.3.** (**a**) Bacterial community composition (relative abundances) by genus* of source reef colonies at $T_0$ and $T_{6m}$ and of corals grown on three different metal propagation materials (Reef Stars, rebar stakes and aluminium frames) from 16S rRNA amplicon sequencing. Pastel colours represent genera with an average relative abundance of < 0.1% in all samples, full legend provided as supplemental data (Supplementary Data S1.). (**b**) Bacterial community structure and relative dispersion of the microbial communities of the source reef colonies at $T_0$ and after six months ($T_{6m}$) and corals propagated on different metal propagation structures after six months ($T_{6m}$). Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of bacterial community structure from 16S rRNA gene taxonomy profiles. * family/class classification were used where genus was unknown.

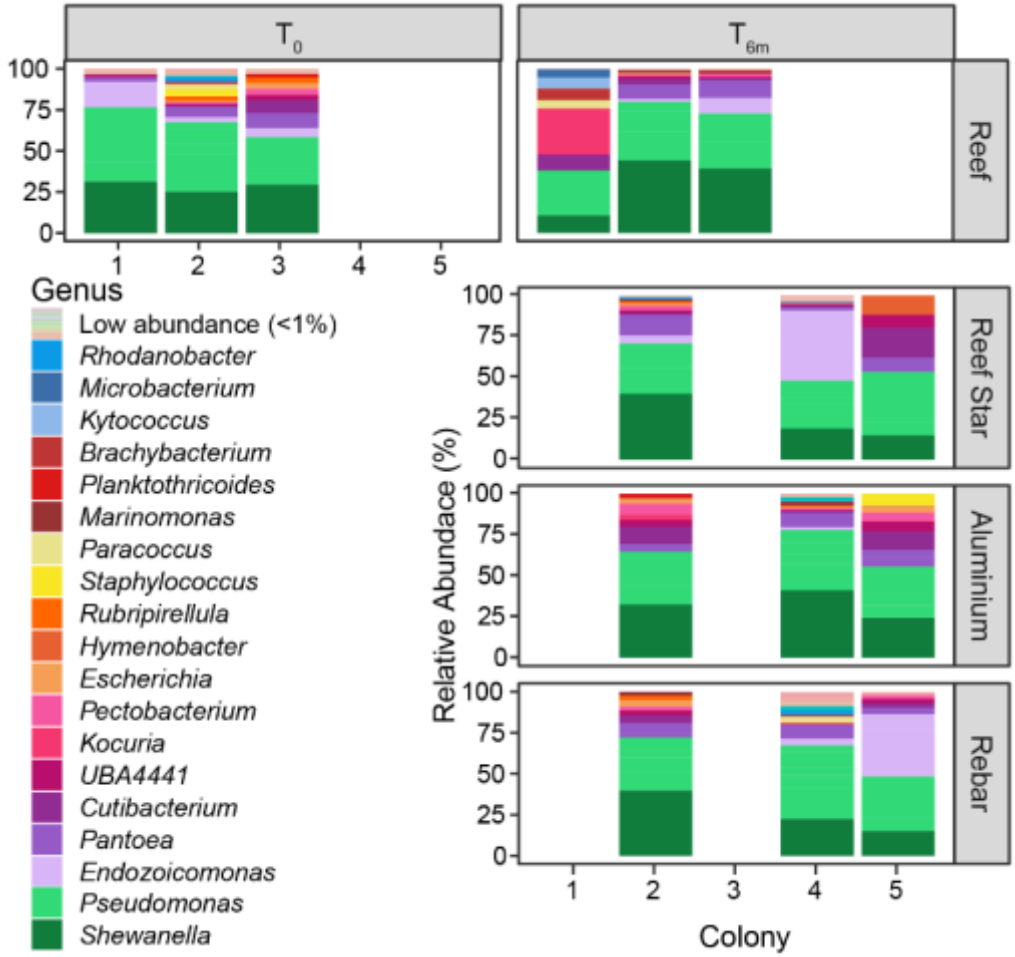


**Figure S5.4**. Bacterial community composition (relative abundances) by genus of source reef colonies at $T_0$ and $T_{6m}$ and of corals grown on three different metal propagation materials (Reef Stars, rebar stakes and aluminium frames) from shotgun metagenomic sequencing. Pastel colours represent genera with an average relative abundance of < 0.1% in all samples, full legend provided as supplemental data (Supplementary Data S1.).

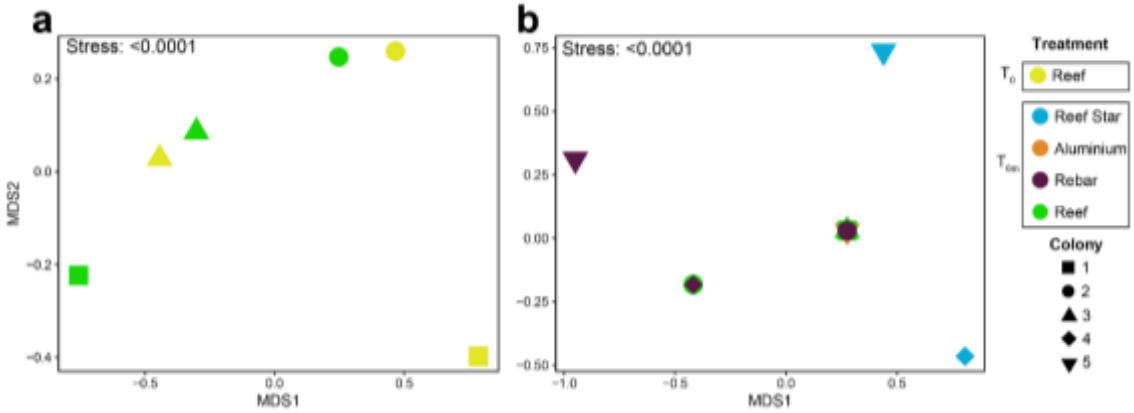


**Figure S5.5.** Bacterial community functional structure of the metagenomes of source reef colonies at Long Bommie, Opal Reef (**a**) at the start of the experiment (February 2022: $T_0$) and after 6 months (August: $T_{6m}$) and (**b**) source reef colonies and propagated corals at $T_{6m}$. Plots are based on non-metric multidimensional scaling (nMDS) of Bray-Curtis distances of KEGG Ortholog (KO) community structure from shotgun metagenomic functional profiles.

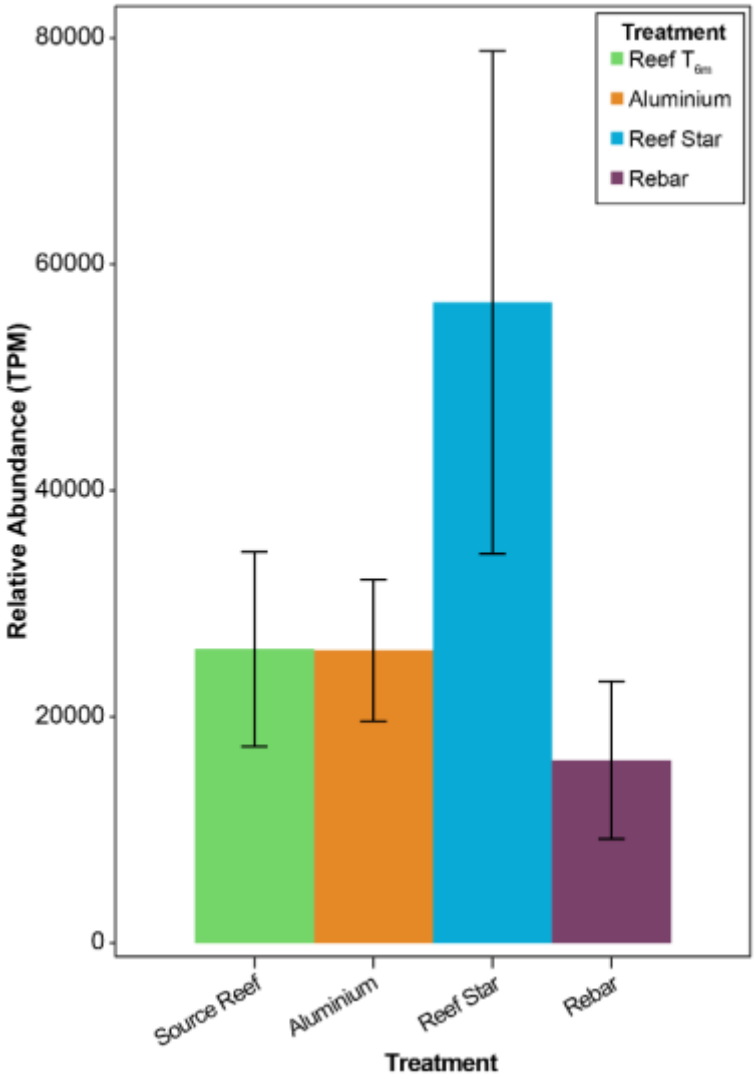


**Figure S5.6.** KEGG energy metabolism (classification 1.1) pathway in the metagenomes of source reef colonies or propagated corals after six months. KOs were grouped by path and a mean of the total transcripts per million (tpm) relative abundance values is plotted with standard error bars.

### *5.9.2 Supplementary Tables*

| **PERMANOVA $T_0$ vs $T_{6m}$ Source Colonies** | | | | | | |
|---|---|---|---|---|---|---|
| | **df** | **Sums Of Sqs** | **$R^2$** | **F** | **Pr(>F)** | |
| treatment | 1 | 0.44634 | 0.14175 | 1.1561 | 0.182 | |
| Residual | 7 | 2.70243 | 0.85825 | | | |
| Total | 8 | 3.14877 | 1 | | | |
| **PERMANOVA All comparisons (main test)** | | | | | | |
| | **df** | **Sums Of Sqs** | **$R^2$** | **F** | **Pr(>F)** | |
| treatment | 3 | 1.2924 | 0.21834 | 1.2104 | 0.1296 | |
| Residual | 13 | 4.6269 | 0.78166 | | | |
| Total | 16 | 5.9194 | 1 | | | |
| **PERMANOVA All comparisons (pairwise)** | | | | | | |
| **pairs** | **df** | **Sums of Sqs** | **F Model** | **$R^2$** | ***p*-value** | **$p_{adj}$** |
| Reef Star vs Aluminium | 1 | 0.5447708 | 1.517821 | 0.232873 | 0.1143 | 0.2286 |
| Reef Star vs Reef $T_{6m}$ | 1 | 0.5424384 | 1.418692 | 0.191232 | 0.034 | 0.0850 |
| Reef Star vs Reef $T_0$ | 1 | 0.5362396 | 1.428690 | 0.222237 | 0.0286 | 0.0850 |
| Reef Star vs Rebar | 1 | 0.6121541 | 1.799960 | 0.204542 | 0.0115 | 0.0680 |
| Aluminium vs Reef $T_{6m}$ | 1 | 0.3231325 | 0.863223 | 0.109780 | 0.5857 | 0.5857 |
| Aluminium vs Reef $T_0$ | 1 | 0.4353771 | 1.185826 | 0.165023 | 0.2343 | 0.3347 |
| Aluminium vs Rebar | 1 | 0.3138753 | 0.927639 | 0.103906 | 0.5065 | 0.5857 |
| Reef $T_{6m}$ vs Reef $T_0$ | 1 | 0.4463388 | 1.156134 | 0.141750 | 0.1885 | 0.3142 |
| Reef $T_{6m}$ vs Rebar | 1 | 0.3237324 | 0.90868 | 0.091706 | 0.5588 | 0.5857 |
| Reef $T_0$ vs Rebar | 1 | 0.602816 | 1.729139 | 0.177728 | 0.0136 | 0.0680 |

**Table S5.1.** Permutational multivariate analysis of variance pairwise test results comparing (taxonomic) structure of coral-associated bacterial communities over time and between treatments from amplicon (16S rRNA gene) sequencing. Adjusted *p*-value = FDR correction

| Treatment | Genus /ASV ID | Relative Abundance (RA) % | | | | | | Avg RA (%) |
|---|---|---|---|---|---|---|---|---|
| | | Colony Number | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| **Reef $T_0$** | Synechococcus _CC9902 /4ea | - | 3.04 | 1.80 | 12.30 | 3.39 | - | 5.13 |
| | *E. acroporae* /c0c | - | 0.09 | 0.00 | 30.82 | 28.81 | - | 14.92 |
| **Reef $T_{6m}$** | Synechococcus _CC9902 /4ea | 1.12 | 1.95 | 0.85 | 0 | 0 | - | 0.79 |
| | *P. pacificensis* /990 | 20.23 | 4.13 | 0.47 | 1.60 | 1.42 | - | 5.57 |
| | *E. acroporae* /c0c | 0 | 0 | 43.12 | 52.64 | 28.65 | - | 24.88 |
| **Rebar** | Synechococcus _CC9902 /4ea | 0.29 | 1.53 | 0.10 | 0.77 | 0.57 | 0.13 | 0.65 |
| | *P. pacificensis* /990 | 1.06 | 7.96 | 0.14 | 1.72 | 0.57 | 0.00 | 2.29 |
| | *E. acroporae* /c0c | 41.21 | 1.65 | 28.74 | 60.43 | 41.94 | 45.61 | 34.79 |
| **Reef Star** | Synechococcus _CC9902 /4ea | - | 1.88 | - | 3.47 | 5.48 | - | 3.61 |
| | *P. pacificensis* /990 | - | 0.48 | - | 1.15 | 0.09 | - | 0.58 |
| | *E. acroporae* /c0c | - | 0.41 | - | 6.73 | 0 | - | 2.38 |
| **Aluminium** | Synechococcus _CC9902 /4ea | - | 3.35 | 0.11 | 6.00 | - | 0.14 | 2.40 |
| | *P. pacificensis* /990 | - | 0.66 | 0.06 | 10.51 | - | 0.05 | 2.82 |
| | *E. acroporae* /c0c | - | 2.93 | 52.37 | 10.02 | - | 68.00 | 33.33 |

**Table S5.2.** Relative abundance (%) of bacterial taxa identified to be 'core' members of the coral-associated bacterial communities for source (reef) colonies at T0 and T6 and for propagated corals after 6 months of growth on different metal propagation structures coated steel (Reef Stars), uncoated steel (rebar) and aluminium. – indicates where bacterial community was not characterised.

| Propagation Structure | KO ID | KEGG Name | KEGG Classification and Pathway |
|---|---|---|---|
| Reef $T_{6m}$ | K00040 | fructuronate reductase | 1.1 Metabolism: Carbohydrate metabolism |
| Rebar | K12489 | Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein | 4.1 Cellular Processes: Transport and catabolism |
| Rebar | K02965 | small subunit ribosomal protein S19 | 2.1 Genetic information processing: Translation |
| Rebar | K07568 | S-adenosylmethionine:tRNA ribosyltransferase-isomerase | 2.4 Genetic information processing: Replication and repair |
| Rebar | K03075 | preprotein translocase subunit SecG | 2.3 Genetic information processing: Folding, sorting and degradation |
| Rebar | K04046 | hypothetical chaperone protein | 2 Genetic information processing |
| Rebar | K02072 | D-methionine transport system permease protein | 3.1 Environmental information processing: Membrane Transport |
| Rebar | K02073 | D-methionine transport system substrate-binding protein | 3.1 Environmental information processing: Membrane Transport |
| Rebar | K08717 | urea transporter | 4.1 Signalling and cellular processes |
| Rebar | K00116 | malate dehydrogenase (quinone) | 1.1 Metabolism: Carbohydrate metabolism |
| Rebar | K01241 | AMP nucleosidase | 1.4 Metabolism: Nucleotide metabolism |

| | | | |
|---|---|---|---|
| Rebar | K11175 | phosphoribosylglycinamide formyltransferase 1 | 1.4 Metabolism: Nucleotide metabolism |
| Rebar | K09933 | MtfA peptidase | 1 Metabolism |
| Rebar | K07153 | high frequency lysogenization protein | n/a |
| Rebar | K09160 | uncharacterised protein | n/a |
| Rebar | K09858 | SEC-C motif domain protein | n/a |
| Reef Star | K12369 | dipeptide transport system permease protein | 3.1 Environmental information processing: Membrane Transport |
| Reef Star | K00335 | NADH-quinone oxidoreductase subunit F | 1.2 Metabolism: Energy metabolism |
| Reef Star | K07071 | uncharacterised protein | n/a |
| Aluminium | K21412 | ankyrin repeat and LEM domain-containing protein 2 | 2 Genetic information processing |
| Aluminium | K06915 | DNA double-strand break repair helicase HerA and related ATPase | 2 Genetic information processing |
| Aluminium | K07788 | multidrug efflux pump | 3.2 Environmental information processing: Signal transduction |
| Aluminium | K07640 | two-component system, OmpR family, sensor histidine kinase CpxA | 3.2 Environmental information processing: Signal transduction |

**Table S5.3.** Details of the KEGG Orthologs (KOs) identified from presence/absence analysis used to identify functions present in coral-associated bacterial communities of propagated corals and absent from source reef colonies.

### *5.9.3 Appendix 5.1*

Homogenisation and phenol-chloroform DNA extraction protocol

Coral tissue pellets were resuspended and homogenised via the following steps (as per Voolstra et al. 2023); 700 µl of 3X PBS was added to the thawed tissue pellets, the pellet was agitated with a pipette tip to resuspend, the tissue slurry was then homogenised for 30 sec and sonicated for 30 sec. Homogenate was transferred to

ceramic (1.4mm) bead tube (Qiagen) and bead beat at 2000 rpm for two rounds of: 30 sec beading and 30 sec on ice. Following all homogenisation steps a 250 µl aliquot of tissue slurry was used for DNA extraction as follows. 25 µl of 10 x Benzonase buffer (200 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$) was added for a total reaction volume of 275 ml, then 1 µl of 250 U Benzonase (Thermo Fisher Scientific) was added the sample and incubated with gentle shaking at 37 °C for 2 h. The Benzonase reaction was stopped by adding 2.75 µl EDTA (0.5 M) and 27.5 µl NaCl (1.5 M). The sample was centrifuged at room temperature for 10 min at 13,000 x G, supernatant was removed, and the pellet was resuspended in 0.5 mL of buffer (0.75 M Sucrose, 40 mM EDTA, 50 mMTris base pH 8.3). 75 µL of lysozyme (100 mg/mL stock) was added followed by an incubation at 37 °C for 1 h. The sample was frozen in $LiqN_2$ and thawed at 70 °C three times. 100 µl of sodium dodecyl sulfate (SDS) (25% solution) was added followed by a 10 min incubation at 70 °C. The sample was cooled to room temperature and 20 µl of proteinase K (20 mg/mL stock) was added followed by incubation at 37 °C for 1 h. The freeze-thaw cycle was repeated three times as before. An equal volume (~ 700 µl) phenol:chloroform:isoamylalcohol (pH 8) was added followed by an incubation at room temperature for 15 min with frequent inversion. The sample was centrifuged at room temperature for 15 min at 10,000 x G. The top layer (DNA in solution) was transferred to a new 2-mL microcentrifuge tube. An equal volume (~ 700 µl) of chloroform:isoamylalcohol was then added followed by an immediate centrifugation at room temperature for 15 min at 10,000 x G. The top layer (DNA in solution) was then transferred to a new 1.5 mL microcentrifuge tube and 50 µl of NaAc (3M) was added. An equal volume of isopropanol (~ 700 µl) was added, and the sample was gently tilted to precipitate the DNA. The sample was then centrifuged at 4 °C for 30 min at 10,000 x G to pellet the DNA, all liquid was subsequently removed and 500 µl molecular grade Ethanol (100%) was added to the pellet. The sample was recentrifuged at 4 °C for 10

min at 14,000 x G before all liquid was removed and the pellet was allowed to air dry for 15 min. Once dry 30 µl of Milli-Q water was added and the pellet was resuspended by gently flicking the microcentrifuge tube. Extracted DNA was quality checked and the concentration was quantified using a NanoDrop spectrophotometer prior to being sent to Ramaciotti Centre for Genomics (University of New South Wales) for metagenomic sequencing.

### *5.9.4 Appendix 5.2*

Four KOs were present in the metagenomes of corals grown on aluminium propagation (but absent from source reef colonies) structures assigned to genetic information processing, and environmental information processing pathways. The KOs included a protein involved in mitosis (K21412), multidrug efflux pump transporter protein (K07788), a two-component signal transduction protein system (K07640) and a DNA repair enzyme (K06915). Three KOs were present in the metagenomes of Reef Star coral (but absent from source reef colonies) that were related to environmental information processing, metabolism and one unknown pathway; the KOs with known pathways included a periplasmic heme-binding transport protein (K12369, Létoffé et al. 2006), and an oxidative phosphorylation protein (energy metabolism, K00335) that shuttles electrons from NADH via iron-sulfur centers in the respiratory chain (Leif et al. 1995). Fifteen KOs were present in rebar coral (but absent from source reef colonies), four KOs were related to genetic information processing pathways, two KOs were related to environmental information processing pathways, four were KOs related to various metabolic pathways, one KO was related to a signalling and cellular processes pathway, and three KOs were of poorly described pathways. The genetic information processing KOs included a ribosomal protein (K02965, small subunit ribosome protein S19) with antibacterial activity (Qu et al. 2020), a tRNA enzyme (K07568, S-adenosylmethionine:tRNA ribosyltransferase-isomerase), a chaperone protein (K04046,

YegD, Stewart and Young, 2004), and a membrane transport protein (KO3075, SecG, Stathopoulos et al. 2000). The signalling and cellular processing pathway KOs included a a urea transporter (K08717) and a membrane trafficking protein (K12489, queA). The environmental information processing KOs included two ABC transporters involved in dissolved methionine (d-methionine) transport (K02073 and K02072) (del Valle et al. 2015). The KOs from metabolic pathways included malate dehydrogenase, an enzyme required for the citrate cycle (TCA cycle) (K00116) (Cunliffe, 2015) and a glucose related MtfA peptidase (K09933) implicated in carbohydrate metabolism and two purine metabolism enzymes (K01241, AMP nucleosidase and K11175, phosphoribosylglycinamide formyltransferase 1) (Parry and Shain, 2011) implicated in nucleotide metabolism. Three KOs from poorly characterised pathways included a SEC-C motif domain protein (K09858) which is part of the secretory machinery transporting proteins across the cytoplasmic membrane and is present in the C terminus of the SecA protein that binds Fe (Cranford-Smith et al. 2020), and a high frequency lysogenisation protein (K07153) implying the presence of bacteriophages (Bonnain et al. 2016) and an uncharacterised protein (K09160).

**Appendix 5.2 References**

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### *5.9.5 Supplementary Data*

The following supplementary data files are within the *Supplementary Data* file online at https://doi.org/10.5281/zenodo.8093503.

**Data S1a** Full legend for Fig 5.4b and Fig S5.3a.

**Data S1b** Full legend for Fig S5.4.

# Chapter 6: General discussion and concluding remarks

## 6.1 Summary

Application of coral propagation and out-planting reef restoration techniques to protect and foster resilience of coral reef ecosystems has increased over the past two decades (Boström-Einarsson et al. 2020). Reef restoration is now widespread (Suggett et al. 2023), including within Australia (McLeod et al. 2022), however, common techniques have unknown potential to impact coral-associated bacterial communities, which are critical to coral reef health (Bourne et al. 2016). It is essential that this fundamental gap in knowledge is filled to influence future successes of reef restoration efforts that continue to be applied across diverse reef habitats. Research conducted in the preceding chapters has produced new insights into the impacts of reef restoration on the coral holobiont; providing foundational knowledge that has advanced the understanding of coral-associated bacterial community dynamics, informed optimisation of current propagation and out-planting processes and provided direction for the application of novel microbiome manipulation approaches. Within this final chapter, I synthesise the findings presented throughout this thesis to outline the knowledge gaps filled, highlight the importance of applying these findings in restoration frameworks and suggest future avenues for research to further optimise reef restoration outcomes.

Throughout this thesis I have identified how restoration techniques impact the coral microbiome by highlighting coral-associated bacterial community dynamics induced by (i) the process of propagation and out-planting using *in situ* nurseries for two coral species (**Chapter 2**; Strudwick et al. 2022), (ii) out-planting across sites with distinct environmental conditions and related survivorship (**Chapter 3**; Strudwick et al. 2023), (iii) the use of different materials to fasten coral in propagation nurseries

(informing the viability of plastic-free alternatives) (Strudwick et al. 2023b in press, **Chapter 4**), and (iv) the use of different metal propagation structures (**Chapter 5**) (Fig 6.1). Across these chapters, I found that coral-associated bacterial communities are differently impacted during propagation within *in situ* nurseries and during subsequent out-planting depending on the coral host species and out-planting site (Strudwick et al. 2022, **Chapter 2**; Strudwick et al. 2023, **Chapter 3**). These results indicate that translocation of coral material between propagation structures and natural reef areas during restoration leads to changes in coral-associated bacterial communities, likely from contrasting environmental conditions at some reef sites and between propagation structures and the source reef. Consequently, other coral species with variable associated bacterial communities may similarly undergo bacterial community changes after translocation during restoration. There is a potential increased susceptibility to coral disease or dysbiosis during transitions in associated bacterial communities (Voolstra and Ziegler, 2020) and practitioners can use such information to optimise restoration outcomes (Fig 6.2.). For example, refraining from translocating coral species with known bacterial community variability to monostands during propagation, or to degraded sites (Kelly et al. 2014) or areas with high macroalgae cover (Haas et al. 2016) that may have higher pathogen loads during out-planting (monostands, Brown et al. 2022; degraded sites, Kelly et al. 2014; high macroalgae cover, Haas et al. 2016).

I further investigated materials used during *in situ* propagation by testing the impacts of various tie materials (Strudwick et al. 2023b in press, **Chapter 4**) and metal structures (**Chapter 5**) on coral-associated bacterial communities. I identified that tie material used to secure coral fragments does not differentially impact propagated coral-associated bacterial communities for the *A. millepora* coral species, yet can have different retention (success) rates and this informed the suitability of biodegradable (plastic-free) zip-ties for use in reef restoration (Strudwick et al. 2023b in press,

**Chapter 4**). Such an outcome provides practitioners with the confidence to migrate from non-degradable plastics in restoration workflows (Fig 6.2.). In contrast, propagating coral on different metal structures lead to subtle differences in individual bacterial taxa and functional potential of coral-associated bacterial communities, however no increases in putatively pathogenic bacteria or associated virulence genes were observed (**Chapter 5**). These results allude to differences in the propagated coral micro-environment between aluminium and steel structures, yet, reassure practitioners that the material of propagation structures does not significantly impact coral-associated bacterial communities over a 6-month period. Collectively these findings provide a 'formula' to understand or predict the dynamics of coral-associated bacterial communities during restoration relative to natural dynamics (in reef environments). This formula can be utilised by practitioners (Fig 6.2.) to enhance restoration outcomes, or to identify where application of novel approaches (such as probiotics) might be most appropriate and effective. Overall, these outcomes indicate that considerations of coral-associated bacterial communities can (and should) be used to optimise restoration workflows, from planning, through to selection of suitable materials, implementation, and evaluation of successes or failures (Fig 6.2.).

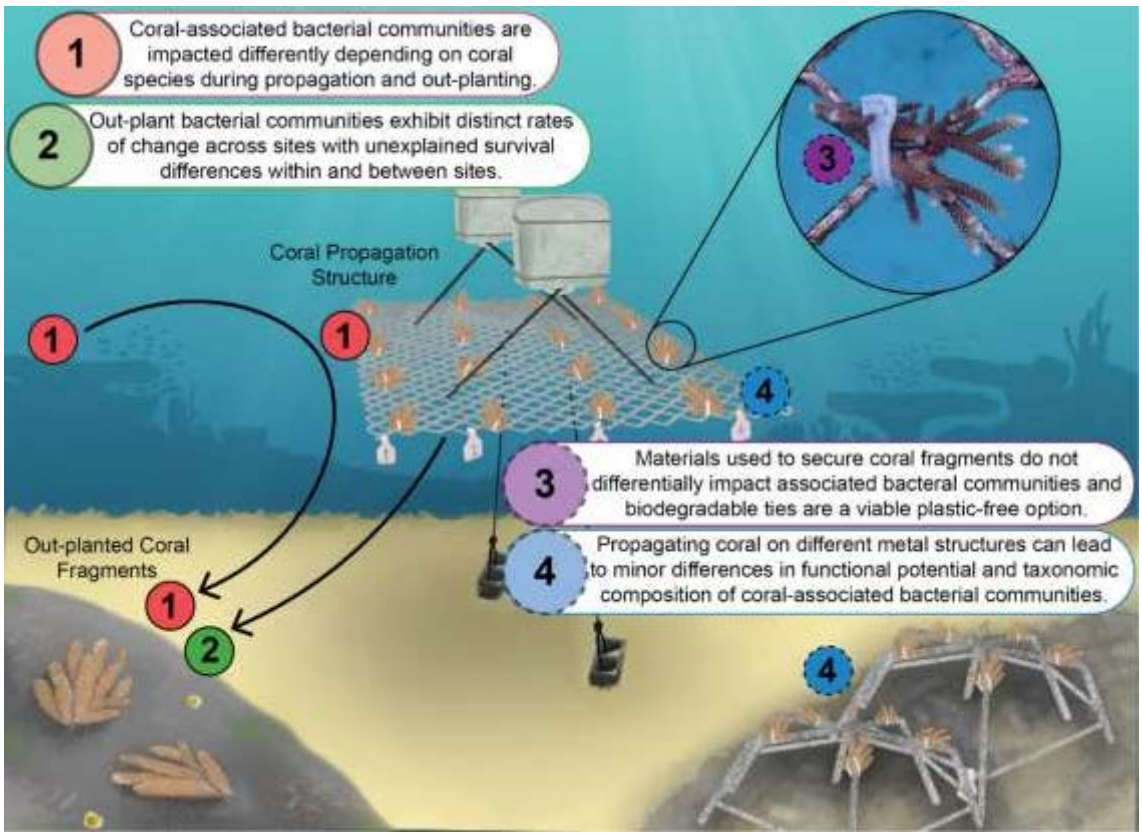


**Figure 6.1.** The overall findings of the four aims addressed in this thesis that filled knowledge gaps regarding either a restoration process or a component of that process (indicated by a solid or dashed circle respectively).

## 6.2 Reef restoration techniques impact essential coral-associated bacterial communities

Coral size, species and attachment technique are all factors postulated to effect coral success (growth and survival) during propagation and out-planting (Yap, 2004; Goergen et al. 2018; Suggett et al. 2018; Munasik et al. 2020; Howlett et al. 2022). Despite protocols developed to consider these aspects, restoration outcomes are still highly variable (Suggett et al. 2019; Boström-Einarsson et al. 2020), indicating there are many still unidentified variables influencing success. Conserving the microbiome by preserving beneficial host-microorganism associations has improved outcomes during agriculture (Kaul et al. 2021), aquaculture (Knipe et al. 2020) and terrestrial restoration (Roper, 2006), yet considerations of coral microbiomes are fundamentally lacking from reef restoration frameworks (Hein et al. 2020; Shaver et al. 2020; Voolstra et al. 2021).

Therefore, a logical first step to conserving the coral microbiome during reef restoration is to encourage stewardship of coral-associated bacterial communities by incorporating the trends identified during propagation and out-planting on the GBR (**Chapters 2-5**, Fig. 6.1.) into restoration planning and implementation (Fig 6.2). Further, proactively characterising coral-associated or environment bacterial communities within routine monitoring (where funding allows) would ensure practitioners are aware of trends in abundance and composition of local bacterial communities, confirming the suitability of continued activity at site and/or highlighting required adjustments of target coral species or activities ahead of potential disease outbreaks (Fig 6.2.).

Maintaining beneficial coral-microorganism relationships and related bacterial community diversity is not only integral to the success of individual corals during restoration, but also for the persistence of restored coral populations and reef ecosystems into the future (Voolstra et al. 2021; Peixoto et al. 2022). Identifying *in situ* coral-associated bacterial community dynamics for all coral species used in reef restoration is currently not feasible due to the related costs (spanning fieldwork, sequencing, and data analysis). However, the bacterial community changes (or lack thereof) observed here (**Chapter 2-5**) suggest that inherent bacterial community characteristics, e.g., variable versus stable recorded in unrelated contexts (Ziegler et al. 2019; Haydon et al. 2021), persist during restoration, but can vary across reef sites (Strudwick et al. 2022, **Chapters 2**; Strudwick et al. 2023, **Chapter 3**; **Chapter 5**). As such, it is possible that known bacterial community characteristics (within or out of a restoration context) of specific coral genera and/or species combined with the fundamental drivers of coral-associated bacterial community changes (primarily environmental conditions), could be locally factored into reef restoration frameworks (Fig 6.2.) to reduce the likelihood of detrimental bacterial community changes. For example, considering known bacterial community trends when tailoring protocols may

improve restoration outcomes e.g., avoiding dense monostands of the *Acropora* coral genus, which is known to generally host highly variable bacterial communities (Strudwick et al. 2022, **Chapter 2**; Strudwick et al. 2023, **Chapter 3**; Strudwick et al. 2023b in press, **Chapter 4**; Voolstra and Ziegler, 2020) due to the potential increased disease susceptibility (Brown et al. 2022). The information gathered through further research (Table 6.1) and/or a meta-analysis of current literature should then be consolidated into a region-specific resource for practitioners to refer to during day-to-day operations.

## 6.3 Materials used in reef restoration have minor effects on coral-associated bacterial communities

As demonstrated in **Chapter 4**, attachment materials used during propagation do not differentially impact coral-associated bacterial communities and propagation structure metals have minor impacts on the taxonomic composition of coral-associated bacterial communities (**Chapter 5**). Materials with negligible impacts on the coral holobiont (Strudwick et al. 2023b in press, **Chapter 4**) represent a potential vector for the introduction of putatively Beneficial Microorganisms for Corals (pBMCs, Rosado et al. 2019). Advances in the manufacture of bioactive nanofiber matrices impregnated with active ingredients has facilitated targeted and sustained delivery of essential nutrients or agrochemicals to enhance agricultural yield (Pirzada et al. 2020; Das et al. 2022). Applying the same technology to engineer pBMC zip-ties (that could easily be interchanged with conventional zip-ties currently used) is an example of how microbiome manipulation could be integrated into current restoration protocols (Fig 6.2.).

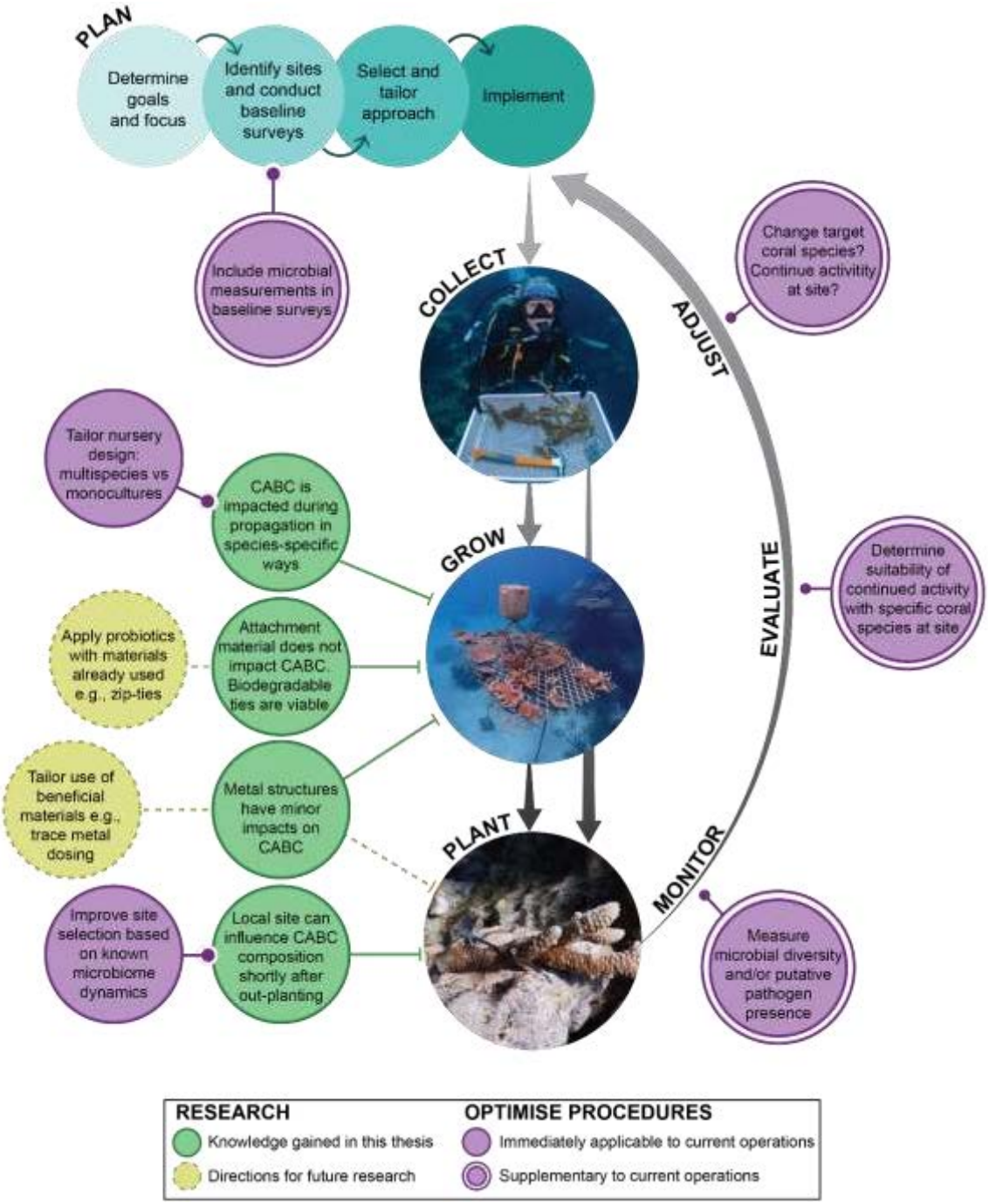


**Figure 6.2.** A reef restoration workflow, with findings from this thesis, suggestions for optimisation, and directions for future research to advance coral-associated bacterial community (CABC) stewardship highlighted.

Unfortunately, the research and development required to produce pBMC zip-ties has similar hurdles to the *in situ* application of probiotics. Notably, there remains uncertain capacity to provide broad stress tolerance, effectiveness of bacterial community changes, longevity of introduced microbial consortia, and affordability

(Peixoto et al. 2021), hence, application is likely years-decades away from implementation (Hein et al. 2020). Instead, practitioners could focus on immediately applicable options e.g., using different metal structures that have the potential to influence the functional potential of coral-associated bacterial communities (**Chapter 5**) and/or other members of the microbiome. Iron (Fe) for example is essential for coral holobiont thermal tolerance, and low-level enrichment can enhance coral thermal performance (Becker et al. 2021); therefore, using steel structures to essentially dose propagated coral with Fe (or other trace elements via the use of alloys) may benefit propagated corals under future climate scenarios (Becker et al. 2021; Blanckaert et al. 2022). However, further research is needed to quantify Fe incorporation into the coral elementome and any associated gains in enhanced stress tolerance during propagation on steel structures.

## 6.4 Future research

During this thesis I have identified a range of coral-associated bacterial community dynamics during propagation and out-planting (**Chapters 2-5**) and collectively evaluating the results of all chapters raises logical key directions for future research (Table 6.1.). The differential responses for the same species across sites (variable, Strudwick et al. 2022, **Chapter 2** and Strudwick et al. 2023b in press, **Chapter 4**, versus consistent bacterial community **Chapter 5**) indicate that research is needed to characterise the spatiotemporal dynamics of coral-associated bacterial communities for more coral species (prioritising those commonly propagated) across diverse sites during restoration. Further, investigating the functional roles of specific coral-associated bacteria (that change in abundance during propagation and out-planting) and their contributions to coral health during restoration, with multi-omics approaches (e.g., transcriptomics, metabolomics and metagenomics), would improve the

understanding of any long-term repercussions for the coral holobiont from bacterial community changes. Although we did not record coral disease, restoration systems can act as reservoirs, or increase the chances of disease emergence (Moriarty et al. 2020; Brown et al. 2022), consequently developing an action plan to account for detrimental changes in coral-associated bacterial communities and/or any cases of disease is key to attenuate negative outcomes. Such a proactive approach to conserving beneficial coral-associated bacterial communities in restoration (Fig 6.2.) would certainly be more effective than defaulting to the historically reactive nature of *in situ* coral microbiome research, typically initiated in response to devastating disease outbreaks (Bourne, 2005; white band disease, Gognoux-Wolfsohn et al. 2020; stony coral tissue loss disease, Rosales et al. 2023).

Long-term similarity of out-planted coral-associated bacterial communities alongside different survival trajectories (Strudwick et al. 2023, **Chapter 3**) between and within sites, suggest factors other than coral-associated bacterial communities influence out-plant survival or were not captured due to fragment mortality. While there is growing popularity of genotype-by-environment (GxE) studies to resolve holobiont-scale drivers of coral success during restoration (Cunning et al. 2021), these studies fundamentally lack any consideration of essential coral-associated microorganisms. As such to further optimise out-plant site selection future research is needed to identify differentiating biotic and abiotic factors between areas of high and low out-plant survival (within sites). Biotic factors that could influence out-plant mortality and/or introduce putatively pathogenic taxa into coral-associated bacterial communities and hence deserve consideration, include corallivores e.g., snails and flatworms (Pausch et al. 2018; Ware et al. 2020), composition of the epilithic algal matrix (Casey et al. 2014) and local fish communities (Renzi et al. 2022). We acknowledge that inter-replicate variability can arise when characterising coral-associated bacterial communities in these

studies which can compromise clarity of conclusions and suggest future research is conducted with larger sample sizes (n > 6).

| Future research area | Aims | Beneficiary outcomes |
|---|---|---|
| Resolve associated bacterial community dynamics for more coral species routinely used in restoration across diverse reef sites. | To identify how coral-associated bacterial communities of different coral species are impacted by propagation and out-planting and how this differs across reef sites. | **Restoration Practitioners**: Tailor protocols to consider coral species-specific associated bacterial community dynamics and reef site characteristics to enhance restoration success. |
| Discern key roles and functions of prominent or variable bacterial taxa to improve understanding of their relationship with the host. | To understand how changes in coral-associated bacterial communities impact holobiont health during restoration and/or contribute to probiotics development. | **Researchers**: Identification of potential taxa with beneficial associations as targets for probiotic application.<br>**Researchers**: Inform routine monitoring/evaluation and inform practitioners whether changes in bacterial communities are detrimental, neutral, or beneficial. |
| Identify differentiating fine-scale environmental conditions between sites with high versus low out-plant survivorship. | To identify specific drivers of different survival of out-planted corals.<br>To improve out-plant success – compounding any improvements gained from using known bacterial community dynamics to inform species handling. | **Restoration Practitioners**: Tailor protocols to select optimal out-planting sites and improve success of out-planted corals. |
| Explore the potential of bioengineering biodegradable zip-ties with probiotics and assess their suitability for other coral growth forms. | To determine whether biodegradable zip-ties can be impregnated or bio-engineered to deliver probiotics* to corals.<br><br>*requires development of effective probiotic consortia and methodology. | **Restoration Practitioners**: Integrate novel/beneficial materials into established protocols to preserve or boost beneficial microbial consortia of corals during propagation. |

| | | |
|---|---|---|
| Test new biodegradable materials as they become available and assess their suitability for other coral growth forms. | To determine the performance of biodegradable zip-ties (e.g., time to degradation). To identify the time-to-attachment for coral taxa of different growth forms to identify whether one material best suits all taxa or different materials are required. | **Restoration Practitioners**: Integrate environmentally positive materials into established protocols to reduce plastic use and or potential (micro-)plastic pollution. |

**Table 6.1.** Future research directions, aims and predicted beneficial outcomes.

## 6.5 Concluding remarks

A future for coral reefs beyond the Anthropocene remains in the balance (Kleypas et al. 2021), yet there is no doubt reef restoration is required to maintain resilience and diversity through periods of environmental uncertainty (Anthony et al. 2020; Kleypas et al. 2021; Vardi et al. 2021; Shaver et al. 2022). However, restoration practitioners need to understand the impacts of coral restoration strategies on the coral-holobiont to optimise approaches and improve restoration outcomes moving forward. This thesis has contributed critical baseline knowledge on the dynamics of coral-associated bacterial communities during the implementation of restoration techniques on the Great Barrier Reef and has provided valuable insights for optimising outcomes, increasing sustainability of practices, and applying novel microbiome manipulation approaches. I have highlighted (i) that various aspects of reef restoration can influence coral holobiont biology, (ii) identified potential factors driving these differences in coral-associated bacterial communities and (iii) evaluated the contribution of materials used in coral propagation to such differences. By continuing to deepen our understanding of coral-associated bacterial community dynamics during reef restoration we can enhance the success of restoration activities and contribute to the preservation and resilience of coral reef ecosystems in the face of ongoing environmental challenges.

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