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Establishing the coral *Galaxea fascicularis* as a model for symbiosis research

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vorgelegt von
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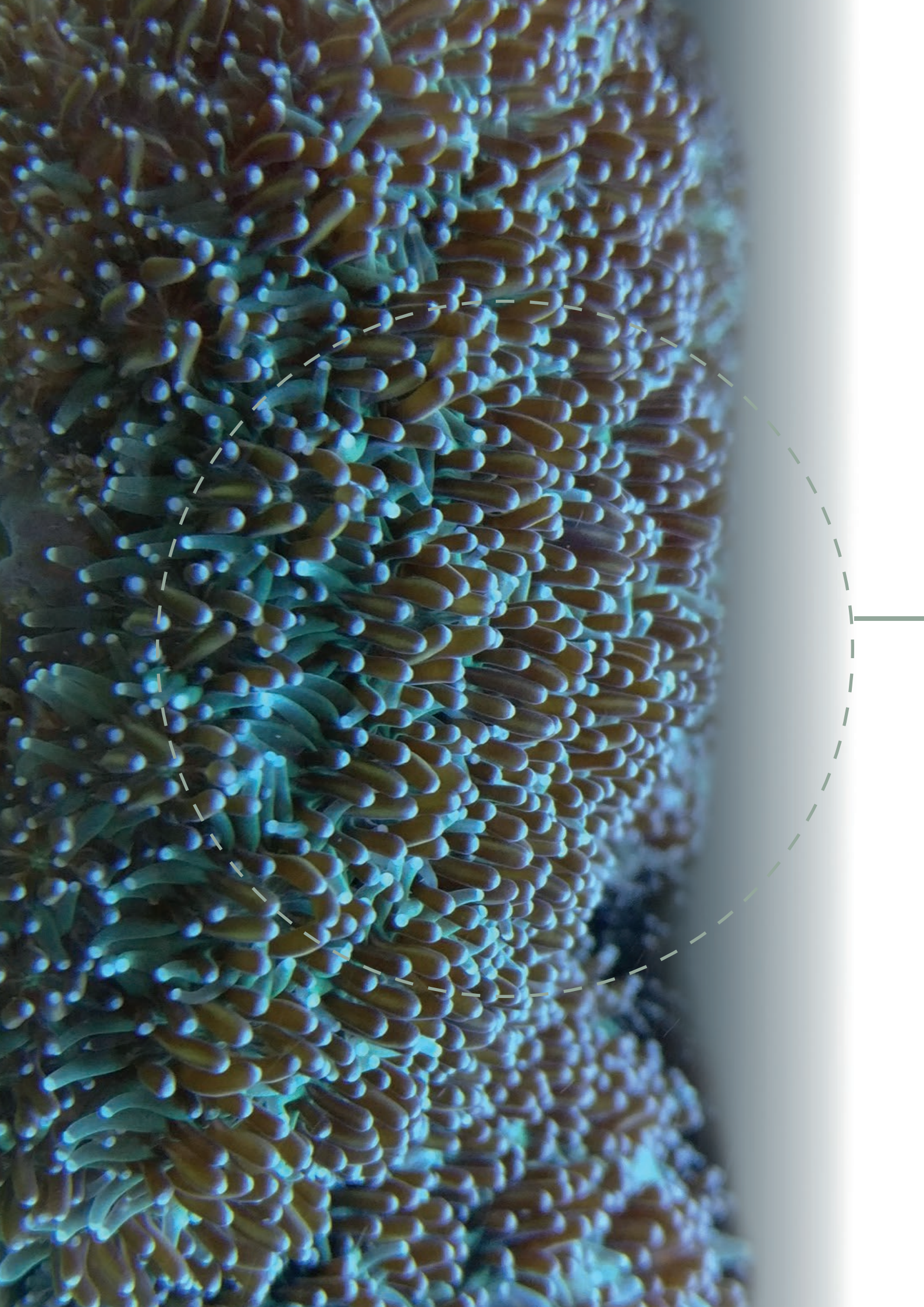

The Missing Link

Establishing the coral *Galaxea fascicularis* as a model for symbiosis research

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Giulia Puntin MSc

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Abstract

Abstract (ENG)

Coral reefs, among the planet's most diverse and productive ecosystems, are under increasing threat from climate change and other anthropogenic stressors. Central to coral reef survival is the health and resilience of the coral holobiont—a complex assemblage comprising the coral animal, its symbiotic microalgae (Symbiodiniaceae), and a dynamic microbial community that includes bacteria, archaea, fungi, and viruses. Despite remarkable research progress, key functional roles of holobiont members remain poorly understood, particularly the mechanisms governing coral health under environmental stress and the factors determining susceptibility to holobiont breakdown, which leads to coral bleaching, disease, and mortality.

To close these knowledge gaps, this thesis aimed to establish a convenient yet representative new model organism to research coral holobiont dynamics through the experimental manipulation of host-microbial associations. As such, *Galaxea fascicularis* (Linnaeus, 1767) was identified as an ideal candidate, serving as the "missing link" between established (non-"true coral") cnidarian models (such as *Hydra*, *Aiptasia*, etc.) and actual reef-building corals, and was evaluated for its suitability as a model system. For this purpose, this work: 1) reviewed the state of model organism-based coral research, identifying the scope of *G. fascicularis* and helping to refine the direction of the experimental chapters of this thesis (Chapter 2); 2) tested the suitability of *G. fascicularis* to laboratory studies by evaluating its tractability in terms of long-term aquarium maintenance, reproduction in captivity, and symbiosis manipulation (Chapter 3); 3) characterized the bacterial microbiome's response to chemically-induced bleaching in long-term aquarium reared colonies (Chapter 4); and 4) discussed and outlined future research directions to further enhance the utility of *G. fascicularis* to address remaining gaps in the understanding of coral holobiont functioning (Chapter 5).

The findings support the practical advantages of using *G. fascicularis* as a model organism. Key achievements include the species demonstrated compatibility with long-term rearing and reproduction in captivity, including compatibility with simplified experimental systems, physiological stress experiments, and symbiosis manipulation. Protocols for producing aposymbiotic fragments through menthol bleaching were refined, enabling reliable production of aposymbiotic fragments for use in experiments, including subsequent re-establishment of symbiosis with novel Symbiodiniaceae strains. Additionally, an initial evaluation of the bacterial microbiome's response to bleaching revealed insights into how rearing conditions, host identity, and symbiont type influence microbial community composition.

Future directions are discussed (Chapter 5) and include refining methodologies for maintaining bleached corals, expanding the range of symbiont strains used for experimental manipulations, and establishing clonal host lines to minimize genetic variability. Further research should also systematically explore holobiont dynamics under varied environmental and rearing conditions to enhance the ecological relevance and reproducibility of findings. These results contribute to a growing body of research aimed at mitigating coral reef decline and highlight the importance of a holobiont-centered approach to coral conservation, where a deeper understanding of microbial roles could inform strategies to enhance coral resilience.

Zusammenfassung (DEU)

Korallenriffe, die zu den artenreichsten und produktivsten Ökosystemen der Erde zählen, sind zunehmend durch den Klimawandel und andere anthropogene Stressfaktoren bedroht. Von zentraler Bedeutung für das Überleben der Korallenriffe sind die Gesundheit und Widerstandsfähigkeit des Korallenholobionten – ein komplexes Gebilde, das aus dem Korallentier, seinen symbiotischen Mikroalgen (Symbiodiniaceae) und einer dynamischen mikrobiellen Gemeinschaft besteht, zu der Bakterien, Archaea, Pilze und Viren gehören. Trotz bemerkenswerter Forschungsfortschritte sind wichtige funktionelle Rollen der Holobiont-Mitglieder nach wie vor kaum verstanden. Dies betrifft insbesondere die Mechanismen, die die Korallengesundheit unter Umweltstress regulieren, sowie die Faktoren, die die Anfälligkeit für einen Zusammenbruch des Holobionts bestimmen, was zu Korallenbleiche, Krankheiten und Sterblichkeit führt.

Um diese Wissenslücken zu schließen, zielte diese Arbeit darauf ab, einen möglichst einfachen, zugleich jedoch repräsentativen neuen Modellorganismus zu etablieren, um die Dynamik von Korallenholobionten durch die experimentelle Manipulation von Wirt-Mikroben-Assoziationen zu erforschen. Als idealer Kandidat wurde *Galaxea fascicularis* (Linnaeus, 1767) identifiziert, der als „fehlendes Bindeglied" zwischen etablierten (nicht „echten Korallen") Cnidaria-Modellen (wie Hydra, Aiptasia usw.) und tatsächlichen riffbildenden Korallen dient und auf seine Eignung als Modellsystem hin untersucht wurde. Zu diesem Zweck wurden im Rahmen dieser Arbeit: 1) der Stand der Modellorganismus-basierten Korallenforschung überprüft, der Anwendungsbereich von *G. fascicularis* identifiziert und die Ausrichtung der experimentellen Kapitel dieser Arbeit präzisiert (Kapitel 2); 2) die Eignung von *G. fascicularis* für Laborstudien getestet, indem seine Handhabbarkeit in Bezug auf die langfristige Haltung im Aquarium, die Fortpflanzung in Gefangenschaft und die Manipulation der Symbiose bewertet wurde (Kapitel 3); 3) die Reaktion des bakteriellen Mikrobioms langfristig im Aquarium gehaltener Kolonien auf eine chemisch induzierte Korallenbleiche charakterisiert (Kapitel 4); und 4) zukünftige Forschungsrichtungen diskutiert und skizziert, um die Nützlichkeit von *G. fascicularis* weiter zu verbessern und verbleibende Lücken im Verständnis der Funktionsweise von Korallenholobionten zu schließen (Kapitel 5).

Die Ergebnisse untermauern die praktischen Vorteile der Verwendung von *G. fascicularis* als Modellorganismus. Zu den wichtigsten Errungenschaften zählt die nachgewiesene Eignung der Art für die langfristige Aufzucht und Fortpflanzung in Gefangenschaft, einschließlich der Eignung für vereinfachte experimentelle Versuchssysteme, physiologische Stressexperimente und Symbiose-Manipulationen. Die Protokolle zur Herstellung aposymbiotischer Fragmente durch Mentholbleiche wurden verfeinert, sodass eine zuverlässige Produktion aposymbiotischer Fragmente für den Einsatz in Experimenten möglich ist, einschließlich der anschließenden Wiederherstellung der Symbiose mit neuen Symbiodiniaceae-Stämmen. Darüber hinaus lieferte eine erste Bewertung der Reaktion des bakteriellen Mikrobioms auf die Bleiche Erkenntnisse darüber, wie die Aufzuchtbedingungen, die Identität des Wirts und der Symbiontentyp die Zusammensetzung der mikrobiellen Gemeinschaft beeinflussen.

Zukünftige Forschungsrichtungen werden diskutiert (Kapitel 5) und umfassen die Optimierung von Methoden zur Erhaltung gebleichter Korallen, die Erweiterung der Palette der für experimentelle Manipulationen verwendeten Symbiontenstämme und die Etablierung klonaler Wirtslinien zur Minimierung der genetischen Variabilität. Weitere Forschungen sollten auch die Dynamik von Holobionten unter verschiedenen Umwelt- und Aufzuchtbedingungen systematisch untersuchen, um die ökologische Relevanz und Reproduzierbarkeit der Ergebnisse zu verbessern. Diese Ergebnisse erweitern die wachsende Forschung, die das Ziel hat, den Rückgang der Korallenriffe zu verlangsamen, und unterstreichen die Bedeutung eines holobiontenzentrierten Ansatzes für den Korallenschutz, bei dem ein tieferes Verständnis mikrobieller Funktionen zur Entwicklung von Strategien zur Stärkung der Korallenresilienz beitragen kann.

Chapter 1

1.1 | Introduction

1.1.1 | Coral reefs: essential ecosystems under threat

Coral reefs represent one of Earth's most biodiverse and ecologically vital ecosystems, sustaining an estimated 25% of marine species while occupying less than 1% of the ocean floor (Reaka-Kudla, 1997). Beyond their ecological significance, these ecosystems provide indispensable services to human populations, including coastal protection, fisheries production, and tourism revenue, collectively valued at over US$30 billion annually (Bartelet et al., 2024; Cesar et al., 2003). In fact, nearly one billion people reside within 100 km of reef systems, many of whom depend directly on these ecosystems for livelihoods and food security (Sing Wong et al., 2022). Alarmingly, anthropogenic pressures—including rising sea temperatures, ocean acidification, pollution, and overfishing—have driven a staggering 50 % global decline in coral cover since the 1950s (Eddy et al., 2021; IPCC, 2024).

Central to this decline is coral bleaching—a breakdown of the symbiosis between corals and their photosynthetic algae (Symbiodiniaceae) that results in the loss of the algal partner and consequently a white appearance of the coral. Bleaching is primarily triggered by elevated sea temperatures (Hughes et al., 2017), but also by other factors such as light stress (Lesser et al., 1990; Lesser and Farrell, 2004), high nutrient concentrations (dissolved inorganic N) (Fabricius, 2005; Wiedenmann et al., 2013), or hypoxia (low oxygen concentrations) (Altieri et al., 2017), low salinity (Kerswell and Jones, 2003), acidification (Anthony et al., 2008), and bacterial infection (Rosenberg et al., 2009; Rosenberg and Falkovitz, 2004). Without Symbiodiniaceae, corals are deprived of their primary energy source, which leads to starvation, increased disease susceptibility, and higher mortality (Hughes et al. 2017b; Hoegh-Guldberg et al. 2018). While corals can re-acquire their symbiont populations, the general strain on physiological processes that follows a bleaching episode can reduce reproductive output and increase vulnerability to subsequent stressors, producing long-term consequences that alter coral species community composition and reef assemblages (Baird and Marshall, 2002; Grottoli et al., 2006; Szmant and Gassman, 1990). Due to climate change, bleaching events have become more frequent, severe, and widespread, posing a critical threat to coral reef ecosystems (Hughes et al., 2017; IPCC, 2024). In numbers, mass bleaching events that recurred approximately every 25-30 years in the 1980s were occurring approximately every six years by 2016 (Hughes et al., 2018), with the Great Barrier Reef having experienced six major bleaching events in the last ten years (2016, 2017, 2020, 2022, 2024 and 2025) (AIMS, 2025). If current trends persist, coral reefs are predicted to disappear entirely by the end of the century, leading to profound ecological and economic consequences (van Hooidonk et al., 2016).

1.1.2 | Symbiosis in action: the coral holobiont

The survival of coral reefs hinges on the health of the coral "holobiont"—a complex and interdependent biological system consisting of the coral animal and its symbiotic partners, including dinoflagellate algae from the family Symbiodiniaceae, bacteria, archaea, fungi, and viruses (Goulet et al., 2020; Rohwer et al., 2002; Rosenberg et al., 2007; Zilber-Rosenberg and Rosenberg, 2008). This association of organisms with different metabolic capacities enables corals to thrive in nutrient-poor tropical waters, and ultimately sustains the productivity of coral reef ecosystems (Bourne et al., 2016; Muscatine and Porter, 1977).

Symbiodiniaceae can be considered the coral's principal symbiotic partners, playing an unquestionably essential role within the holobiont

(Muscatine and Cernichiari, 1969; Roth, 2014). Residing inside coral cells within specialized organelles, known as symbiosomes, Symbiodiniaceae provide the host with energy through photosynthetically fixed carbon (Muscatine and Cernichiari, 1969). This relationship is mutualistic and obligate—the coral depends on the algae for a significant portion of its energy source, while the algae benefit from a stable environment and access to essential nutrients from the host, including inorganic carbon, nitrogen, phosphate, sulfur, and trace elements (Davy et al., 2012). The coral host tightly regulates this partnership by controlling nutrient availability, and effectively regulates symbiont population size by modulating algal reproductive rates, and selectively digesting, killing, or expelling algal cells (Davy et al., 2012; Helgoe et al., 2024; Rädecker et al., 2023; Titlyanov et al., 1996; Wooldridge, 2010).

Besides Symbiodiniaceae, the coral-associated microbiome includes a wide diversity of bacteria, archaea, fungi, and viruses, which also contribute to essential processes in the coral holobiont (Pogoreutz et al., 2020). Among these, bacteria play a central role in nutrient cycling, immune regulation, and environmental adaptation (Bourne et al., 2016; Marangon et al., 2021; Pogoreutz et al., 2017; Raina et al., 2009). For example, bacterial taxa are involved in sulfur cycling (Raina et al., 2009; Robbins et al., 2019) and play an important role in nitrogen metabolism, facilitating the conversion of otherwise inaccessible nitrogen sources into bioavailable forms essential for holobiont function (Moynihan et al., 2022; Rädecker et al., 2015). Additionally, bacteria contribute to pathogen defense, for instance by producing a range of antimicrobial compounds such as antibiotics or lytic enzymes that directly target and suppress the proliferation of harmful microbes, or by outcompeting pathogens for space and resources within the coral mucus layer (Glasl et al., 2016; Ritchie, 2006; Shnit-Orland and Kushmaro, 2009). Certain bacterial groups have also been hypothesized to be linked to enhanced thermal tolerance in corals, either by modulating oxidative stress responses or by influencing host metabolic pathways (Doering et al., 2021; Grottoli et al., 2018).

Furthermore, the coral-associated microbiome might confer ecological flexibility and adaptive capacity in response to environmental changes (Marangon et al., 2021; Voolstra and Ziegler, 2020; Ziegler et al., 2017). In fact, the holobiont is a dynamic system that changes over time and in response to changes in life stages (Epstein et al., 2019; Van Oppen and Blackall, 2019), morphology (Morrow et al., 2022), and environmental conditions including temperature, light, and nutrients (Fabricius, 2005; Thurber et al., 2009; Ziegler et al., 2017). Shifts in microbial communities may help corals acclimate by acquiring new beneficial partners to cope with the adverse effects of environmental stressors (Peixoto et al., 2021; Voolstra et al., 2024; Zhang et al., 2021). However, these shifts are not always beneficial—perturbations can destabilize microbial communities, potentially leading to a detrimental imbalance known as dysbiosis (Boilard et al., 2020; Bourne et al., 2016). Dysbiosis refers to a state where the composition and function of the microbial community shift from a typically beneficial or neutral state to one that is harmful to the host, often resulting in increased susceptibility to disease and stress-related mortality (Boilard et al., 2020; Zaneveld et al., 2016). A deeper understanding of the complex interactions within the coral holobiont and its adaptive capacity is essential for predicting how corals will respond to future ocean conditions and for informing conservation strategies.

1.1.3 | Coral holobiont complexity: challenges and opportunities

Despite significant progress in our understanding of coral reef biology, critical gaps remain, particularly in the context of the coral holobiont and its response

to environmental stress. Research has provided foundational insights into the structure and composition of the coral microbiome, highlighting the pivotal roles of Symbiodiniaceae and bacterial symbionts in coral health (Muscatine and Cernichiari 1969; Roth 2014; Pogoreutz et al. 2020). However, the precise functions of many microbial partners remain largely unexplored, and their interactions with the coral host and with one another are still unclear (Puntin et al., 2022; Van Oppen and Blackall, 2019). Additionally, while the role of certain microbial functions (e.g., nitrogen cycling and immunity processes) is becoming clearer, the interplay between the coral's microbial partners and the host's physiological performance is still only partially understood (Matthews et al., 2020). In other words, while correlations between microbial shifts and host health are often observed, the precise mechanistic links remain largely unknown.

A major obstacle to unraveling the complexities of the coral holobiont is its extraordinary microbial diversity (Blackall et al., 2015). Each coral colony harbors a vast array of microbial taxa—likely numbering in the tens of thousands (Blackall et al., 2015; Galand et al., 2023; O'Brien et al., 2020; Ziegler et al., 2016)—present across distinct microhabitats, including the surface mucus layer, tissue, and skeleton (Apprill et al., 2016; Sweet et al., 2010). These microbial communities exhibit considerable spatial and temporal variability, influenced by environmental conditions, coral species, and within-species inter-colony differences (Hernandez-Agreda et al., 2018; Pollock et al., 2018; Van Oppen and Blackall, 2019). Such variability complicates the identification of functionally significant microbes, because it complicates the distinction between true symbionts and transient or opportunistic taxa (Ainsworth et al., 2015; Hernandez-Agreda et al., 2018). Moreover, microbial interactions within the holobiont are intricate, with cooperative and competitive dynamics shaping community function (Matthews et al., 2020; Voolstra and Ziegler, 2020). Symbiodiniaceae themselves also host bacterial microbiomes that could influence their metabolism and stress responses, for example through the production of B vitamins and antioxidants (Maire et al., 2023, 2021). This, in turn, impacts the holobiont response to environmental conditions, such as heat tolerance, exemplifying the multi-layered nature of holobiont dynamics (Maire et al., 2023). Parsing out the roles of individual taxa amidst this ecological complexity is critical to understanding how microbial partnerships contribute to coral health, resilience, and susceptibility to stressors.

However, mechanistic studies are hindered by the lack of workable frameworks to test hypotheses that balance ecological relevance and experimental tractability—a paradigm achievable only through standardized systems, such as model organisms.

1.1.4 | Towards a coral model: the case for *Galaxea fascicularis*

Model organisms have long been central to scientific research, providing simplified systems to investigate complex biological processes (Galliot, 2012; Weis et al., 2008). In coral biology, cnidarian model organisms such as Hydra (*Hydra* spp.), Aiptasia (the sea anemone *Exaiptasia diaphana* (Rapp, 1829)), and Cassiopea (the "upside-down" jellyfish *Cassiopea xamachana* (Bigelow, 1892)) have been instrumental in advancing our understanding of cnidarian-algal symbiosis (e.g., Fraune et al. 2015; Ohdera et al. 2018; Weis 2019). These organisms offer tractable systems to study cellular and molecular mechanisms, with the added advantages of relative ease in maintenance, manipulation, and use in controlled laboratory experiments. However, while these cnidarian models share certain biological traits with reef-building corals, they lack important features that are central to corals, including the ability to form calcium carbonate skeletons and the obligate nature of coral-algal symbiosis (Falkowski et al., 1984; Gattuso et al., 1999). As a result, while these models are valuable for studying general aspects of cnidarian biology and symbiosis, they fall short of capturing unique coral-specific traits that are fundamental to reef ecosystems.

Recognizing the limitations of non-coral

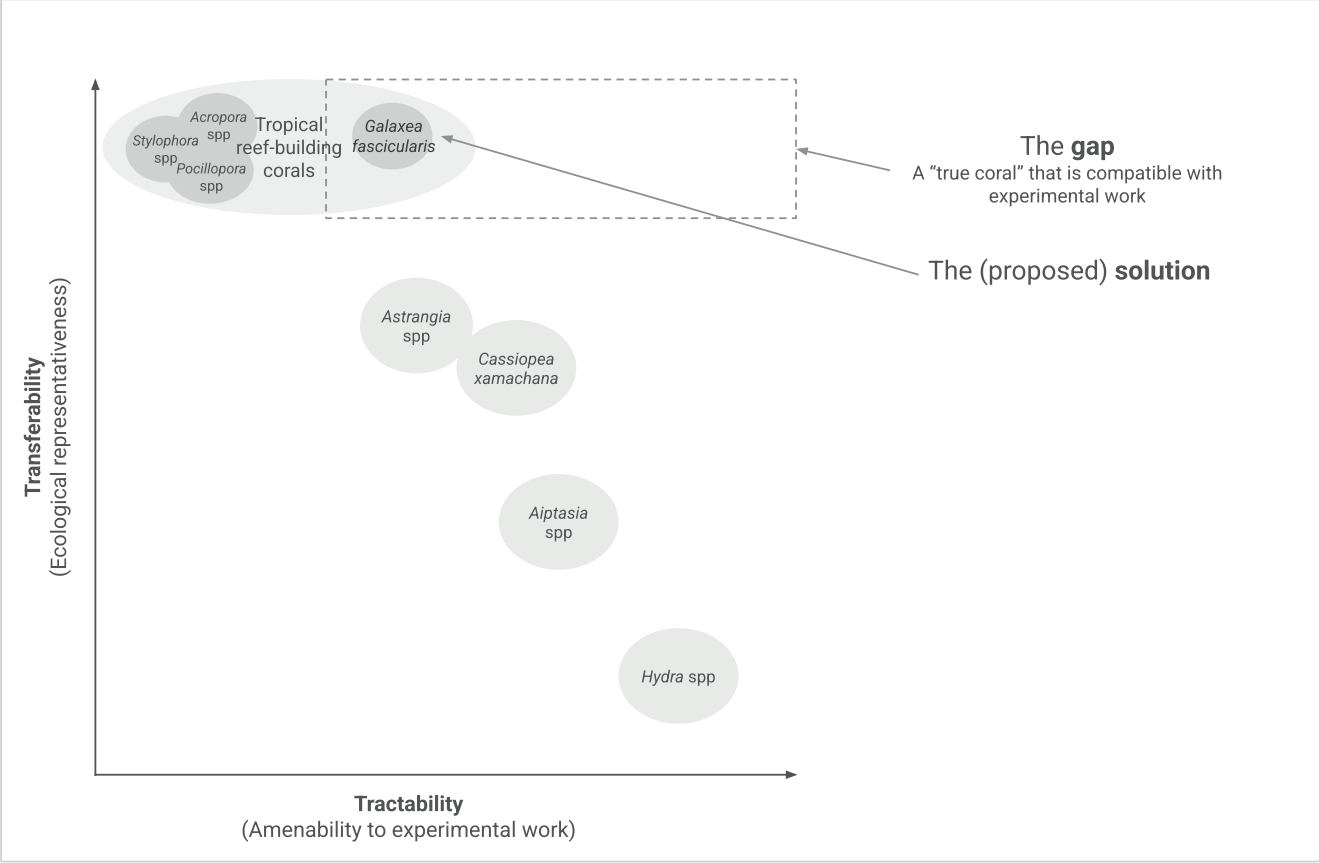

Figure 1 | The landscape of cnidarian model organisms and the role of *Galaxea fascicularis*

This figure illustrates the scientific rationale for establishing *G. fascicularis* as a "true" coral model system. A conceptual plot of cnidarian model systems based on the trade-off between "Tractability" (e.g., ease of laboratory maintenance, amenability to symbiosis manipulation, ex situ reproduction) and "Transferability" (i.e., transferability of findings to tropical, reef-building corals, or ecological representativeness). Established non-coral models (e.g., Aiptasia, Hydra) offer high tractability but lack key coral traits like calcification and obligate Symbiodiniaceae symbiosis, limiting their ecological transferability. Conversely, major reef-building corals (e.g., *Acropora* spp., *Pocillopora* spp.) are highly representative but often possess low tractability, hindering mechanistic studies. This framework reveals a "research gap" for a species that is both representative and experimentally tractable. *G. fascicularis* is positioned to fill this gap, combining essential reef-building traits with a demonstrated high amenability to laboratory rearing, symbiosis manipulation (e.g., menthol bleaching and re-establishment), and ex situ sexual reproduction, as validated by this thesis.

cnidarian models, *Galaxea fascicularis* (Linnaeus, 1767)—a tropical, reef-building coral—is proposed as a candidate "true" coral model system (Puntin et al., 2023) (Figure 1). This choice was guided by the consideration of several aspects. *G. fascicularis* is relatively common across the Indo-Pacific region (Veron et al., 2016), with its phylogeny well resolved (Wepfer et al., 2020) and a draft genome already available (Liew et al. 2016; Niu et al. 2016; http://www.gfas.reefgenomics.org/), making it an accessible and well-understood species for research. It has been frequently used in heat stress and calcification studies, and its practical advantages include ease of growth and propagation in aquaria, which is also reflected in high stress tolerance in the field (e.g., Marshall and Baird 2000; Al-Horani et al. 2002). Additionally, *G. fascicularis* possess large polyps that can be easily isolated—a feature that could be further exploited in research and experimental settings as it allows for reduced structural complexity and high clonal replication (Al-Horani et al., 2003; Pavia

and Estacion, 2018; Puntin et al., 2023). Importantly, *G. fascicularis* retains key biological characteristics essential to understanding reef-building coral biology, while offering greater tractability compared to many coral species. This combination makes *G. fascicularis* a valuable "middle ground" between simplified cnidarian models and less tractable coral species. Notably, *G. fascicularis* (clade: Complexa, family: Euphyllidae) occupies a distinct phylogenetic position within scleractinians, complementary to other widely studied corals such as *Pocillopora damicornis* and *Stylophora pistillata* (both clade: Robusta, family: Pocilloporidae) (e.g., McLachlan et al. 2020; Venn et al. 2020). This thesis builds on these preliminary insights by testing the suitability of *G. fascicularis* as a model to address coral-specific questions surrounding holobiont composition, stability, and microbial interactions.

1.2 | Scope and aims

The main aim of this thesis was to establish *G. fascicularis* as a viable and effective model organism for studying coral symbiosis and holobiont interactions. This aim was pursued through the following specific **objectives**:

i | **To critically review** the landscape of model organism-based coral research. This objective sought to identify the key knowledge gaps that current cnidarian models cannot fill, thereby establishing the scientific rationale and defining the specific research niche for *G. fascicularis* as a novel, reef-building coral model system (Chapter 2).

ii | **To experimentally validate** the suitability of *G. fascicularis* as a model for coral symbiosis. This objective was addressed by systematically evaluating its tractability under controlled laboratory conditions, specifically assessing: (i) its capacity for long-term aquarium maintenance, (ii) its potential for completing its reproductive cycle ex-situ, and (iii) its amenability to protocols for experimental symbiosis manipulation (Chapter 3).

iii | **To provide a foundational characterization** of the bacterial microbiome's response to holobiont manipulation. This aim involved assessing the impacts of menthol-induced bleaching (Symbiodiniaceae removal) on the microbial community structure of long-term aquarium-reared corals, providing an initial baseline for understanding coral-Symbiodiniaceae-bacteria dynamics within the Galaxea model (Chapter 4).

iv | **To synthesize** the findings and propose a research roadmap for the future development of *G. fascicularis* as a model system. This involved critically discussing the model's limitations and outlining key research priorities required to enhance its utility for addressing unresolved questions in coral holobiont functioning (Chapter 5).

1.3 | Publication outlines

Chapter 2 | Model organisms in coral research: what works, what's missing, and the role of *Galaxea fascicularis*

Published in Microbiology and Molecular Biology Reviews (MMBR):
Puntin G, Sweet M, Fraune S, Medina M, Sharp K, Weis VM, Ziegler M **(2022) Harnessing the Power of Model Organisms To Unravel Microbial Functions in the Coral Holobiont.** Microbiol Mol Biol Rev MMBR 86:e0005322

Personal contribution: GP helped conceive the article and was the leading contributor to writing and revising the manuscript.

This article reviews the current state of model organisms in coral research to evaluate the scope for establishing *Galaxea fascicularis* as a new reef-building coral model system. Recognizing the need for a comprehensive approach that integrates diverse models, the review highlights how Galaxea could

play a critical role in overcoming practical challenges that hinder a comprehensive understanding of coral holobiont functioning.

To decipher coral holobiont complexity, research must address key questions such as identifying holobiont members, characterizing their interactions, and understanding how these interactions shape the coral phenotype. Different experimental approaches are required to answer these questions, necessitating a diverse range of research models. The review advocates for a multi-model strategy: tractable but less representative models (e.g., distantly related cnidarians) are ideal for studying basal biological mechanisms, while more representative but challenging models (actual tropical reef-building corals) are essential to validate findings. To provide a roadmap for future research, the review evaluates both reductionist (simplifying holobiont components) and manipulative (producing novel partner combinations) approaches. It first examines established and emerging non-reef-building cnidarian models (such as Hydra, Aiptasia, Cassiopea, and *Astrangia poculata*) and then explores direct experimentation methods in reef-building corals through simplified systems, including (experimentally produced) aposymbiotic hosts or cell and tissue culture models. Finally, it presents a framework for evaluating tropical reef-building corals as model species based on tractability (ease of laboratory use) and transferability (relevance and applicability).

Through this evaluation, *Galaxea fascicularis* emerges as one of the most promising reef-building coral models, given its availability, pre-existing knowledge, and amenability to laboratory study and experimental manipulation. This review also highlights the need for further research into the suitability of the Galaxea model for symbiosis manipulation and the broader impacts on holobiont compartments, topics addressed in the following chapters of this thesis.

Chapter 3 | Experimental validation of *Galaxea fascicularis* as a model system: laboratory rearing, symbiosis manipulation, and reproduction in captivity

Published in Coral Reefs:
Puntin G, Craggs J, Hayden R, Engelhardt KE, McIlroy S, Sweet M, Baker DM, Ziegler M **(2023) The reef-building coral *Galaxea fascicularis*: a new model system for coral symbiosis research.** Coral Reefs 42:239–252

Personal contribution: GP helped conceive and design the study, produced and analyzed data, and led the manuscript writing and revision.

Recognizing the limitations of traditional cnidarian models for studying coral-specific phenomena, this study tested the suitability of *Galaxea fascicularis* as a novel coral model system. The results experimentally validated *G. fascicularis* amenability to long-term laboratory rearing, experimental handling (i.e., use in physiological experiments), symbiosis manipulations, and *ex-situ* reproduction.

To address these aspects, this investigation included three independent experiments, each employing corals from a distinct geographic origin: the Red Sea, Hong Kong, and the Great Barrier Reef. In the first experiment, polyps from the Red Sea were reared in simplified laboratory systems, rendered aposymbiotic using menthol bleaching, and maintained in this state for three weeks under controlled thermal stress conditions. In the second experiment, corals from Hong Kong were bleached and subsequently inoculated with a mix of two non-native *Symbiodiniaceae* strains; successful re-establishment and characterization of the symbiosis were confirmed. In the third experiment, colonies from the Great Barrier Reef were reared in artificial systems that simulated natural environmental cues for spawning, and their reproductive performance was monitored to evaluate *ex-situ* breeding potential.

The results validated *G. fascicularis* as a promising coral model system: (1) *G. fascicularis* polyps can be reliably maintained in simplified laboratory conditions, surviving extended periods

post-bleaching and tolerating experimental handling; (2) menthol bleaching allows re-establishment of symbiosis with cultured *Symbiodiniaceae* strains, demonstrating flexibility in symbiotic associations; and (3) colonies can be induced to spawn in artificial settings, successfully producing viable offspring (F1 generation). Together, these findings demonstrate that *G. fascicularis* could offer a solution to practical limitations in coral research, particularly regarding the challenges of experimental handling and symbiosis manipulation. The Galaxea model has the potential to enable new research avenues to uncover coral symbiosis mechanisms and improve our understanding of coral holobiont functioning.

Chapter 4 | Menthol bleaching and the bacterial microbiome in the Galaxea model: comparative analysis of symbiotic and menthol-bleached polyps

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Puntin G, Wong JCY, Röthig T, Baker DM, Sweet M, Ziegler M **(2024) The bacterial microbiome of symbiotic and menthol-bleached polyps of long-term aquarium-reared *Galaxea fascicularis*.** Peer Community J 4:e59

Personal contribution: GP helped conceive and design the study, produced and analyzed data, and led the manuscript writing and revision.

This study investigated bacterial microbiome dynamics in *Galaxea fascicularis* under both symbiotic and menthol-induced aposymbiotic conditions, aiming to understand how microbial communities respond to symbiosis disruption in this emerging coral model. This study addressed a critical gap by exploring the response of the bacterial microbiome to menthol bleaching—a foundational step in coral symbiosis manipulation.

In this experiment, *G. fascicularis* polyps from Red Sea and Hong Kong colonies, which were long-term reared in controlled aquaria in separate facilities, were bleached using menthol to remove Symbiodiniaceae, followed by 16S rRNA gene sequencing to profile the bacterial microbiome. The results show that bleaching does not significantly alter overall microbial diversity (species richness), but bleached polyps show stochastic shifts in community composition indicative of a dysbiotic state. On the other hand, symbiotic polyps maintained distinct colony-specific microbiomes despite being hosted in shared aquaria. Additionally, differences in microbial community responses across rearing facilities highlighted the potential influence of aquarium and rearing conditions on microbial dynamics, pointing at this direction for further investigation. This study also identified potential core bacterial associates in *G. fascicularis*, providing a basis for future studies on essential microbial partners. Furthermore, the relatively low diversity of bacterial taxa across both symbiotic and bleached states suggests a simplified microbiome, which could facilitate the discovery of essential microbial players and facilitate experimental microbial manipulations.

These findings improve our understanding of bacterial dynamics during menthol bleaching in captive corals, a fundamental aspect of coral symbiosis research. This work also suggests areas for future research, particularly regarding rearing conditions, necessary to consolidate the role of Galaxea as a model for coral symbiosis research through holobiont manipulations.

Additional co-authored publications

Non-coral cnidarian model illustrates the role of the microbiome in holobiont stability through nutrient regulation

Published in Microbiology:
Röthig* T, **Puntin* G**, Wong JCY, Burian A, McLeod W, Baker DM **(2021) Holobiont nitrogen control and its potential for eutrophication resistance in an obligate photosymbiotic jellyfish.** Microbiome 9:127–127

Personal contribution: GP contributed to the study design and execution, sample processing, data analysis, manuscript writing and revision.

Eutrophic conditions disrupt the dynamics of the coral-Symbiodiniaceae association, making corals more

prone to bleaching. In contrast, the photosymbiotic upside-down jellyfish *Cassiopea xamachana* can maintain a stable association even in high-nutrient environments. To understand this stability, nitrogen cycling within the holobiont was investigated using stable isotope labeling (^{13}C, ^{15}N) and microbial community analysis (16S rRNA gene). Results show that while the host meets its nitrogen needs through heterotrophic feeding, the nitrogen available to algal symbionts—as dissolved inorganic nitrogen (DIN)—remains limited, even when environmental DIN is high. This restriction, likely mediated by the microbiome through processes such as denitrification, might be what allows the Cassiopea-Symbiodiniaceae symbiosis to thrive in eutrophic conditions that would be detrimental for corals.

These findings support the broader thesis objective of assessing cnidarian models to understand holobiont complexity (Objective 2). Specifically, this work illustrates how microbial communities contribute to holobiont stability through nutrient regulation. Insights from this study highlight the importance of microbiome dynamics, which could be further explored within the Galaxea model to clarify microbial roles in coral stability.

Expanding the menthol bleaching toolkit: a comparative study across six reef-building coral species

Preprint at bioRxiv:
Bauer L, Ferrara E, **Puntin G**, Paulus A-L, Reiser M, Schmidt F, Ziegler M **(2025) Establishment of menthol bleaching protocols for six stony coral species.** bioRxiv: https://doi.org/10.1101/2025.07.01.662491

Personal contribution: GP contributed to the study design and execution, data analysis, manuscript writing and revision.

This work directly builds upon the methodological aspect, central to this thesis (Objective 2), to validate protocols for the experimental manipulation of the coral-Symbiodiniaceae association in *G. fascicularis* (Chapter 3). Bauer et al. (2025) extends this foundational work by performing a comparative study to establish effective menthol bleaching protocols across six different stony coral species, including *G. fascicularis*. This analysis revealed high species-specific tolerance to menthol. Notably, it demonstrated that while the protocol adapted in Chapter 3 was effective for *G. fascicularis* single polyps, a more intensive six-day protocol was required to successfully bleach *G. fascicularis* fragments. This work refines the "menthol bleaching toolkit" for Galaxea and other species, providing a methodological guide for future comparative symbiosis research and highlights the importance of considering species-specific responses to holobiont manipulations.

Validating a non-destructive sampling method for holobiont monitoring

Published in Environmental Microbiology :
Million WC, Voolstra CR, Perna G, **Puntin G**, Rowe K, Ziegler M **(2025) Resolving Symbiodiniaceae Diversity Across Coral Microhabitats and Reef Niches.** Environ Microbiol 27:e70065

Personal contribution: GP contributed to the study design and execution, data analysis, manuscript writing and revision.

The work by Million et al. (2025) contributes to the establishment of the Galaxea model system (Objective 2) by validating a non-destructive monitoring technique. While the study's primary focus was on reef-wide Symbiodiniaceae diversity, a key component involved using the *G. fascicularis* colonies from the Ocean2100 facility to test mucus sampling as a proxy for tissue sampling. The study showed that Symbiodiniaceae communities in the surface mucus layer (SML) are "largely identical" to those in the tissue and that the sampling method is non-invasive. This result is relevant for the Galaxea model, as it validates mucus sampling as a reliable, non-destructive technique for the long-term monitoring required in the experimental manipulations proposed by this thesis.

Chapter 1

Refining non-invasive monitoring: validating RGB analysis as a proxy for bleaching

Preprint at bioRxiv:
Ferrara EF, Bauer L, **Puntin G,** Bautz F, Celayir S, Do M-S, Eck F, Heider M, Wissel P, Arnold A, Wilke T, Reichert J, Ziegler M **(2024) RGB color indices as proxy for symbiont cell density and chlorophyll content during coral bleaching.** bioRxiv: https://doi.org/10.1101/2024.12.20.629333
Personal contribution: GP contributed to data collection and manuscript revision.

Ferrara et al. (2024) validates a rapid and non-invasive method to quantitatively assess bleaching. The study systematically tested 19 different RGB-derived color indices from digital photographs against direct physiological measurements (Symbiodiniaceae cell density and chlorophyll content). This was tested across three coral species exposed to different bleaching conditions, including the menthol-bleaching method used in Chapter 3. The results demonstrated a strong correlation between the 'Red index' (Red channel) and both symbiont density and chlorophyll. This work provides a robust, quantitative, and objective alternative to the more subjective visual assessments used to confirm bleaching, thereby strengthening the methodological toolkit for future symbiosis manipulations available to coral research and the Galaxea model (Objective 2).

1.4 | References

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CHAPTER 2

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REVIEW

Harnessing the Power of Model Organisms To Unravel Microbial Functions in the Coral Holobiont

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SUMMARY Stony corals build the framework of coral reefs, ecosystems of immense ecological and economic importance. The existence of these ecosystems is threatened by climate change and other anthropogenic stressors that manifest in microbial dysbiosis such as coral bleaching and disease, often leading to coral mortality. Despite a significant amount of research, the mechanisms ultimately underlying these destructive phenomena, and what could prevent or mitigate them, remain to be resolved. This is mostly due to practical challenges in experimentation on corals and the highly complex nature of the coral holobiont that also includes bacteria, archaea, protists, and viruses. While the overall importance of these partners is well recognized, their

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Model organisms in coral research: what works, what's missing, and the role of *Galaxea fascicularis*

specific contributions to holobiont functioning and their interspecific dynamics remain largely unexplored. Here, we review the potential of adopting model organisms as more tractable systems to address these knowledge gaps. We draw on parallels from the broader biological and biomedical fields to guide the establishment, implementation, and integration of new and emerging model organisms with the aim of addressing the specific needs of coral research. We evaluate the cnidarian models Hydra, Aiptasia, Cassiopea, and *Astrangia poculata*; review the fast-evolving field of coral tissue and cell cultures; and propose a framework for the establishment of "true" tropical reef-building coral models. Based on this assessment, we also suggest future research to address key aspects limiting our ability to understand and hence improve the response of reef-building corals to future ocean conditions.

KEYWORDS model organisms, metaorganism, reef-building corals, microbial functions

INTRODUCTION

Scleractinian or stony corals build the framework of coral reefs, which are the most biodiverse and productive marine ecosystems (1). Coral reefs provide important ecosystem services and a livelihood to over 500 million people globally (2). Climate change, together with the local stressors of pollution and overexploitation have heavily impacted coral reefs around the world, causing major habitat loss and threatening the survival of these ecosystems (3–5) (Fig. 1). Preserving the biological and ecological functions of coral reefs requires drastic reductions of global and local stressors (6) together with active conservation and restoration interventions (7, 8). The effectiveness of such interventions depends upon a deep, accurate, and comprehensive understanding of coral biology (9, 10). However, while speed is imperative, research progress is challenged by the difficulties associated with working with corals.

Holobiont Diversity and Complexity

Corals are complex metaorganisms. The coral animal hosts a vast array of microorganisms encompassing unicellular algae of the family *Symbiodiniaceae*, bacteria, archaea, fungi, and other protists, as well as viruses, which collectively constitute the so-called coral holobiont (see Box 1) (11–13). Each coral colony represents a rich and diverse microecosystem often hosting several *Symbiodiniaceae* species (14, 15), hundreds to tens of thousands of bacterial taxa (16, 17), and probably at least as many archaea, viruses,

BOX 1: CORAL HOLOBIONT MEMBERS AND FUNCTIONS—AN OVERVIEW OF CURRENT KNOWLEDGE

***Symbiodiniaceae* and coral bleaching.** The endosymbiotic dinoflagellate algae (*Symbiodiniaceae*) are the most extensively studied and better characterized members of the coral holobiont (13). The coral-algal symbiosis is obligate and based around nutritional exchange, where the metabolic contribution of the photosynthetic algae supports high productivity under oligotrophic conditions—far beyond the capacity of the coral animal alone (24, 25). This symbiosis supports the building of the structural foundation of coral reefs and represents the engine of these ecosystems (26). However, this symbiosis is under threat, primarily due to global warming and other anthropogenic stressors on both a local and a global scale. The loss of the algae from coral tissue—coral bleaching—weakens the coral host and often leads to its death (27). Increasing frequency of and decreasing recovery time between bleaching events (28) make coral bleaching the largest challenge for the persistence of reef ecosystems, which has received much attention over the last 3 decades (29). Nonetheless, a complete and detailed understanding of the underlying cellular mechanisms is still lacking.

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Chapter 2

Bacteria and coral disease. Bacteria are the best studied of the microbial coral holobiont members and are known to play an important role in holobiont health (30). They are known to exist in highly diverse communities, which appear to vary in composition depending on coral and *Symbiodiniaceae* genotype (31, 32), environmental conditions (33), anatomical compartments (21, 34), and even colony age (35). They have been accredited as controlling or governing key functions for the coral host including, nutrient cycling (36, 37), and immunity (38, 39), and they have even been hypothesized to facilitate rapid environmental adaptation (40).

Imbalances in the microbiome, or dysbioses, compromise coral health and can lead to the emergence of disease (30). Due to climate change and anthropogenic activities, coral diseases are increasing in frequency and number, e.g., black band disease, now occurring in coral reefs around the world (41), and gray patch disease in the Indo-Pacific (42). Some researchers are now arguing that disease rivals coral bleaching as a major cause of coral reef decline on a global scale (43, 44).

Manipulating the coral microbiome has recently been shown to increase the tolerance of corals to a number of stressors, for example, by enriching the holobiont in members with beneficial functions and traits (45–48). Research on coral probiotics aims at understanding how to perform such manipulations to accelerate the rate of coral adaptation to global change (45, 47, 49). However, although our understanding of the likely functional role many bacteria play in coral health is rapidly advancing, we still lack a mechanistic understanding of the dynamics and functions of the majority of the coral associates (reviewed in reference 50). While there are limitations inherent to culture-based methods, a recent study has shown that diverse members across many phyla can and have already been cultured and highlighted how these could be further expanded in the coming years from the adoption of more diverse culturing approaches (51).

Understudied microbial partners. The remainder of the coral's microbiome—i.e., the "other" (endolithic) microalgae, protists, archaea, fungi, and viruses—is comparatively less well understood. However, these microbes constitute a nonnegligible proportion of the coral microbiome. Archaea were found to constitute up to half of the prokaryotic fraction in absolute abundance, fungi were the most abundant microorganism in metagenomes of *Porites astreoides*, for example (18, 19), and viruses have been shown to be present in abundances of upwards of $\sim 10^7$ viruses per mL of mucus (52). Fungi and endolithic algae specifically appear to at least spatially dominate in the coral aragonite skeleton, where they have been shown to be directly involved in carbon and nitrogen cycling and may metabolically interact with each other and the coral host (53, 54). Archaea also appear to be involved in nutrient metabolism, in particular ammonia oxidation, carbon metabolism, and the synthesis of essential vitamins (55). Viruses, however, remain the most elusive members of the coral holobiont, and both their pathogenic and their beneficial roles are currently being investigated (reviewed in reference 56).

and protists (18–20). This high diversity of microorganisms can be partially explained by coral colony morphology consisting of a dynamic surface mucus layer, the coral gastrodermal and epidermal tissues, the mesoglea, and the skeleton. Each of these represents a microhabitat or niche populated by distinct microbial communities (21–23).

Many of the associated microorganisms are likely to be involved in holobiont metabolism, immunity, and environmental adaptation and may therefore contribute to the health and performance of the metaorganism (reviewed in references 30 and 50). The holobiont phenotype thus results from the combination of the long-term stable host genotype and the more flexible genotypes of the associated microbes (57–60). In addition, the environment (e.g., temperature, light, and salinity) and host characteristics (e.g., trophic state and

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FIG 1 Coral bleaching and coral diseases as major threats to coral reefs. (A) Aerial view of coral bleaching in the Great Barrier Reef (Australia) during the 2017 mass bleaching event. (B) *Acropora cytherea* affected by white syndrome (WS), a tissue loss disease of unknown etiology. (C) *Orbicella annularis* suffering from stony coral tissue loss disease (SCTLD), a new lethal disease alarmingly spreading through the Caribbean. (D) *Goniopora* sp. infected with black band disease (BBD) during a bleaching event (visible loss of pigmentation). BBD is caused by a microbial consortium dominated by filamentous cyanobacteria. Image credits: A, Ed Roberts/ARC Centre of Excellence for Coral Reef Studies; B, C, and D, Dr. Greta Aeby.

age) modulate the cross-kingdom interactions between holobiont members (i.e., host-microbe and microbe-microbe) in a complex and underexplored framework (35, 61, 62).

Our understanding of what makes a coral "tick" has recently expanded exponentially and now the majority of researchers acknowledge the importance of the holobiont as a whole rather than focus on any one aspect (30). However, we still struggle to disentangle holobiont complexity and fall short in our understanding of coral functioning from a holistic perspective (Fig. 2 and Table 1). Several fundamental questions, such as those listed below, therefore remain either fully or partially unanswered.

Who is there and where? The coral microbiome has certainly not been fully characterized yet. The current knowledge of the coral microbiome is highly skewed toward *Symbiodiniaceae* and bacterial members, and less is known about the other microbial partners that constitute a large proportion of the microbiome in both biomass and absolute abundance (18, 19, 53). Thousands of taxa are likely yet to even be described and characterized.

Who does what? While it is well recognized that the microbiome plays an important role in fundamental physiological functions such as nutrition, development, and immunity, the exact contribution and involvement of each microbial taxon remains to be resolved.

Who interacts with whom? Besides coral-*Symbiodiniaceae* dynamics, very little is known about interactions between other members of the holobiont (63, 64). For instance, do bacteria interact with each other, the coral host, the *Symbiodiniaceae*, archaea, fungi, and/or viruses? Furthermore, can microbial communities in different anatomical compartments interact with each other? If so, then our next question would be as follows.

How do they interact with each other? Individuals may affect or impact others within the community via positive (e.g., mutualistic symbiosis and facilitation), negative (e.g.,

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CHAPTER 2

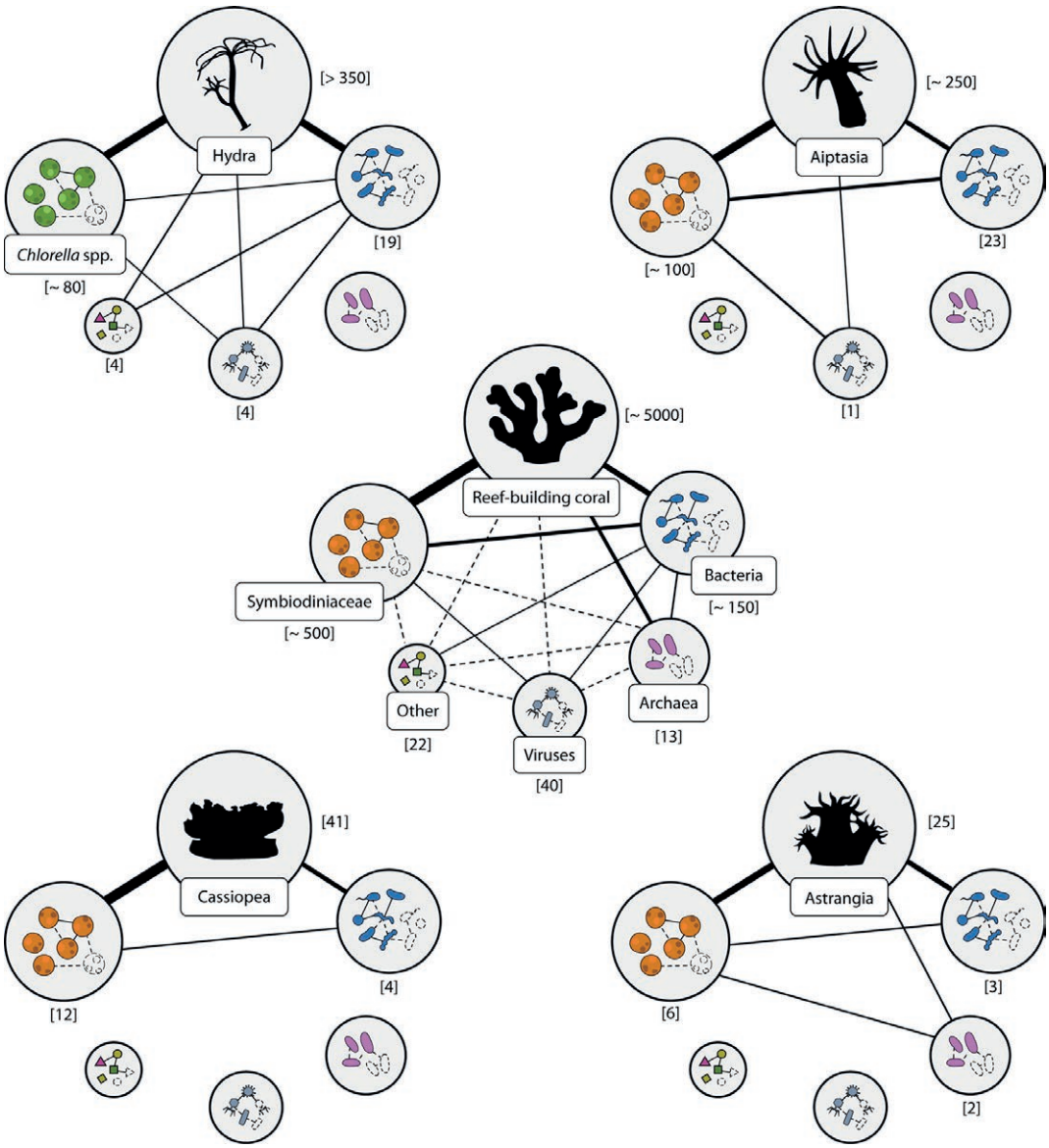

FIG 2 Overview of the state of knowledge on cnidarian holobionts regarding composition and functional interactions among their members. Cnidarian holobionts are disassembled into their major taxonomic compartments (gray circles). Within each taxonomic compartment, dashed-outlined microbes represent hypothesized, yet currently unidentified, taxa. Lines connecting compartments indicate known relationships, while absence of connecting lines indicate lack of information. Lines connecting individual microbes indicate known (solid), hypothesized (dashed), and presumed lack of functional relationships (no lines). The size of the taxonomic compartments and the thickness of the connecting lines approximate the assumed importance of each holobiont member or relationship. "Other" includes fungi and other protists (e.g., unicellular algae other than *Symbiodiniaceae* and *Chlorella* spp.). Numbers in square brackets report the numbers of peer-reviewed publications on the model organisms and their compartments (see the supplemental material for additional details).

competition, predation, and parasitism), or neutral (e.g., commensalism) interactions. Resolving the network of interactions between individual members of the microbiome and across Kingdoms is extremely challenging, yet it will yield very valuable information on ecological and coevolutionary processes (42, 65).

Model Organisms Unravel Complex Biological Principles

Model organisms by their very nature facilitate research because they are practically and/or ethically "more convenient to study" than the organisms of interest, and at the same time they are similar enough so that discoveries can be meaningfully transferred.

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TABLE 1 Summary of the most relevant literature on functional interactions between members of the cnidarian model organisms

	Observation(s)[a]			
Interaction	**Hydra**	**Aiptasia**	**Cassiopea**	***Astrangia* spp.**
Host-symbiotic algae	↔ Facultative endosymbiosis with *Chlorella* spp. (only *H. viridissima*); metabolic complementarity. The host provides CO_2, N, P, and S. The algae provide photosynthates and amino acids (reviewed in references 84, 85, and 296). → Host controls *Chlorella* population size through algal cell cycle modulation, expulsion, or digestion (reviewed in references 84, 85, and 296).	↔ Bleaching is associated with cellular and molecular responses in both partners (127, 297–300). ↔Onset of symbiosis as a modulation of the host immune response (137, 301–304). ↔ Species specific; association with non-native algal types results in altered expression patterns for metabolic exchanges, oxidative stress response, and immunity processes (119, 122, 136). ↔ Metabolic complementarity; Transfer of org. and inorg. nutrients between partners (135, 140, 305–307). ← Species-specific; association with non-native algal types is less stable and results in lower host growth and reproduction rates (132, 133).	↔ Partner specificity: Association with non-native thermotolerant algal strain produces more heat-sensitive holobionts (164) ↔ Coupling of host and symbiont metabolism through translocation and recycling of C and N compounds (308, 309). → Host can restrict N (nitrate) availability to symbiont (170, 173). ← Symbiosis-driven development (161, 169, 171). ← Unable to heterotrophically compensate lack of alga-derived nutrients under low illumination (310) or in aposymbiotic state (162). ← Alga-fixed and translocated C satisfies >100% of host metabolic demand (311).	↔ Facultative association; the symbiont provides mild advantages (nutrition, host growth and healing) or nondetectable effects (calcification rate) (180, 190, 312–314). → Host regulates symbiont population density through expulsion (315). → Host trophic conditions drive mutualistic/parasitic shift (181).
Host-bacteria	→ Host influences bacterial microbiome through antimicrobial compounds and by altering bacterial quorum-sensing signaling (95, 97–99, 106). ← Bacteria protect the *Hydra* host against fungal infections (100). ← Bacterial involvement in host body developmental regulation (101). ← Bacterial role in spontaneous body contraction regulation (105).	↔ Species specificity in the host-bacterium association (111). → Host state linked to bacterial microbiome structure and composition (141, 145). ← Potential role of bacteria in host defensive tissues (316).	← Bacteria as larvae settlement cue (161, 317).	
Host-virus	↔ Viromes are species specific; heat stress linked to altered viromes and altered predicted cellular functions involved in (DNA and C) metabolism and defense (318).	↔ Diverse but stable viral assemblage (319).		
Host-fungi	← Lethal fungal pathogen (100).			

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TABLE 1 (Continued)

	Observation(s)[a]			
Interaction	**Hydra**	**Aiptasia**	**Cassiopea**	***Astrangia* spp.**
Symbiotic algae-bacteria	→ Symbiotic algae provide host with colonization resistance and community-immunity against an invasive bacterium (320).		↔ Symbiotic state linked to bacterial community composition; potential bacterial involvement in modulating N availability (170).	→ Recovery of prokaryotic microbiota following antibiotic treatment is more consistent among symbiotic (than aposymbiotic) individuals (195). × Prokaryotic microbiome unaffected by seasonality or symbiotic state (77, 321).
Symbiotic algae-archaea				→ Recovery of prokaryotic microbiota following antibiotic treatment is more consistent among symbiotic thann aposymbiotic individuals (195).
Symbiotic algae-virus	←? Consistent presence of virus in Chlorella suggests functional role (322).	→ Relative abundance of viral taxa linked to symbiotic state (319).		
Bacterium-virus	← Bacteriophages dominate *Hydra* virome (318). ← Bacteriophage role in bacterial population dynamics (323, 324).			

[a]References are indicated parenthetically where applicable.

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Interestingly, there is no apparent fixed set of rules to define a model organism or its validity (66). Instead, the models are usually chosen based on the suitability of the organism to investigate a specific phenomenon or set of questions needing to be addressed, namely, its tractability and its informative power (67, 68). For example, tractability is typically associated with small size, fast growth rate, short reproductive cycle, broad availability, ease of maintenance under laboratory settings, and simplicity of some traits (e.g., small number of genes or simple body plan) (68). In addition to tractability, many models possess what could be perceived as "odd" or "unusual" features, which make them stand out among similar organisms. Typically, these oddities have a great informative power if harnessed for research purposes (67, 68). For example, the high regenerative capacity of Hydra or the ability to survive extreme conditions of tardigrades helped shed light on the mechanisms of aging, transdifferentiation, and *de novo* generation of biological patterns (69, 70) and on protection against damage of biological structures (71, 72).

Simplicity is also a very important feature for biological investigation that traditionally follows a reductionist approach. Compared to more complex or derived systems, simpler systems possess all of the fundamental features but lack much of the "extra noise", such as additional biochemical pathways or regulatory processes, and therefore facilitate understanding of fundamental biological mechanisms. Thus, researchers exploring the mechanisms of gene expression and regulation found it more convenient to study the yeast *Saccharomyces cerevisiae*, a single-celled eukaryote with a simple genomic structure and comparably little non-coding DNA (73), rather than something as complex as a human cell. The same fundamental principles apply to both, to the point that essential yeast genes can be replaced by their human orthologs (74).

As scientific knowledge grows and new questions arise, new model organisms are being added to a growing list (75). Technological progress is increasing the insight from each model organism rather than replacing them. Easy access to high-throughput sequencing technologies expands the number of sequenced genomes available and facilitates the development of new and customized molecular tools (67, 68). Model organisms are not only growing in number, but they are also used in new and more varied applications. While model organisms have been extensively used to investigate fundamental biological principles (e.g., organismal development, behavior, and evolution [69]), phenomena directly affecting human health (i.e., to understand and treat diseases) or to generate economic benefits (agricultural crops and livestock), more recently, nature and biodiversity conservation represents a new niche for model organism-based research (76).

Coral research is challenged by a set of important questions that could benefit from the use of model organisms (76–79) (Fig. 2). Disentangling holobiont complexity to shed light on the mechanisms underlying coral responses to global change (e.g., coral bleaching and diseases; see Box 1 and Fig. 1) and how these can be prevented or mitigated, is one of the most pressing challenges faced today (8). To reverse the decline of coral reefs (and maintain the services they provide), scientists have been embracing efforts to increase corals resilience through approaches that span many levels of intervention, from the microscopic level (cellular and molecular, i.e., assisted evolution) to the macroscopic level (ecosystem scale, i.e., assisted gene flow), and from laboratory-based to field deployment (7). Here, we provide a comprehensive overview of the state-of-play of model organisms and systems that can be utilized to move coral holobiont research into the next stage. Our aim for this review is to (i) highlight each model system's advantages and disadvantages and (ii) synthesize open research questions and how establishment of new model systems could address them. We review established and emerging cnidarian model systems, including the freshwater hydroid Hydra, the anemone Aiptasia, the jellyfish Cassiopea, and the temperate coral *Astrangia poculata* (Fig. 3). Moreover, we provide a comparative overview of their attributes, including distribution, ease of rearing in aquaria, life cycle, amenability to manipulate symbiotic states, existing knowledge base, and resources. We further introduce the different approaches that can facilitate direct experimentation on tropical stony corals, a

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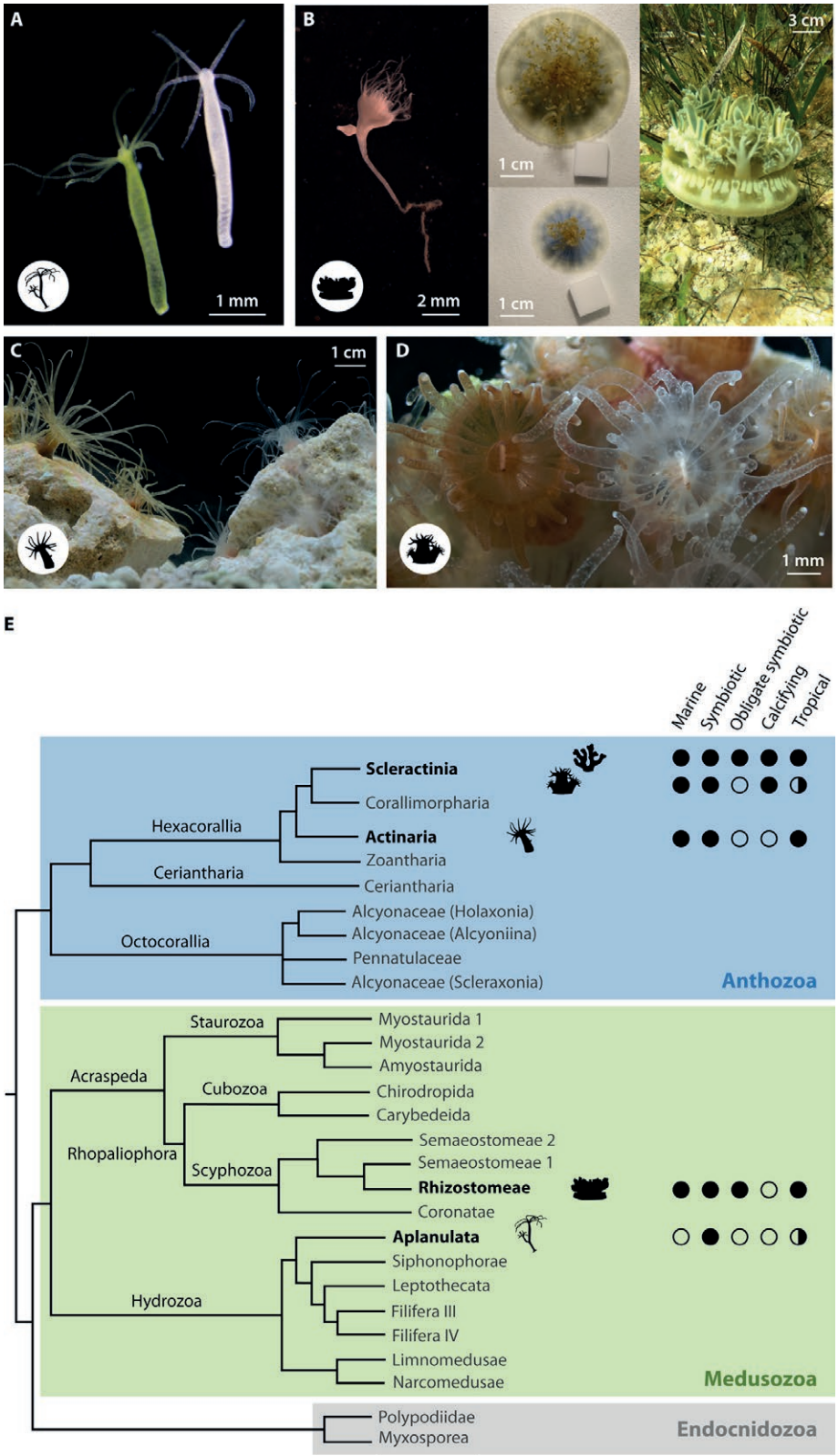

FIG 3 Cnidarian model organisms and systems for reef-building corals. (A) Freshwater hydroid Hydra, symbiotic with the chlorophyte *Chlorella* spp. (left) and aposymbiotic (right). (B) *Cassiopea xamachana* scyphistoma (polyp,

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necessary step to validate discoveries made on laboratory model systems (10, 80). These approaches include the establishment of tropical stony coral species as model organisms, as well as simplified systems such as cell and tissue cultures.

CNIDARIAN MODEL ORGANISMS AS CORAL HOLOBIONT MODELS

Freshwater Hydroid Hydra

Hydra is the oldest cnidarian model organism and arguably the best known. Hydra species belong to the class Hydrozoa, in the anthozoan sister subphylum Medusozoa (Fig. 3E). They inhabit freshwater ecosystems worldwide (81–83) and while most do not associate with microalgae, one species (*H. viridissima*) can establish facultative endosymbiosis with the chlorophyte *Chlorella* spp. (84, 85). Hydra has a simple life cycle that can be easily completed in the lab. Under normal laboratory conditions, solitary polyps reproduce asexually by budding, while environmental shifts in temperature or food induce sexual reproduction (86, 87). This ease of rearing combined with its exceptional regenerative capacity, was already appreciated in the 1700s (88) and contributed to the birth of experimental zoology (89). This is evidenced by early fundamental discoveries using the Hydra model, such as Ethel Browne's discovery of induced formation of a secondary axis by transplanting a head onto a polyp (90), 15 years before Mangold and Spemann published their observation of the organizer activity of the dorsal lip of the amphibian embryo (91). Hydra then grew into a model for developmental biology that helped answer questions of pattern formation at the theoretical (92) and molecular levels (93). Later, the adoption of *H. viridissima* as a model organism contributed to the understanding of fundamental processes in cnidarian-algal nutrient exchange and symbiosis regulation (see reference 85 and references therein).

Over the last 15 years, Hydra has become an important model for host-bacterium research. Initial analyses of the bacterial microbiome of different Hydra species revealed a high degree of species specificity reflecting the phylogenetic relationships of its Hydra host species (94, 95). Subsequent research identified that the epithelial cells produce specific antimicrobial peptides (96–99) which act as key innate immune factors responsible for shaping the species-specific bacterial associations (95).

The Hydra model has shed light on the involvement of the bacterial microbiome in shaping the holobiont phenotype. For example, the removal of the intact microbiome revealed that bacteria protect the Hydra host against fungal infections (100). Other recent results indicate that bacteria associated with Hydra are able to modify the Wnt-signaling pathway, a central signaling cascade in development (101). This pathway is involved in several developmental processes in Hydra, such as head (93) and bud (102, 103) formation, and the differentiation of stem cells (104). Functional analyses of bacterium-regulated genes revealed that the corresponding peptides have an antagonistic function to Wnt-signaling and influence stem cell differentiation (101). Studies have also shown that specific bacterial species are involved in regulating the frequency of spontaneous body contractions, which is reduced by ~50% in germ-free animals (105). Although the mechanisms underlying these responses are still unclear, studying the host-microbe signaling in detail revealed a direct interaction between Hydra and its associated bacteria based on quorum-sensing signaling molecules (106). This investigation identified a fundamental mechanism whereby the host-modified bacterial signal molecule promotes symbiosis establishment, while the non-modified signal molecule

FIG 3 Legend (Continued)
early life stage, left), young symbiotic medusa (center top), young aposymbiotic medusa (center-bottom), and adult symbiotic medusa (right). (C) Sea anemone Aiptasia, symbiotic (left) and aposymbiotic (right) polyps. (D) *Astrangia poculata* naturally occurring in symbiotic (left) and aposymbiotic (right) state. (E) Phylogeny showing the relative phylogenetic distance within cnidarians and between tropical scleractinians (including reef-building corals) and the cnidarian model organisms discussed in this review. Specifically, from top to bottom are shown: tropical stony corals (Scleractinia), *Astrangia* spp. (Scleractinia), *Exaiptasia* spp. (Actinaria), *Cassiopea xamachana* (Rhizostomeae), and *Hydra* spp. (Aplanulata). Phylogeny was modified from reference 159. Image credits: A, Jay Bathia; B, Victoria Sharp, Claudia Tatiana Galindo, and Andre Morandini; C, Samuel Begood; D, Alicia Schickle.

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represses it (106). This demonstrates that Hydra is able to alter quorum-sensing controlled behavior of its bacterial symbionts to promote metaorganism assembly and resilience (106).

Within the bacterial communities of Hydra, one particular bacterium (a *Curvibacter* sp.) stands out as it is the most abundant member (95). *Curvibacter* sp. appears to populate and accumulate in a mucus-like layer of the ectodermis (100) and can easily be cultivated and reproduced in the lab (100). Genomic and transcriptomic data are available for both *Curvibacter* and its Hydra host (106–108), which facilitates functional studies in both symbiosis partners (109–111). Importantly, due to the transparent appearance of the animals, the host's cells and those of the *Curvibacter* can be transgenically labeled (109, 110). This means microscopic analysis can be achieved in an *in vivo* context on single cells, as well as whole tissue levels across space and time. It is now even possible to generate germ-free Hydra polyps via antibiotic treatment and repopulate them with single or multiple bacterial strains (95, 100, 112). Despite differences in the surface topography between Hydra and stony corals (113), their relatively distant phylogenetic relationships (Fig. 3E), and differences in life history (Table 2), the Hydra model can help to understand mucosal host-microbe interactions in corals by providing a roadmap to controlled symbiosis-reestablishment experiments in cnidarians.

Sea Anemone Aiptasia

The sea anemone Aiptasia is also among the original models utilized for the study of coral-dinoflagellate symbiosis, with some publications dating back to the 1960s and early 70s (e.g., reference [114]). The name Aiptasia is a common name for *Exaiptasia diaphana* (Rapp, 1829), which was previously named *Exaiptasia pallida* (115). Similar to Hydra, Aiptasia is fast growing, hardy, clonal, and it is extremely amenable to laboratory culture. Indeed, it is considered an aquarium pest by the hobby industry due to its rapid growth and propagation, often quickly overgrowing corals and other sessile reef fauna. Animals can be purchased from animal supply companies, collected from nature with relative ease, and acquired from the growing global network of Aiptasia laboratories that commonly share strains. Its strengths and limitations as a model have been extensively documented elsewhere (e.g., see references 76, 116, and 117), so we will only summarize them here.

With Aiptasia's popularity growing, the model has been adopted by an increasing number of laboratories around the world (117, 118). Historically, studies were primarily performed on animals of unknown or mixed genetic background, with different laboratories using different populations, strains, and possibly even different species (115). Since the early 2000s, however, the Aiptasia community has rallied and increasingly turned to clonal populations, allowing for the control (to some degree) of genetic background noise (119, 120). Two specific clonal lines, CC7 and H2 (originating from Florida and Hawaii, respectively), are now shared widely among research groups in the United States, Europe, and the Middle East (118, 121). However, different clonal populations are in laboratory culture in both Australia (117) and New Zealand (122) since these countries have strict limitations on importing non-native organisms. The genomes of CC7 (123), H2, and a clonal line from the Red Sea are available, as are numerous transcriptomes from other clonal lines (see Table 2).

The Aiptasia-*Symbiodiniaceae* model is a powerful system to unravel the cellular and molecular mechanism underlying coral bleaching. In contrast to tropical corals, Aiptasia can be easily bleached experimentally in a standardized and controlled manner, maintained in this aposymbiotic state indefinitely (typically under dark conditions to prevent repopulation from accidentally introduced algae) and subsequently repopulated with *Symbiodiniaceae* algae. Aposymbiotic animals will grow and continue to undergo pedal laceration to reproduce asexually if fed frequently (124), suggesting that the health of the animal is not impeded to any major degree. Bleaching can be achieved via a number of methods, including heat stress (125), cold shock (often in combination with the herbicide DCMU [126]), or by incubation in menthol (126). Examining aspects of the heat stress response and accompanying loss of symbionts

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TABLE 2 Summary of relevant features of cnidarian model organisms[a]

	Observation(s)					
Feature	**Hydra**	**Aiptasia**	**Cassiopea (polyp)**	**Cassiopea (medusa)**	**Astrangia**	**Tropical scleractinians**
Species	*Hydra* spp.	*Exaiptasia diaphana* (Rapp, 1829)	*C. xamachana* (Bigelow, 1892)		*A. poculata* (Ellis & Solander, 1786)	~800 species (symbiotic)
Body features						
Form	Sessile	Sessile	Benthic	Benthic but not sessile	Benthic	Sessile
Motility	Limited	Limited	No	Yes	No	No
Calcifying	No	No	No	No	Yes	Yes
Colonial	No	No	No	No	Yes	Yes
Polyp size	5 to 15 mm	~1 mm to 5 cm	~0.1 to 2 mm	~2 mm to 20 cm	<10 mm	~1 mm to 20 cm
Environment and availability						
Water/medium	Freshwater	Seawater	Seawater		Seawater	Seawater
Latitudinal distribution	Temperate	Tropical and subtropical	Tropical and subtropical		Temperate to tropical	Tropical and subtropical
Trophic environment	Medium nutrients (mesotrophic)	Low nutrients (oligotrophic)	High nutrients (eutrophic)		Medium nutrients (mesotrophic)	Low nutrients (oligotrophic)
Habitat	Streams and lakes	Mangroves, coral reefs	Mangroves	Mangroves, coral reefs, seagrass beds	Shallow to deep hard substrates	Coral reefs
Geographic area	Circumglobal	Shallow seas in the intertropical belt	Caribbean and Gulf of Mexico		Atlantic	Shallow seas in the intertropical belt
Life cycle and propagation						
Full/closed life cycle in lab (*ex situ*)	Yes	No	Yes		Yes	Yes
Mode of clonal propagation	Budding	Pedal laceration	Budding	Regeneration	Fragmentation	Budding and fragmentation
Time to maturity or (sexual) generation time	Wks	NA	Mos		Mos	Yrs
Rearing and maintenance in artificial settings	Easy	Easy	Easy	Moderate	Moderate/demanding	Demanding
Algal symbiont						
Algal symbiont: identity	*Chlorella*	*Symbiodiniaceae*	*Symbiodiniaceae*		*Symbiodiniaceae*	*Symbiodiniaceae*
Algal symbiont: culturable	No	Yes	Yes		Yes	Yes
Needs algal symbiont to complete development	No	NA	Yes		No	Yes
Obligate symbiosis at adult stage	No	Yes	Yes		No	Yes
Survival in aposymbiotic state	Yrs	Indefinitely	Indefinitely	>3 wks	Indefinitely	Wks
Algal symbiont acquisition	Mixed	Horizontal (env)	Horizontal (env)		Horizontal (env)	Depends on species

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TABLE 2 (Continued)

Feature	Observation(s)					
	Hydra	Aiptasia	Cassiopea (polyp)	Cassiopea (medusa)	Astrangia	Tropical scleractinians
Practical aspects						
Consortium	OpenHydra	Aiptasia Symbiosis Resource	CassiopeaBase		Temperate Coral Research Working Group	No
Online open access resources	Hydra 2.0 Genome Project Portal	protocols.io, Reefgenomics	Medina Lab		Coral Microbiome Portal	Reefgenomics, SymPortal
Established strain(s)	Yes	CC7, H2, EM5, JK, JKA2, VW9, VWA12, PLF3, PLF5, and PLF8	12 strains: T1–A, B, C, D, E, and F and T2–A, B, C, D, E, and F		No	No
Genome(s) available	Assembled	3	Casxa1		Draft	>45 (05.10.2022)
Cites regulation	No	No	No		No	Appendix II

[a]This summary can help evaluate the suitability of a system of interest based on similarities and dissimilarities with tropical scleractinian corals. NA, not applicable; mos, months; wks, weeks; yrs, years.

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over time in Aiptasia provides a powerful analog to coral bleaching responses (e.g., references 125, 127, and 128). However, for rendering animals aposymbiotic for use in experiments on symbiosis reestablishment, menthol bleaching is the most rapid and the most effective at eliminating essentially all symbionts from host tissues (126).

Depending on host origin, Aiptasia harbors different *Symbiodiniaceae* species. Most laboratory animals contain *Breviolum minutum* (including host strain H2), or *Symbiodinium linuchae* (including host strain CC7). Both of these symbiont species have been successfully brought into culture and the genomes sequenced (129, 130). In addition, Aiptasia is tolerant of a variety of non-native symbiont species, including *Symbiodinium microadriaticum* and *Durusdinium trenchii*, two species with differing susceptibility to environmental perturbation (131–134). These species can enter hosts but are less successful than native species at populating them, and the metabolic exchange and interpartner homeostasis is perturbed (122, 135, 136). Similar to polyps, Aiptasia larvae can take up a variety of algal species and subsequent persistence and proliferation appears to depend on the ability of the microalgae to suppress host innate immune response and escape expulsion (vomocytosis) (137). This diversity in specificity allows for the study of comparative *Symbiodiniaceae* repopulation dynamics, mechanisms of recognition, specificity and regulation, and differential susceptibility to heat stress or other perturbation (e.g., see references 133 and 138–140).

The Aiptasia microbiome has now been described in a variety of strains from around the world (141–144). Overall, there is congruence between the different studies, showing a similar microbiome makeup among animals in culture and similarity in the taxonomic diversity of microbiomes between Aiptasia and corals. These baseline descriptions of the Aiptasia microbiome set the stage for future studies on the effect of heat stress and other environmental perturbation on the Aiptasia holobiont, the interaction of the algal symbionts with the microbiome, and the possibility of manipulating the microbiome to aid in building coral resilience. Indeed, some of this work has already begun (113, 145).

The life history characteristics of Aiptasia contain both key strengths, and at present, significant limitations to its value as a model system. For example, asexual reproduction (pedal laceration) facilitates clonal propagation, but sexual reproduction has not yet been achieved in captivity. Aiptasia is gonochoristic, although there is some evidence that animals can switch sex (146). Animals spawn non-symbiotic gametes and therefore onset of symbiosis must occur anew with each host generation. Further, researchers have now developed culturing conditions that result in predictable and repeated spawning of gametes, successful fertilization and subsequent rearing of F1 larvae (147–150). These larvae can establish symbiosis with algae from culture, which again provides a powerful system to examine mechanisms of recognition and specificity. To date however, despite considerable effort by several research groups, there has been no success in achieving larval settlement and metamorphosis into juvenile polyps (unpublished data). This presents a major barrier to conventional genetics, gene editing, or other gene knockdown techniques in Aiptasia (151) that would revolutionize our ability to discern host gene function in the symbiosis.

There are other aspects of Aiptasia biology that warrant further development to cement this organism as a key model. The host processes of pedal laceration and subsequent patterning that results in development of clonal juveniles has been described (152–154). However, the role of symbiosis in these processes is just beginning to be described (155) and is a topic ripe for future work. Finally, although we believe animals harboring *B. minutum* occur pan-tropically, whereas those containing *S. linuchae* appear restricted to the Florida Keys (156, 157), there is a lack of global sampling studies describing natural symbiosis states. Such surveys could be further expanded to include other symbiotic anemone species within the Aiptasiidae. This would extend the relevance of the model system into a comparative genomic and ecological framework and explore conservation in the molecular evolution of marine endosymbioses.

Jellyfish Cassiopea

The Upside-down Jellyfish *Cassiopea xamachana* (Bigelow, 1892) has been a powerful model to study developmental symbiosis for more than four decades (reviewed in

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reference 158). In recent years, the "Cassiopea" model has gained increasing attention as a system to study cnidarian symbiosis (78, 158), with a complete genome now available (159, 160). All nine species in the genus are found in tropical and subtropical waters around the world, although the distribution range of *C. xamachana* is limited to the Caribbean and Gulf of Mexico (summarized in reference 158), and therefore the adoption of this species as a model organism might need to overcome transport restrictions (as previously discussed for Aiptasia). Like corals, Cassiopea establishes an obligatory association with *Symbiodiniaceae* algae (161, 162) and is therefore employed to study the bleaching response of cnidarian-algal symbiosis to heat stress (163, 164). Cassiopea is suited to laboratory rearing and investigations since it can tolerate a broad range of environmental conditions (163, 165, 166), is noncalcifying, and has a short life cycle that can be completed in the laboratory in four to six months. Embryos can be collected daily from the brooding region of female medusae, a trait which facilitates genetic (e.g., microinjection) and developmental studies (e.g., embryogenesis) (reviewed in reference 158).

The Cassiopea life cycle allows easy access to different life-stages that also differ in their dependence on *Symbiodiniaceae*. Female medusae constantly release brooded swimming larvae that settle and metamorphose into polyps upon encountering microbial settlement cues (161). These polyps (scyphistomae) reproduce by budding, and large clonal aposymbiotic populations can easily be maintained under laboratory conditions with regular feeding as "immortal lines" (158). The establishment of symbiosis with *S. microadriaticum* triggers strobilation, a metamorphic transition into sexual ephyra (i.e., free-living juvenile medusae), termed symbiosis-driven development (167). Interestingly, polyps can establish symbiosis with a broad range of *Symbiodiniaceae* species (168), but only some elicit metamorphosis (169). This system enables developmental, genetic, and physiological comparisons of the onset of symbiosis in the same host genetic background. On the contrary, adult medusae depend on their *Symbiodiniaceae* partner (162, 170) toward which they show a high degree of selectivity and specificity (164, 169, 171), not unlike differences in symbiotic specificity between juvenile and adult corals (172).

These characteristics make Cassiopea particularly suitable for symbiosis manipulations. Both polyps and medusae can be bleached through temperature stress (162, 171) or menthol treatment (170) and, although lack of the algal symbionts eventually leads to death (162), aposymbiotic medusae can survive for more than 3 weeks (170). Comparison between symbiotic states can help unravel each partner's contribution to holobiont functioning, such as nutrient uptake and dynamics, and the effect of *Symbiodiniaceae* presence or absence on the bacterial microbiome (170). Further, aposymbiotic polyps can reestablish symbiosis with native or non-native *Symbiodiniaceae* strains (164, 169, 171). The ability to obtain different polyp-*Symbiodiniaceae* associations will in turn allow the production of clonal polyps that harbor different microbiomes. This, together with efforts to develop axenic and gnotobiotic animals, will also open doors to systematically explore host-microbiome interactions with the Cassiopea model system (158).

Another outstanding feature of Cassiopea is its ability to maintain a functional symbiosis across a wide range of environmental stressors (163, 165, 166). This trait can therefore help identify mechanisms that confer tolerance to changing environmental conditions in reef-building corals (170, 173). For example, Cassiopea can withstand high temperatures, showing onset of bleaching between 37 and 40°C (163). Also, high nutrient loads, that typically destabilize the coral-algal symbiosis and lower their bleaching threshold (174), are well tolerated by Cassiopea (175). Tracking of uptake and translocation of isotopically labeled nutrients suggest that Cassiopea is able to exert control over its algal symbionts' capacity to access N, specifically by restricting nitrate (170, 173), the N species linked to decreased heat tolerance in corals (176).

The Cassiopea system is marked by peculiarities that further distinguish it from stony corals and that contribute to its large environmental tolerance. While corals host *Symbiodiniaceae* in their gastrodermal cells, in Cassiopea these are predominantly located inside amoebocytes (177). Amoebocytes are motile cells found in the

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mesoglea, which can be actively redistributed to meet energetic demands across different body parts (173). In addition, Cassiopea has a greater capacity to enrich its nutrient environment compared to corals (178). Rather than relying on currents to transport particle and solutes, Cassiopea uses bell pulsation to generate flows that draw particles (e.g., zooplankton) from the surrounding seawater to its feeding appendages and mobilize nutrients from the underneath sediments (178). Discoveries made on Cassiopea therefore need to be contextualized considering these aspects.

Temperate Coral *Astrangia poculata*

The temperate coral *Astrangia poculata* (Ellis & Solander, 1786) is one of the very few calcifying cnidarian model organisms currently available. Since this species is more amenable to rearing in aquaria (compared to the majority of tropical scleractinians), it represents an attractive, and increasingly popular, model system. Colonies are easy to collect since they are abundant in coastal, easily accessible locations across the western Atlantic. On average, colonies carry about 20 to 100 polyps, can grow to ~10 cm in diameter, and are gonochoric (carry separate sexes). Spawning is synchronous and inducible in the laboratory throughout the period of late July to early October, mirroring patterns of gametogenesis (179). In addition, *A. poculata* are gaining attention not only as an emerging model system but also as an emblem for coral and climate change research, demonstrated by its designation in 2021 as the Official State Coral of RI, USA.

Above all, two aspects (and their implications) are particularly remarkable about *A. poculata*: the nature of its photosymbiosis and its outstanding thermotolerance. *A. poculata* facultatively engages in symbiosis with the photosymbiont *Breviolum psygmophilum*. Sympatric colonies can be found in different symbiotic states (symbiotic, aposymbiotic, and patchy/mixed) across all seasons (77, 180), and photosymbiont density can be artificially manipulated (increased with high light intensity; decreased with low light intensity) (R. Rotjan, unpublished data). Because tropical corals often cannot be decoupled from *Symbiodiniaceae* without imposing stress, many critical, basal questions regarding this symbiosis are difficult to address in corals directly. Here, the *A. poculata* model can be particularly advantageous. Tracking nitrogen uptake and translocation in both symbiotic and aposymbiotic *A. poculata*, for example, helped elucidate how nutrient availability modulates the coral-algal relationship, specifically suggesting that nitrogen and carbon limitation shift the coral-photosymbiont mutualism toward parasitism (181). Similar to Cassiopea, *A. poculata* can tolerate extreme temperatures—withstanding what is among the largest temperature ranges that any hard coral has been documented to experience in its natural habitat. In the species' northernmost distribution (southern New England), seawater temperature seasonally fluctuates over a range exceeding 20°C, with average temperatures spanning from 4 to 29°C (77). This annual temperature range compares to that of the Persian Gulf, the region with the most extreme environmental conditions where tropical reef-building corals persist (with recorded extremes spanning from ~11 to 36°C [182, 183]). Although the thermal environment of *A. poculata* is much colder than that of coral reefs, this ability to cope with such a large temperature range makes *A. poculata* an excellent experimental system for identification of genes and critical mechanisms of thermal tolerance. For example, comparison of gene expression between symbiotic states of *A. poculata* under thermal stress demonstrated that many stress-response genes previously identified in tropical corals likely belong to the host, as these were also present in aposymbiotic specimens (184), while transcriptional profiles of *Symbiodiniaceae* remain relatively unaffected by heat stress in corals (185). These experiments mirror physiological and metabolic patterns of the coral holobiont under stress (186, 187) and underline the potential to directly transfer insights gained from studying *A. poculata* to tropical reef-building corals. Furthermore, explorations of *A. poculata* in its natural environment across seasons and along latitudinal gradients can be used to test the influence of symbiosis and seasonality on microbe-microbe interactions within the holobiont. Across the year, *A. poculata* experiences shifts in photosymbiont density similar to those described for stony corals (188, 189), and the onset of a state of cold-induced

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quiescence (dormancy) during the winter months (190–192). Recent research efforts have utilized individuals from these naturally occurring gradients to identify microbes and multipartner (*Symbiodiniaceae*-bacterial) interactions important in the cnidarian response to environmental changes. Recent work on wild *A. poculata* colonies showed that the influence of photosymbiont density on the taxonomic structure and activity of the bacterial and archaeal community was smaller than that of seasonality (77). These findings largely agree with the stable bacterial communities found in cold shock bleached Aiptasia (141) and heat-stress bleached *Porites lobata* and *Pocillopora acuta* corals (193, 194) and support the generalization that external (environmental) factors have a stronger effect on microbiome structuring than photosymbiont density alone. Interestingly however, the presence of *B. psygmophilum* appears to facilitate consistent recovery of the bacterial and archaeal communities in *A. poculata* after antibiotics treatment (195). In these studies, the *A. poculata* bacterial community shares similarities in taxonomic structure with those of tropical corals but is remarkably less species-rich and more predictable (51, 77). As next steps, development of protocols is under way for spawning, embryonic development, larval rearing, larval settlement, and post-settlement growth to enable experimental examination of processes governing multipartner symbioses, including symbiont recruitment, establishment, and succession.

Increasing the Power of Cnidarian Model Systems

The traits that make the discussed cnidarian model organisms convenient study systems (and ecologically successful species) also set them apart from tropical reef-building corals. For example, features that greatly facilitate experimental investigation such as the lack of a carbonate skeleton, facultative photosymbiosis, and broad environmental tolerance (or "hardiness" of a species) have relevant physiological implications, and ignoring them might leave important biological mechanisms unaddressed. Therefore, to be informative for coral reef conservation, discoveries made from model systems should not be viewed as standalones but contextualized within a broader framework. To increase the power of these experimental model organisms, it is therefore necessary to adopt combined research approaches that use multiple models chosen for the complementarity of their features and that rely on multi-institutional collaborations (see "A Trait-Based Approach To Identify Suitable Coral Species" below and Table 2 for a summary of similarities and dissimilarities between the discussed organisms).

DIRECT TESTING AND EXPERIMENTATION ON CORALS THROUGH HOLOBIONT SIMPLIFICATION

Tissue Cultures as Structural Simplification

Structural simplifications offered by tissue cultures and cell lines allow for direct miniaturization of tropical stony coral systems. They eliminate skeletal components, which increases optical transparency aiding visualization, and liberates sample processing from the interferences of the aragonite particles and Ca^{2+} ions. As in the examples of the cnidarian model systems, a small sample size here aids high replication, translates into faster and less expensive workflows, and uses available live material efficiently.

The first step of structural simplification of the coral host involves the isolation or explantation of tissues or cells and maintenance of these as "primary cultures" or "tissue explants." This can be undertaken in a number of ways (Table 3). So-called "destructive approaches" affect tissue organization by breaking down cell-cell or cell-ECM (extracellular matrix) adhesion, through the removal of divalent cations (Ca^{2+}, Mg^{2+}), enzymatic digestion of ECM components, and single-cell isolation through gravimetric fractionation or sieving of digested tissues (reviewed in reference 196). Isolated coral cells then reaggregate into multicellular structures. Nondestructive approaches preserve the original tissue organization and can be achieved mechanically by cutting (197, 198) or peeling off coral tissues (199, 200), or physiologically by inducing a stress response mechanism called polyp bail-out (201–203). Destructive approaches remain prone to microbial contamination that hinders long-term survival (196) (Table 3).

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TABLE 3 Chronological overview of studies on coral cell and tissue cultures with synthesis of culture origin and type, viability, and use of antimicrobials

Tropical scleractinian coral species group(s)	Other cnidarian species	Life stage	Suspended and/or adherent[a]	Viability[b]	Proliferation[c]	Isolation approach[d]	Antibiotic and/or antimycotic[e]	Year of publication	Reference(s)
Fungia scutaria, Pocillopora damicornis	*Boloceroides sp., Cassiopea xamachana, Exaiptasia diaphana (formerly Aiptasia pulchella), Zoanthus sociatus*	Adult	S	5 h	NO	Des		1992	252
Acropora microphthalma, Montipora digitata, Pocillopora damicornis, Porites sp., Seriatopora hystrix, Stylophora pistillata		Adult	S	12 days	NO	Des	AB, AM	1999	204
Pocillopora damicornis		Adult	A, S	7 to 12 days	NO	Des	AB, AM	2001	207
Pocillopora damicornis, Stylophora pistillata		Adult	A	7 days	NO	Des	AB, AM	2004	202, 211
Montipora digitata	*Xenia elongata*	Adult	A	21 days	NO	Des	AB, AM	2007	208
Acropora millepora		Planula	S	10 wks	CP	Des	AB, AM	2009	214
Fungia sp.,Pavona divaricata		Adult	S	4 days	NO	Des		2009	205
Pocillopora damicornis		Adult	S	9 days to 2 mos	NO	Des		2010	325
Favia favus, Fungia granulosa	*Oculina patagonica*	Adult	A, S	3 mos to >3 yrs	After	NDes		2011	199
Stylophora pistillata		Adult	A	6 to 8 wks	NA	Des	AB, AM	2012	209
Pocillopora damicornis		Adult	S	>2 days	NS	Des	AB, AM	2013	206
Pocillopora damicornis		Adult	S	5 to 11 days	NA	NDes		2014	197
Fungia granulosa		Adult	S	>2 mos	NA	NDes	AB	2015	200
Pocillopora damicornis, Seriatopora hystrix, Stylophora pistillata		Adult	A, S	Wks to mos	After	NDes		2016	203
Stylophora pistillata		Adult	A	>12 days	NA	Des	AB, AM	2017	210
Goniopora lobata		Adult	S	NA	NA	NDes		2017	198
Pocillopora damicornis	*Nematostella vectensis*	Adult	S	12 to 13 days	Reported	Des	AB	2021	216
Pocillopora acuta		Adult	S	7 to 10 days	NO	Des	AB, AM	2021	213
Acropora tenuis		Planula	S	>8 mos	CP	Des	AB, AM	2021	212

[a]S, suspended; A, adherent.
[b]wks, weeks; mos, months; yrs, years.
[c]NA, not applicable; CP, clear proliferation; NS, not significant; NO, not observed; After, after settlement and differentiation; Reported, reported but time limited.
[d]Des, destructive; NDes, nondestructive.
[e]AB, antibiotic; AM, antimycotic.

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Nondestructive approaches only require minimal treatment to control contamination and have, on average, longer viability (Table 3).

Coral tissue explants can be maintained in either suspended (204–206) or adherent cultures (202, 207–209). Suspended aggregates are ball-shaped and present a tissue organization similar to coral colonies (204–206). These also maintain a photophysiology comparable to that of the parental colony (200). They have been used to investigate the involvement of light and oxidative stress in coral bleaching (205) and to study ecotoxicology of cryoprotectants (197). Interestingly, the method developed by Vizel et al. (199) produced explants that can be kept for several months in an undeveloped state or induced to develop into a polyp that calcifies and ultimately regrows into a colony. In addition, *Symbiodiniaceae*-free tissue balls potentially provide a tool to complement investigation on the coral-algal symbiosis (198).

Adherent aggregates are generally flat, but spatial relationships between the diverse cell types are not well resolved between isolation protocols (202, 207–209). Only these adherent aggregates (as opposed to tissue balls) calcify *in vitro*, likely owing to the presence of the ECM and skeletal organic matrix which are secreted *de novo* (207, 208). The easy access to the site of calcification in adherent cultures has allowed researchers to reveal important details of the calcification process, such as its conditional independence from photosynthetic activity of the *Symbiodiniaceae* partner (209), the intracellular commencement of the biomineralization process (209), and its dependence on multicellularity (e.g., see references 207, 208, and 210). The use of adherent cultures also produced the first evidence of interactions between coral cells and an associated endolithic fungus (211). Although it appears to be limited to pocilloporid corals, polyp bail-out induced through controlled salinity stress is arguably the most successful approach for obtaining adherent coral micropropagates (201, 203). The resulting micropropagated polyps fit inside a microfluidic platform, the "coral-on-a-chip", representing one of the most advanced tools for live study of coral physiology (203). This made it possible to observe microscopic phenomena in real time, such as calcification, coral-pathogen interactions, and coral bleaching (203).

Untapped Potential of Coral Cell Cultures

The next stage in structural simplification of the coral host can be achieved with secondary cultures and cell lines (cultures that can be propagated indefinitely). These are less-representative versions of the original biological system because they filter out some cell types, but they overcome the time limitation of primary cultures and the need to continuously source from living organisms. To date, most coral-derived cell cultures have only short viability and coral cell lines have been established only very recently (212, 213) (Table 3). The most remarkable achievements in terms of culture longevity rely on cells originating from larvae (212, 214, 215). This approach resulted in mixed cell cultures visibly proliferating and remaining viable for between 10 weeks (214) and upwards of 8 months, with the possibility of restarting the culture after cryopreservation and rewarming (212, 215). To this point proliferating cultures either contained mixed, often unidentified, cell types (214, 216), or homogeneous, but not *a priori* specifically selected for, cell populations (212). Nevertheless, cell lines with specific properties could be selected *a posteriori* from the pool of available established cell lines, each of which reportedly expressed specific and consistent sets of genes (over time) reminiscent of different cell types (e.g., gastrodermis, epidermis, and secretory, undifferentiated, and neuronal cells) (212). This was demonstrated (in principle) after cells from a line showing endoderm-like properties successfully established endosymbiosis (*in vitro*) when exposed to cultured *Symbiodiniaceae* (215).

Among the various cell types, stem-cell like cells are particularly sought after because of their potential to initiate persistent cell lines. Currently all immortal animal cell lines originate from tumors or from experimentally reprogrammed cells (217). These behave substantially differently than physiological cell populations (218). For that matter, tropical corals (and other cnidarians) are an attractive subject for cell cultures, owing to their longevity (219) and regenerative capacity, which suggests that stem-cell like cell populations remain abundant throughout the life of these organisms (220). While adult somatic cells of hydrozoans have been described to dedifferentiate (i.e., return to a pluri- or totipotent state) and

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transdifferentiate (i.e., differentiate into different cell types) (220–222), a mechanistic understanding of coral self-renewal is still lacking (223). Stem-cell like cell lineages have been identified and characterized in cnidarians (class Hydrozoa) as interstitial cell lines (or I cells) (220, 224). In corals (class Anthozoa), protease-treated larval-derived cells assumed amorphous shapes with extended pseudopodia capable of proliferating *in vitro* indefinitely. These appeared morphologically similar to endoderm precursor cells (212) and resemble amoebocytes, which are hypothesized to play a similar role to I cells in non-hydrozoan cnidarians (223). Gene expression patterns, enzymatic activity assays such as applied to human hematopoietic stem cells based on aldehyde dehydrogenase, and fluorescence-activated cell sorting (FACS) could help locate stem-cell like cells in corals (225, 226), and some coral cell lines have indeed shown properties of progenitor cells (212). However, even single-cell RNA sequencing of FACS-sorted cells from swimming larvae, primary polyps, and adult colonies of *Stylophora pistillata* could not identify any stem-cell like cell populations (227). The cellular basis underlying coral regenerative capacity thus remains elusive.

Another limitation in establishing coral cell cultures is the lack of coral-specific culturing formulations (213). The culture media used presently seem to favor growth of contaminants rather than of coral cells, and dilution leads to better results (196). In addition, for many coral cell types, proliferation likely requires initial adhesion to a substrate (196, 213). Therefore, lack of knowledge about the structure and function of regenerative systems and of appropriate culturing conditions hinder the culturability of coral tissues and cells (196, 213). This field might benefit from a change of perspective that focuses on the peculiarity of corals rather than on their similarity with terrestrial metazoans. Culturing techniques could be improved through reverse-engineered approaches that use (meta)genomic, transcriptomic, and proteomic information to guide the design of culturing media and protocols, as pioneered in bacterial culturing (228, 229).

Simplifying the Coral Holobiont by Disassembling Its Members

In the intact coral holobiont, the interdependence and complementarity of processes underlying fundamental functions hamper the understanding of contributions of individual members (80). Hence, as a complementary strategy to structural simplification, the complexity of the coral holobiont can also be simplified by disassembling and isolating its members (host and microorganisms, respectively). Such approaches can guide studies aimed at clarifying partner dynamics, and generate predictions that will then ultimately need validation through the controlled reassembly of metaorganism components (80, 100).

Possibility of gnotobiotic coral hosts. Axenic (germ-free) or gnotobiotic hosts (where all associated microbes are known), are powerful tools to study the role of the microbiome in health and fitness (as reviewed in reference 80). Comparison between symbiotic and axenic individuals allows us to explore the contribution of the microbiome to host physiology, and targeted inoculation with selected microorganisms can help identify causative links to their function (100, 101, 105). By combining established *Symbiodiniaceae* and bacterial depletion protocols, the generation of gnotobiotic coral hosts can be broken down into sequential steps.

The removal of *Symbiodiniaceae* from the gastrodermal tissue by bleaching produces aposymbiotic hosts and can be seen as a first step in holobiont simplification. Ideal methods maximize bleaching efficacy while they minimize the impact on the host and the remaining microbiota. Temperature stress was among the first methods utilized to bleach corals in artificial systems (27); however, it may result in high host mortality, not be fully effective, and potentially influence thermotolerance in subsequent studies (126, 230). The herbicide Diuron or DCMU [*N*′-(3,4-dichlorophenyl,-*N*,*N*-dimethylurea)] overcomes most of these limitations, but it also does not lead to complete bleaching and it is a hazardous substance (231, 232). Menthol is considered a more "gentle," yet effective, bleaching agent that has now been applied to many cnidarians (170, 232, 233). However, the exact mechanism triggering the bleaching response remains to be fully elucidated (232, 234). Menthol-bleached hosts remain aposymbiotic (for at least 15 weeks, in Aiptasia) after the cessation

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of the treatment (126) and can subsequently be employed in experiments and reestablish symbiosis with *Symbiodiniaceae* (126, 232). Of note, while a fully aposymbiotic state can be difficult to achieve (and prove), removing ~98% of the symbiont population appears sufficient to allow inoculated non-native symbionts to establish and repopulate the host (235). How these chemical bleaching agents affect other microorganisms in the coral holobiont, however, remains unknown.

Completing a fully sterile life cycle is the gold standard of developing true axenic animals (236). Similarly, protocols for creating axenic Hydra polyps make use of sterile rearing techniques and its closed life cycle in the laboratory (112), a prospect that may become attainable in research on corals given advancements in artificially producing larvae from brooding species (237, 238) and *ex situ* techniques for spawning coral species (239, 240). Among cnidarians, Hydra, Nematostella, and Aiptasia adult polyps can be rendered gnotobiotic through antimicrobial treatment (100, 113, 241). This represents a complementary or alternative approach, which has been successful in eliminating more than 99% of the microbial load of corals (242), when a closed life cycle is not (yet) available (100, 236, 241). However, microbial load can recover in as little as 96 h once dosing stops (242). Although no protocol for long-term maintenance of gnotobiotic corals after antimicrobial treatment is available yet, it could be argued that organisms that are allowed to develop naturally (i.e., with their native microbiome), and only later undergo microbial depletion, represent more realistic models of wild-type organisms (100, 236).

The efficacy of the holobiont disassembly process will vary between coral life histories. First, coral species have different strategies of acquiring their microbiome and vary in microbiome flexibility at early life stages (243) and as adults (33). The main division occurs between broadcast spawners and brooders (244), with the former acquiring *Symbiodiniaceae* and likely bacteria horizontally from the environment (244, 245), while the latter mostly inherit them vertically from the parental colony (172). Second, in contrast to axenic mice and Hydra, raising axenic corals may be challenging as it remains unclear whether corals require the presence of specific microorganisms to complete development (246). Finally, antimicrobial treatment might be detrimental as it was shown to cause disaggregation of tissue in coral larvae (214) and adults in some cases (216). Since this might be a feature of the specific antimicrobials employed, testing substances with different mechanisms of action (e.g., azoles) is warranted. Nevertheless, antimicrobial treatment may not be effective in the long-term due to difficulties of antibiotics reaching the inner skeleton (21, 242). Early-life stages after settlement may therefore be particularly suitable for manipulation, as they have more dynamic microbial communities than do adult corals (45, 243).

***Symbiodiniaceae* cultures.** *Symbiodiniaceae* cultures represent the first established and most advanced cell culturing technique in coral research. *Symbiodiniaceae* are routinely studied for their properties in culture (247, 248) and are used to study early symbiont acquisition by larvae, symbiosis establishment and reestablishment (e.g., following bleaching) dynamics, and comparative physiology in the host (133, 149, 169). While historically only a small proportion of coral-associated *Symbiodiniaceae* have been considered culturable (249), new approaches such as isolation and culturing from single cells promise innovation in the field (250).

Symbiodiniaceae in culture experience substantially different conditions compared to those *in hospite* (within the host) and therefore do not perfectly replicate endosymbiotic dynamics (215, 251, 252). Many cultures utilize antibiotics to keep bacterial contamination to a minimum, but such practices could affect *Symbiodiniaceae*, either through side effects of the antibiotics or through the induced loss of their bacterial associates (253, 254).

Bacterial cultures. The majority of our knowledge on coral-associated bacteria is based on 16S rRNA gene amplicon sequencing, while metabolic pathways and interactions in the holobiont are less well-explored (55). The use of culture-based approaches may be one option which will likely provide additional insight into microbial functions (e.g., reference 255), but which has largely been forgotten about in favor of next generation culture-independent methods. Sweet et al. (51) recently curated data of the

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diversity and function of cultured bacteria (both published and unpublished) from tropical, temperate, and cold-water corals. This resulted in a catalog (isolates.reefgenomics.org) of 3,055 unique isolates that spanned 138 species and 12 putatively novel bacterial genera across the *Pseudomonadota* (*Proteobacteria*), *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla. Available genomes from these bacteria were considerably sparse, but those available (74 at the time of writing) allowed the researchers to analyze biosynthetic gene clusters underlying the production of secondary metabolites important in host health and symbiosis (51, 256). Despite this promising start, most bacteria have yet to be cultured, and some have even been deemed unculturable (257). Indeed, a metadata analysis of SSU rRNA gene sequences from bacteria and archaea associated with corals found that only 6.5% of these were generated from cultured isolates (258). Unlocking at least part of this additional diversity is likely to be achieved with alternative isolation and cultivation procedures, inspired by advancements in the broader microbiological field (259, 260). For example, the gradients of physicochemical growth conditions could be widened within the 'culturomics' framework (238), and implementing microfluidics systems (261–263). The growth of obligate symbiotic or syntrophic bacteria could be achieved through co-culturing (264), and growth media and sorting methods could be developed through omics-guided approaches (228, 229).

Microbial contaminants in cultures: friends or foes? Invasion of cell cultures by microbial contaminants is a universal issue. Antimicrobial agents routinely employed in terrestrial animal cell cultures are largely ineffective against coral microbial contaminants, which are known to overgrow and cause the termination of coral cell cultures (200, 214, 216, 265). While there is clearly a need for coral-specific antimicrobial treatments, corals are exceptional in hosting microbes not only on external surfaces and mucosa but also in all other tissue compartments and in the skeleton (21, 53). In addition, although cultured *Symbiodiniaceae* strains are often treated with antimicrobials (249) and can in some cases be maintained axenically (118), *Symbiodiniaceae* cultures also harbor abundant and characteristic bacterial microbiomes (266). Recently, bacteria have also been reported to associate with *Symbiodiniaceae* intracellularly as well as extracellularly (267). This suggests a tight involvement of the coral microbiome in holobiont functioning and regulation. Indeed, the longest viability of coral explants was achieved from protocols that did not use antimicrobials (199, 203) (Table 3). In contrast, the first coral cell lines were grown in media containing antimicrobials (212, 215). This indicates a potential connection between the presence of associated bacteria and the formation of complex structures (tissues), and represents an incentive to investigate whether so-called microbial contaminants might comprise key coral associates.

Reassembling the Metaorganism for Hypothesis Testing

The study of isolated holobiont members is necessary to generate hypotheses on their functions and the dynamics of their interactions (100, 241); however, these hypotheses then need to be tested in a metaorganism context (80). For this purpose, reassembling metaorganisms represents the ultimate testing ground. Practically speaking, the inoculation of axenic or gnotobiotic hosts with cultured or "transplanted" (46, 268) microbial isolates will elucidate the intra- and interkingdom interactions underpinning holobiont functioning. This approach borrows from the field of human gut microbiome, where studies on animal models could demonstrate causative links between the presence of specific bacteria and the host phenotype. For example, the introduction of a single gut-residing bacteria in axenic mice led to the development of autoimmune arthritis (269) and the presence or absence of a bacterial consortium modulated food allergy in the host (270). More recently, several authors have proposed to adopt this type of approach based on success in other cnidarian models (100, 105, 110, 113, 241, 271, 272) and on promising first applications in some reef-building coral species (46).

TROPICAL STONY CORALS AS CANDIDATE MODEL SPECIES

The validation of laboratory results from model systems on true corals remains irreplaceable in the transition from controlled experiments to practical implementation of

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conservation activities. Non-coral model organisms lack important features such as the aragonite skeleton, obligate nature of the symbiosis with *Symbiodiniaceae*, and adaptation to oligotrophic conditions. Although the term "coral model" is found in the literature and attributed to a number of species (see reference 213 and references therein), to date there is no formally established or universally agreed-upon true coral model organism. Establishment of such models should start by identifying a group of promising species which possess characteristics that maximize amenability to experimentation (tractability) and informative power (transferability of knowledge to ecologically relevant contexts). Because tractability and transferability often can be antithetic, we discuss the most relevant factors that affect these two properties in selected candidate coral model species (see, e.g., Table 4).

Trait-Based Approach To Identify Suitable Coral Species

Tractability. Tractability is the sum of a set of characteristics that make a coral species a practically more convenient study subject (Table 4). These characteristics include: (i) broad availability in geographic distribution and abundance in the reef (however, this aspect may only be relevant in the initial exploratory stage: once the species becomes established as a model organism, research will focus on a few selected strains/clonal lines shared between research groups, overcoming the need to source colonies from the wild); (ii) compatibility with growth under aquarium conditions to maintain long-term cultures or living collections (this includes for example colonies of *Stylophora pistillata* that have been kept in aquarium culture for more than 3 decades) (273); (iii) amenability to bleaching, maintenance in a bleached state, and symbiosis reestablishment to disentangle host and *Symbiodiniaceae* contribution to holobiont functioning (85); and (iv) larger polyps (e.g., in *Galaxea fascicularis*) offer the option to isolate a single polyp, reducing complexity from the colony to the individual level (274, 275). Corals with larger polyps are also easier to visually examine (276, 277), not to mention separating the tissue from the skeletal matrix (197–200). (v) Finally, further case-specific advantageous traits could also include the continuous release of larvae (237, 238) with the long-term prospect of achieving a closed axenic life cycle or a consistent budding and polyp bailout response to reliably produce tissue cultures (203, 278, 279). Some of these traits are correlated and tend to co-occur. For example, survival in the bleached state is linked to polyp size because it generally correlates with heterotrophic feeding capacity (280).

Transferability. Coral model species with high tractability will be representative of only a subsample of the scleractinian taxon. Therefore, their representativeness and transferability should be considered targeting a diverse suite of model systems. We suggest identifying coral model species that aim at collectively covering the broadest range possible of the following aspects (Table 4): (i) geographic distribution, where at both larger and smaller scales, different regions are dominated by different species of both coral host and associated *Symbiodiniaceae* (15, 281), and (ii) phylogeny, where metabolism and microbiome composition differ between the two major coral clades (282, 283) and thermal tolerance varies between coral families (284). Of the hundreds of extant tropical reef-building coral species, only a few have been extensively investigated, with the majority of studies focusing on members of just a few families (29). Although this might seem like an underrepresentation, it is noteworthy that the three most abundant families (Acroporidae, Pocilloporidae, and Poritidae) make up ~70% of corals on the coral reefs worldwide (see reference 285 and references therein). In addition, *Symbiodiniaceae* identity should also be considered, since it is known to affect metabolism and thermotolerance (286, 287). The following aspects should also be taken into account: (iii) colony morphology, which plays an important role in thermotolerance (284, 288) and is influenced by external conditions (289); (iv) trophic strategy, which can range from more autotrophic to more heterotrophic and also affects holobiont thermal tolerance (280); (v) habitat preference, which determines the exposure and acclimatization potential to stressors (290, 291) and can vary with depth, distance from shore, and reef type and topography, among others; (vi) reproductive mode,

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TABLE 4 Relevant features to identify and evaluate the suitability of candidate coral species to the establishment of true coral model organisms[a]

	Observation(s)[b]				
Characteristics	***Pocillopora damicornis***	***Stylophora pistillata***	***Acropora millepora***	***Galaxea fascicularis***	***Orbicella faveolata***
Tractability					
Distribution (% of global ecoregions according to CotW)	Very broad (85.3)	Broad (68.7)	Broad (56.7)	Broad (67.3)	Localized (5.3)
Suitability to aquarium rearing	High, aquarium culture >10 yrs (326)	High, aquarium culture >30 yrs (273)	High, completed life cycle *ex situ* (240)	High (327, 328)	High (329)
Amenability to experimental bleaching	Unknown	High, effective with menthol (232)	Unknown	High, effective with menthol (328)	Unknown
Polyp size range in mm (corallite diam)	0.8–1 (WoRMS, CTD)	0.9–1.4 (CTD)	0.4–1.6 (CTD)	5–10 (CotW)	2–3 (CotW)
Others	Polyp-bailout stress response (203, 330, 331)	Polyp-bailout stress response (203)	First broadcast spawning coral to produce F2 fully *ex situ* (240)	Protruding corallites allow easy isolation of individual polyps; polyps remain extended during daytime; large egg size (332)	Major reef builder in the Caribbean (333)
Transferability					
Natural occurrence: oceanic basin	IndoPacific (CotW)	IndoPacific (CotW)	IndoPacific (CotW)	IndoPacific (CotW)	Atlantic (CotW)
Taxonomy: host clade	Robusta (334)	Robusta (335)	Complexa (334)	Complexa (334)	Robusta (336)
Taxonomy: host family	Pocilloporidae (WoRMS)	Pocilloporidae (WoRMS)	Acroporidae (WoRMS)	Euphylliidae (WoRMS)	Merulinidae (WoRMS)
Taxonomy: *Symbiodiniaceae* genus	*Cladocopium* spp. most common, *Symbidinium* and *Durusdinium* spp. also found (CTD)	*Cladocopium* spp. (CTD)	*Cladocopium* spp. (CTD)	*Cladocopium* and/or *Durusdinium* spp. (337–341)	*Brevioulum* spp. predominant; *Symbidinium*, *Cladocopium*, and *Durusdinium* spp. also found (CTD)
Colony morphology	Branched, usually <30 cm tall (WoRMS)	Branching to submassive (CofW)	Corymbose cushions or clumps (CotW)	Massive (often dome-shaped) or columnar (CotW)	Massive, sizes up to 10 m (CotW)
Trophic strategy			Relatively autotrophic (280)	Mixotrophic but with great variability (342)	
Habitat preference depth	0 to >40 m (particularly abundant at 5 to 20 m) (WoRMS)	1 to 65 m (343)	2 to 30 m (CTD) (344)	2 to 20 m (CotW, WoRMS)	0.5 to 40 m (CTD)
Reproductive mode	Brooder (predominantly) and broadcast spawner (244)	Brooder (peculiarity: protandrous simultaneous hermaphrodites) (345)	Broadcast spawner (244, 346)	Broadcast spawner (peculiarity: pseudogynodioecious) (347)	Broadcast spawner (348)

[a]Tractability refers to traits that facilitate experimental work, while transferability indicates the most relevant aspects to consider to address the broad variation encompassed by the scleractinian taxon. This list can be considered a template or guide to be applied beyond the species listed here.

[b]Abbreviations: CotW, Corals of the World (www.coralsoftheworld.org); CTD, Coral Trait Database (https://www.coraltraits.org/); WoRMS, World Register of Marine Species (https://www.marinespecies.org). Reference sources are indicated parenthetically where applicable.

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which determines the mode of microbiome transmission between generations and affects the stability of the microbiome and the potential for transgenerational adaptation (23, 292, 293); and (vii) host-symbiont flexibility, since the ability of the coral host to associate with different *Symbiodiniaceae* strains and species has been linked to adaptive capacity (281, 294). However, to allow for comparisons between host species, a framework for the quantification of this trait is needed. Namely, it is necessary to define a methodology for symbiont characterization together with a metric to quantify flexibility (or specificity), as well as to explore within-species variation through balanced sampling efforts that account for temporal, spatial, and environmental variability (295). Further, this approach could be expanded to the other components of the microbiome (e.g., bacteria [33]).

JOINING FORCES: EXPANDING COMMUNITY-BASED APPROACHES TO CNIDARIAN MODEL ORGANISMS

While it is necessary to establish multiple and diverse model systems (67, 68), strategic focus on a limited number of species will increase efficiency of resource use. A clearly stated or universally agreed-upon selection of "best candidate" species for the establishment of true coral model organisms seems to be lacking; however, the coral field clearly has its favorites. For example, over the last 30 years the three species *Pocillopora damicornis*, *Stylophora pistillata*, and *Acropora millepora* were preferentially used in coral heat stress experiments (29). Although these may be excellent candidates for a true coral model, preexisting knowledge is not *per se* a requirement nor an indicator of the validity of species as model organisms (68), but a widespread appreciation might be a good indicator of their practical advantages that should be taken into account.

Cnidarian model systems can accelerate the rate of discovery necessary to develop solutions for coral reef preservation, and these tools can become particularly powerful when part of a structured framework with defined goals and strategies (10, 76, 80). The idea of a community-based coordinated effort to develop and establish cnidarian model systems was put forward with a "call to arms" (10, 76). The main recommendations included (i) a reframing of researchers' attitude to put collective achievements above individual accomplishments, together with (ii) cooperation between working groups to improve efficiency, through active communication and resource sharing, and (iii) the adoption of a targeted approach that prioritizes the most pressing issues pertinent to corals and coral reef adaptation to future ocean conditions.

Examples that show encouraging progress in this direction can be found among the existing cnidarian model organism working groups and open resource networks. Researchers and educators interested in working with Hydra can find a comprehensive collection of best practices and resources through the OpenHydra (http://openhydra.org/) platform, and the Hydra 2.0 Genome Project Portal (https://research.nhgri.nih.gov/hydra/) provides easy access to the data generated from the Hydra genome sequencing projects. The Aiptasia community has developed a web site (https://aiptasia-resource.org/) and a Protocols.io group (https://www.protocols.io/workspaces/aiptasiasymbiodiniaceae-model-system) for researchers and educators who are interested in using Aiptasia in the laboratory and classroom (Table 2). Current resources for the Cassiopea model include a publicly available draft genome (https://mycocosm.jgi.doe.gov/Casxa1/Casxa1.home.html) and 10 clonal lines available upon request (http://medinalab.org/new/). The genome line T1-A is kept by multiple labs around the world (https://cassiopeabase.org/). The bacterial species, *Pseudoalteromonas* sp., that is an active inducer of settlement is available upon request as well as other *Cassiopea* spp. bacterial isolates from different developmental stages (medinalab.org [A. H. Kerwin et al., unpublished data]). Research on *A. poculata* has accelerated rapidly in recent years, thanks to the development of a large research collaborative focused on the temperate coral genera *Astrangia* and *Oculina*. The Temperate Coral Research Working Group (https://sites.bu.edu/astrangia/), which now exceeds 100 researchers, has met annually since 2016 (with the exception of 2020) and consists of researchers, educators, and journalists who work with *Astrangia* and *Oculina*, all of whom are addressing long-standing questions of coral-microbe symbiosis, as well as climate change education

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and outreach. A draft genome of the host has been assembled and is currently under annotation. *Breviolum psygmophilum*, the intracellular photosymbiont thought to be present in all *A. poculata* populations, is in culture, and draft transcriptomes of *B. psygmophilum* and other congeners exist. 16S rRNA sequences from *A. poculata* specimens from two populations in the United States (Jamestown, RI, and Woods Hole, MA) have been deposited and are publicly available on the Coral Microbiome Portal (https://www2.whoi.edu/site/amy-apprill/coral-microbiome-portal/). Protocols for spawning, embryonic development, larval rearing, larval settlement, and CRISPR protocols have been developed and will be shared so that they are publicly available for researchers. Regarding the establishment of true coral models, however, there is still a general lack of coordination, and we can draw inspiration from the work done on the other cnidarian model systems.

Community-based and coordinated efforts may further include the integrated use of the different model systems, for example, in a validation sequence where first explorations are conducted on more tractable systems and are subsequently validated on systems with higher transferability, improving both speed and efficiency. In this context, the protocols for drug development and approval provide a valuable example of integrated approaches that combine the use of several model systems selected for the complementarity of their features. Similar to preclinical trials in human medical applications, test series are performed on model systems of increasing complexity starting from *in vitro* assays, and proceeding on to animals that possess anatomical and physiological features comparable to humans regarding the effects of a particular drug. Only drugs that pass all of the sequential validations are considered for testing on humans in clinical trials. In analogy, coral-related biological features can first be assayed in tissue or cell cultures and/or non-coral models, to be later corroborated by direct testing on corals (80). This process has already been successfully implemented to explore the involvement of cellular and immunity responses in coral bleaching, by first using Aiptasia and subsequently verifying on stony corals (reviewed in reference 10). Therefore, joining forces and coordinating efforts among groups working on different cnidarian model systems represents a promising approach to accelerate the development of solutions for coral reef conservation.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

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The cnidarian and microbe pictograms were created by Giulia Puntin (licensed under CC BY 4.0, https://github.com/sPuntinG/Coral_stuff) and modified for use here.

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CHAPTER 2

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CHAPTER 2

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CHAPTER 3

Experimental validation of *Galaxea fascicularis* as a model system: laboratory rearing, symbiosis manipulation, and reproduction in captivity

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REPORT

The reef-building coral *Galaxea fascicularis*: a new model system for coral symbiosis research

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Abstract Reef-building corals owe their evolutionary success to their symbiosis with unicellular algae (Symbiodiniaceae). However, increasingly frequent heat waves lead to coral mass-bleaching events and pose a serious threat to the survival of reef ecosystems. Despite significant efforts, a mechanistic understanding of coral–algal symbiosis functioning, what leads to its breakdown and what can prevent it, remains incomplete. The main obstacles are low amenability of corals to experimental handling and, owing to its obligatory nature, the difficulties of manipulating the coral–algal association. Indeed, many studies on the symbiotic partnership are conducted on other cnidarian model organisms and their results may therefore not be fully transferable to tropical reef-building corals. Here, we identify the tropical stony coral species *Galaxea fascicularis* as a novel candidate coral model system. Individual polyps of this species can be separated, enabling highly replicated genotype studies, and are well suited to experimental investigation of the symbiosis as they can be easily and effectively rid of their algal symbionts (bleached). We show that bleached adult individuals can reestablish symbiosis with non-native symbionts, and we report the completion of the gametogenic cycle *ex situ*, with the successful spawning in aquaria over multiple years. These achievements help overcome several of the major limitations to direct research on corals and highlight the potential of *G. fascicularis* as an important new model system for investigations of symbiosis functioning and manipulation.

Keywords Model organisms · Reef-building corals · Menthol bleaching · Symbiosis manipulation · Ex situ sexual reproduction

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Introduction

Reef-building corals form an obligate endosymbiotic association with unicellular algae of the family Symbiodiniaceae. This association is key to their evolutionary success, but it is also at the heart of corals' susceptibility to global climate change, which manifests in coral bleaching—the breakdown of the coral–algal symbiosis (Hoegh-Guldberg 1999). Bleaching is mostly driven by marine heatwaves which are predicted to worsen, causing the loss of virtually all coral reefs by the end of this century (van Hooidonk et al. 2016).

The interaction between heat stress and bleaching has been studied for more than 30 years (Gates et al. 1992; McLachlan et al. 2020), but a detailed understanding of the mechanisms underlying coral bleaching is still missing. Progress in coral symbiosis research is hampered by two main aspects. First, corals are challenging to maintain in aquarium settings as they tolerate only a narrow range of environmental conditions, i.e., bleaching can be triggered by just 1–2 °C increases above summer mean temperatures

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(Glynn and D'Croz 1990). Second, the obligatory nature of the coral–algal association makes it particularly challenging to physically and functionally separate the partners, an approach often necessary to unravel the mechanisms underlying symbiosis breakdown and what could prevent it (Weis et al. 2008; Voolstra 2013).

Model organisms are integral for understanding fundamental biological principles, and symbiotic cnidarians have successfully been used as "coral models," advancing our understanding of coral bleaching and holobiont functioning (Weis et al. 2008). These model organisms share important traits with corals, such as being cnidarians that associate with microalgae, yet they also lack other features that normally represent obstacles to research work such as the calcium carbonate skeleton. In addition, the ability to study the animal host and its algal symbiont in isolation is more easily achieved with facultatively symbiotic organisms such as *Hydra* spp., Aiptasia (*Exaiptasia diaphana*), and *Astrangia poculata* (Dimond and Carrington 2007; Weis et al. 2008; Galliot 2012). Cnidarian model organisms allow the study of basal or shared traits or phenomena at a speed that would not be possible with reef-building corals, but testing on the latter remains necessary for the understanding of coral-specific or ecologically relevant aspects. The obligatory symbiosis with Symbiodiniaceae in reef-building corals has important biological implications (Falkowski et al. 1984; Hoegh-Guldberg 1999), similar to the calcification process that is deeply intertwined with coral physiology and ecology (Gattuso et al. 1999). It is therefore important to establish a "true" tropical reef-building coral model species.

The richness of the scleractinian taxon offers a vast array of species to choose from in the quest for a suitable candidate coral model species. These can be evaluated for their tractability (as amenability to experimental work) considering several aspects which, as proposed by Puntin et al. (2022), should include: (1) pre-existing knowledge: baseline information of the organism's biology is necessary to interpret and contextualize results; (2) compatibility with aquarium rearing: the possibility to maintain the organism and preferably to complete its life cycle in the laboratory is essential to increase its availability, reduce confounding effects such as unknown life history, improve reproducibility, and lower the pressure on threatened wild populations; (3) amenability to symbiosis manipulations is necessary to help unravel complex coral–algal functional interactions and can be broken down into the organism's suitability to be rendered aposymbiotic (bleached), to be maintained aposymbiotic for a certain time, and to then be re-populated with Symbiodiniaceae.

We have identified the coral species *Galaxea fascicularis* (Linnaeus, 1767) (clade: Complexa, family: Euphyllidae) as a promising candidate coral model system. The species is relatively common across the Indo-Pacific region (Veron et al. 2016), where the phylogeny of the genus *Galaxea* is geographically well resolved (Wepfer et al. 2020b). It is also one of the more commonly used species in heat stress experiments (McLachlan et al. 2020) and in studies on coral calcification (e.g., Marshall and Clode 2004; Al-Horani 2005). Importantly, other community resources such as an annotated draft genome are available (Liew et al. 2016; Niu et al. 2016; http://www.gfas.reefgenomics.org/). In addition, *G. fascicularis* is known for its tolerance to stressors as it is "largely unaffected by bleaching" in the field (Marshall and Baird 2000) and for being easy to grow and propagate in aquaria (Pavia and Estacion 2019). Further, its morphology provides additional practical advantages with relatively big polyps, which can be easily isolated (Pavia and Estacion 2019). This conveniently allows reduction in the complexity of the organism from colonial to individual, enables high clonal replication, and facilitates visualization (Al-Horani et al. 2003; Marshall and Clode 2004). Its association with Symbiodiniaceae is relatively well characterized (Huang et al. 2011; Wepfer et al. 2020a). Furthermore, the species' reproductive mode and spawning patterns in the wild are well documented (Babcock et al. 1986; Harrison 1988; Keshavmurthy et al. 2012). However, its amenability to symbiosis manipulation, to experimental handling of bleached individuals, and the ability to complete gametogenic cycles and spawning in closed aquaria have not yet been described.

The overarching aim of this study was to explore the potential of *Galaxea fascicularis* as a coral laboratory model. Specifically, we investigated: (1) the feasibility to render polyps aposymbiotic by eliminating Symbiodiniaceae (bleaching), (2) the amenability to experimental manipulation of symbiotic and aposymbiotic polyps in a simplified system, (3) the re-establishment of the symbiosis with different species of Symbiodiniaceae, and (4) the possibility to induce full gametogenic cycles ex situ, with subsequent spawning under aquarium conditions. These aspects are central to overcoming the two main obstacles to coral research work: low tractability of corals and their complex nature.

Materials and methods

Coral collection and long-term aquarium rearing

Colonies of *Galaxea fascicularis* were collected from three geographical locations: the Red Sea, Hong Kong, and the Great Barrier Reef (Fig. 1). To increase comparability, rearing conditions were adjusted to be similar between laboratories, with few exceptions pertaining to the particular experimental needs or feeding regime, which was based on the long-term rearing arrangements in each facility (Tab. S1). Red Sea colonies were collected at 9–13 m depth at the North-Eastern protected end of the reef "Al Fahal" in

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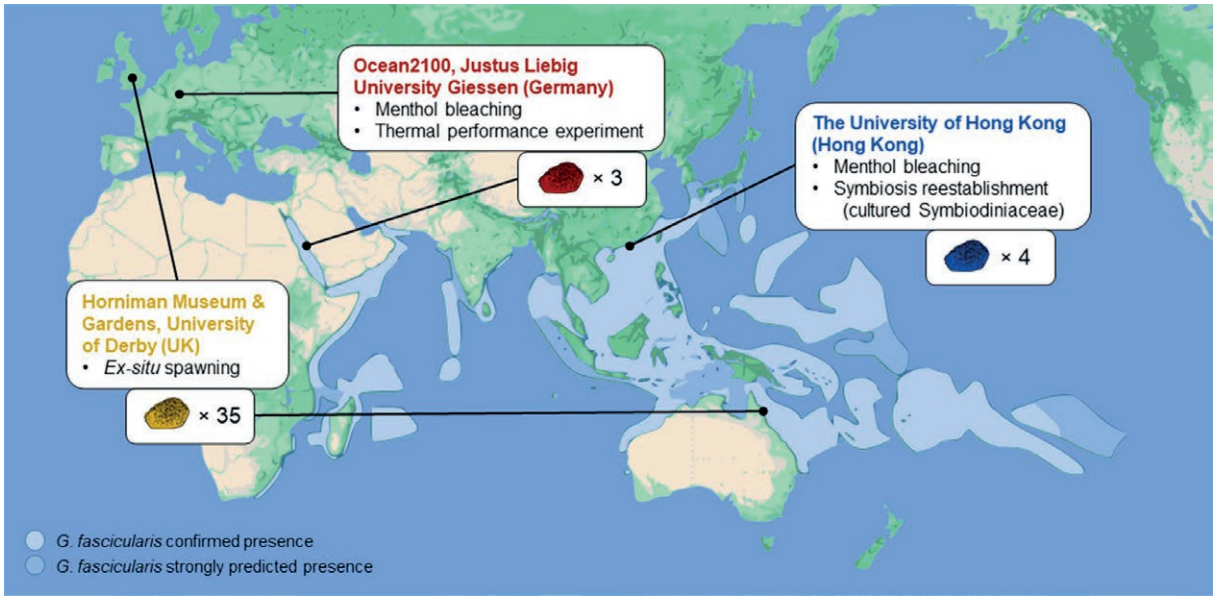

Fig. 1 Summary of *G. fascicularis* coloniy origins, participating laboratories, and experiments described in this study. Colonies were collected from three distant locations (Red Sea, Hong Kong, and the Great Barrier Reef) and separately employed in different experiments (menthol bleaching, thermal performance, symbiosis reestablishment, and ex situ spawning). Map modified from Corals of the World (www.coralsoftheworld.org, Veron et al. (2016))

the central Saudi Arabian Red Sea (22.3054°, 38.9655°) in March 2019 and transported to the Ocean2100 aquarium facility at Justus Liebig University Giessen (Germany) where they are part of the live collection (CITES permit 19-SA-000096-PD). In the aquarium system, light was provided by white and blue fluorescent lamps with a light:dark cycle of 12:12 h at 130 -160 µmol photons m^{-2} s^{-1}, which approximates light condition at the collection site (Ziegler et al. 2015). Salinity was maintained around 35 and temperature at 26 °C. Colonies were fed daily with a combination of frozen copepods, *Artemia*, krill, and *Mysis*.

Hong Kong colonies were collected from 5 m (max. depth) from Crescent Island (22.5308°, 114.3150°) in November 2020 and transported to the University of Hong Kong, where they were fragmented and acclimated to aquarium conditions for one month. The aquarium system consisted of 6-L acrylic tanks placed within a plant growth chamber (Panasonic MLR-352H-PA). Each tank held eight ~ 3 cm-fragments and was fitted with a submersible pump (Atman AT301) and a small water filter (Shiruba PF120). The fragments used in symbiosis reestablishment were fed twice-weekly with a powdered blend of marine plankton (ReefRoids, Polyp Lab) followed by a partial water change. Light, salinity, and temperature conditions were consistent with those maintained in the Ocean2100 facility.

For ex situ spawning, 35 colonies (10.1 cm ± 2.5 (mean ± sd)) were collected from Arlington Reef, Great Barrier Reef, Australia (-16.7000°, 146.0500°), in September 2019, and transported to the Horniman Museum and Gardens (United Kingdom; CITES permit 585,319/01) with the 'inverted submersion method' as described by Craggs et al. (2018). Colonies were distributed into four coral spawning systems (www.coralspawninglab.org) that replicated the natural environmental parameters (seasonal temperature, photoperiod, solar irradiance, and lunar cycle) required to stimulate reproduction (Tab. S1). Aquarium design and coral husbandry protocol was based on the mesocosms described by Craggs et al. (2017), and the seasonal temperature profile was based on a non-sequential eight year average (1998—2017) from Moore Reef (-16.8667°, 146.2334°') (http://data.aims.gov.au/aimsrtds/datatool.xhtml?site=931&param=water%20temperature) (Fig. S7). Colonies were fed daily on a mix of phytoplankton (*Tisochrysis lutea*, *Chaetoceros calitrans* and *Rhodomonas salina*) and zooplankton (newly hatched *Artemia salina* nauplii, frozen red plankton, rotifers, and lobster / fish eggs) (Tab. S1). During feeding, the broodstock tank was isolated from the filtration for two hours to aid prey capture and uptake.

CHAPTER 3

Thermal performance experiment on single polyps

Preparation of single polyps

For the menthol bleaching and thermal performance experiment, we used single polyps from three Red Sea colonies each showing distinct coloration (Fig. S1). Replicate clonal polyps were isolated from each colony using an electric rotary cutter, mounted on coral glue (Grotech, Cora-Fix SuperFast), and allowed to heal for two weeks in the Ocean2100 aquarium facility (Schubert and Wilke 2018).

Menthol bleaching to remove algal symbionts

An equal number of polyps from each colony were randomly assigned to the control group (maintained in a symbiotic state) and the bleached group (chemically bleached with menthol). Menthol bleaching followed a protocol modified from Wang et al. (2012) and consisted of three days of treatment in 0.38 mM menthol solution in seawater, followed by a day of rest and a fourth day of menthol treatment. Each day the polyps were incubated in menthol for 8 h under light and stirring (Fig. S2). After, the polys were rinsed and kept in clean containers with air bubbling and stirring. During menthol treatment, the treated polyps were only exposed to filtered (1.2 µm) artificial seawater (FASW) to prevent exposure to Symbiodiniaceae. Following, all polyps (including, for consistency, the symbiotic group) were maintained in FASW.

To confirm bleaching, half of the polyps from each group (bleached and symbiotic) and each colony were visually assessed with a fluorescence stereomicroscope (Leica MZ16 F) 10 days after the termination of the menthol treatment. Representative pictures were taken under natural light (brightfield, light source: 2950 K) and under UV light with GF2 filters allowing visualization of the green fluorescent proteins and chlorophyll (Fig. 2a). Following the same bleaching protocol, menthol-bleached polyps were also documented using a compound epifluorescence microscope to reach higher resolution (Leica DM 5500B, TX2 filter) (Fig. 2b).

Thermal performance experimental design

Symbiotic and bleached polyps were moved to the experimental tanks on the day after the last menthol exposure and allowed to recover and acclimate for 10 days. The experimental system consisted of eight 5-L glass tanks (20 cm × 30 cm) equipped with a small pump (Resun SP-500) in a common temperature-controlled water bath (26 °C). White fluorescent lamps were used to maintain the light cycle and intensity consistent with long-term rearing conditions. Bleached and symbiotic polyps were kept in separate tanks, with four replicate tanks per treatment filled with FASW. Each tank contained one polyp from each colony (n = 3) that were used for the thermal performance experiment. Additional polyps were kept in each tank as back-up (Fig. S3). Polyps were fed each day after the end of the dark incubation, followed by 10% water change after 2–3 h. Each polyp was fed one small frozen adult Artemia pipetted directly on top of the oral opening. The polyps were cleaned every two to three days after the incubations

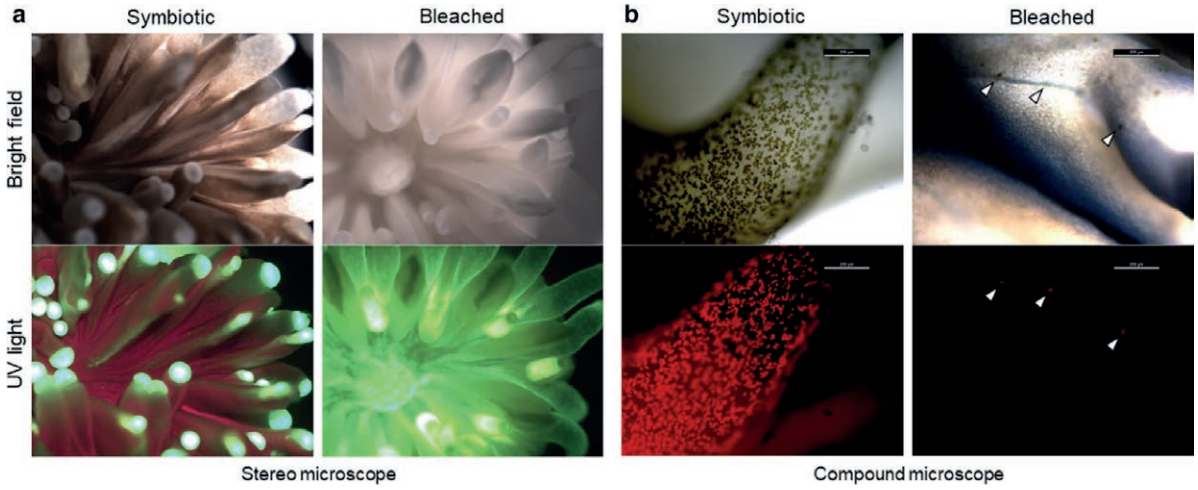

Fig. 2 Microscopic comparison between symbiotic and menthol-bleached polyps. Representative stereo and compound micrographs in bright field and UV light, with filters for chlorophyll (red) and coral tissue (green) autofluorescence. **a** Symbiodiniaceae cells are abundant in symbiotic polyps while not detectable in bleached polyps. **b** At higher magnifications, few algal cells are still detectable in bleached polyps only at the base of the tentacles (arrows)

to remove fouling that would interfere with respirometry measurements.

To test the amenability to experimental handling of single polyps and to compare metabolic rates of bleached and symbiotic polyps, we conducted a thermal performance experiment. Photosynthesis and respiration of *G. fascicularis* polyps were measured across a 12 °C temperature range over 10 days. For this, the polyps were acclimated to a different temperature each day, in the following order: 20, 22, 23, 24, 25, 26, 27, 28, 30 to 32 °C. The temperature of the water bath was changed overnight, and incubations took place the subsequent day at the respective temperature.

Photosynthesis and respiration of symbiotic and bleached polyps

We measured oxygen evolution of individual *G. fascicularis* polyps in light and dark incubations to calculate net photosynthesis (PN) and dark respiration (R), respectively. For these measurements, four bleached and four symbiotic polyps from three colonies were used ($n = 12$). Every day, each polyp was placed in individual 160-mL glass incubation jars (Weck, Germany), equipped with a magnetic stirrer (~ 5 cm s^{-1}). Three additional jars containing only seawater and stirring bars were used to control background biological activity. Light intensity of 230—280 µmol photons m^{-2} s^{-1} during light incubations (ATI 80 W Aquablue Special) and temperatures were maintained through thermostat-controlled water baths placed on a custom-made multiplate stirring system (Rades et al. 2022).

Dissolved oxygen (DO) was measured at the start and at the end of each incubation using a handheld multiparameter probe (WTW Multi 3620 IDS set). Light incubations lasted 2 h around mid-day followed by 1.5 h of dark incubation. Net photosynthesis and respiration were calculated using the following formula (Schneider and Erez 2006):

$$\mathrm{PN}\,or\,R\left[\mathrm{mg\,O_2 \times cm^{-2} \times h^{-1}}\right] = \frac{\Delta \mathrm{O_2}\left[\mathrm{mg} \times L^{-1}\right] \times V_{\mathrm{incubation}}[L]}{T[\mathrm{h}] \times \mathrm{SA}\left[\mathrm{cm^2}\right]}$$

where ΔO_2 is the difference in dissolved oxygen between the end and the start of the incubation ($DO_{end} - DO_{start}$) corrected for the controls' ΔO_2. This is multiplied by the volume of the incubation chambers in L ($V_{incubation}$), and divided by incubation time (T) in hours and polyp surface area (SA) in cm^2.

Surface area measurements

Polyp surface area was measured through photogrammetry. Between 40 and 50 pictures of each polyp were taken with a phone camera two days before the beginning of the incubations. These were used to create 3D models with 3DF Zephyr Free (v.4.523), cleaned on Artec3D (Studio 11 Professional v.11.2.2.16), and loaded on MeshLab (v.2016.12, Cignoni et al. 2008) for size scaling and calculation of live coral surface area.

Thermal performance data analysis

All analyses were conducted in the R statistical environment (v.4.1.0, R Core Team 2021) and plotted with ggplot2 (v.3.3.5, Wickham 2016). The effects of symbiotic state ('symbiotic' vs. 'bleached') or colony identity on net photosynthesis (PN) and respiration (R) were analyzed using linear mixed-effect models considering light and dark incubations separately. For this, 'symbiotic state,' 'colony identity,' and 'temperature' were set as fixed factors, and 'polyp identity' as random factor. The package 'lmerTest' (v.3.1–3, Kuznetsova et al. 2017) was used to construct the models and calculate p-values. The package 'performance' was used to check the residuals and to compare alternative models (v.0.7.3, Lüdecke et al. 2021). Differences between colonies were tested as pairwise comparisons of estimated marginal means (Searle et al. 1980) with Bonferroni correction with the 'emmeans' package (v.1.6.2–1).

Symbiosis reestablishment after menthol bleaching

Native Symbiodiniaceae characterization

Galaxea fascicularis colonies were collected in March 2019 from Crescent Bay in Hong Kong. DNA was successfully extracted from two colonies using the Qiagen DNeasy 96 Blood & Tissue kit. The ITS2 marker region was amplified with the primers SYM_VAR_5.8S2 and SYM_VAR following the PCR protocol by Hume et al. (2018), followed by paired-end sequencing on the Illumina MiSeq platform (2 × 300 bp). Raw sequencing data were analyzed using the SymPortal workflow remote instance (Hume et al. 2019), with results reported as post-MED ITS2 sequences in relative abundances.

Menthol bleaching and inoculation of cultured Symbiodiniaceae

We explored the possibility of returning adult corals to the symbiotic state after menthol bleaching. For this, we inoculated menthol-bleached coral fragments from Hong Kong with cultured Symbiodiniaceae. Bleaching followed the same protocol described for the Red Sea colonies. Two species of symbionts, *Cladocopium goreaui* and *Durusdinium trenchii,* were chosen for targeted exposure of the bleached fragments, as species from both of these genera are known to form stable symbiosis with *G. fascicularis* across the South China Sea (Tong et al. 2017). Batch cultures of *C.*

goreaui (AIMS-SCF-055, ITS2 type-sequence C1, isolated from *Acropora tenuis*) and *D. trenchii* (AIMS-SCF-088, ITS2 type-sequence D1-4, isolated from *A. muricata*) were obtained from the Symbiont Culture Facility at the Australian Institute of Marine Science and maintained in f/2 media (Guillard 1975) under the same conditions as the corals. A total of 16 fragments were inoculated with a concentration of 200 symbiont cells ml^{-1} in each 6-L aquarium. The concentration of symbionts was divided in a 90:10 ratio (180 cells ml^{-1} *D. trenchii* : 20 cells ml^{-1} *C. goreaui*). This ratio was chosen because members of *Cladocopium* are the most prevalent symbionts found in *G. fascicularis* in Hong Kong (Huang et al. 2011; Tong et al. 2017) and across its wide geographic range from the Red Sea to the South China Sea, where *Durusdinium* is only occasionally found and only in comparably warm habitats (Dong et al. 2009; Ziegler et al. 2017; Zhou et al. 2017). The symbiont aliquots were centrifuged to remove the culture media and washed once with FASW before final resuspension in 10 mL FASW. The bleached fragments were first fed before being exposed to the chosen symbionts. For this, a suspension of ReefRoids in ASW was pipetted directly into the oral opening of each polyp. The same process was then immediately repeated with the suspended symbionts. Aquarium filters were turned off for 1 h to allow the corals to feed and to prevent removal of symbiont cells from the water column. Fragments were inoculated twice a week for 6 weeks, until symbiosis reestablishment was visually confirmed. Symbiodiniaceae populations *in hospite* were then characterized with fluorescent in situ hybridization (FISH) and flow cytometry (McIlroy et al. 2020).

Fluorescent In Situ Hybridization (FISH)

After 6 weeks of inoculation, a single polyp was removed from each fragment with a chisel. Tissue was removed from the skeleton with an airbrush containing deionized water. The resulting slurry was homogenized using a 20-µl pipette tip. 1.5 mL of homogenate was transferred to a 2-mL Eppendorf tube and centrifuged at 500 rpm for seven minutes, after which the supernatant was discarded. The remaining pellet was washed once before being preserved in 5X SET buffer and stored at −80 °C for further analysis.

Samples were prepared following an adapted protocol from McIlroy et al. (2020). Briefly, after washing with 5X SET in IGEPAL at 0.4 and 0.1% vol/vol samples were split equally between three 1.5-mL black Eppendorf tubes. Each sample had one tube treated with a SymC probe, one tube with a SymD probe and one without any probe as a control. Both SymC (5'-CTACCCAAGAACTTGCAGG-3') and SymD (5'-CCACCCAAGAACTCGCGTG-3') probes were designed to have genus-level specificity for *Cladocopium* and *Durusdinium*, respectively, and were labeled at 5' end with Alexa Fluor ® 532. After overnight hybridization at 45 °C (5X SET, 0.1% vol/vol IGE- PAL, 10% vol/vol formamide and 100 pmol of the relevant probe), samples were washed once in warm 1X SET, vortexed, and resuspended in 500 µl of 1X SET for same-day cytometric analysis on a BD FACSAria Fusion flow cytometer (BD Biosciences, CA). A gating hierarchy was used to isolate cells of interest. Cells were screened for size and presence of doublets (multiple cells stuck together) based on light scatter parameters (forward-scatter (FSC) and side-scatter (SSC) (Fig. S6a-c). Probe-positive cells were then distinguished based on their position within the 582 nm vs SSC-A scatterplot (Fig. S6d,e). FlowJo™ software (v.10.8.1, BD Life Sciences) was used to determine the percentage of probe-positive cells (either *Cladocopium* or *Durusdinium*), to identify the presence and the relative abundance of each algal genus within the sample.

Completing gametogenic cycles and ex situ spawning

Galaxea fascicularis in the GBR spawns between October and December, 0.05–200 min after sunset (MAS) and 1–8 nights after full moon (NAFM) (Babcock et al. 1986; Baird et al. 2021). To determine if the ex situ reproductive pattern remained in synchrony with the wild, we followed 35 colonies over three consecutive reproductive seasons (2019–2021).

To ascertain gamete development in each colony, two months prior to predicted spawning, and two to four days prior to the full moon, whole polyps were removed from each colony and longitudinal sections imaged (Canon 5d MKIII, Fig. 5a,d). Based on the stage of gamete development in these sections, ex situ spawning activity was predicted.

To record spawning observations each year, colonies were observed with red head torches during the predicted spawning months. Observations commenced two NAFM and 30 min prior to the predicted spawning time, during which time the broodstock aquariums were isolated from the filtration system. In addition, all internal water pumps were also turned off leaving the aquariums' water static. Observations continued over multiple consecutive nights until all colonies that had developed gametes, during that reproductive season, spawned. Spawning date and time of each colony were recorded, with onset of spawning being denoted as the time of first gamete release. Following release, gametes were pooled to allow fertilization to occur, and subsequent embryos were reared and larvae settled following the method described by Craggs et al. (2019).

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Results

Production and long-term rearing of bleached polyps

We produced single polyps (clonal replicates) of *G. fascicularis* colonies from the Red Sea and bleached them with menthol. All polyps ($n = 24$, including additional polyps not used in this experiment) survived the menthol treatment and appeared visually completely white. Similarly, all menthol-bleached coral fragments from Hong Kong ($n = 16$) survived the treatment. Visual inspection under a fluorescent stereomicroscope revealed no detectable algal cells in the bleached polyps (Red Sea colonies, Fig. 2a), but inspection at higher magnification (compound fluorescent microscope, × 400) revealed the presence of few scattered algal cells, limited to the base of the tentacles (Fig. 2b).

All polyps that were employed in the thermal performance experiment ($n = 12$) remained visually completely bleached and viable throughout the 10 days of temperature treatment, with the exception of two polyps that appeared dead on the 7th and 8th day of incubation, respectively. These polyps showed atrophic and unresponsive tentacles, and overall thinning of the soft tissue until it was not recognizable. Overall, we successfully maintained the bleached polyps for three weeks from the termination of the menthol treatment.

Amenability to experimental manipulation and broad thermal tolerance

Symbiotic (untreated) polyps had positive net photosynthesis across all temperatures from 21 to 32 °C (Fig. 3). Net photosynthesis peaked at 23 °C (mean 13.05 µg O_2 cm^{-2} h^{-1}) and reached the minimum at 32 °C (mean 3.26 µg O_2 cm^{-2} h^{-1}). In contrast, respiration overall increased with temperature with minimum values at 21 °C (mean 5.77 µg O_2 cm^{-2} h^{-1}) and maximum at 32 °C (mean 25.70 µg O_2 cm^{-2} h^{-1}; Fig. 3). Bleached polyps, as expected by their lack of photosymbionts, exhibited a net oxygen depletion. Similar to symbiotic polyps, respiration in bleached polyps increased with temperature, both in light and dark incubations (Fig. 3). Overall, respiration in symbiotic polyps was higher than in bleached polyps ($P_{lmer} < 0.0001$).

While net photosynthesis was similar between colonies ($P_{lmer} > 0.05$), respiration was significantly higher in one colony ($P_{lmer} < 0.001$) compared to the other two (RS2-RS1: $P_{Bonf} < 0.01$; RS2-RS3: $P_{Bonf} < 0.01$; RS1-RS3: $P_{Bonf} > 0.05$; Fig. S4).

Symbiosis re-establishment after bleaching

Inoculation with cultured symbionts resulted in successful symbiosis re-establishment for 100% of the samples ($n = 16$), which were macroscopically visibly symbiotic. Of these, nine samples were successfully FISH hybridized, with the genus-specific probes identifying the presence of both target genera in each sample. Individual variation in the relative

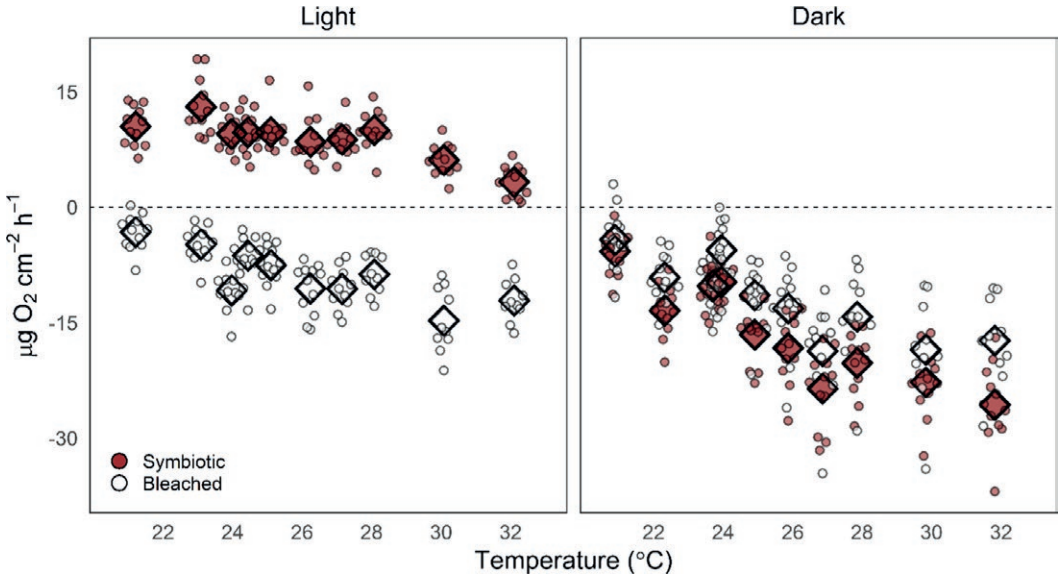

Fig. 3 Net photosynthesis and respiration rate of symbiotic states in *Galaxea fascicularis*. Symbiotic (brown symbols) and bleached (white symbols) polyps in light and dark incubations. Noise added (jittering) for ease of visualization, means per group depicted as diamonds. $n = 12$

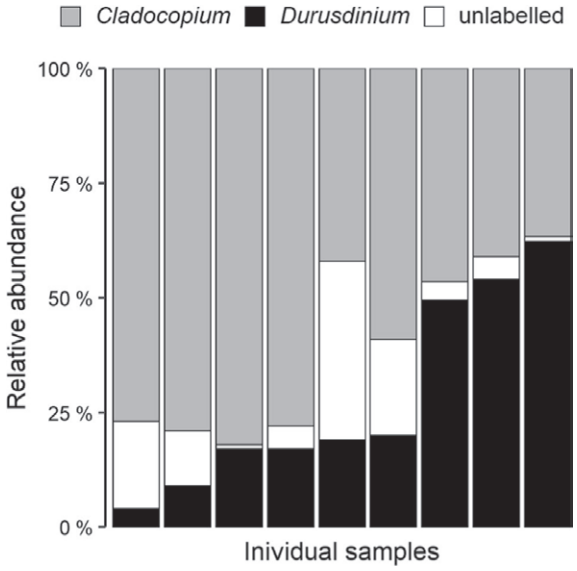

Fig. 4 Symbiosis reestablishment in menthol-bleached adult corals inoculated with cultured Symbiodiniaceae of the genera *Cladocopium* and *Durusdinium*. The presence and relative abundance (%) of both genera were confirmed and quantified through fluorescent in situ hybridization (FISH) and flow cytometry

ratio of each genus was evident among the fragments, with *Durusdinium* contributing to 4–63% of cells and *Cladocopium* to 37–82% (Fig. 4, Tab. S2). A small, but significant proportion of symbionts remained unlabeled in some samples. These may represent additional symbiont genera or be the result of variable probe efficiency. *G. fascicularis* colonies from the same location were found to harbor *Cladocopium* symbionts only (dominant ITS2 sequence types: C1 and C1c, Tab. S3).

Ex situ spawning

Spawning was monitored each year over the three-year observation period for all 35 colonies. Annual fecundity, as a percentage of colonies completing a full gametogenic cycle, was on average 56.2% during these three spawning cycles (2019: 48.6%, 2020: 45.7%, and 2021: 74.3%).

Spawning occurred in synchrony with wild predictions, with gametes being released between October and January each year, between 5–259 MAS and 4–13 NAFM (Fig. 5b-c, e–f, Fig. 6). Following embryo rearing, Symbiodiniaceae were observed within primary polyps 14 days post-settlement (Fig. 5g). These originated from the adult colonies housed in the same system and taken up via the water column. Polyps were fully developed 40 days post-settlement (Fig. 5h) and grew into small colonies within the first six months (Fig. 5i).

Discussion

Here, we explored the potential of *G. fascicularis* as a novel coral model for symbiosis research by assessing its amenability to aquarium rearing, experimental handling, and symbiosis manipulation.

Successful bleaching with menthol

We showed that *G. fascicularis* can be readily and reliably rendered aposymbiotic through menthol bleaching, similar to other cnidarian model systems (e.g., Matthews et al. 2015; Röthig et al. 2021). We lowered the menthol concentration in the protocol developed by Wang et al. (2012) to further limit stress on the host, and indeed, all coral polyps survived this bleaching procedure on two independently replicated occasions. Overall, menthol treatment was very effective, with only a few algal cells remaining at the base of the tentacles. From a photophysiological perspective, these scant remnants can be considered negligible, as illustrated by the light and dark oxygen production data. Furthermore, bleached polyps remained aposymbiotic for weeks and we therefore considered this bleaching protocol suitable for experimental investigation of aposymbiotic *G. fascicularis* in physiological studies and symbiosis manipulation.

Notably, besides Symbiodiniaceae, reef-building corals also associate with other microorganisms (i.e., bacteria, viruses, and other unicellular algae and protists) which constitute the so-called coral holobiont (Rosenberg et al. 2007). Although this has only been appreciated more recently, holobiont composition and dynamics are now understood to underlie coral health and adaptability (Pogoreutz et al. 2020). As menthol has photo-inhibitory (Wang et al. 2012; Clowez et al. 2021) and antimicrobial properties (İşcan et al. 2002), further studies should investigate the effects of menthol bleaching on the remaining members of the microbiome.

Tractability of symbiotic and aposymbiotic *G. fascicularis*

Tractability, as amenability to experimental work and laboratory conditions, is one of the most important requirements for model organisms. The *G. fascicularis* polyps and fragments could be maintained for three and ten weeks, respectively, in simple systems consisting of small independent tanks (5–6 L) with regular feeding and basic water quality care. These time frames match the requirements for most experimental designs, and we believe could be further extended. Indeed, symbiotic individuals looked healthy and

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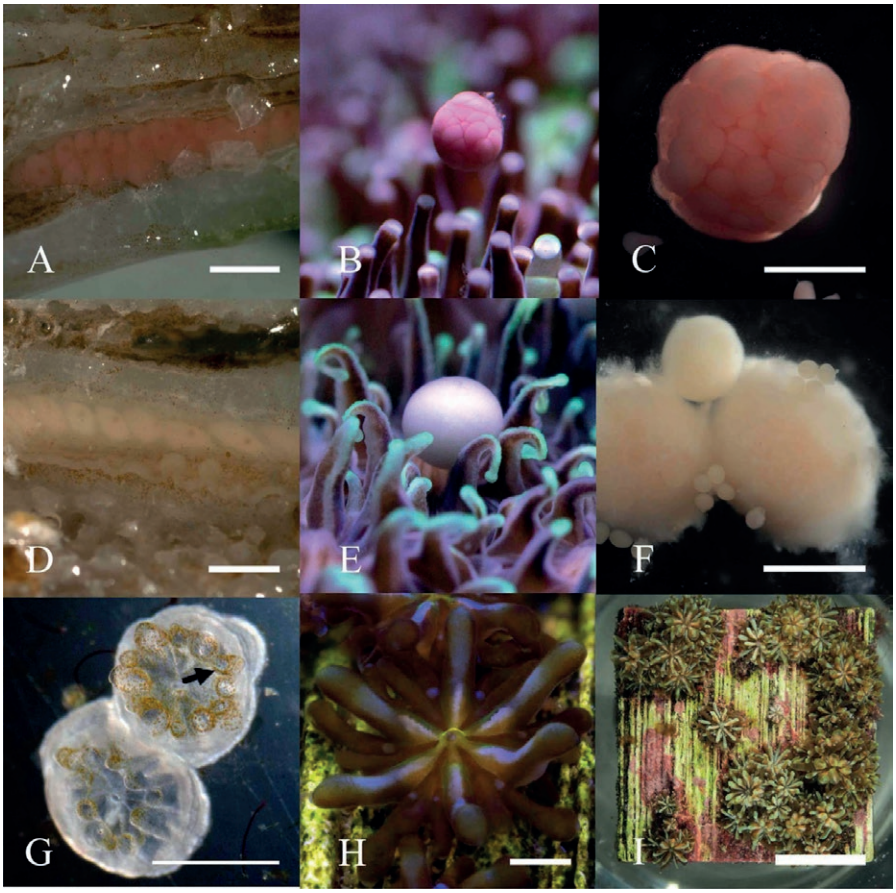

Fig. 5 Representative images of female and male colonies of *Galaxea fascicularis* spawning ex situ and gamete development. **a** Longitudinal section of female polyp with pinkish-red pigmented oocytes. **b** Female colony releasing oocyte bundle ex situ. **c** Close-up of female bundle post-release. **d** Longitudinal section of male polyp with white oocytes. **e** Male colony releasing oocytes/sperm bundle ex situ. **f** Close-up of male gamete bundles post-release undergoing disassociation. **g** Newly settled *G. fascicularis* primary polyps showing initial Symbiodiniaceae (arrow) uptake 14 days post-settlement. **h** Primary polyp 40 days post-settlement. **i** Multiple colonies six months post-settlement. Scale bar: a-h = 1 mm, i = 1 cm

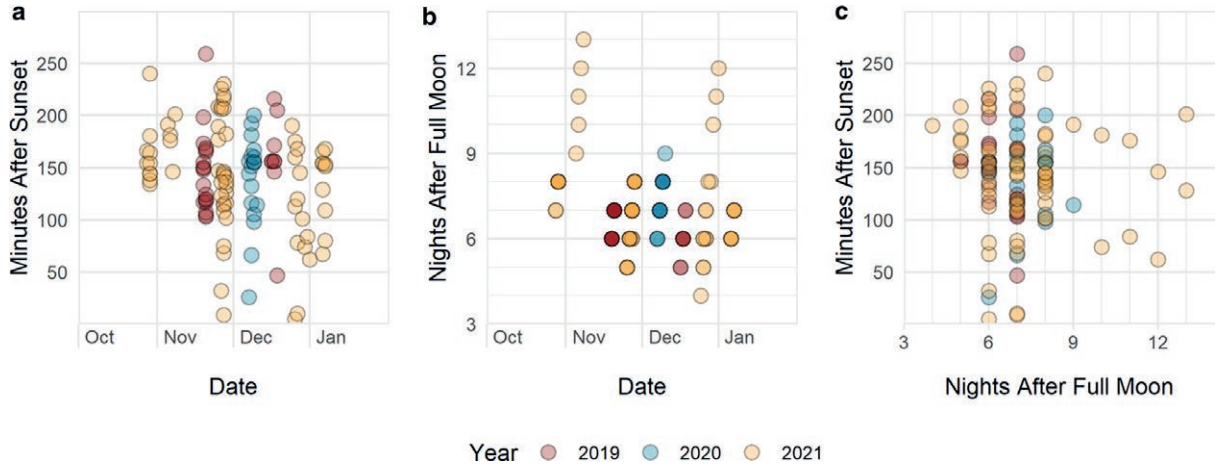

Fig. 6 *Galaxea fascicularis* ex situ spawning periodicity over three spawning cycles (2019 – 2021). **a** Spawning time, recorded as the first gamete release in Minutes After Sunset (MAS) by date. **b** Night After Full Moon (NAFM) of gamete release by date. **c** Comparison of MAS and NAFM values over three ex situ spawning cycles. 2019 $n = 28$, 2020 $n = 19$, 2021 $n = 69$

responsive throughout the whole period, testifying to a comparatively hardy coral with only low demands on rearing conditions. Additionally, the positive net photosynthetic rate across a large temperature range confirmed the broad

thermal tolerance of this species (Al-Horani 2005; McIlroy et al. 2019), which was retained in the simplified single polyp application.

Moreover, bleached polyps could be maintained in the simple experimental system for three weeks even with the additional stress of the thermal experiment. However, they eventually showed signs of deteriorating condition indicating that not all nutritional needs were met. Thus, one priority is to develop bespoke feed for long-term rearing of bleached corals (Wang et al. 2012).

Thermal performance of symbiotic and aposymbiotic *G. fascicularis*

Studies that compare symbiotic and aposymbiotic coral holobionts are particularly informative for understanding coral response to climate change, and therefore, we investigated the comparability between symbiotic and aposymbiotic *G. fascicularis* polyps. As expected, these differed in their photosynthetic rates, where the latter were net oxygen consumers even under illumination. Overall, symbiotic polyps had higher respiration rates than aposymbiotic polyps, which could be ascribed to the additional metabolic burden of the associated Symbiodiniaceae, as well as a greater metabolic capacity derived from the availability of photosynthates (Muscatine et al. 1981; Al-Horani et al. 2003). For all polyps, respiration rates overall increased with temperature in line with optimum thermal performance of corals and macroalgae from the central Red Sea (27–33 °C) (Anton et al. 2020).

Interestingly, we observed intraspecific variability in physiological performance. One colony (RS2) had significantly higher respiration rates than the others (Fig. S4). This manifested exclusively under dark conditions and was therefore more likely linked to host rather than symbiont identity. This variability warrants further investigations to better characterize host genotypes, as in the long term it will be necessary to establish representative host clonal lines.

Symbiosis reestablishment in adult corals

We showed that adult bleached *G. fascicularis* can successfully reestablished symbiosis with cultured Symbiodiniaceae. The experimental repopulation of adult corals with cultured non-native symbionts has only recently been demonstrated (Scharfenstein et al. 2022), and it is considered particularly relevant in the context of symbiosis manipulations to enhance heat tolerance in reef-building corals (van Oppen et al. 2015). Notably, here this was further explored with two non-native algal cultures during simultaneous exposure. The first, *D. trenchii* (ITS2 type D1-4), has never been found in *G. fascicularis* from Hong Kong (Huang et al. 2011; Tong et al. 2017) nor in our characterization of Symbiodiniaceae from that site specifically (two other colonies from the same location, Tab. S3) and can therefore confidently be considered to be non-native. The second, *C. goreaui* (ITS2 type C1), was isolated from the coral species *A. tenuis* from Australia. Even though the dominant C1 ITS2-sequence is also present in *G. fascicularis* from Hong Kong, the high coral host-Symbiodiniaceae specificity, large radiation of C1-like Symbiodiniaceae (LaJeunesse 2005; Thornhill et al. 2014), and the consistent occurrence of the minor sequence C1c in addition to C1 in our samples implicate the *C. goreaui* culture as non-native in *G. fascicularis*. However, the higher similarity with the native symbionts likely explains the more efficient establishment of the *C. goreaui* culture than the *D. trenchii* culture despite a reversed (10:90) ratio in supply.

Galaxea fascicularis has generally been reported to host taxa from the genera *Cladocopium* and *Durusdinium*, both exclusively and simultaneously, and with an equatorial-ward shift from *Cladocopium* dominance to *Durusdinium* dominance (Huang et al. 2011 and references therein). At Hong Kong's latitude and at our rearing temperature, *G. fascicularis* is expected to be exclusively associated with *Cladocopium* (Huang et al. 2011; Tong et al. 2017; Zhou et al. 2017), yet the targeted inoculation produced mixed genus associations. This demonstrated how targeted inoculation can successfully shift Symbiodiniaceae community composition toward a higher proportion of heat tolerant strains without thermal treatment (Silverstein et al. 2015). Although the longevity of these partnerships is still unclear, these are promising results for future development of *in hospite* studies on the role of temperature on inter-partner symbiosis dynamics and symbiont recognition mechanisms (Bove et al. 2022). The success and potential insights from such experimental approaches will rely on future efforts to establish a variety of coral host-specific Symbiodiniaceae cultures (including the establishment of specific cultures from *G. fascicularis*) for further study.

Of note, repopulation is possible even with lower concentrations of Symbiodiniaceae, as we observed from earlier qualitative trials (details in Supplementary Text). There, we exposed the menthol bleached polyps after they were used in the thermal performance experiment to Symbiodiniaceae through the water medium (symbionts shed by nearby symbiotic colonies) or additionally in combination with a one-time inoculum of freshly isolated symbionts from symbiotic conspecifics. The success rates of these qualitative trials of ~ 30% and 43%, respectively, were lower than in the Hong Kong experiment (100%) yet remarkable considering the poorer health conditions of the polyps at the time of re-exposure to symbionts (i.e., after repeated incubations through the 21–32 °C temperature excursion) and the expectedly smaller number of symbionts available for uptake. In these trials, bleached hosts retained the ability to feed, which is

known to facilitate symbiont uptake (Fitt and Trench 1983) and likely contributed to symbiosis reestablishment. In the long-term, the polyps that reestablished symbiosis grew into small colonies and became visually indistinguishable from the untreated controls (Fig. S5). Taken together, these observations attest to the suitability of *G. fascicularis* to experimental symbiosis manipulation.

Ex situ spawning

Galaxea fascicularis is a good model species candidate for sexual reproductive work due its high survivorship in aquarium conditions. As demonstrated herein, a combination of seasonal programming and husbandry enabled the species to complete a full gametogenic cycle ex situ over multiple years, which closely mimics that observed in the wild (Babcock et al. 1986; Baird et al. 2021). Furthermore, we showed in vitro fertilization, embryological development, and ex situ larval settlement resulting in spat of known ages. As the age at which the species reaches sexual maturity is currently unknown, future studies should focus on this aspect, which is also important for producing an F2 generation and for closing the species' life cycle ex situ (Craggs et al. 2020). Access to a F2 generation of known parental lineage will provide a platform to conduct experiments in areas such as mapping quantitative trait loci (Zhang 2012) or phenotypic traits such as growth, disease resistance, or thermal tolerance (van Oppen et al. 2015). As *Galaxea* must acquire their symbionts from the seawater (horizontal transmission, with symbionts visible after the development of tentacles in settled polyps, Lin et al. (2022)), ex situ spawning also opens possibilities for studies on the dynamics of initial symbiosis establishment and how this is affected by symbiont identity, heritability in symbiont selection, and for rearing of axenic or gnotobiotic coral lineages, paralleling procedures established for *Hydra* (Fraune et al. 2015).

Conclusions

The study of coral–algal symbiosis has been constrained by the difficulties of maintaining corals ex situ and of manipulating the coral–algal association to unravel symbiosis functioning. Here, we showed that adopting *G. fascicularis* as a model system can alleviate these limitations. Its demonstrated compatibility to rearing in simplified systems and experimental applications, together with the repeated spawning in aquaria, confirm the high tractability of this coral species. Further, the possibility to readily obtain aposymbiotic individuals suitable for detailed study and symbiosis reestablishment set the stage for exciting future developments in coral symbiosis research which is necessary to understand corals' potential to cope with changing oceans.

Besides these strengths, our study also highlights areas worth future efforts on the path to developing the *G. fascicularis* model system further. These include: i) characterization of the effect of menthol on the rest of the coral microbiome (e.g., bacteria); ii) development of custom feed for long-term maintenance of bleached individuals; iii) assessment of long-term persistence of non-native Symbiodiniaceae, and testing of a variety of symbiont strains to understand the breadth of symbiont diversity that can be accommodated by the *G. fascicularis* host; iv) further characterization of host colonies and genotypes with the aim of establishing clonal lines, and from these, isolate and establish Symbiodiniaceae cultures, mirroring for example the development of the Aiptasia model; and v) development of collaborative platforms and open-access community resources around this emerging model system to accelerate research and discovery.

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Author contributions GP and MZ conceived and designed the study. GP, JC, RH, KEE, SM produced and analyzed data. MS, DMB, MZ contributed reagents/materials/analysis tools. GP and MZ wrote the first draft with contributions from all authors.

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Data and code availability Data and R scripts used for this study are available at: https://github.com/sPuntinG/Galaxea_Coral_Model/

Declarations

Conflict of Interest The authors declare no conflict of interest for this submission.

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Chapter 3

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CHAPTER 4

Menthol bleaching and the bacterial microbiome in the Galaxea model: comparative analysis of symbiotic and menthol-bleached polyps

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The bacterial microbiome of symbiotic and menthol-bleached polyps of long-term aquarium-reared *Galaxea fascicularis*

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Abstract

Coral reefs support the livelihood of half a billion people but are at high risk of collapse due to the vulnerability of corals to climate change and local anthropogenic stressors. While understanding coral functioning is essential to guide conservation efforts, research is challenged by the complex nature of corals. They exist as metaorganisms (holobionts), constituted by the association between the (coral) animal host, its obligate endosymbiotic algae (Symbiodiniaceae), and other microorganisms comprising bacteria, viruses, archaea, fungi and other protists. Researchers therefore increasingly turn to model organisms to unravel holobiont complexity, dynamics, and how these determine the health and fitness of corals. The coral *Galaxea fascicularis* is an emerging model organism for coral symbiosis research with demonstrated suitability to aquarium rearing and reproduction, and to manipulation of the host-Symbiodiniaceae symbiosis. However, little is known about the response of the *G. fascicularis* microbiome to menthol bleaching—the experimental removal of the Symbiodiniaceae which represents the first step in coral-algal symbiosis manipulation. For this, we characterized the bacterial microbiome of symbiotic and menthol-bleached *G. fascicularis* originating from the Red Sea and South China Sea (Hong Kong) that were long-term aquarium-reared in separate facilities. We found that the coral-associated microbiomes were composed of relatively few bacterial taxa (10-78 ASVs). Symbiotic polyps (clonal replicates) from the same colony had similar microbiomes, which were distinct from those of other colonies despite co-culturing in shared aquaria. A pattern of seemingly differential response of the bacterial microbiome to menthol bleaching between the two facilities emerged, warranting further investigation into the role of rearing conditions. Nevertheless, the changes in community composition overall appeared to be stochastic suggesting a dysbiotic state. Considering the importance of bleaching treatment of captive corals for symbiosis research, our results—although preliminary—contribute fundamental knowledge for the development of the Galaxea model for coral symbiosis research.

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Chapter 4

Introduction

By the end of this century, the livelihood of more than half a billion people will largely depend on the ability of corals to cope with changing ocean conditions (Hughes et al. 2017). Corals are vulnerable to climate change, pollution, and overfishing, which have caused the loss of half of the world's coral cover since the 1950s (Eddy et al. 2021; Eakin et al. 2022). As such, the reef structures they build and the multi-billion dollar ecosystem services they provide are at high risk of collapse (Costanza et al. 2014; van Hooidonk et al. 2016). Considering the high stakes, understanding coral functioning is essential to predict future scenarios and guide management and conservation efforts.

Corals exist as metaorganisms, or so-called holobionts (Rohwer et al. 2002), where complexity hinders our ability to unravel coral functioning and how it ultimately affects coral physiology and ecology (Rosenberg et al. 2007; Jaspers et al. 2019). Indeed, the coral holobiont comprises the animal host, obligate intracellular algal symbionts (Symbiodiniaceae), a rich and diverse bacterial community, together with other microorganisms such as archaea, fungi, viruses, and protists (Bourne et al. 2016; Pogoreutz et al. 2020). Even richer than their taxonomy is the potential diversity of relationships and interactions among members, and how these could contribute to holobiont health and resilience (Thompson et al. 2015; Pogoreutz et al. 2020).

Besides the coral host, the best understood member of the coral holobiont is the algal symbiont, as its photosynthesis-derived energy fuels the calcification process that builds reefs and allows corals to thrive in nutrient-poor waters (Muscatine and Porter 1977; Muscatine 1990). Bacteria are also involved in nutrient cycling and metabolism (Robbins et al. 2019; Tandon et al. 2020), as well as other essential physiological processes such as development (Webster et al. 2004; Tebben et al. 2015) and immunity (Certner and Vollmer 2018; Miura et al. 2019). Interestingly, bacteria also mediate host-Symbiodiniaceae dynamics through the mitigation of thermal and light stress on the algal symbiont (Motone et al. 2020; Connelly et al. 2022), and they produce antimicrobial agents from an organosulfur compound released by Symbiodiniaceae (Raina et al. 2016, 2017). A growing body of knowledge suggests a central role of complex multipartite interactions between bacteria, Symbiodiniaceae, and the coral host in nutrition, health and fitness (reviewed in Matthews et al. 2020). However, given the intricacy of players' diversity and their metabolic capacities, linking partner identity to function and holobiont phenotype proves particularly challenging.

Model organisms can help unravel holobiont complexity through manipulation. Comparison of different host-Symbiodiniaceae combinations in the sea anemone Aiptasia revealed that heat-tolerance of the symbiont is not linearly transferred to the host (Chakravarti et al. 2017; Gabay et al. 2019; Herrera et al. 2020). Thus complex mechanisms where holobiont properties cannot be predicted as the "sum of its parts" require a more holistic approach (Goulet et al. 2020). Such empirical testing of multi-partner interactions relies on the ability to study the holobiont upon experimental manipulation, namely by removing and/or adding members (Jaspers et al. 2019). Most of such studies in the field were conducted with non-calcifying species such as Aiptasia or the hydroid polyp *Hydra*, which are well established, tractable model organisms (Weis et al. 2008; Galliot 2012). Yet, while these models have proven instrumental for breakthrough discoveries in the cnidarian symbiosis field (e.g., Weis 2008; Murillo-Rincon et al. 2017; Pietschke et al. 2017; Gabay et al. 2018), they lack key features of reef-building corals such as calcification and the obligate endosymbiosis necessary to understand the ecology of coral functioning. To include these critical features, research with corals is irreplaceable (Puntin et al. 2022b).

The reef-building coral *Galaxea fascicularis* (Linnaeus, 1767) has been proposed as a model species for coral symbiosis research (Puntin et al. 2022a). This species is well represented in the literature, featured in studies that characterize the coral gastric cavity (Agostini et al. 2012; Zhou et al. 2020) and the calcification

processes (Al-Horani et al. 2003, 2005, 2007), among others (Ferrier-Pagès et al. 1998; Niu et al. 2016; Miura et al. 2019). Introducing *G. fascicularis* as a model system, we previously demonstrated its ease of rearing in simplified systems (closed, small volume), compatibility with *ex-situ* reproduction, effective removal of the algal symbiont through menthol bleaching, and subsequent reestablishment of the symbiosis with both cultured and environmental Symbiodiniaceae in adult individuals (Puntin et al. 2022a). This demonstrated the potential to experimentally produce a variety of coral-Symbiodiniaceae combinations to study symbiosis functioning and partner compatibility in a true reef-building coral. While recent coral probiotic approaches rapidly expand our knowledge on the functions of the bacterial fraction (Rosado et al. 2019; Peixoto et al. 2021), untangling the complexity in the holobiont requires detailed knowledge of the interrelationships that consider all partners.

One of the main knowledge gaps to advance mechanistic symbiosis research in the Galaxea model system is the effect of menthol bleaching on the remaining coral microbiome. Menthol is becoming increasingly common in manipulative experiments due to its efficacy in removing Symbiodiniaceae while causing virtually no host mortality (Wang et al. 2012; Matthews et al. 2015; Puntin et al. 2022a). To date, menthol bleaching has been used with a range of symbiotic cnidarians, including jellyfish (Röthig et al. 2021), anemones (Matthews et al. 2015; Dani et al. 2016), corallimorpharia (Lin et al. 2019), and nine species of reef-building corals (Wang et al. 2012, 2019; Puntin et al. 2022a; Scharfenstein et al. 2022; Chan et al. 2023). Yet, its impact on their bacterial fraction remains unknown. Menthol is known to have antimicrobial activity against several human pathogens (Trombetta et al. 2005; Mahzoon et al. 2022) and can select against certain bacteria, thus impacting microbial community composition, as seen in other contexts (Chopyk et al. 2017). To address this knowledge gap, we characterized the bacterial microbiome of symbiotic and menthol-bleached *G. fascicularis* polyps from the central Red Sea and the South China Sea that were maintained in two separate facilities for several months.

Materials and Methods

Coral collection and long-term aquarium rearing

Colonies of *Galaxea fascicularis* were collected from two locations: the Red Sea (hereafter referred as "Red Sea") and Hong Kong in the South China Sea (hereafter referred as "Hong Kong"). Red Sea colonies (n = 3) were collected from the central Saudi Arabian Red Sea at "Al Fahal" reef (N 22˚18.324' E 38˚57.930'), at 9-13 m depth in March 2019 (CITES permit 19-SA-000096-PD) and transported to the Ocean2100 aquarium facility at Justus Liebig University Giessen (Germany) where they were maintained in a 7,000 L closed aquarium system composed of several tanks (100-265 L) including a technical tank fitted with protein skimmer, active charcoal filter, phosphate adsorber, algal refugium (*Chaetomorpha* sp.) with reverse light cycle, and calcium reactor (Schubert and Wilke 2018). In the aquarium system, light was provided by white and blue fluorescent lamps with a light:dark cycle of 12:12 h at 130-160 µmol photons m^{-2} s^{-1} to approximate light conditions at the collection site (Ziegler et al. 2015). Salinity was maintained around 35 and temperature at 26 °C. Colonies were fed daily with a combination of frozen copepods, Artemia, krill, and Mysis. Hong Kong colonies (n = 2) were collected from ≤ 5 m depth from Crescent Island (N 22° 31' 51.035", E 114° 18' 53.888) in June 2019 and transported to the University of Hong Kong (HKU), where they were maintained in a 500-L aquarium equipped with a filtration system and protein skimmer, and fed daily with Reef-Roids (Polyplab) and frozen artemia. Light intensity, salinity, and temperature conditions were consistent with those maintained in the Ocean2100 facility.

Menthol bleaching

At both locations, individual (clonal) polyps were mechanically isolated from their colony, mounted on coral glue (Red Sea colonies, JLU-Ocean2100; Grotech, Cora-Fix SuperFast) or attached to small ceramic tiles (Hong Kong colonies, HKU; Aron Alpha, GEL-10) (Fig. 1). After 10-14 days of healing, polyps were randomly divided between a 'symbiotic' and a 'bleached' group at each location. Both groups were maintained under the same conditions until healed, then the 'bleached' group was treated with menthol to chemically induce bleaching. Menthol treatment started after approximately seven (Red Sea) and three (Hong Kong) months of captivity, and it was replicated at the two facilities following a protocol modified from Wang et al. (2012). Specifically, three days treatment in 0.38 mM menthol solution in filtered (1.2 µm) artificial seawater (FASW) was followed by one day of rest and another day of menthol treatment. Menthol incubations lasted 8 h during the light period.

Bleaching was assessed in the Red Sea polyps by visual inspection under a fluorescence stereomicroscope (Leica MZ16 F) 10 days after the menthol treatment, when algal cells were not detectable in any polyp. At the same time point, Hong Kong polyps also appeared fully bleached under microscopic inspection (Olympus Optical, mod. CHK at 400×).

Post-bleaching rearing conditions

To prevent coral from Symbiodiniaceae exposure and symbiosis re-establishment, all polyps were kept in simplified (see below for details) systems with FASW (1.2 µm) after the menthol bleaching treatment. Here, polyps were fed daily with one small frozen adult Artemia each, followed by partial (~10 %) water change after 2-3 h. At both facilities, temperature, light, and salinity were maintained consistent with the long-term rearing conditions, while the setups differed. Specifically, at the Ocean2100 facility, the polyps were distributed among eight 5-L glass tanks (20 cm × 30 cm) (four per treatment), each equipped with a small pump (Resun SP-500) in a temperature-controlled water bath. At HKU, symbiotic and bleached polyps were maintained in separate 600 ml glass jars, each holding ~6 polyps and equipped with magnetic stir bars for water flow inside a Plant Growth Chamber (Panasonic MLR-352H-PA).

Sampling for microbial analysis

16S rRNA amplicon sequencing was employed to characterize the bacterial communities of symbiotic and menthol-bleached polyps of *G. fascicularis* colonies from the two geographic locations (Fig. 1).

At both locations, three polyps per colony (5 colonies: RS1, RS2, RS3, HK1, HK2) per state (2 states: symbiotic, bleached) were sampled on the 13th day after the menthol treatment when they visually appeared completely bleached and otherwise healthy (n = 15 bleached and 15 symbiotic polyps). The polyps were rinsed with seawater, separated from the substrate, placed in sterile tubes, and stored at -80 °C. For transport, polyps were stored in RNAlater (R0901-100ML, Sigma-Aldrich, Hong Kong S.A.R.). Two samples from Hong Kong (1 symbiotic and 1 bleached) were damaged during shipping and therefore excluded from processing. All samples were processed together for DNA extraction (University of Derby's Aquatic Research Facility, UK) and subsequently sequenced in the same sequencing run (Bart's and the London Genome Centre, Queen Mary, University of London).

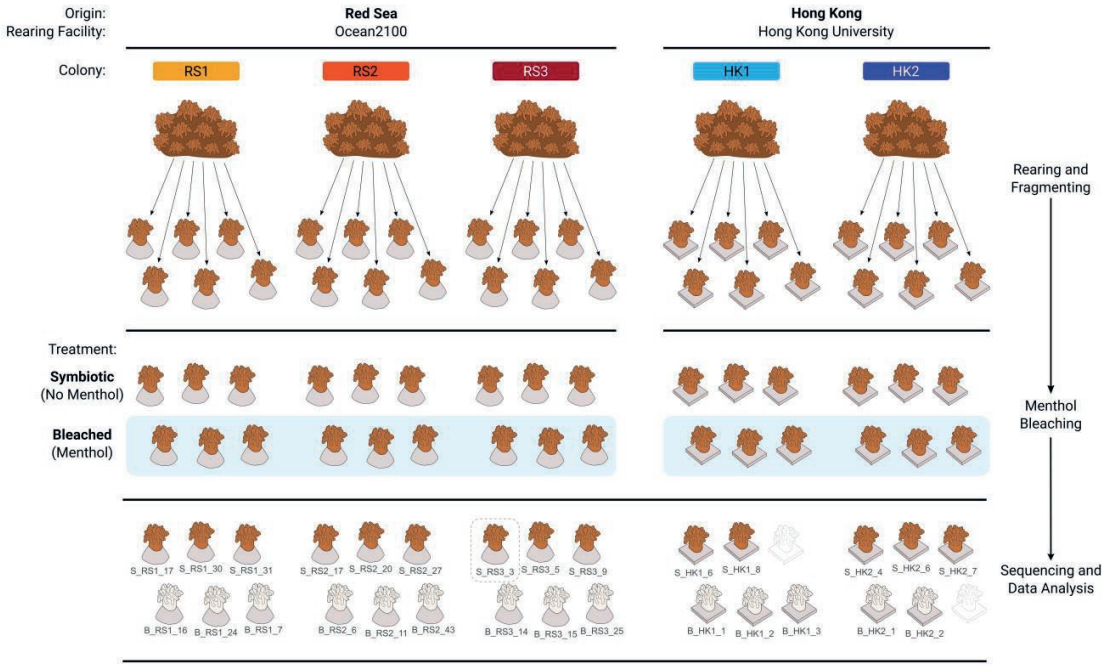

Figure 1 – Visual summary of the experimental design and data processing. Two samples (outlined polyps) were excluded from processing due to damage during shipping, while one sample (dashed box) was omitted after rarefaction for alpha and beta diversity analysis owing to low sequencing depth.

Bacterial community analysis

DNA was extracted using the Qiagen DNeasy 96 Blood & Tissue kit from crushed polyps (containing skeletal and tissue material) with about 10 mg of coral tissue per sample as starting material. Extractions followed the user manual with centrifugations at working steps 10, 12, and 16 performed at 1500 *g* at doubled centrifugation times. Extracted DNA was sent to the sequencing facility for quality control, PCR, library preparation, and pair-end sequencing with Illumina MiSeq platform v3 (2 × 300 bp). The 16S rRNA gene region V5/V6 was amplified using the primers 784F and 1061R (Andersson et al. 2008). A contamination control consisting of pure RNAlater buffer was included in all steps.

Bacterial sequencing data were processed in Qiime2 (v.2021.11, Bolyen et al. 2019) and analyzed in R (v.4.1.0, R Core Team 2021). After primer removal, forward and reverse reads were truncated to 232 and 234 nt respectively, paired, dereplicated, quality checked, cleaned, and clustered to amplicon sequence variants (ASVs) using the denoise-paired method in DADA2 (Callahan et al. 2016). This resulted in a total of 138,620 sequences and 547 ASVs. ASVs were taxonomically assigned using a weighted classifier trained against the SILVA 138 database (99 % clustering, full length) (Yilmaz et al. 2013) with the classify-sklearn method from ‘q2-feature-classifier’ plug-in (Bokulich et al. 2018; Kaehler et al. 2019; Robeson et al. 2020). Then, sequences assigned to “mitochondria”, “chloroplast”, “Archaea”, “Eukaryota”, or “unknown” at the phylum level were removed. Sequences found in the control sample were considered potential lab contaminants and evaluated based on presence/absence across the coral samples and habitat description (e.g., known contaminants) of the closest BLASTn matches (GenBank) (for full details see “01_find_contaminants.R” in

https://doi.org/10.5281/zenodo.10551928; Puntin 2024a). This led to the removal of five ASVs and their 18,990 sequences, and resulted in a final data set of 112,789 sequences and 515 ASVs across 28 samples (after excluding the contamination control).

Rarefaction and alpha and beta diversity calculations were performed with the R package 'phyloseq' (v.1.38.0, McMurdie and Holmes 2013), 'metagMisc' (v.0.0.4, Mikryukov 2023) and 'btools' (v.0.0.1, Battaglia 2022). Samples were rarefied to 2,690 sequences (based on 1,000 iterations of random subsampling without replacement), which caused the exclusion of one sample (symbiotic colony "RS3") due to low sequencing depth. Rarefaction curves showed that for most samples the number of ASVs plateaued before the rarefaction depth indicating that most of the diversity was captured and retained after rarefaction (Fig. S1). Alpha diversity was estimated through multiple indices chosen for their complementarity and comparability with previous studies (observed richness, Chao1, Shannon diversity, Simpson diversity, Pielou's evenness, and Faith's phylogenetic diversity). Differences in alpha diversity between symbiotic and bleached individuals were tested with t-tests or Mann-Whitney U tests depending on data distribution and variance. Beta diversity based on Bray-Curtis distances was visualized with non-metric multidimensional scaling (nMDS). The differential relative abundance of bacterial taxa between symbiotic states (symbiotic, bleached) was tested at the ASV and family level across the whole data set and by geographic origin (Red Sea, Hong Kong) using the Mann-Whitney U test with Benjamini-Hochman correction for multiple comparison. Relative abundance bubble plots of bacterial community composition at bacterial family level and of core ASVs (occur in all groups by state and geographic origin), and the UpSet plot (Lex et al. 2014) were generated from non-rarefied data. The UpSet plot was created with the package 'UpSetR' (v.1.4.0, Conway et al. 2017). All other plots were created in 'ggplot2' (v.3.3.5, Wickham 2016).

Results

Microbial diversity and richness were unaffected by menthol bleaching

When considering all colonies together, alpha diversity remained similar between symbiotic and menthol-bleached samples across all diversity and richness indices tested (incl. observed richness, Chao1, Shannon diversity, Simpson diversity, Pielou's evenness, and Faith's phylogenetic diversity, see Table S1, S2), and regardless of their origin ($P_{t\text{-test}}$ or $P_{Mann\text{-}Whitney\ U\text{-}test} > 0.05$; Fig. 2A). When considering each individual colony, alpha diversity was remarkably and consistently low in symbiotic RS1 polyps, while in their bleached counterparts it was in range with the other colonies (Fig. 2A, Fig. S2, Tab. S2). Within-colony difference between symbiotic and bleached polyps could only be tested for RS1 and RS2, and it was significant in RS1 across all alpha diversity indices tested ($P_{Welch} < 0.05$, Tab. S3).

Bacterial communities were generally uneven

A small number of ASVs dominated the bacterial communities, where the 3 and 9 most abundant ASVs accounted for > 25 % and > 50 % of the total number of sequences, respectively (Fig. 2B). Evenness was on average lower among the symbiotic polyps. Specifically, in the symbiotic samples, the 5 (for Red Sea) and 4 (for Hong Kong) most abundant ASVs account for > 50 % of total reads, while in the bleached samples it took 7 (for Red Sea) and 12 (for Hong Kong) ASVs to pass the 50 % relative abundance threshold. Of note, microbiomes were made of a small number of bacterial taxa, ranging from 10 to 78 ASVs, and with symbiotic RS1 polyps having the smallest number of ASVs (10, 11, and 13 respectively).

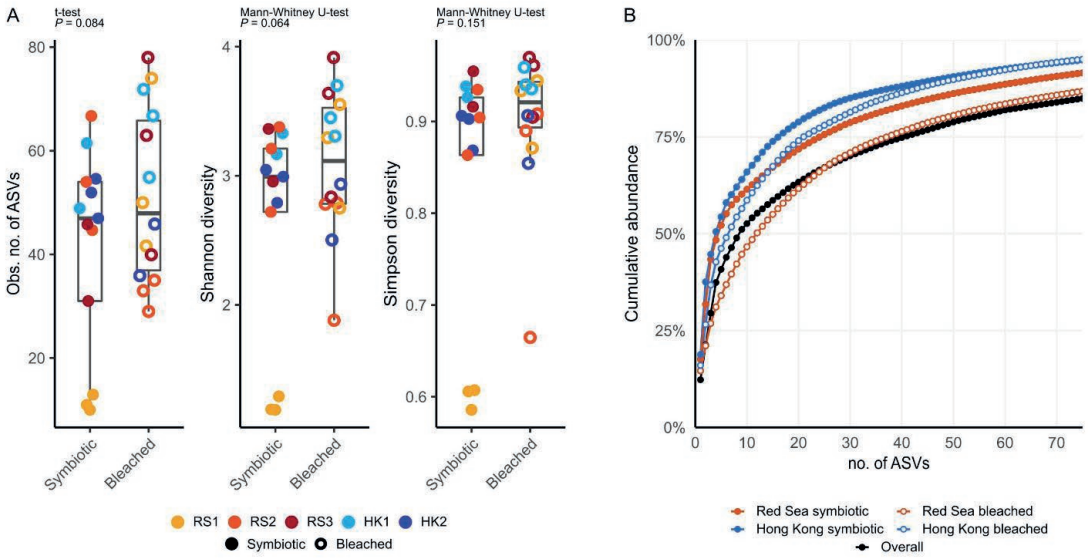

Figure 2 - Diversity and evenness of the bacterial communities of symbiotic and menthol-bleached polyps of *Galaxea fascicularis*. (A) Comparison of observed number of ASVs, and Shannon and Simpson diversity index between symbiotic and bleached samples; (B) ASV accumulation curve for the whole data set, and separated by symbiotic state and geographic origin.

Menthol-bleaching seemed to elicit stochastic changes in the microbial communities

Changes in community composition between symbiotic states showed different trends across the two facilities, however dissimilarity was generally higher in menthol bleached polyps. While symbiotic polyps clustered by colony for both Red Sea and Hong Kong (indicating similar microbial communities, Fig.3A), bleached polyps showed no such clear grouping (Red Sea, Fig.3C) or larger scattering compared to symbiotic polyps (Hong Kong, Fig.3D), and collectively had a significantly higher within-colony dissimilarity ($P_{Mann\text{-}Whitney}$ = 0.0001, Fig. 3A,B, Fig. S3), seemingly indicating random changes in the communities of the menthol-bleached polyps.

Additionally, no bacterial taxa (neither at ASV nor at bacterial familiy level) showed significantly different relative abundance between symbiotic and bleached samples, neither across the whole data set nor by geographic origin (i.e., considering Red Sea and Hong Kong samples separately) (Mann-Whitney U test with Benjamini-Hochman correction, all P > 0.05). This includes bacteria previously reported to be associated with Symbiodiniaceae cultures (Fig. S4; Supplementary Materials and Methods).

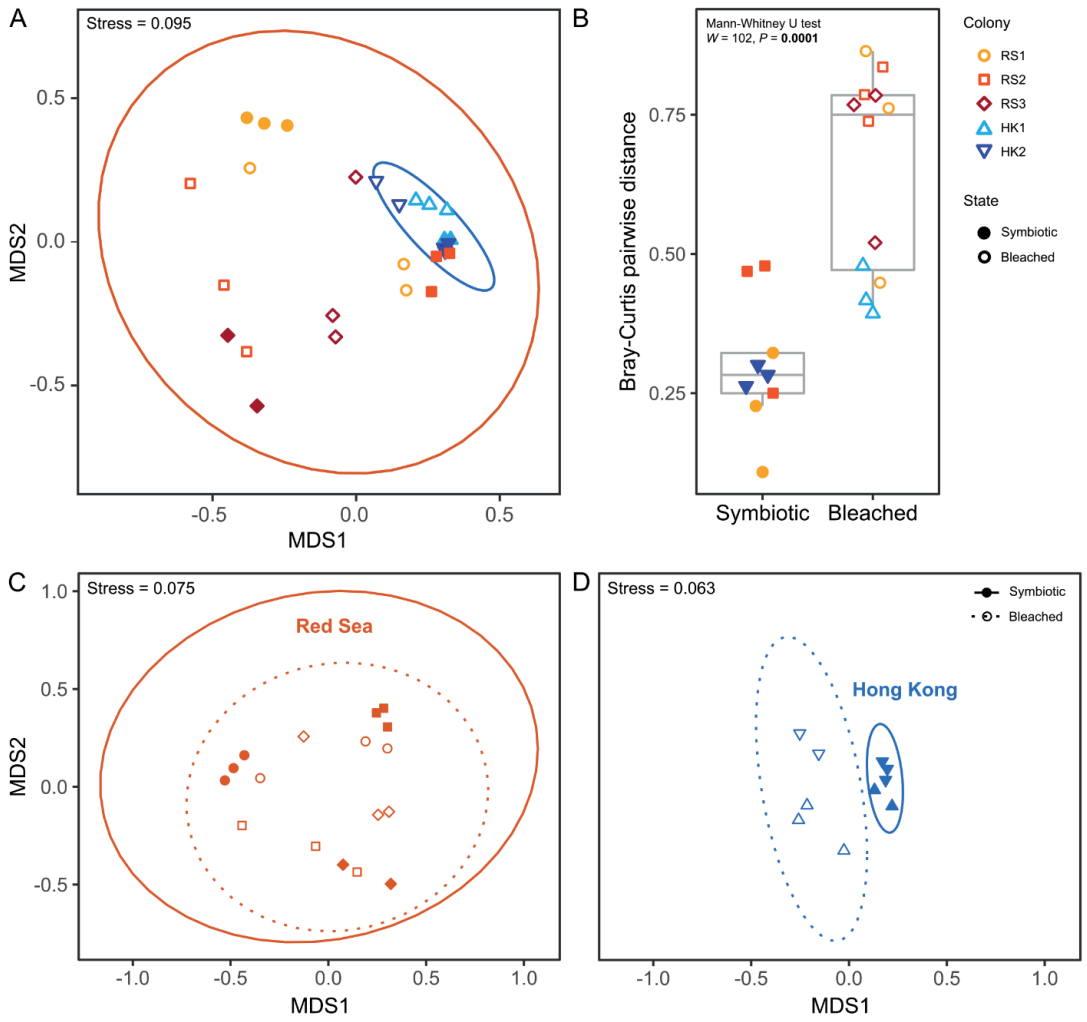

Figure 3 – Microbial community structure of *Galaxea fascicularis* from the Red Sea and Hong Kong. Non-metric multi-dimensional scaling (nMDS) plot of bacterial community composition based on Bray-Curtis dissimilarity for all polyps (A), Red Sea polyps (C), and Hong Kong polyps (D), and comparison of pairwise dissimilarity between symbiotic and bleached polyps of the same colony (only groups with $n = 3$ considered, but comparable results were found considering groups with $n < 3$, see Fig. S3) (B). Ellipses = 95 % confidence intervals (A, C, D); colors (A, D) or shapes (C, D) denote colony identity, filled symbols = symbiotic polyps, hollow symbols = bleached polyps.

Three bacterial families dominated most microbial communities

A total of 138 bacterial families were found across the 28 sampled polyps. Of these, 104 bacterial families occurred in symbiotic (74 in Red Sea, 66 in Hong Kong) and 109 in bleached (89 in Red Sea, 69 in Hong Kong) polyps. The three most abundant families *Rhodobacteraceae* (25.8 %), *Alteromonadaceae* (14.6 %), and

Moraxellaceae (10.1 %) together represented > 50 % of all sequences, and the 11 most abundant families represented > 75 % of all sequences. Symbiotic colonies were largely dominated by members of the two bacterial families *Rhodobacteraceae* and *Alteromonadaceae*. Except for the two Red Sea colonies RS1 and RS3 that were dominated by *Moraxellaceae* and *Bacillaceae*, or that showed no consistently dominant family across individual polyps, respectively.

Bleached polyps were dominated by varying bacterial families, with inconsistent patterns between and within colonies and regions. In addition to the dominant families in symbiotic colonies, *Endozoicomonadaceae*, unclassified *Cellvibrionales*, and *Amoebophilaceae* became dominant in some bleached polyps. While members of the family *Endozoicomonadaceae* were the most abundant fraction in one bleached polyp (39 %; RS3), they were only present in 15 of the 28 polyps (7 symbiotic, 8 bleached), and with low relative abundance (mean 2.2 % in symbiotic, and 8.9 % in bleached).

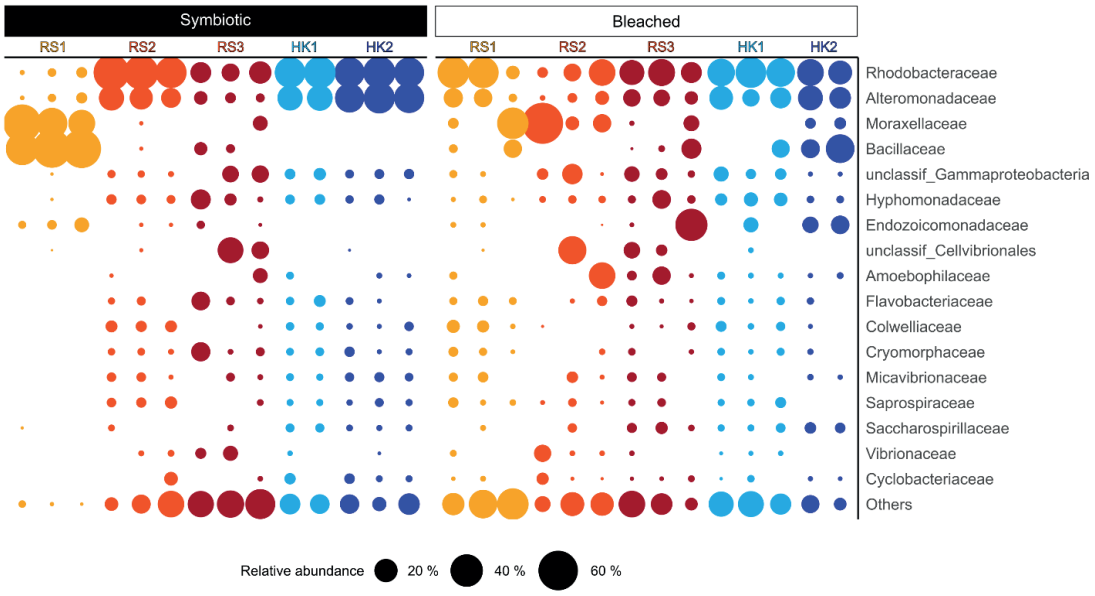

Figure 4 – Relative abundance of bacterial families in symbiotic and bleached *Galaxea fascicularis* polyps from the Red Sea and Hong Kong. Bubble size is proportional to the relative abundance per polyp of the 17 most abundant families, which account for 83.7 % of the total number of reads; all other 121 families that had a relative abundance < 1 % are grouped as 'Others'.

28 core ASVs were shared by all coral colonies and symbiotic states

Altogether, there were more exclusive than shared ASVs between experimental groups (Fig. 5A). Specifically, 33.4 % and 52.9 % of ASVs were exclusively found in symbiotic or bleached samples, respectively, while 5.4 % of ASVs (28) were found in all groups (symbiotic state and geographic origin) and were considered core taxa. Although no ASV was found in all samples (nor in all samples of the same experimental group, Tab. S4), three ASVs occurred in ≥ 70 % of samples, and five additional ASVs occurred in ≥ 60 % of samples (Fig. 5B). The sequences of these eight ASVs, which also corresponded to the most abundant of the 28 core ASVs, were BLAST-searched against the NCBI Nucleotide collection (Tab. 1), which

revealed that most of the matching sequences were from samples associated with marine environments and/or organisms.

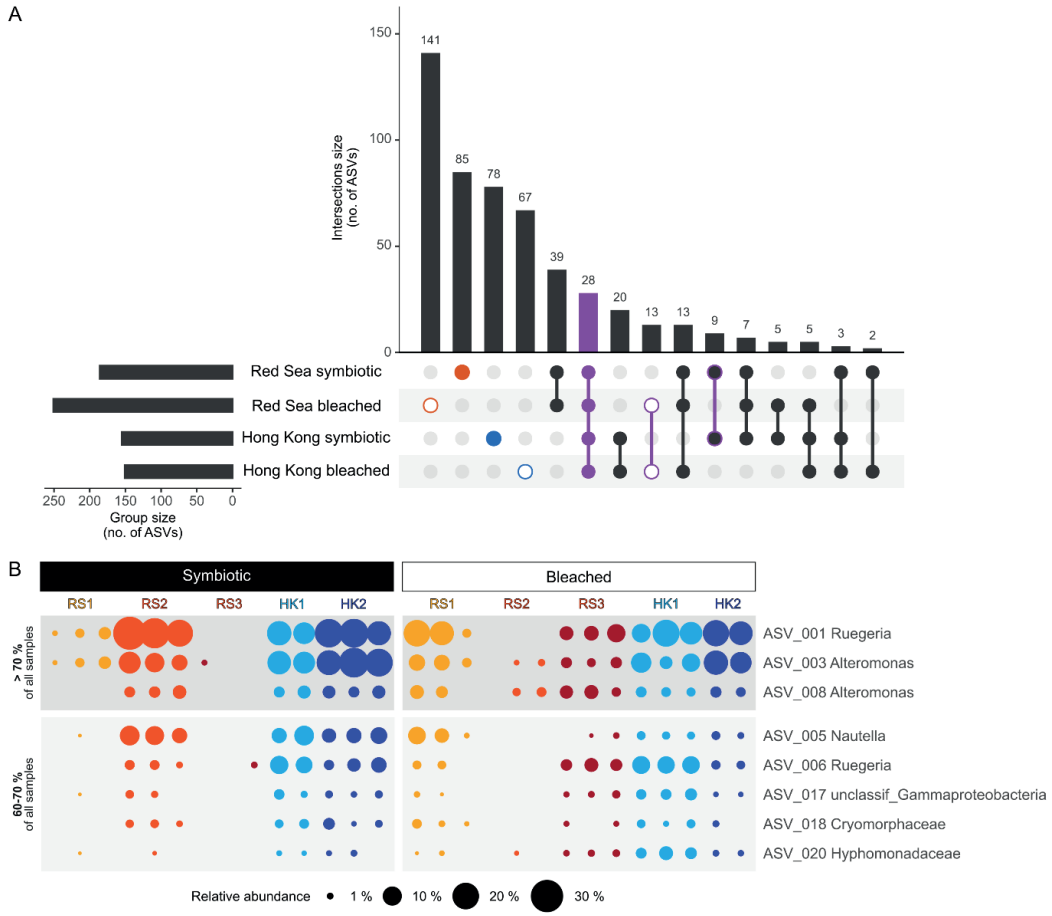

Figure 5 - Overview of total and core ASVs in *Galaxea fascicularis* symbiotic and menthol-bleached polyps from the Red Sea and Hong Kong. (A) UpSet diagram showing the number of observed ASVs per group and per intersection. (B) Relative abundance of core bacterial ASVs by host origin and symbiotic state, considering only ASVs present in ≥ 70 % (top-darker grey block) and between 60 % and 70 % of samples (bottom-lighter grey block); bubble size is proportional to the relative abundance of ASVs in each sample.

Among these, the most abundant bacterial sequences belonged to the genera *Alteromonas*, *Ruegeria*, and *Nautella* (Fig. 5B, Tab. 1). Interestingly, two ASVs (ASV_001 and ASV_006), both assigned to the genus *Ruegeria*, matched with strains isolated from aquarium-reared *Galaxea fascicularis* from the South China Sea (collected in Hainan Island, China, Zhou et al. 2020) and Japan (Miura et al. 2019). Further, three ASVs (ASV_006, ASV_018, ASV_020) matched with bacteria identified in *Acropora* spp. and *Pocillopora* spp. from the central Red Sea, amplified with the same primer set used for this study (Tab. 1).

Table 1 - Summary of NCBI BLASTn matches for the eight most common core ASVs (occur in both symbiotic and bleached samples from both Red Sea and Hong Kong, and in > 60 % of all samples) in order of abundance. For brevity, only the top three matches are reported (100 % query cover) with information on isolation source and location, where coral species are highlighted in bold. Taxonomy is reported as the lowest taxonomic level assigned by the Qiime2 classifier.

Taxonomy	ASV no.	GenBank Accession no.	Identity (%)	Isolation source (location)
Ruegeria	ASV_001	MK967091	100.00	Jellyfish, *Aurelia aurita* polyp (Kiel Bight, Baltic Sea)
		MT187950	100.00	***Galaxea fascicularis*** (Hainan, South China Sea)
		MT187656	100.00	***Galaxea fascicularis*** (Hainan, South China Sea)
Alteromonas	ASV_003	MT525287	100.00	Marine sediment (South Korea)
		MK967157	100.00	Artificial Seawater (Kiel Bight, Baltic Sea)
		MT515801	100.00	Marine sediment (South Korea)
Nautella	ASV_005	LC543501	100.00	Coastal seawater (Japan)
		MH556771	100.00	Ciliate, *Euplotes vannus* in Yantai Institute of Coastal Zone Research (Shandong, China)
		MK801649	100.00	Unspecified coral from South China Sea (Guangdong, China)
Ruegeria	ASV_006	MK736137	100.00	***Acropora hemprichii*** (Central Red Sea)
		MK736054	100.00	***Pocillopora verrucosa*** (Central Red Sea)
		MH807604	100.00	***Galaxea fascicularis*** (Aquarium, Japan)
Alteromonas	ASV_008	MT472680	100.00	Shrimp gut, *Penaeus vannamei* (India)
		MN704564	100.00	Undescribed source at marine molecular ecology lab (Zhejiang, China)
		MH556744	100.00	Ciliate, *Euplotes vannus* in Yantai Institute of Coastal Zone Research (Shandong, China)
unclassified ***Gammaproteobacteria***	ASV_017	AB294979	99.61	Microbial mat at shallow submarine hot spring (Okinawa, Japan)
		KU629853	99.23	Seaweed, *Asparagopsis* sp. (Portugal)
		GU061986	99.23	Oceanic water (South China Sea)
unclassified ***Cryomorphaceae***	ASV_018	KY374050	100.00	***Acropora hyacinthus*** (Central Red Sea)
		KF185498	99.60	Marine snow (Adriatic Sea)
		KU648372	95.65	Seawater (Gullmarsfjorden, Sweden)
unclassified ***Hyphomonadaceae***	ASV_020	KY455482	100.00	***Acropora hemprichii*** (Central Red Sea)
		KY373570	100.00	***Acropora hyacinthus*** (Central Red Sea)
		CP017718	100.00	Algal culture, coral reef substrate (Palmyra Atoll, Pacific Ocean)

Discussion

We employed 16S rRNA gene amplicon sequencing to characterize the bacterial communities of symbiotic and menthol-bleached *G. fascicularis* polyps from the Red Sea and Hong Kong that were long-term aquarium reared in separate facilities.

Menthol bleaching seemingly led to stochastic changes in the microbiome of Galaxea

Menthol bleaching appeared to be associated with changes in the bacterial communities that differed between individual polyps and produced stochastic configurations. Unlike previous studies on Aiptasia that

compared bleached and symbiotic individuals and found significantly different microbiomes (Röthig et al. 2016; Curtis et al. 2023), we could not identify a "signature of bleaching" as no bacterial taxa showed differential abundance between symbiotic states in our Galaxea colonies. This surprisingly included Symbiodiniaceae-associated bacteria that we were expecting to be reduced after the removal of Symbiodiniaceae (Fig. S5; Supplementary Materials and Methods). Such results could be an artifact of whole-tissue sampling in this study. As different coral compartments host distinct bacterial communities (Sweet et al. 2011; Ainsworth et al. 2015; Apprill et al. 2016), changes at the level of the gastroderm, where Symbiodiniaceae and their bacterial communities are located, might have been masked. Alternatively, it might indicate that Symbiodiniaceae-associated bacteria only repesent a small proportion of the community in Galaxea, or that they were able to persist in the absence of Symbiodiniaceae.

The lack of significant changes in bacterial taxa abundance between symbiotic and menthol-bleached polyps is surprising also given the known antimicrobial activity of menthol. Despite its effectiveness against various bacteria (i.e., *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* spp., *Lactobacillus* sp.) and its demonstrated selective activity on cigarette microbiomes (Trombetta et al. 2005; Chopyk et al. 2017; Mahzoon et al. 2022), direct impact of menthol on coral-associated bacteria may have been limited. Factors such as concentration, exposure time, and the varying susceptibility of bacterial species, alongside interactions with the coral environment and other microorganisms, likely contribute to the lack of an observable effect. These aspects, as well as further exploration of onset, duration, and specific mechanisms of action of menthol represent important areas for future research to elucidate its impact on coral-associated microbial communities.

A stochastic response to bleaching aligns well with the concept of an obligatory nature of the coral-algal symbiosis. In facultatively symbiotic cnidarians (such as the anemone Aiptasia), symbiotic and bleached can constitute alternative stable states, and it is thus possible to identify distinct and characteristic symbiotic and bleached microbiome configurations (Röthig et al. 2016; Curtis et al. 2023). Corals however depend on their algal symbiont for energy and other essential metabolic processes (Muscatine and Porter 1977; Muscatine 1990), and therefore the bleached state is not a stable alternative to the symbiotic state. In the bleached state the weakened coral host becomes progressively unable to regulate its microbial community, leaving room for the establishment of opportunistic bacteria, producing novel and stochastic combinations (Zaneveld et al. 2017).

The response of the bacterial microbiome to menthol bleaching seemed to differ between the two facilities. While we cannot draw definitive conclusions due to low replication, it is possible that differences in patterns of microbial response to menthol bleaching were linked to differences in rearing conditions. While these were largely replicated between facilities, feed type, tank volume and filtration systems differed. We therefore suggest that future studies incorporate an adequately replicated "facility" factor in their design, as well as food and seawater samples to better characterize these aspects.

The microbiome of long-term aquarium-reared *Galaxea fascicularis*

The *G. fascicularis* polyps hosted simple microbiomes which were composed of a relatively small number of bacterial taxa (10-78 ASVs). While direct comparisons across studies that employed different PCR, sequencing, and analysis pipelines should generally be avoided, such numbers still appear small compared to previous characterizations of wild *G. fascicularis* from the South China Sea which reported 646-1,459 OTUs (Li et al. 2013) and compared to most other coral species, which typically harbor 100s to 1000s of bacterial taxa (e.g., Ziegler et al. 2016; Hernandez-Agreda et al. 2018; Pollock et al. 2018; Galand et al. 2023). We are unable to tell whether captivity caused a reduction in bacterial diversity as we lack direct comparison with the original wild colonies. However, we suspect that captivity favored a simplification of the microbiome, as

stable and homogenous environmental conditions decrease both chances and need for the association with functionally and taxonomically diverse microbial partners. In fact, decreases in metabolic diversity and species richness have consistently been reported for tropical reef-building corals reared in closed systems (Kooperman et al. 2007; Vega Thurber et al. 2009; Pratte et al. 2015; Damjanovic et al. 2020), and for the anemone Aiptasia already after a few days of captivity (Hartman et al. 2020). Such effects could have been exacerbated by the use of filtered seawater during the bleaching phase, which largely reduced the pool of available microbes (Dungan et al. 2021b), and by the reduced structural complexity of the polyps that, compared to colonies, provide fewer micro-environments and ecological niches (Putnam et al. 2017; Morrow et al. 2022).

Regardless of the causes, simple (or simplified) microbiomes present the opportunity to identify essential associates and facilitate the development of microbial manipulation protocols to unravel holobiont functioning (Jaspers et al. 2019; Puntin et al. 2022b). Culturing corals in sterile seawater may help to limit the horizontal acquisition of transient microbes and thus favor proliferation of core or stable members for detailed characterization (Dungan et al. 2021b). Also, a simple microbiome facilitates the elimination of bacterial populations to produce gnotobiotic or axenic hosts, which could subsequently be re-inoculated to produce a range of host-bacteria combinations to test microbial functions and inter-partner dynamics (Fraune et al. 2015; Murillo-Rincon et al. 2017; Jaspers et al. 2019; Taubenheim et al. 2020). Reduced microbial complexity—whether due to captivity or other factors—might therefore provide advantages for these specific experimental approaches with the Galaxea model.

Interestingly, the coral colonies tested here maintained distinct bacterial microbiomes even after long-term co-culturing, which supports a degree of host genotype effects controlling the microbiome composition, as previously reported from Hydrozoan and other coral species in the field (Pollock et al. 2018; Dubé et al. 2021). Surprisingly, the microbiome of one Red Sea colony was highly similar to that of Hong Kong colonies. This appears counterintuitive as colonies from the Red Sea and Hong Kong may belong to different *Galaxea* lineages (*sensu* Wepfer et al. 2020), and considering the large differences in environmental conditions at their origin. While these colonies were also maintained in separate facilities, rearing conditions were similar at both locations (i.e., temperature, salinity, illumination) and may have induced convergence of microbial community composition (Dubé et al. 2021). On the other hand, *G. fascicularis* is a polyphyletic species that contains several morphologically cryptic lineages (Wepfer et al. 2020) and the dissimilarity between colonies from the Red Sea could reflect host phylogentic differences (i.e., cryptic species within the same region).

Besides host genotype, Symbiodiniaceae community composition could also explain differences in bacterial community composition between colonies (Littman et al. 2010; Bernasconi et al. 2019). To investigate this aspect, we also characterized the Symbiodiniacea communities of the same polyps (Supplementary Materials and Methods). While only a small proportion of samples were successfully sequenced, we noticed emerging patterns of Symbiodiniaceae-bacteria co-occurrence that warrant further investigation (Fig. S5). We therefore recommend that future studies characterize a larger number of Galaxea holobionts at multiple locations across the species distribution range to explore links between host-Symbiodiniaceae-bacteria associations. This could elucidate the influence of each member on coral holobiont compositions and functioning.

Core bacterial associates of Galaxea

We defined core or stable microbial associates based on prevalence of ASVs across treatment groups and polyps. Notably, no taxa was present in 100 % of the samples, suggesting a certain degree of microbiome variability within this coral host species. Among the 28 core ASVs (occuring in all groups), the five most

frequent and abundant were assigned to the genera *Alteromonas* and *Ruegeria*. Both *Alteromonas* and *Ruegeria* are common coral-associates reported from at least 20 other coral species, and sequences assigned to these two genera ranked 6th and 33rd most abundant in the Coral Microbial Database (Huggett and Apprill 2019).

Ruegeria spp. are commonly and consistently found in association with *G. fascicularis* in wild and aquarium-reared colonies, from Hong Kong to Japan and across seasons (Cai et al. 2018a, 2018b; Miura et al. 2019; Tang et al. 2020; Kitamura et al. 2021). Indeed, the two *Ruegeria* core ASVs had identical sequences with *Ruegeria* from *G. fascicularis* from Hainan and Japan that were maintained under aquarium conditions comparable to ours (Miura et al. 2019; Zhou et al. 2020). This shows that the Galaxea-*Ruegeria* association is highly conserved and therefore putatively biologically relevant. *Ruegeria* strains isolated from *G. fascicularis* were indeed previously identified as potential probiotics, through inhibitory activity towards the coral pathogen *Vibrio coralliilyticus* (Kitamura et al. 2021). The ubiquitous and persistent Galaxea-*Ruegeria* association thus warrants attention in future investigations.

The potential role of *Alteromonas* spp. in the Galaxea holobiont functioning also deserves attention. Despite their high abundance and prevalence in this study (and in corals in general), the role of *Alteromonas* spp. remains controversial. They have been considered pathogenic, owing to their co-occurrence with coral diseases (e.g., Sunagawa et al. 2009), but also listed as candidate probiotic for their free radical scavenging abilities (Raina et al. 2009; Dungan et al. 2021a), which could be linked to their consistent association with Symbiodiniaceae (Lawson et al. 2018; Nitschke et al. 2020).

Bacteria in the family *Endozoicomonadaceae* are the most prominent members of coral microbiomes in a range of coral species (Morrow et al. 2012; Bayer et al. 2013; Neave et al. 2017; Pogoreutz et al. 2022), which have been investigated for their involvement in holobiont metabolism, for example in the C and S cycles (Neave et al. 2016; Ide et al. 2022). Yet, *Endozoicomonadaceae* were only present in approximately half of Galaxea polyps, at between 0.06 and 39.12 % (mean 5.78 %) relative abundance. This is slightly lower than in wild *G. fascicularis* from Hong Kong (< 10 % relative abundance) in which, however, *Endozoicomonas* spp. were present in all samples (Cai et al. 2018a, 2018b). Such scarcity and inconsistent presence therefore suggest that *Endozoicomonas* spp. might not play an essential role in the *G. fascicularis* under captive conditions. If further proven, a lack of reliance on *Endozoicomonas* spp. in captivity could offer insights into which functions benefit from microbial help in the wild, hence highlight the role of this bacterial associate in the coral holobiont in the wider context.

Conclusions

Model organisms provide powerful tools for unraveling holobiont complexity. These models can be used to test hypotheses of functional relationships and inter-partner interactions through holobiont manipulation. To complement current cnidarian model systems such as Aiptasia and Hydra, we recently proposed the adoption of *Galaxea fascicularis* as a true coral model owing to its suitability to aquarium rearing and reproduction, and manipulation of its association with Symbiodiniaceae following menthol bleaching (Puntin et al. 2022a). However, how this bleaching treatment affected the bacterial microbiome remained to be explored.

In this study, we provided the first baseline assessment of the response of the Galaxea bacterial microbiome to menthol bleaching. Overall, menthol bleaching appeared to induce stochastic changes in the microbiome seemingly indicating dysbiosis. The response of the bacterial microbiome to bleaching differed in trend between the two facilities, possibly reflecting differences in rearing conditions, which remain to be addressed. Despite co-culturing in shared aquaria, symbiotic polyps originating from different colonies

maintained distinct community assemblies. This suggests links to host and/or Symbiodiniaceae identity that warrant further investigation.

Bacterial communities of the captive Galaxea colonies were composed of a small number of taxa. A simple microbiome could facilitate both characterization and experimental manipulation, and guide the identification of essential ("core") members among the retained associates. In this regard, we identified *Ruegeria* spp. and *Alteromonas* spp. as candidate associates for further functional interrogation. In conclusion, our study contributes valuable information towards a better characterization of the Galaxea holobiont, as well as its continued development and establishment as a coral model system.

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Data, scripts, code, and supplementary information availability

Data are available online:

- Raw sequencing data submitted to NCBI SRA BioProject PRJNA947274;
- Representative sequences (ASVs) submitted to NCBI GenBank Accession numbers OQ677536 to OQ677992;
- Additional data, scripts, and code are available online at https://doi.org/10.5281/zenodo.10551928 (Puntin 2024a);
- Supplementary Tables, Figures, and Materials and Methods are available online at https://doi.org/10.5281/zenodo.11651776 (Puntin 2024b).

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

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Chapter 5

This thesis sets out to establish *Galaxea fascicularis* as a representative model for coral research, addressing limitations of existing cnidarian models and therefore proposing the establishment of a true reef-building coral as model organism. Each chapter contributes essential findings toward validating the potential of Galaxea as a research tool for studies aiming at understanding coral holobiont functioning (Figure 2). First, *G. fascicularis* is contextualized within the broader field of coral research by identifying the gaps that current models leave unaddressed (Chapter 2). Then, experimental work tested the compatibility of Galaxea with laboratory rearing, manipulation of coral-Symbiodiniaceae symbiosis, and sexual reproduction in captivity (Chapter 3). Finally, investigations provide insights into the microbiome dynamics of

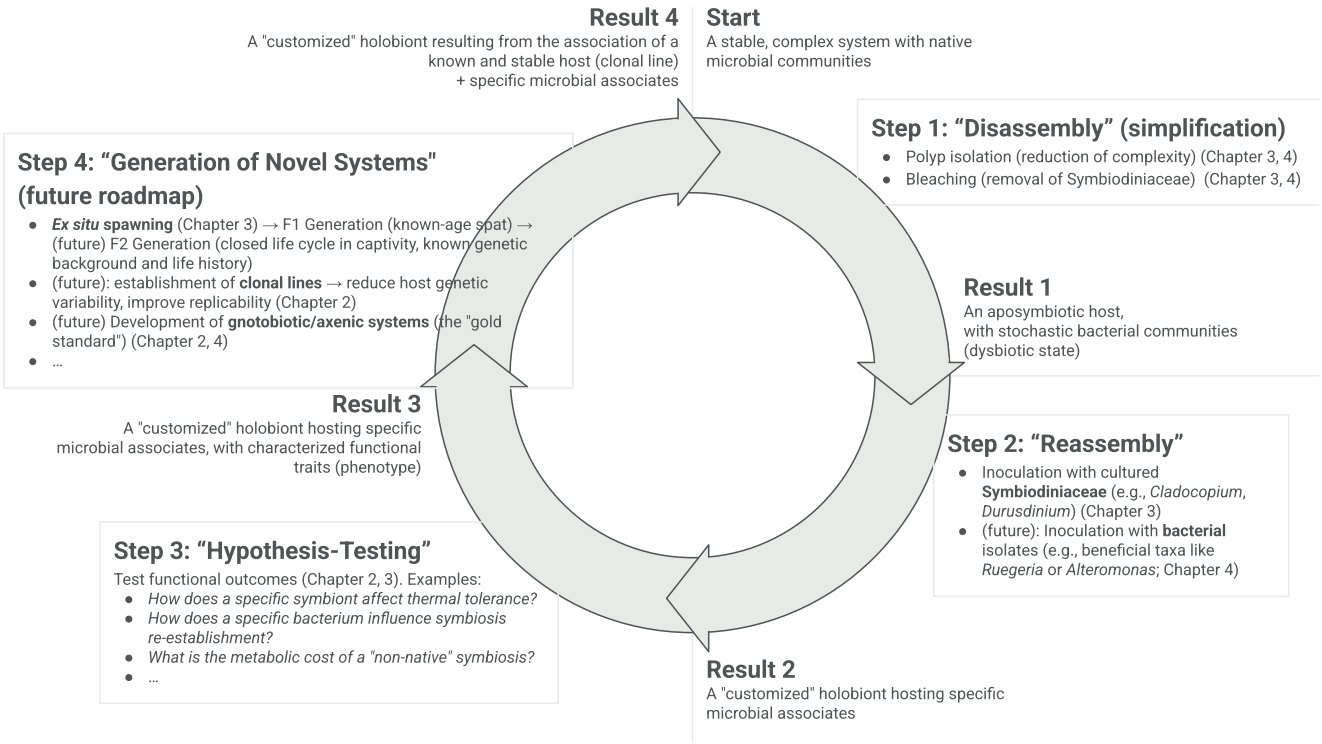

Figure 2 | The Galaxea model system: an experimental framework for symbiosis research

This diagram synthesizes the primary contributions of this thesis, illustrating a complete "disassembly-reassembly" workflow that facilitates hypothesis-driven experimentation on a reef-building coral. The process begins with the **0) stable holobiont**, represented by long-term aquarium-reared colonies that maintain stable, host-specific microbiomes. This thesis validated methods for experimental **1) "Disassembly"**, primarily using menthol bleaching to reliably produce viable, aposymbiotic hosts (Chapters 3, 4). These simplified, aposymbiotic hosts are amenable to **2) "Reassembly"**, as demonstrated in Chapter 3 by the successful re-establishment of symbiosis with non-native cultured Symbiodiniaceae strains (*Cladocopium* spp and *Durusdinium* spp). This capacity to create "customized" holobionts establishes a platform for **3) "Hypothesis-Testing"**, allowing for controlled experiments to test the functional consequences (e.g., thermal tolerance, metabolic performance) of specific host-microbial pairings. Finally, the framework is completed by the demonstrated potential for **4) "Generation of Novel Systems" (future roadmap)**. This pathway is initiated by the successful induction of *ex situ* spawning (Chapter 3), which produces a known-age F1 generation. This provides the crucial first step towards closing the life cycle in captivity, thereby sexually producing a new generation (F2) with known genetic background and life history. Such advancements are necessary for the future establishment of standardized clonal lines to reduce host genetic variability and improve experimental replicability (Chapter 2). Ultimately, this provides the foundation for developing gnotobiotic or axenic systems—the "gold standard" for mechanistic studies—to fully dissect holobiont partner functions (Chapter 2, 4).

G. fascicularis under symbiotic and bleached states, setting a basis for further holobiont studies (Chapter 4). While these combined findings position *G. fascicularis* as a versatile and promising system for advancing controlled experimental frameworks in coral holobiont research, limitations that remain to be addressed and further needed developments are also discussed to guide future development of the Galaxea model.

5.1 | Suitability of *Galaxea fascicularis* as a coral model

The central contribution of this thesis is the validation of *Galaxea fascicularis* as a robust model system for coral holobiont research. This section evaluates its suitability by assessing two fundamental criteria for any model organism: tractability, which covers its practical utility in experimental settings, and transferability, which considers the ecological representativeness and broader relevance of the findings (Table 1).

5.1.1 | Tractability: Suitability of *Galaxea fascicularis* to experimental work

Tractability, the ease of maintaining and manipulating an organism in laboratory settings, is crucial for establishing *G. fascicularis* as a model for coral holobiont research (Puntin et al., 2023, 2022; Weis et al., 2008). This section examines its compatibility with captive rearing, reproductive success in controlled environments, amenability to holobiont manipulations, and generally practical advantages for experimental work.

5.1.1.1 | Compatibility with captivity and experimental conditions

Long-term maintenance

The coral species *G. fascicularis* has proven well-suited for long-term aquarium maintenance. Colonies collected from the Red Sea in 2019 (Puntin et al., 2023) have thrived in captivity within the Ocean2100 system for over five years. During this time, they underwent multiple propagation cycles and participated in numerous experiments (beyond this thesis work), underscoring their adaptability to artificial conditions (Bauer et al., 2025; Ferrara et al., 2025, 2024; Million et al., 2025). While some coral species, like *Stylophora pistillata*, have been successfully maintained in aquaria for decades (Venn et al., 2020, p. 202), many reef-building corals are difficult to sustain long-term due to their sensitivity to fluctuations in water quality, temperature, light, and nutrient levels (Craggs et al., 2017; Schubert and Wilke, 2018). These requirements demand significant expertise and resources. Within this context, *G. fascicularis* stands out as a robust and adaptable candidate, capable of enduring and thriving under artificial conditions.

Besides long-term compatibility with aquarium rearing, Galaxea can also be maintained in simplified systems (Chapters 3 and 4). Fragments (nubbins) and individual polyps have been successfully kept for several weeks in small-scale setups, such as 5-L tanks with basic water circulation and 6-L tanks equipped with simple filtration systems, regular feeding, and water changes (Puntin et al., 2024, 2023). Furthermore, coral fragments and polyps have survived for weeks in basic beaker setups with air bubbling for oxygenation and circulation, alongside temperature-controlled environments and consistent maintenance routines. However, a limitation of these approaches was that they only confirmed short-term success, and the question of whether long-term viability of months to years could be achieved in such minimalist settings remained untested by this thesis work.

This question has recently been addressed by the development of the "coral-on-a-laboratory dish" (CLD) system, which allows for the long-term culturing of coral micro-fragments (~5 mm) in standard petri dishes within a laboratory incubator (Shikina et al., 2023). This approach was successfully used to maintain microcolonies of five common coral species for several months, with the corals retaining key biological properties, including reproduction, calcification, and symbiosis (Shikina et al., 2023).

Although this method has not yet been tested on *G. fascicularis*, its proven robustness and adaptability, as demonstrated in this thesis, make it a strong candidate for this new rearing and study system. The CLD system represents a technological advancement that could further enhance the utility of the Galaxea model by enabling the scaling up of standardized micro-scale investigations.

5.1.1.2 | Reproduction in captivity

A key component of tractability is the ability to sustain reliable sexual reproduction in captivity. *G. fascicularis* has partially demonstrated this capability through successful gametogenic cycles (spawning events), induced under controlled aquarium conditions, and enabled by seasonal programming and targeted husbandry (Puntin et al., 2023; Wei et al., 2023) (Chapter 3). Over multiple years, *ex situ* spawning has been reliably achieved, and followed by successful *in vitro* fertilization, embryological development, and larval settlement, hence demonstrating the possibility to sexually produce a new (first) generation of corals in captivity (F1). These efforts have enabled the production of spat with known ages, providing a unique opportunity to study early developmental stages under controlled conditions (Puntin et al., 2023; Wei et al., 2023). The ability to track larval development *in vitro* offers valuable insights into coral developmental biology and onset of symbiosis, as learnt from the Aiptasia model (Bucher et al., 2016; Hambleton et al., 2014).

Completing the life cycle in captivity, however, requires that these F1 corals reach sexual maturity and undergo their own gametogenic cycle, fertilization and settlement to produce an F2 generation (Craggs et al., 2020). This has not yet been achieved for *G. fascicularis*, primarily due to time constraints: corals generally require at least 2–3 years to reach sexual maturity, depending on species and growth rates (Baria et al., 2012; Guest et al., 2023). In fact, the exact timing for *G. fascicularis* remains unknown. However, these preliminary results are encouraging. Achieving a closed life cycle would be pivotal for research, eliminating the need for wild collection—reducing costs, saving time, and addressing ethical concerns (Craggs et al., 2020). Importantly, it would enable comprehensive investigations into all life stages, providing researchers with a continuous supply of specimens for various studies (discussed in more detail in 5.3 Future avenues and perspectives).

5.1.1.3 | Amenability to holobiont manipulations

Holobiont manipulations are essential for dissecting the complex interrelationships within the coral metaorganism (Jaspers et al., 2019). Simplifying the holobiont by selectively removing specific partners, such as Symbiodiniaceae, allows researchers to investigate the individual contributions of each member and test hypotheses about their functional roles (Weis, 2019).

This thesis successfully employed and refined a menthol-based bleaching protocol to selectively remove Symbiodiniaceae from *G. fascicularis*, producing aposymbiotic corals without inducing direct host mortality, on multiple occasions (Bauer et al., 2025; Puntin et al., 2024, 2023) (Chapters 3 and 4). This chemical approach offers a controlled means of inducing an aposymbiotic state, addressing significant challenges associated with traditional heat-stress methods (Gates et al. 1992; Wang et al. 2012). Namely, heat-induced bleaching often yields inconsistent outcomes, causes high mortality rates, and introduces confounding physiological stress on the host, complicating the interpretation of results (Dani et al. 2016). In contrast, menthol treatment decouples symbiont loss from thermal stress by acting more specifically on the photosynthetic apparatus of the algae, thereby minimizing collateral damage to the host (Clowez et al., 2021; Wang et al., 2017, 2012). Furthermore, this method avoids the pre-selection of heat-tolerant holobionts, ensuring a more representative sample for experimental studies

(Wang et al. 2012; Silverstein et al. 2014; Matthews et al. 2015). The work in this thesis also highlights the species-specific nature of menthol bleaching, a critical consideration for establishing standardized protocols. The protocol used in Chapters 3 and 4 (an adaptation of Wang et al., 2012) proved highly effective for *G. fascicularis*. However, subsequent research by our team, which tested four different protocols on six coral species, demonstrated that bleaching tolerance varies greatly among species (Bauer et al., 2025). For instance, while species like *Stylophora pistillata* bleached efficiently with just two days of menthol exposure, *G. fascicularis* required a longer (six days) treatment to achieve a comparable reduction in symbiont density (> 95 %) (Bauer et al., 2025). This confirms that menthol is a versatile tool, and its application could be further improved after empirical optimization for target species to ensure maximum efficacy and host survival.

Importantly, the aposymbiotic corals and polyps produced in this thesis were amenable to subsequent re-establishment of symbiosis with various cultured Symbiodiniaceae strains (Chapter 3). This ability to "disassemble and reassemble" the holobiont is fundamental for experimental hypothesis testing (Jaspers et al., 2019; Weis, 2019). The findings here align with parallel work from Scharfenstein et al. (2022), who provided compelling evidence that adult corals from six species across both the Complexa and Robusta clades can acquire novel, cultured symbionts from the environment after chemical bleaching (menthol combined with DCMU). This confirms that the phenomenon is not limited to the shuffling of rare, pre-existing symbionts but involves the genuine acquisition of new partners from the environment (Puntin et al., 2023; Scharfenstein et al., 2022). Together, these studies demonstrate that chemical bleaching is a viable method for manipulating adult coral symbioses (Puntin et al., 2023; Scharfenstein et al., 2022). This capacity makes *G. fascicularis* a versatile platform for studying symbiotic dynamics, partner specificity, stress tolerance, and recovery processes in a controlled experimental setting—all of which are critical for understanding the fundamental aspects of coral biology and developing strategies to enhance resilience (Jaspers et al. 2019; Weis 2019).

5.1.2 | Transferability: informative power and ecological representativeness

While the previous section established the experimental tractability of *G. fascicularis*, its ultimate value as a model system hinges on the transferability of research findings. This concept assesses how well insights from a single species can be generalized to the broader group it is intended to represent—in this case, the approximately 800+ species of tropical reef-building corals (Scleractinia) (DeVantier and Turak, 2017; Rassmussen et al., 2025; Veron et al., 2016). These corals exhibit huge diversity in their phylogeny, colony morphology, habitat preference, reproductive strategies, and symbiotic relationships (Madin et al., 2016; Veron et al., 2016). Given this vast functional and ecological variety, no single species can serve as a perfect analogue for all others. Therefore, this section critically examines the key traits of *G. fascicularis* to delineate its representativeness, clarifying both the strengths that make it an informative model and the limitations that define the scope of its applicability (Table 1).

5.1.2.1 | Geographic distribution

G. fascicularis is broadly distributed across the Indo-Pacific region, ranging from the Red Sea and East African coastlines to the Pacific islands east of Australia. This extensive range encompasses approximately 67% of global coral ecoregions (as defined by Corals of the World (Veron et al., 2016)) and includes biodiversity hotspots such as the Coral Triangle, where the highest coral diversity is found (DeVantier and Turak, 2017). However, it is important to note that *G. fascicularis* does not occur in the Atlantic, home to several endemic species and providing critical ecological roles threatened by climate change (Evangelista et al., 2023;

Table 1 | Summary of advantages and limitations of *Galaxea fascicularis* as a model system

Traits are evaluated based on experimental Tractability (practical advantages) and Transferability (generalizability of insights).

Suitability as a coral model	Trait	Explanation	*G. fascicularis*: Advantages	*G. fascicularis*: Limitations
Tractability What makes the chosen species a practically more convenient study subject	Availability	Abundance and broad geographic distribution facilitate the initial acquisition of individuals.	Broad geographic distribution, occupying ~67% of the global coral reef eco regions, and ~76% of the Indo-Pacific eco regions (CotW).	Not a good representation of the Atlantic region.
	Compatibility with Aquarium Rearing	Ability to thrive in controlled, simplified environments for long-term rearing, reducing variability and reliance on wild colonies.	Thrives in aquaria, also compatible with closed and simplified systems.	Might not represent well a large porportion of corals that typically tolerate only a narrow range of conditions.
	Amenability to Symbiosis Manipulations	Includes bleaching, maintenance in an aposymbiotic state, and reestablishment of symbiosis to enable detailed studies of coral-algae relationships.	Demonstrated ability to undergo menthol bleaching and reestablish symbiosis with both native and non-native Symbiodiniaceae.	Microbiome composition post-bleaching is rather unpredictable. Unclear whether this is specific to ***G. fascicularis*** or to corals in general.
	Polyp Size	Larger polyps simplify experimental manipulations (reduce complexity from colonial to individual), and visual examinations.	Large polyps that can be easily separated, reduce complexity of colony to single-polyps.	Morphological simplicity of polyps may limit representativeness of structural and functional complexity of typical coral colonies, possibly effecting physiological responses. Single polyps are not suitable for hanging (need to be glued to a support), requiring more maintence effort (cleaning).
	Reproductive & Propagation-Related Traits	Includes: potential for closed life cycles in captivity and controlled spawning; polyp bailout. Provide easy access to different life stages, facilitate tissue culturing and propagation.	Completes gametogenic cycle in aquaria (***ex situ*** spawing and production of F1 generation) enabling access to various life stages and allowing reproductive studies.	Clonal propagation is less straightforward than in branching corals, requiring more effort and resulting in higher resource use.
Transferability How well insights from the model organism can be generalized to the taxonomic or ecological group it is intended to represent	Geographic Distribution	Ensures relevance across various ecological contexts by selecting species broadly distributed and abundant in key regions.	Relatively common across its distribution range (although not typically dominat).	Distribution is limited to specific regions in the Indo-Pacific, reducing applicability to other coral reef ecosystems.
	Phylogeny	There are > 800 spp of reef-building corals (Phylum: Cnidaria, Class: Anthozoa, Order: Scleractinia). The choice of model organisms species should be representative of this diversity.	Member of the Complexa clade. Family Euphylliidae.	Coral species belonging to the clade Robusta are not represented.
	Colony Morphology	Branching versus massive growth forms; correlates with thermotolerance and environmental conditions (i.e., flow, light).	Massive or columnar; can be several m large, but usually small and cushion-shaped.	Does not represent branching and massive colony types (and typically not the main structural contributor to coral reefs).
	Trophic Strategy	Reflects a spectrum from relatively autotrophic (highly reliant on photosymbiont) to heterotrophic (less reliant on photosymbiont); underlies symbiosis synamics and sensitivity to fluctuations in external conditions.	Presumably rather on the heterotrophic side of the spectrum given the large poly size and peculiarly develop prey-capturing ability (sweeping tentacles). However, positioning on the tropich spectrum might depend on environmental conditions.	Presumably not a goood representative of more autotrophic species (e.g., ***Acropora*** spp.). Note that tropich strategy correlates to thermal tolerance (and possibly other physiological traits).
	Habitat Preference	Preference and ability to tolerate variability with regard to depth, salinity, temperature, light availability, flow, ect.; determines potential acclimatization to stressors.	Tolerates a wide range of habitats, but prefers inshore fringing reefs and environments protected from strong wave action (COTW).	Not a good representation of coral species adapted to more exposed reef environments.
	Reproductive Mode	Broadcast spawning versus brooding, which affects microbiome transmission and transgenerational adaptations.	Broadcast spawning, like most coral species, although with but with peculiarity (pseudogynodioecious). Larvae likely to acquire most of their microbial associates horizontally (from the environment), linked to higher plasticity and flexibility in microbial association.	Not representative of brooding species.
	Symbiosis Flexibility	Ability to associate with diverse Symbiodiniaceae strains; linked to adaptive capacity to environmental changes. However, a metric to quantify symbiosis flexibility/specificity is still lacking.	Known to naturally associate with Cladocopium and/or Durusdinium spp.; Adults able to establish symbiosis with non-native strains (experimental manipulation). Conducive to studies on host-symbiont compatibility.	Might not represent well species with high symbiont specificity.

Kuffner and Toth, 2016; Principe et al., 2021). To fill this representativity gap, species such as *Orbicella faveolata*, a major reef builder in the Caribbean (Puntin et al., 2022; Szmant and Miller, 2006), as well as other representative and extensively studied species as for example *Mussismilia hispida*, *Montastraea cavernosa*, *Siderastrea* spp., and *Favia* spp. (Adam et al., 2024; Godoy et al., 2021; Gutierrez et al., 2024; Picciani et al., 2016; Principe et al., 2021) should also be included in the collective effort to establish new coral models (Chapter 2).

5.1.2.2 | Phylogeny

As a member of the Complexa clade (family Euphylliidae), *G. fascicularis* represents one of two deep evolutionary lineages within Scleractinia, which diverged from the Robusta clade over 245 million years ago (Romano and Palumbi, 1996). This fundamental split is supported by distinct genomic, morphological, and physiological traits with significant implications for research transferability (Han et al., 2022; Kerr, 2005; Thobor et al., 2024). For example, robust corals like *Pocillopora* possess a complete histidine biosynthesis pathway that is absent in complex corals, implying different nutritional and symbiotic strategies (Ying et al., 2018). Morphological differences in skeletal porosity and polyp-canal systems, along with divergent gene expression for calcification and stress response, further underscore this divide (Li et al., 2023). Consequently, while *G. fascicularis* is an excellent model for the Complexa lineage, its metabolic and symbiotic traits cannot be directly extrapolated to Robusta corals. This phylogenetic limitation necessitates a multi-model approach, complementing this work with species of the Robusta clade like *Pocillopora damicornis* or *Stylophora pistillata* (Kerr, 2005) to ensure findings have broader relevance.

5.1.2.3 | Colony morphology

G. fascicularis exhibits diverse colony morphologies that vary with size and age. Small colonies are typically cushion-shaped or low domes, while larger colonies can become columnar or massive, with some exceeding 5 meters in diameter in the wild (Crabbe and Smith, 2006; Madin et al., 2016; Veron et al., 2016). Corallites are relatively large, reaching up to 10 millimeters in diameter, which provides unique advantages for certain studies (Al-Horani et al., 2003; Marshall and Clode, 2004). However, this morphology differs significantly from other coral forms, such as the branching structures of *Acropora* or the encrusting and massive forms of *Porites*, both of which feature much smaller polyps (1 millimeter or less) (Madin et al., 2016; Veron et al., 2016). These morphological traits have important functional consequences. Colony shape dictates mass transfer, self-shading, and even the composition of the microbiome (Álvarez-Noriega et al., 2016; Morrow et al., 2022; van Woesik et al., 2012). Additionally, the large polyps of *G. fascicularis* enhance its heterotrophic feeding capacity, a trait correlated with increased resilience to thermal stress (Conti-Jerpe et al., 2020). This distinction is important, as species from the families Acroporidae, Pocilloporidae, and Poritidae constitute approximately 70% of reef-building corals worldwide (Reichert et al., 2022), making them critical targets for research due to their structural importance.

It is also important to consider that while wild colonies of *G. fascicularis* can grow to several meters in diameter, colonies raised in captivity are inherently constrained by the size of their aquarium tanks. Such limitations on colony size can affect the rates and relationships of key physiological processes such as calcification and biomass production, which in turn modulate the coral response to stressors, including thermotolerance (Álvarez-Noriega et al., 2016; Edmunds and Burgess, 2016; van Woesik et al., 2012). More broadly, regardless of the coral species under study, it is important to recognize that biological features observed in nubbins (simple and small) do not linearly scale to whole colonies (complex and large) and therefore cannot be accurately extrapolated

(Edmunds et al., 2022). Therefore, while the tractability of smaller-scale fragments is invaluable for laboratory work, researchers must be cautious when extrapolating findings to the complex, large-scale dynamics of wild colonies.

5.1.2.4 | Trophic strategy

G. fascicularis, like all corals, is mixotrophic, relying on both autotrophic feeding via photosynthesis and heterotrophic feeding through prey capture (Muscatine and Porter, 1977). However, *G. fascicularis* presents a rather pronounced trophic plasticity, with the ability to expand either its heterotrophic or autotrophic capacity depending on environmental conditions (Crabbe and Smith, 2006) (see more detailed discussion at 5.2.1.1 | Effects of Symbiodiniaceae removal on the coral host). This flexibility likely enhances its resilience and adaptability in laboratory settings, making it well-suited for experimental studies.

Despite these advantages, the trophic strategy of *G. fascicularis* may not fully represent the feeding behaviors of less flexible coral species, such as the more autotrophic *Acropora* species (Conti-Jerpe et al., 2020; Shinzato et al., 2011). Heterotrophic capacity is particularly relevant because it is often associated with higher thermal tolerance and increased resistance to thermal bleaching (Conti-Jerpe et al., 2020), an important factor to consider particularly in the context of stress response and resilience studies.

5.1.2.5 | Habitat preference

G. fascicularis predominantly inhabits inshore fringing reefs and areas sheltered from strong wave action (Madin et al., 2016; Veron et al., 2016). In aquarium settings, it similarly thrives under low to moderate flow conditions but suffers under high flow, which tends to cause damage to its soft tissues (personal obs.).

While this habitat preference provides valuable insights into corals adapted to similar environments, it limits the applicability of findings to species that dominate habitats with different conditions, such as deeper reefs or high-flow environments. Corals from families like Acroporidae, for example, often prefer and perform better in areas with strong water movement (Lee et al., 2017; Madin et al., 2016; Veron et al., 2016).

5.1.2.6 | Reproductive mode

G. fascicularis reproduces through broadcast spawning, a common strategy among the majority of corals (Baird et al., 2009). However, Galaxea stands out as the only genus exhibiting pseudo-gynodioecious reproduction, where female colonies produce red eggs and hermaphroditic colonies produce sperm and white eggs (Harrison, 1988; Suwa and Nakamura, 2018). This reproductive pattern has unclear implications, and distribution. While this reproductive mode was first described in colonies from Australia (Harrison, 1988), a study on colonies from Taiwan found that both red and white eggs could be fertilized and developed into viable larvae, prompting a reconsideration of its reproductive mode as gynodioecious (Keshavmurthy et al., 2012a). Nevertheless, the broadcast spawning mode suggests that larvae acquire most of their microbial associates horizontally from the surrounding environment (rather than vertically from their parents) (Baird et al., 2009; Sharp et al., 2010), likely contributing to higher microbial plasticity and flexibility, which could enhance the species' adaptability to varying environmental conditions (Voolstra and Ziegler, 2020; Ziegler et al., 2017).

5.1.2.7 | Symbiosis flexibility

G. fascicularis is considered a "flexible" species in terms of host-Symbiodiniaceae associations owing to its ability to associate with multiple Symbiodiniaceae species, typically from the genera *Cladocopium* and *Durusdinium*, which can be hosted exclusively or simultaneously (Million et al., 2025; Zhou et al., 2017; Zhou and Huang, 2011). Such flexibility could confer adaptive capacity (Voolstra and Ziegler, 2020). In fact, in *G. fascicularis* colonies, *Cladocopium* spp. are the dominant symbiont at more temperate locations, while

Durisdinium spp., which are generally associated with thermal tolerance (Berkelmans and van Oppen, 2006; LaJeunesse et al., 2009), becomes dominant at warmer sites at both regional and local scale (Tong et al., 2017; Zhou et al., 2017; Zhou and Huang, 2011). A similar pattern of shifting dominance along thermal gradients has been observed in other coral species (Bongaerts et al., 2010; Keshavmurthy et al., 2012b; Ziegler et al., 2015). Additionally, adult *G. fascicularis* colonies demonstrated the ability to establish symbiosis with non-native strains through experimental manipulation (chapter 3), suggesting that this species might be particularly adaptable in terms of symbiotic association. On the contrary, species like *Acropora cervicornis* and *Acropora palmata* show high fidelity to their dominant symbiont species across temperature gradients (Muller et al., 2018; Parkinson et al., 2018; Thornhill et al., 2006). This difference in symbiotic flexibility could affect the transferability of findings, as corals with strict symbiont associations may be more susceptible to stressors both in experimental settings and in the field.

However, a standardized framework for quantifying symbiosis flexibility (or specificity) is still lacking. In fact, there is still no generic agreement in the research community on how to interpret Symbiodiniaceae genetic data and hence characterize communities composition and diversity (Davies et al. 2023). For example, while the vast majority of corals appear to associate with one dominant microalgal symbiont, the functional importance of rare, background symbionts remains to be determined (Ziegler et al. 2018; Davies et al. 2023). Even more complicated is the assessment of flexibility and its functional relevance when considering the prokaryotic microbiome. As opposed to the relatively stable and exclusive nature of coral-Symbiodiniaceae associations, coral-associated bacterial communities are commonly much more diverse and more evenly distributed (Galand et al., 2023; Pollock et al., 2018). This disparity makes it difficult to draw meaningful comparisons between species and determine how the flexibility of *G. fascicularis* compares to that of other corals. The same issue also applies to the other, less characterized components of the holobiont, such as archaea and viruses (Pernice et al., 2020; Thurber et al., 2017; Wegley et al., 2007). Future research should aim to develop standardized methods for evaluating microbial flexibility across the entire holobiont (Chapter 2).

5.1.3 | Necessity of a multi-model approach

Given the extensive diversity within the coral group, relying solely on *G. fascicularis* as a model organism will not be insufficient to capture the breadth of coral biology and ecology. A multi-model approach, incorporating multiple coral species with varied traits and ecological niches, is essential to ensure comprehensive and representative insights (Puntin et al., 2022; Weis, 2019). This strategy leverages the strengths of different models to cover the diversity in phylogeny, morphology, and ecological adaptations. Complementing *G. fascicularis* with other species as coral models is necessary to provide a more holistic understanding of coral holobiont dynamics (Chapter 2).

To facilitate this, Table 1 summarizes the traits defining tractability and transferability, outlining the advantages and limitations of *G. fascicularis* as a model organism while highlighting areas where alternative species may be required. This synthesis helps identify research areas that would benefit from a broader suite of coral models. By developing and employing multiple model organisms, researchers can tackle the multifaceted challenges of coral biology, ensuring findings are robust and applicable across the diverse reef-building coral group.

5.1.4 | Challenges of representing a group that lacks clear definition and scope

Defining what constitutes "coral" presents a significant challenge due to the unresolved taxonomy within

the group. Many coral families and genera are poorly delineated, and misidentifications or misclassifications frequently arise from overlapping morphological traits and limited genetic data (Rassmussen et al., 2025). This taxonomic ambiguity complicates efforts to select a representative model species, and raises fundamental questions about the scope and applicability of generalizations drawn from such models.

Further complicating matters is the lack of clarity around the primary objectives of coral reef conservation initiatives (Boström-Einarsson et al., 2020). Is conservation aimed at protecting species (and if so, all coral species or just a prioritized subset?), ecosystems as a whole, or specific ecosystem functions and services (Bellwood et al., 2019a; Mulà et al., 2025; Vardi et al., 2021)? Not all coral species contribute equally to reef structure or function: some are keystone species that underpin biodiversity and ecosystem services, while others have more limited or redundant ecological roles (Bellwood et al., 2019b, 2004). To add to the complication is a lack of comprehensive data on these contributions, hampering our understanding of the matter (Bellwood et al., 2019a).

This uncertainty poses significant challenges when assessing the utility of the Galaxea model (and of any model organism). If the target organisms or conservation goals are undefined, it becomes difficult to evaluate representativeness or determine what the model should be measured against. While *G. fascicularis* may offer valuable insights into specific aspects of coral biology, its applicability to species or functions critical for reef resilience and conservation may be more limited. This calls for a strategic approach: balancing the need for broadly representative models with the prioritization of specific ecological and conservation goals that are most critical for coral reef preservation (Bellwood et al., 2019a; Mulà et al., 2025; Vardi et al., 2021).

5.2 | Insights into coral holobiont dynamics

Beyond assessing *G. fascicularis* as a model organism, this work also helped gaining insights into holobiont functioning from manipulations of the Galaxea-Symbiodiniaceae association. Specifically, as we disassembled and reassembled the holobiont by first removing and then reintroducing Symbiodiniaceae, it was possible to study how this affected the coral host and the associated bacterial microbiome, and tested hypotheses on symbiosis re-establishment.

5.2.1 | Disassembling the holobiont: removal of Symbiodiniaceae

5.2.1.1 | Effects of Symbiodiniaceae removal on the coral host

Bleached (aposymbiotic) polyps of *G. fascicularis* could survive for up to three weeks without their Symbiodiniaceae, relying on energy reserves and heterotrophic feeding to meet their metabolic demands (Chapter 3). Unlike facultatively symbiotic models such as Aiptasia, which can survive indefinitely without their algal symbiont (Matthews et al., 2015; Röthig et al., 2016), corals, as obligate symbiotic, are much less amenable to such manipulations. Yet the notable trophic plasticity of *G. fascicularis* contributes to its resilience.

While all corals are mixotrophic, their reliance on autotrophy versus heterotrophy varies significantly (Conti-Jerpe et al., 2020; Grottoli et al., 2006; Houlbrèque and Ferrier-Pagès, 2009). *G. fascicularis* exemplifies a species with relatively high heterotrophic capacity, a trait linked to its large polyps (up to 10 mm) that provide a greater surface area for prey capture compared to predominantly autotrophic species like *Acropora* spp. (~1 mm polyps), as supported by quantifications of feeding rates (Saper et al., 2023). This heterotrophic potential is not static; *G. fascicularis* actively modulates its physiology and morphology in response to environmental cues. For instance, under low light, it can optimize for heterotrophy by altering its corallite structure (Crabbe and Smith, 2006), and it

can leverage heterotrophic inputs to maintain skeletal growth and symbiont health at the expense of asexual reproduction, demonstrating a robust capacity to acclimate to variable resources (Yu et al., 2023). This adaptability confers distinct advantages, especially in nutrient-variable environments (Radice et al., 2019). Ultimately, this plasticity likely underpins the species' notable stress tolerance (Marshall and Baird, 2000), making it an insightful model for investigating the mechanisms of coral resilience.

However, despite the temporary mitigation of energy deficits through heterotrophic feeding, *G. fascicularis* could not fully compensate for the metabolic contributions of Symbiodiniaceae. Indeed, while the aposymbiotic polyps survived for three weeks and maintained feeding responses, they showed clear signs of physiological stress, such as the thinning of soft tissues (Puntin et al., 2022) (Chapter 3), and exhibited a destabilized, dysbiotic microbiome (Puntin et al. 2023) (Chapter 4). This suggests that extended survival beyond three weeks in an aposymbiotic state may be unlikely without additional interventions. The provided diet was insufficient to meet the host's full metabolic demands—a finding consistent with other obligate symbiotic cnidarians like the jellyfish model *Cassiopea xamachana*, which also loses mass even when fed abundantly in the aposymbiotic state (Röthig et al., 2021). This is not just an energetic limitation: Symbiodiniaceae provide a suite of structurally and metabolically essential compounds. For instance, corals are sterol auxotrophs, meaning they cannot synthesize essential sterols (like cholesterol) *de novo* and must therefore acquire them from their symbionts or prey to maintain functions involved in cell membrane structure and signaling (Hambleton et al., 2019). Similarly, several essential amino acids (EAAs) are synthesized and translocated by the symbiont to the coral host (Ferrier-Pagès et al., 2021; Wall et al., 2021). However, the degree of reliance on symbiont-derived EAAs can vary between coral species, with some showing more trophic plasticity and the ability to supplement their amino acid needs through heterotrophy (Ferrier-Pagès et al., 2021; Wall et al., 2021). The reliance on a single food source (Artemia) in our experiments likely failed to provide these and other vital micronutrients, leading to a significant nutritional deficit. This highlights the critical need for future investigations into optimized feeding regimens for maintaining aposymbiotic corals. Such studies could explore diets incorporating a wider variety of zooplankton and supplementing them with specific lipids and amino acids, thereby better supporting coral health and resilience during symbiosis manipulation experiments. For example, Wang et al. (2012) found that providing aposymbiotic *Isopora palifera* with a nutrient supplement containing a mixture of free amino acids, glycerol, and vitamins successfully restored metabolic indices to levels comparable to their symbiotic counterparts. Their work provides a clear precedent and a methodological foundation for developing the refined husbandry protocols necessary to advance the *G. fascicularis* model system.

Comparing the thermal performance of symbiotic and aposymbiotic polyps of *G. fascicularis* provided insights into the metabolic contributions of the host and Symbiodiniaceae. While respiration rates for both states increased with temperature as expected (consistent with thermal performance of corals from the region of origin of the colonies (Anton et al. 2020)), symbiotic polyps consistently exhibited higher holobiont respiration rates across the entire thermal range (21–32°C) (Chapter 3). This difference is likely a direct reflection of the total metabolic activity of the holobiont, encompassing the combined respiratory demands of both the coral host and its population of Symbiodiniaceae, with the latter enhancing the host's metabolic capacity by provision of energy as photosynthates (Muscatine et al. 1981; Al-Horani et al. 2002). However, the metabolic response to the loss of Symbiodiniaceae is not uniform and varies considerably among coral species. Indeed, several studies have reported no significant

difference in host respiration rates between symbiotic and aposymbiotic states in corals such as S*tylophora pistillata*, *Isopora palifera*, and the temperate coral *Astrangia danae* (Szmant-Froelich and Pilson, 1984; Wang et al., 2012). Similarly, (nighttime) respiration remained unchanged in bleached *Montipora capitata* and *Pocillopora compressa* (Rodrigues and Grottoli, 2007). These divergent outcomes can be attributed to both methodological and biological factors. Methodologically, the present study measured total holobiont respiration, whereas some studies, such as Wang et al. (2012), aimed to isolate host respiration *per se* by mathematically correcting for the respiratory contribution of the symbionts. Additionally, the host's nutritional strategy is also relevant: *Astrangia*, for instance, being facultatively symbiotic may possess a greater capacity to compensate heterotrophically for the loss of symbionts compared to obligate species like *G. fascicularis* (Sharp et al., 2017; Szmant-Froelich and Pilson, 1984).

Beyond these inter-specific differences, this thesis also revealed intraspecific variability, with polyps from one colony (RS2) exhibiting significantly higher respiration rates under dark conditions compared to the others, in both symbiotic states (Chapter 3). This finding suggests that host-specific factors, likely genetic, play a relevant role in shaping baseline physiological responses. This aligns with studies on corals like *Acropora hyacinthus*, where distinct host genotypes from the same reef exhibit inheritable differences in their thermal tolerance (Naugle et al., 2024). The principle of strong host-genotypic influence is further supported by the microbiome data from this thesis, where different *G. fascicularis* colonies maintained distinct bacterial communities even when reared together in a shared aquarium system (Chapter 4). This supports a degree of host control over the microbiome, a pattern previously reported from wild Hydrozoan and other coral species (Dubé et al., 2021; Pollock et al., 2018).

However, disentangling direct host effects from the influence of its symbiotic partners is complex given their long history of co-evolution (Thornhill et al., 2014). Intriguingly, the molecular characterization of the Symbiodiniaceae community revealed that the high-respiration colony (RS2) was the only one to host symbionts from the genus *Durusdinium* (ITS2 types D1, D4) prior to bleaching (Chapter 4). Associations with *Durusdinium* are generally understood to affect coral metabolism by conferring higher thermotolerance (Baker, 2003; Kemp et al., 2023; Silverstein et al., 2014). The higher respiration rate displayed by RS2 polyps, which was maintained in the bleached polyps, could therefore represent a physiological legacy or a "carry-over" effect of its former association with *Durusdinium*, and suggest that the deep metabolic integration may not be immediately erased upon the symbiont's removal. This however requires further testing as the current investigation was very limited in sample size (RS2: n = 2; RS3: n = 3). Nevertheless, these results highlight the importance and need of characterizing both host genotypes and their native symbiont communities, reinforcing the value of establishing representative clonal lines for future studies.

5.2.1.2 | Effects of Symbiodiniaceae removal on the bacterial microbiome

It is well-established that coral bleaching coincides with profound disruptions in the bacterial microbiome. A large body of research on corals bleached by heat stress, both in natural and laboratory settings, consistently reports a shift from a stable, symbiotic microbiome to a dysbiotic state (Boilard et al., 2020; Sweet and Bulling, 2017). This dysbiosis is typically characterized by an increase in opportunistic and potentially pathogenic bacteria and a decline in core symbiotic (putatively beneficial) taxa like *Endozoicomonas* (Kusdianto et al., 2021; Sajid et al., 2025; Sun et al., 2022; Zou et al., 2022). A major limitation of these studies is that thermal stress acts as a confounding variable, making it difficult to disentangle whether microbiome shifts

are a direct temperature response, a result of host physiological decline, or a consequence of losing the regulatory influence of the algal symbiont.

While previous studies characterized the bacterial microbiome of menthol-bleached cnidarians, such as Aiptasia and the jellyfish *Cassiopea xamachana* (Röthig et al., 2021), this thesis provides the first such characterization in a reef-building coral. By employing menthol to induce an aposymbiotic state in *G. fascicularis*, this work directly assesses how the absence of Symbiodiniaceae alone impacts the bacterial community, thereby decoupling symbiont loss from heat stress (Chapter 4). The results suggest that the removal of the algal symbiont is, by itself, sufficient to destabilize the bacterial microbiome. While symbiotic clonal polyps maintained distinct and stable microbiomes, the communities of bleached polyps became highly variable and diverged from one another (Chapter 4). This shift towards increased variability and seemingly stochastic community configurations is a classic hallmark of dysbiosis, where stressed systems become unpredictable as regulatory controls break down (McDevitt-Irwin et al., 2017; Zaneveld et al., 2017a).

This finding suggests that Symbiodiniaceae act as a key regulator of the holobiont's microbial landscape. This regulation likely occurs through several interconnected mechanisms. First, via indirect control: Symbiodiniaceae removal leads to host starvation, where a weakened state likely compromises host immunity and its ability to control its microbial population (Zaneveld et al., 2017b). Second, Symbiodiniaceae engage in direct niche provisioning by altering their immediate microenvironment (the "Symbiodiniaceae phycosphere") (Garrido et al., 2021). They release a significant amount of dissolved organic carbon and modulate local oxygen and pH levels, creating metabolic niches for specific bacterial populations, both inter- and intracellularly (Barott et al., 2015; Davy et al., 2012; Maire et al., 2021; Matthews et al., 2020). The loss of the algae eliminates these niches, contributing to the collapse of the established bacterial community structure (Claar et al., 2020; Lawson et al., 2020).

However, it is important to acknowledge that the menthol treatment itself may be a confounding factor. While the direct antimicrobial activity of menthol against coral bacterial communities remains uncharacterized, its efficacy against various human-pathogenic and other non-marine bacteria has been documented (Chopyk et al., 2017; Trombetta et al., 2005). Consequently, the observed stochastic shifts in the microbiome could result not only from the loss of the algal symbiont but also from a direct, selective antimicrobial action, or an interaction between the two. Therefore, a rigorous and standardized assessment of menthol's impact on coral-associated bacteria is warranted. The recent expansion of cultured coral-associated bacteria provides a timely opportunity to address this knowledge gap (Sweet et al., 2021). Future studies should systematically test the antimicrobial activity of menthol *in vitro* against a panel of representative bacterial isolates from *G. fascicularis* (Miura et al., 2019; Motone et al., 2020) or from other coral models. Employing standardized methods, such as minimum inhibitory concentration (MIC) assays, would allow for a quantitative assessment of potency and selectivity (Hamed and Hussein, 2020), thereby clarifying the role of menthol in shaping the microbiome during experimental bleaching.

Furthermore, the response to bleaching appeared to differ between the two rearing facilities, suggesting that extrinsic factors such as feed type, tank volume, and filtration systems play a significant secondary role in shaping microbial dynamics following a disturbance (Chapter 4). Intriguingly, we did not identify a consistent "bleaching signature"—a specific set of bacterial taxa that consistently changed—but rather a move towards stochasticity. Notably, even bacterial taxa typically associated with Symbiodiniaceae (i.e., *Labrenzia* spp., *Alteromonas* spp., *Marinobacter* spp.) were not significantly depleted post-bleaching in *G.*

fascicularis colonies (Puntin et al., 2024) –although it should be noted that there are inconsistencies among studies regarding the presence and abundance of "core" Symbiodiniaceae-associated bacteria (Camp et al., 2020; Lawson et al., 2017) which should be considered when interpreting their presence/absence. This lack of a distinctive pattern in the microbiome across different symbiotic states in Galaxea contrasts with what described for the facultatively symbiotic *Aiptasia*, where alternative stable microbial configurations (Curtis et al., 2023; Röthig et al., 2016), but aligns with results from the obligate symbiotic *C. xamachana* (Röthig et al., 2021). In an obligate symbiont like a coral, the bleached state is inherently unstable, which may favor random colonization over the establishment of a defined "aposymbiotic microbiome" (Puntin et al., 2024).

Ultimately, this work provides crucial evidence for the reciprocal nature of holobiont interactions. More and more studies highlight the importance of host-Symbiodiniaceae-bacterial interactions in determining holobiont health and function (Bernasconi et al., 2018; Claar et al., 2020; Haydon et al., 2021). However, most studies attempting to unravel the interplay between Symbiodiniaceae and bacteria tend to focus on how bacteria affect Symbiodiniaceae (Camp et al., 2020; Connelly et al., 2022; Matthews et al., 2023), and not the other way around. The findings from this thesis suggest that the health and stability of the bacterial community are also likely dependent on the presence of the algal partner–an area of research that warrants further investigations. This underscores the importance of viewing the holobiont as a complex network of multi-directional interactions, where symbiotic partners are intricately linked to the presence and function of one another.

5.2.2 | Reassembling the holobiont: re-establishing symbiosis with Symbiodiniaceae

A crucial step in testing hypotheses about the functional contributions of individual symbiont partners is the ability to reassemble the coral holobiont after experimental simplification. This thesis demonstrated the successful re-establishment of symbiosis in bleached adult *G. fascicularis* through inoculation with cultured, non-native Symbiodiniaceae strains (Chapter 3). This capacity for holobiont "disassembly and reassembly" provides a powerful experimental platform within a true reef-building coral (Chapter 2).

A key achievement was the targeted inoculation of aposymbiotic *G. fascicularis* polyps with *Durusdinium trenchii* (ITS2 type D1-4) and *Cladocopium goreaui (*ITS2 type C1) (Chapter 3). Both symbiont types successfully established, but *C. goreaui* became dominant despite being introduced at a lower concentration (9:1 ratio favoring D. trenchii). Although the culture was non-native, *C. goreaui* belongs to the same genus that dominates in wild *G. fascicularis* from Hong Kong, the origin of the host colonies (Huang et al., 2011; Puntin et al., 2024; Tong et al., 2017; Zhou and Huang, 2011). This outcome highlights the role of host-symbiont specificity in partner preference, in alignment with the general consensus in the field (Baker, 2003; Hume et al., 2020; Thornhill et al., 2006, p. 20006). Particularly noteworthy was the successful association with *D. trenchii*, a symbiont genus typically associated with higher thermal tolerance but absent from *G. fascicularis* in Hong Kong waters (Berkelmans and van Oppen, 2006; Huang et al., 2011; Palacio-Castro et al., 2023). The ability to establish such an atypical pairing experimentally suggests that host-symbiont combinations can be extended beyond their natural boundaries, creating opportunities to study the functional consequences of novel symbioses (Puntin et al., 2023; Scharfenstein et al., 2022; van Oppen et al., 2015).

However, the long-term persistence and functional stability of these novel partnerships remain to be assessed. Long-term monitoring is essential to determine whether these associations persist and translate into measurable physiological benefits. For instance, a long-term study outplanted *Acropora*

palmata colonies harboring a novel Durusdinium symbiont acquired in *ex situ* nurseries back onto the reef, and found that the majority of corals maintained this heterologous association for two years, supporting the potential for long-term stability (Elder et al., 2023). Yet, their study also revealed the complexity of these dynamics, as 15% of the corals showed some degree of symbiont shuffling or reversion to the native symbiont, even in the absence of bleaching stress (Elder et al., 2023). This indicates that even established novel symbioses may not be permanent. Furthermore, assuming functional benefits based on taxonomy alone can be an overgeneralization, as not all members of a given genus confer the same traits (Davies et al., 2023). Non-native associations can be less stable and may come with metabolic costs or perturbations to the host-symbiont nutrient exchange that compromise, rather than enhance, overall holobiont fitness (Cunning and Baker, 2020; Jones and Berkelmans, 2011; Pettay et al., 2015).

The experiments in this thesis were short-term and did not assess functional outcomes like calcification rate, growth, or stress response (Puntin et al., 2023). Therefore, a critical next step is to conduct long-term studies to determine if the novel associations established in *G. fascicularis* are stable over time and whether their presence translates into tangible benefits for the holobiont. Additionally, expanding the available Symbiodiniaceae cultures, particularly with strains isolated directly from *G. fascicularis*, will be essential for dissecting these intricate coral-algal interactions with greater precision.

Ultimately, this work confirms that *G. fascicularis* is a highly versatile model for symbiosis manipulation. Its demonstrated capacity for symbiosis re-establishment opens new avenues for investigating the fundamental mechanisms of partner recognition, compatibility, and the potential for corals to adapt through symbiotic flexibility.

5.3 | Future avenues and perspectives

The findings of this thesis support the value of *G. fascicularis* as a model for coral holobiont research; however, further development is essential to fully realize its potential. This section provides a roadmap for advancing the Galaxea model, highlighting key areas where focused research could expand its utility and further establish it as a standardized system for coral holobiont studies.

5.3.1 | Establish *Galaxea fascicularis* clonal lines

Developing well-characterized clonal lines of *G. fascicularis* is a crucial step in the development of model organisms (Chapter 2). Clonal lines provide a stable genetic baseline for the coral host, reducing genetic and life-history related variability and hence enabling more reproducible and comparable results across studies and laboratories (Dungan et al., 2020; Medina et al., 2021; Xiang et al., 2013). This, in turn, allows for deeper and more meaningful insights into coral holobiont dynamics under controlled experimental conditions (Weis, 2019; Weis et al., 2008).

To establish such lines, comprehensive genotyping of the coral host is the first essential step (Dubé et al., 2021; Pollock et al., 2018), followed by the characterization of its associated microbiome, including *Symbiodiniaceae* and key bacterial taxa (Bernasconi et al., 2018; Littman et al., 2010). Ideally, this characterization should expand to include all the microbiome components—archaea, viruses, fungi, and other protists—to develop a holistic understanding of the coral holobiont (Bourne et al., 2016; Pogoreutz et al., 2020). Over time, it will also be necessary to monitor genetic and microbial profiles to ensure the stability and integrity of the established clonal lines (Damjanovic et al., 2020; Hartman et al., 2020; Pratte et al., 2015).

Baseline physiological characterizations should also be conducted, encompassing traits such as growth rates, tolerance to temperature and light, and susceptibility to bleaching (Bauer et al., 2025; Marshall

and Baird, 2000; Pavia and Estacion, 2018) (among others, see section 5.1.2 | Transferability: informative power and ecological representativeness). These metrics will provide essential reference points for assessing experimental outcomes. For example, linking physiological traits with microbiome composition could allow functional studies of microbial communities—such as their contributions to nutrient cycling, immunity, or stress resilience (Doering et al., 2021; Grottoli et al., 2018; Röthig et al., 2021)—and help to identify potential keystone taxa that may drive holobiont health and stability.

In *G. fascicularis*, the absence of a consistent, species-wide "core" microbial community (as a set of bacterial taxa consistently found associated with *G. fascicularis*) complicates the application of microbiome-related findings (Chapter 4). This variability raises important questions about the functional roles of microbial taxa and whether shared functional capacities (redundancies) might underlie holobiont stability, despite taxonomic differences (Hernandez-Agreda et al., 2018; Jaspers et al., 2019; Voolstra and Ziegler, 2020). Such insights will become more accessible only with a comprehensive understanding of the interactions between the microbiome of *G. fascicularis* and its physiological traits, emphasizing the necessity of detailed characterizations as a foundational step.

Closing the life cycle of *G. fascicularis* in captivity represents another major avenue (Chapter 3). Beyond eliminating the costly and time-consuming reliance on wild coral collection, achieving sexual reproduction in corals with well-defined genetic and life-history backgrounds would open the door to mapping genes associated with critical physiological traits (Craggs et al., 2020). Moreover, a closed life cycle would enable more advanced holobiont manipulations, allowing corals to be grown under axenic or gnotobiotic conditions and facilitating the controlled introduction of specific microbes at precise developmental stages (Bucher et al., 2016; Costa et al., 2021; Domin et al., 2018; Wein et al., 2018).

Prioritizing the development of genetically and microbially stable clonal lines, alongside closing the life cycle in captivity, will significantly enhance the utility of *G. fascicularis* as a model system.

5.3.2 | Characterize the effects of captivity on holobiont composition and coral physiology

Establishing *G. fascicularis* as a reliable model for holobiont research requires a thorough understanding of how long-term laboratory conditions influence holobiont composition and dynamics. While stable aquarium conditions are advantageous for experimental control and reproducibility, they may inadvertently simplify the holobiont by reducing microbial diversity, symbiotic plasticity, and ecological representativeness (Chapters 2, 3, 4).

In their natural reef habitats, corals are subjected to a dynamic environment characterized by fluctuating physicochemical parameters and a constant influx of diverse microorganisms (Terzin et al., 2024; Ziegler et al., 2021). In contrast, the stability of aquarium systems—with their filtered seawater, controlled nutrient inputs, and limited microbial reservoirs—might act as a selective pressure that streamlines the holobiont. The relatively simple bacterial microbiomes found in the long-term aquarium-reared *G. fascicularis* (Chapter 4) align with a well-documented trend. Multiple studies on tropical, reef-building corals have demonstrated a significant reduction in bacterial alpha-diversity following transfer from the wild to aquaria. For instance, a drop in microbial diversity in the mucus of *Fungia granulosa* was observed after three weeks in captivity (Kooperman et al., 2007). Similarly, another study reported a clear decrease in the diversity of *Acropora loripes*-associated bacteria within the first week of aquarium acclimation (Damjanovic et al., 2020), and a decrease in diversity was also reported for *Siderastrea siderea* within days (Pratte et al., 2015). This suggests that the simplified microbiomes observed in our study are not a peculiar characteristic

of *G. fascicularis* but are likely a direct consequence of long-term rearing in a controlled environment.

However, it is important to note that this trend is not universal across all cnidarians. Research on non-tropical or non-reef-building cnidarians reveals a more complex picture. For example, studies on the cold-water corals *Madrepora oculata* and *Lophelia pertusa* have documented an increase in bacterial diversity under aquarium conditions (Galand et al., 2023; Schöttner et al., 2009), while the deep-sea coral *Eguchipsammia fistula* showed a depletion of functions based on putative functional profiling of microbial communities (Röthig et al., 2017). Finally, the microbiome of the lab-maintained anemone *Exaiptasia diaphana* had lower richness compared to the wild counterparts, which the authors identified as a "significant tank effect" (Hartman et al., 2020). Interestingly, such variation underscores the ecological and physiological differences that exist between tropical reef-building corals and other cnidarians. The mechanisms driving these divergent responses are likely tied to fundamental differences in metabolism, holobiont composition, and adaptation to their native environments (e.g., oligotrophic reefs vs. nutrient-richer deep-sea environments).

Importantly, the composition, not just the diversity, of the microbiome might be altered in captivity. For instance, the absence of *Endozoicomonas* as a consistent "core" microbial taxon in aquarium-reared *G. fascicularis* contrasts with field studies where it has been identified as low-abundance but ubiquitous associate (Cai et al., 2018a, 2018b). A similar decrease in relative abundance of *Endozoicomonas* **spp**. in captivity compared to wild counterparts was also observed in *Stylophora pistillata* (Barreto et al., 2021). Further studies are needed to clarify whether such absences are artifacts of captivity or represent natural variability.

Adding another layer of complexity, findings from this thesis also highlight the issue of inter-facility variability, where bacterial microbiomes responded differently to menthol bleaching across facilities (Chapter 4). While symbiotic polyps consistently maintained more stable microbiomes than their bleached counterparts, the specific taxonomic shifts after bleaching varied between laboratories. This difference in response illustrates the relevance of variables such as tank size, filtration methods, water sources, and feeding regimes. These husbandry-related variables, while critical, are often under-reported in the literature, a gap that presents a significant challenge for cross-study comparisons (McLachlan et al. 2020). Furthermore, their specific impacts on holobiont dynamics remain poorly characterized; indeed, to my knowledge, multi-facility replication of coral microbiome experiments has not been systematically undertaken. Future research could systematically analyze the microbial communities of food, source water, and filtering systems to disentangle these inputs.

Finally, to bridge the gap between laboratory findings and ecological reality, future research must prioritize direct comparisons of holobiont composition and physiological functions between wild and laboratory-reared *G. fascicularis* (Grottoli et al., 2021; Voolstra, 2013; Ziegler et al., 2021). The use of well-characterized clonal lineages, as advocated throughout this thesis, will be invaluable in this effort (Chapter 2). By providing a stable genetic baseline, clones will allow us to precisely quantify the physiological and microbiological shifts attributable to the aquarium environment and better characterize the "captivity effects" (Chapter 4).

5.3.3 | Opportunities for hypothesis testing through holobiont manipulation

A key advantage of *G. fascicularis* as a coral model lies in its potential for controlled holobiont manipulation, offering a platform to investigate the roles and interactions of holobiont partners within a stable laboratory environment (Chapters 2, 3, 4).

If successful, efforts to establish "customized" holobionts in *G. fascicularis* would pave the way for

experiments that probe microbial functions (Jaspers et al., 2019; Puntin et al., 2022). While axenic (microbe-free) or gnotobiotic (defined microbiome) rearing of *G. fascicularis* has not yet been achieved, this goal is made more attainable by the species demonstrated tolerance for long-term aquarium culturing and its ability to reproduce in captivity (Pavia and Estacion, 2018; Puntin et al., 2023; Venn et al., 2020). The recent success in generating F1 individuals under controlled conditions represents a crucial step toward achieving closed life cycle maintenance, which would provide the foundation for advanced holobiont manipulations (Craggs et al., 2020; Wein et al., 2018).

These future advancements could enable researchers to create controlled holobionts by introducing specific microbial taxa in isolation or combination (Murillo-Rincon et al., 2017; Rahat and Dimentman, 1982). Custom-designed microbial assemblages would allow for direct testing of causative links between microbial partners and functions such as nutrient cycling, immunity, and symbiosis stability (Augustin et al., 2017; Doering et al., 2021; Franzenburg et al., 2013).

Additionally, *G. fascicularis* presents opportunities to study microbial acquisition mechanisms. With the potential to rear successive generations in captivity, researchers could manipulate vertical (inherited) and horizontal (environmental) microbial transmission at various life stages (Greer et al., 2016; Wein et al., 2018). Introducing microbes at specific points in the life cycle would help unravel how early microbial exposure influences development, holobiont stability, and host-symbiont interactions (Puntin et al., 2023, 2022). Drawing from methodologies established in other model organisms, such as *Hydra*, these approaches could yield valuable insights into host-microbe relationships (Augustin et al., 2017; Baldassarre et al., 2022; Fraune et al., 2015; Murillo-Rincon et al., 2017).

In summary, *G. fascicularis* represents a promising platform for hypothesis-driven research. Its potential for advanced holobiont manipulation—enabled by progress in captive rearing—offers a pathway for detailed mechanistic studies in coral biology that explore microbial roles, holobiont stability, and resilience.

5.4 | Concluding remarks

A central challenge to a mechanistic understanding of coral bleaching is the gap between the complexity of the coral holobiont and the tractable, simplified systems required for experimental biology. While foundational cnidarian models (i.e., *Aiptasia*, *Hydra*) have provided invaluable insights, their lack of essential coral traits defines their limitations. This thesis sought to bridge this gap by systematically evaluating the reef-building coral *Galaxea fascicularis* as a new, more representative model system. The primary contribution of this work is not a singular discovery, but rather the development of a robust experimental "toolkit" that demonstrates the tractability of this species and, in doing so, provides a new platform to advance symbiosis research.

A specific achievement was the successful validation of a "disassembly-reassembly" framework for the *G. fascicularis* holobiont. This thesis demonstrated that adult polyps can be reliably rendered aposymbiotic ("disassembled") using menthol bleaching, producing viable hosts that can be maintained in simplified laboratory systems for weeks. This work also confirmed the "reassembly" potential of the model, showing that these aposymbiotic corals can be successfully re-infected with cultured, non-native *Symbiodiniaceae* strains, which demonstrates the model utility for directly investigating the mechanisms of partner specificity and preference. Further, this work provided insights into microbiome dynamics by decoupling symbiont loss from thermal stress and showing that the removal of *Symbiodiniaceae* induced a dysbiotic state. This dysbiosis was not characterized by a predictable shift to a defined "bleached" community, but rather by a significant increase in stochasticity and inter-

individual variability—a finding that contributes to our understanding of holobiont stability. Furthermore, the successful induction of ex situ spawning and the rearing of an F1 generation laid the groundwork for a self-sustaining laboratory population and future transgenerational studies.

The significance of this thesis work lies in its potential to shift coral symbiosis research from a discipline dominated by correlative field observations to one capable of mechanistic, hypothesis-driven experimentation on a true coral. This model provides a platform to test the specific functional contributions of individual algal and bacterial partners to holobiont resilience. However, the scope of this model must be clearly defined, including its limitations. As a single species, the physiological and microbial traits of *G. fascicularis* cannot be uncritically extrapolated to, nor can they represent, the full functional diversity of the whole Scleractinia taxon, which comprises hundreds of species adapted to a range of environmental niches. These challenges to transferability are coupled with the inherent trade-offs of controlled experimentation. The effect that captivity itself introduces on the holobiont composition and functioning is an unavoidable trade-off, where enhanced control is gained by sacrificing ecological representativeness. Similarly, the use of menthol, while effective, remains a potential confounding variable for microbiome studies that warrants further, direct investigation.

This thesis, therefore, should be viewed as a foundational contribution. It provides the scientific community with a validated toolkit and a critical baseline understanding of the *G. fascicularis* model. The value of this work lies in the research it now makes more attainable: the development of characterized clonal lines, the pursuit of gnotobiotic systems, and the systematic testing of holobiont dynamics. These future avenues are essential for uncovering the fundamental mechanisms of coral resilience and for developing the informed interventions necessary to support the persistence of coral reefs in a changing world.

5.5 | References

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Chapter 5

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APPENDIX

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Microbiome

RESEARCH Open Access

Holobiont nitrogen control and its potential for eutrophication resistance in an obligate photosymbiotic jellyfish

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Abstract

Background: Marine holobionts depend on microbial members for health and nutrient cycling. This is particularly evident in cnidarian-algae symbioses that facilitate energy and nutrient acquisition. However, this partnership is highly sensitive to environmental change—including eutrophication—that causes dysbiosis and contributes to global coral reef decline. Yet, some holobionts exhibit resistance to dysbiosis in eutrophic environments, including the obligate photosymbiotic scyphomedusa *Cassiopea xamachana*.

Methods: Our aim was to assess the mechanisms in *C. xamachana* that stabilize symbiotic relationships. We combined labelled bicarbonate (^{13}C) and nitrate (^{15}N) with metabarcoding approaches to evaluate nutrient cycling and microbial community composition in symbiotic and aposymbiotic medusae.

Results: C-fixation and cycling by algal Symbiodiniaceae was essential for *C. xamachana* as even at high heterotrophic feeding rates aposymbiotic medusae continuously lost weight. Heterotrophically acquired C and N were readily shared among host and algae. This was in sharp contrast to nitrate assimilation by Symbiodiniaceae, which appeared to be strongly restricted. Instead, the bacterial microbiome seemed to play a major role in the holobiont's DIN assimilation as uptake rates showed a significant positive relationship with phylogenetic diversity of medusa-associated bacteria. This is corroborated by inferred functional capacity that links the dominant bacterial taxa (~90 %) to nitrogen cycling. Observed bacterial community structure differed between apo- and symbiotic *C. xamachana* putatively highlighting enrichment of ammonium oxidizers and nitrite reducers and depletion of nitrogen-fixers in symbiotic medusae.

Conclusion: Host, algal symbionts, and bacterial associates contribute to regulated nutrient assimilation and cycling in *C. xamachana*. We found that the bacterial microbiome of symbiotic medusae was seemingly structured to increase DIN removal and enforce algal N-limitation—a mechanism that would help to stabilize the host-algae relationship even under eutrophic conditions.

Keywords: Stable isotope analysis, Tracer, Bacterial profiling, Environmental resilience, 16S rRNA gene

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Establishment of menthol bleaching protocols for six stony coral species

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Abstract

The mutualistic symbiosis between stony corals and unicellular algae of the family Symbiodiniaceae forms the base of coral reef ecosystems. However, anthropogenic stressors, such as rising seawater temperatures, cause a breakdown of the coral-algal symbiosis, so-called coral bleaching, which leads to mass mortalities and a rapid loss of coral reefs. To functionally disassemble the coral-algal symbiosis, corals have been artificially rendered aposymbiotic using temperature stress, DCMU, or menthol. As menthol has proven to be an efficient and gentle bleaching agent, we tested four menthol treatments with six commonly investigated stony coral species. The overarching aim was to establish a broadly efficient bleaching protocol as a guide for future investigations. Menthol-induced bleaching was traced with chlorophyll fluorescence and tissue color analyses over time and confirmed as final symbiont cell density two weeks after the last day of menthol treatment. Here we found that the coral species varied greatly in their menthol bleaching tolerance, underlining the importance of establishing bespoke bleaching approaches. *Acropora muricata* and *Stylophora pistillata* were efficiently bleached within two days of menthol exposure with symbiont cell reductions of 94 % to 98 %. For *Galaxea fascicularis, Montipora digitata* and *Porites cylindrica*, six days of menthol exposure proved most successful in reducing symbiont density by 92 to 97 %. While these coral species suffered no mortality, fragments of *Pocillopora verrucosa* died or suffered severe necrosis in most of the protocols, making the tested menthol treatments unsuitable for this species. We demonstrate that repeated menthol treatment at low concentrations renders most coral species aposymbiotic within few days without visual or physiological damage. Our study, therefore, provides a guideline for efficient and customized application of menthol bleaching treatments for future coral symbiosis research.

APPENDIX

Environmental Microbiology

ENVIRONMENTAL MICROBIOLOGY

BRIEF REPORT OPEN ACCESS

Resolving Symbiodiniaceae Diversity Across Coral Microhabitats and Reef Niches

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Keywords: environmental reservoirs | free-living | microhabitat partitioning | surface mucus layer | symbiosis

ABSTRACT

Dinoflagellates of the family Symbiodiniaceae are important symbionts of diverse marine animals and they also occupy different environmental niches on coral reefs. The link between diversity at ecosystem-scale to microhabitats of Symbiodiniaceae within the coral holobiont is largely unknown. Using ITS2-amplicon sequencing, we compared Symbiodiniaceae communities across four environments (seawater, near-reef vs. distant sediments and turf algae) and two coral microhabitats (tissue, mucus) on a coral reef in the Red Sea. We found that coral and environmental habitats were both dominated by the genera *Symbiodinium*, *Cladocopium* and *Durusdinium*, but environmental habitats additionally harboured *Fugacium*, *Gerakladium* and *Halluxium*. Each environmental habitat harboured a distinct Symbiodiniaceae community. Nonetheless, 17 ITS2 sequences were shared among coral and environmental habitats and were also part of nearly half of the ITS2 type profiles in coral-based communities. Tissues and mucus of 49 coral colonies from 17 genera had largely identical Symbiodiniaceae communities. Together with the large difference between environmental Symbiodiniaceae communities and those in the coral tissue and mucus, our results indicate a clear barrier between host-associated and environmental Symbiodiniaceae communities marked by only few shared complete type profiles. Monitoring coral colonies after mucus sampling confirmed its suitability for long-term monitoring of coral-associated Symbiodiniaceae communities.

1 | Introduction

The coral holobiont describes the consortium of eukarya, bacteria, archaea and viruses that interact through a range of symbioses with their animal host, and each other (Rohwer et al. 2001; Thompson et al. 2014). Among the most studied of these relationships is that between the coral and its photosynthetic dinoflagellates of the family Symbiodiniaceae. This symbiosis supports the productivity of the holobiont and the ecosystems they build in otherwise oligotrophic waters (Muscatine and Porter 1977; Stanley Jr. 2006). In exchange for a protective, high nutrient habitat, the algal symbionts can meet the majority of the coral energy requirements (Yellowlees et al. 2008; Tremblay et al. 2012) while the remaining budget can be met through heterotrophy (Mills et al. 2004; Houlbrèque and Ferrier-Pagès 2009; Hoogenboom et al. 2010). Despite this capacity, prolonged or severe disruption of the exchange between members, such as those caused

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RGB color indices as proxy for symbiont cell density and chlorophyll content during coral bleaching

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Abstract

Coral bleaching, the breakdown of the symbiosis between the coral host and endosymbiotic microalgae, is the main cause of widespread coral reef degradation. Current methods for assessing coral health based on visual appearance, such as the use of color reference cards, are limited by subjective human color perception and low resolution. Digital photography with RGB (Red, Green, Blue) color channel analyses offers a fast, non-invasive, and standardized alternative to estimate physiological parameters. However, the link between coral color and physiological parameters during bleaching may vary depending on the type of stressor. While such approaches are extensively used in plant studies, their application in estimating Symbiodiniaceae cell density and chlorophyll content in corals requires further attention. In this study, we analyzed the correlation between Symbiodiniaceae cell density and chlorophyll content across three coral species (*Acropora muricata*, *Pocillopora verrucosa,* and *Stylophora pistillata*) with 19 color indices derived from the RGB channels currently established as predictors of chlorophyll content in plants. Corals were exposed to three bleaching conditions (acute short-term and chronic long-term heat stress and menthol bleaching) to identify the best color indices for assessing coral health through image analysis. We found that the Red index had the strongest linear correlation with symbiont cell density and chlorophyll content across species (R^2 up to 0.97), so that relative changes in this color index can be directly interpreted as corresponding changes in tissue parameters. To train a model that predicts symbiont densities of a distinct sample set using the Red index, we found 10 to 12 samples to be sufficient to achieve an accuracy of > 95 % of the models trained on the full datasets. This research contributes to improved image analysis as a reliable and non-invasive tool for monitoring, by providing guidelines for a systematic use of RGB data to interpret coral health.

Keywords: Coral bleaching, RGB colors, symbiont density, chlorophyll content, image analysis, coral health monitoring

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