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A Multi-disciplinary Approach towards Sexual Propagation of Caribbean Corals in the Anthropocene

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Marine Biology

by

Emily Nixon

Committee in charge:

Linda Wegley Kelly, Chair
Eric Allen
Stuart Sandin
Martin Tresguerres

2026

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The Dissertation of Emily Nixon is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2026

DEDICATION

I would not be the woman I am today without the mentorship of the wonderfully whimsical Dr. Tierney Thys. I was just a teenager that came knocking out-of-the-blue, captivated by the ever-magnificent ocean sunfish, and she opened the door to science and a life perspective driven by curiosity. Tierney, your lessons in wonder and observation have played a fundamental role in shaping me though early adulthood. Even though my passion for the largest teleost has shifted down to the wee'est of corals, I know you are proud of who I have become today. I love you, I thank you, and I will miss our time together, but I am at peace knowing you are somewhere in the sea, with a *Mola mola*, *Mola tecta*, or maybe *ramsayi.*

EPIGRAPH

Excerpt from *In Response to a White Wild Fire*
…

Coral colonies have a way of doing that after all,
of befriending you.

A wave of tentacles, a squeeze of polyps, a shimmer of fluorescent proteins.
These sentient sentinels capture the human heart.

Regardless of my position on the ever-shifting baseline,
regardless of my grief,

I am overcome with gratitude for my time underwater,
for the privilege to form connection with such magnificent beings.

I take solace in knowing I am bound to the corals for the remainder of my life,
committed to the point where all else feels quite trife.

I know I'm not alone.
This is the perk and peril of falling in love with a system in decline.

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ABSTRACT OF THE DISSERTATION

A Multi-disciplinary Approach Towards Sexual Propagation of Caribbean Corals in the Anthropocene

by

Emily Nixon

Doctor of Philosophy in Marine Biology

University of California San Diego, 2026

Linda Wegley Kelly, Chair

Coral reef systems host a diverse array of life, ranging from large megafauna to unicellular microbiota and viruses. This spectrum of life exists and interacts within an amalgamation of physical and chemical processes. Together, this biochemical landscape is a major driver of coral health, although these complexities are often overlooked in a coral restoration framework. This dissertation assesses settlement and survival outcomes, as well as holobiont trajectories, of early life history corals in the Caribbean from both a restoration and a molecular lens. Its results aim to inform applied restoration techniques and advance molecular tools for more accurately examining early life history corals and their associated bacterial communities. I begin with an ecological and economic evaluation of three rearing investment strategies for sexually propagated corals, finding that *in situ* nurseries confer significantly higher survival in comparison to direct outplanting or aquaculture facility rearing. Notably, survival is low at four years across species (0% - 1.8%), highlighting the current limitations when outplanting on degraded reefs. Next, I explore the capacity of non-biological cues to induce settlement on engineered settlement substrates; monitoring coral larval behavior, I find that the presence of inorganic magnesium and strontium increase both motility and settlement. I then shift to a microbial lens, first developing sampling and statistical techniques to more accurately identify coral-bacteria associations in early life stages. I conclude by utilizing these methodologies to investigate how distinct benthic biofilms influence coral settlement and initial microbiome development, showing that three distinct biofilms conferred distinct settlement patterns and that substrate biofilms shape both the coral larval and post-settlement microbiome.

INTRODUCTION

Scleractinian corals are both resilient sentinels and sources of immense resources and ecosystem services. Corals have subsisted for an estimated 200-400 million years (Stanley 2003), surviving extreme changes in climate as well as innumerable stochastic stress events. Once described by the naturalist Sir John Murray as "myriads upon myriads of outstretched and hungry mouths" (Murray 1888), coral populations of today, paradoxically, sustain the hungry mouths of millions. Holistically, coral reefs are estimated to provide $9.8 trillion in ecosystem services (de Groot et al 2012; Costanza et al 2014), and support an estimate of over 25% of all marine biodiversity (Reaka-Kudla 1997).

This resilience and continued ability to support such productive systems is due in large part to the successful renewal of reef populations following disturbance events. While population regeneration is achieved via both adult colony fragmentation and sexual reproduction (Linares et al. 2011; Doropolous et al. 2015), this body of work focuses on the latter mode. Sexual reproduction is compulsory in fostering novel genetic diversity, a fundamental tenet in promoting population resilience under changing environmental conditions (Reusch et al. 2005; Baums 2008; Baums 2013). Specifically, this dissertation focuses on Caribbean broadcast spawning corals, which are regionally considered as dominant reef builders and providers of reef system stability via mass larval supply (Szmant 1986).

Broadcast spawning occurs synchronously: individuals of a coral species spawn concurrently in coordination with environmental cues, namely season and lunar cycle (Kaniewska et al. 2015). Following fertilization, successful recruitment to the population hinges on the survival of larvae to competency and successful settlement on the reef in response to an array of visual, audio, and chemical cues (reviewed in Gleason & Hofmann 2011). Once settled

and metamorphosed, this coral mote then accretes and outcompetes benthic neighbors, contributing to reef habitat structure and population reseeding (Babcock & Mundy 1996; Arnold & Steneck 2011). These pillars of persistence and pertinent processes, however, are increasingly threatened by human-derived stressors. Anthropogenic climate change, pollution, overfishing, and coastal development, to name a few, have led to the loss of coral reefs globally (Hughes et al. 1994; Hughes et al. 2003; Huang et al. 2012; Hoegh-Goldberg et al. 2017). From a reproduction standpoint, this loss has diminished fertilization rates due to both reduced connectivity (Baums et al. 2019) and synchronicity (Schlesinger & Loya 2019). Further, successful settlement and post-settlement survivorship are impaired by stressors such as increased algal cover (Arnold et al. 2010), sedimentation (Moeller et al. 2017), and heat stress (Randall & Szmant 2009). Combined, these stressors have led to the breakdown in mass larval supply to Caribbean reefs; a decline disturbingly described as near-to-full recruitment failure (Hughes et al. 2000; Vermeij et al. 2011).

In response to this sharp decline in reef health, assisted sexual propagation of corals holds promise for bolstering coral populations by enhancing larval supply and genetic diversity. Advances in gamete collection, fertilization, and larval culturing have been prolific (Banaszak 2023). However, while hundreds of thousands of larvae can now be reared in culture, post-settlement survivorship persists as a massive bottleneck. Coral recruit mortality remains high with 90-100% mortality occurring once outplanted to the reef (e.g. Chamberland et al. 2015, 2017; Nixon et al. 2025). Innovative work to address this impediment spans disciplines. Restoration groups have designed low-cost rearing solutions to increase survival; for example, the benefits of *in situ* nursery rearing for sexually propagated corals are widely upheld in the Pacific (e.g. Ligson et al. 2020; Humanes et al. 2021; Guest et al. 2023), as well as the Caribbean

(e.g. Chamberland et al. 2015; Henry et al. 2021; Nixon et al. 2025). Engineers have optimized material designs to enhance natural reef recruitment and growth: on a large-scale, artificial reefs are being designed to be specifically hospitable to coral larval recruitment (Lee et al. 2018), while on a finer scale, settlement substrates featuring inorganic and organic chemical compounds have been fabricated to bolster coral settlement, growth, and post-settlement survival (e.g. Levenstein et al. 2022; Kundu et al. 2025). In parallel, microbiologists have identified settlement cues (e.g. Tebben et al. 2011) and have even investigated the viability of directed inoculations (Damjanovic et al. 2019; Matsuda et al. 2022). Examples are extensive and knowledge is advancing, yet despite these collective efforts, the post-settlement bottleneck remains in place.

In this thesis, I span the disciplines of restoration ecology, materials engineering, and microbiology to examine the intricacies of early life history corals. Beginning with a broad lens, I conduct a long-term ecological and economic assessment to determine optimal rearing environment and monetary investment. With a finer lens, I explore settlement ecology from both an abiotic and biotic perspective. I first examine various substrate compositions and inorganic compounds as potential settlement inducers, then move towards the biological to refine the accuracy in which we define early coral-bacteria associations. I use this refined method to elucidate the influence of algae-promoted biofilms as drivers of coral settlement and initial coral microbiome development, positing the question: can the biofilm community, so vital for attracting a coral to settle, also influence the formation of the early coral microbiome? While ultimately microbial in focus, this thesis seeks to progress knowledge surrounding coral early life history stages with the overarching aim of addressing the post-settlement bottleneck.

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Chapter 1 MULTI-YEAR EVALUATION OF REARING TECHNIQUES FOR THREE SEXUALLY PROPAGATED CARIBBEAN CORALS IN A RESTORATION SETTING

Abstract

In response to declining coral populations worldwide, conservation groups are increasingly applying restoration strategies to bolster abundance and diversity, including sexual propagation of corals. Collection and fertilization of coral gametes as well as larval rearing and settlement have been successful. However, post-settlement stages remain a bottleneck (80-100% mortality), which makes this technique costly to implement at scale. To address this challenge, we compared survival and colony size of three sexually propagated Caribbean coral species, *Diploria labyrinthiformis*, *Pseudodiploria strigosa*, and *Orbicella faveolata*, reared at three levels of investment: direct outplant to reef, *in situ* field nursery rearing, and *ex situ* aquaculture facility rearing. As part of coral sexual propagation work in St. Croix, United States Virgin Islands (USVI), recruits were reared for one year before being outplanted to reef plots and were monitored annually for three subsequent years. Cost-effectiveness of each rearing strategy was calculated at each monitoring timepoint via coral seeding unit yield and cost per seeding unit. Although survival was low at four years (0% - 1.8%), corals reared in the *in situ* nursery displayed significantly higher survival and therefore lower cost per seeding unit than the other two investment strategies. These results highlight the benefits of an *in situ* nursery stage to increase long-term juvenile survival and cost-effectiveness. The return-on-investment of corals reared in the *in situ* nursery suggests that outplanting sexually propagated corals may be a viable restoration strategy; however, the low proportion of corals surviving at four years highlights current limitations when outplanting on degraded reefs.

Introduction

Coral reefs are in stark decline worldwide due to an array of natural and anthropogenic stressors, compounded by increasingly severe disturbances related to human-derived climate change (Hughes 1994; Hughes et al. 2003; Hoegh-Guldberg et al. 2017). These stressors have synergistically shifted coral reefs towards an altered steady state of algae-dominance (Jackson et al. 2014), characterized by increased microbialization and altered reef water chemistry, which further promote algal growth and scleractinian coral decline (McDole et al. 2012; Haas et al. 2016; Nelson et al. 2023). Current models estimate that 70-90% of coral reefs will be lost by 2050 if average global temperatures continue to warm by 1.5°C (Hoegh-Guldberg et al. 2019; Intergovernmental Panel on Climate Change 2022). Given that coral reefs are among the most biodiverse ecosystems and provide valuable ecosystem services by supporting fisheries, tourism, and shoreline protection (Gattuso et al. 2014; Burke & Spalding 2022), efforts to enhance reefs through active coral restoration have grown over recent decades (reviewed in Boström-Einarsson et al. 2020).

When approaching reef restoration, it is important to consider how coral populations naturally recover following episodic disturbances through variable reproductive strategies, that is via adult colony regeneration and larval recruitment (Linares et al. 2011; Doropoulos et al. 2015). Colony regeneration, or asexual propagation, whereby fragments from wild donor colonies are repeatedly fragmented and grown-out in nurseries, has been utilized as a restoration technique for over 40 years (Bowden-Kerby et al. 2001; Young et al. 2012). While straightforward to implement and successful on small scales, this approach relies on existing coral biomass and does not foster novel genetic diversity, thought to be a key factor in promoting population resilience under changing environmental conditions (Reusch et al. 2005; Baums

2008; Baums et al. 2013). An alternative technique that addresses these demographic and genetic limitations is sexual propagation, which harnesses the production of millions of gametes by broadcast spawning corals to mass fertilize and produce coral propagules *ex situ* (reviewed in Randall et al. 2020).

Broadcast spawning corals are dominant reef-builders in the Caribbean and are key to reef persistence via mass larval supply (Szmant 1986). Following mass spawning and fertilization, successful recruitment to the population is dependent on availability of competent larvae, successful settlement, and survival through early juvenile stages (Babcock & Mundy 1996; Arnold & Steneck 2011). In the Caribbean this influx of new individuals to sustain coral populations has declined substantially in recent decades (Hughes & Tanner 2000; Vermeij et al. 2011), impaired by stressors such as increased algal cover (Arnold et al. 2010), heat stress (Randall & Szmant 2009), and lower fertilization rates due to reduced connectivity (Baums et al. 2019) and synchronicity (Schlesinger & Loya 2019) among metapopulations. In the face of natural recruitment failure, sexual propagation provides a promising approach to generating millions of diverse coral propagules for seeding reefs. Many advances in gamete collection, fertilization, and larval culture methods have been made over the past two decades (Banaszak et al. 2023), however, low recruit survival combined with the cost of sexual coral propagation impedes its application as an effective restoration method on ecologically meaningful scales.

Given that coral recruit mortality is high when outplanted to reefs shortly after settlement, a key question is whether a period of nursery rearing can increase recruit survival and thus optimize cost-effectiveness. Several studies in the Pacific have demonstrated survival and growth benefits conferred by *in situ* nursery rearing for sexually propagated acroporids (e.g., Guest et al. 2014; Ligson et al. 2020; Humanes et al. 2021) and massive species (Guest et al. 2023), whereas

a dearth of information exists surrounding the long-term return-on-investment of nursery rearing in the Caribbean (Chamberland et al. 2015; Henry et al. 2021). Therefore, we aimed to determine if juvenile survival of three Caribbean coral species can be increased by investing in an intermediate nursery rearing stage for the first year post-settlement and whether this intermediate increases cost-effectiveness.

Here we examine the effect of three levels of rearing investment (low, medium, high) on survival and colony size of *Diploria labyrinthiformis* (Grooved brain coral; Linnaeus 1758), *Pseudodiploria strigosa* (Symmetrical brain coral; Dana 1846), and *Orbicella faveolata* (Mountainous star coral; Ellis and Solander 1786). Species chosen are current or recommended targets for sexual restoration efforts (Baums et al. 2019), contribute to reef structure, and have suffered major declines in the United States Virgin Islands (USVI) in the past two decades (Miller et al. 2009; Grove 2023). These species represent a range of proficiency in natural recruitment, with *Orbicella spp.* exhibiting severely low natural recruitment (Hughes & Tanner 2000), while recruitment of brain coral species such as *P. strigosa* remains robust (Baums et al. 2019). Sexually propagated coral recruits were settled onto seeding units and either directly outplanted onto the reef (low investment, high mortality risk), reared for one year in an *in situ* field nursery (medium investment, potential reduction in risk), or reared for one year in an *ex situ* aquaculture facility (high investment, low risk of predation, competition, and disease). After one year all surviving corals were outplanted to the reef, and survival and maximum diameter (mm) of outplanted corals were measured annually for the subsequent three years. A cost evaluation of each investment strategy was completed based on cost per seeding unit retaining a minimum of one coral.

Methods

Study site. This study was conducted at The Nature Conservancy's (TNC) Coral Innovation Hub in St. Croix, USVI. The *in situ* nursery and outplant reef site are located at Channel Rock (Lat / Long: 17.77°N, 64.59°W), a patch reef at approximately 7.5 m depth located within the protected East End Marine Park. Channel Rock receives low exposure to anthropogenic impact due to its location outside of St. Croix's northeastern barrier reef complex, neighboring a low population density shoreline, but has been impacted by the emergence of stony coral tissue loss disease (Brandt et al. 2021), bleaching (Miller et al. 2009), and the recent invasion of *Ramicrusta* species (Edmunds et al. 2019).

Gamete collection, larval rearing, and settlement. Gametes were collected by SCUBA divers during a succession of spawning events in 2020 on coral reefs in two bays on the north side of the island, Cane Bay (Lat-Long: 17.77°N, 64.81°W) and Tamarind Reef (17.76°N, 64.67°W) (Table S1.1, Fig. S1.1). Weighted nylon mesh nets attached to plastic funnels and 50 mL polypropylene vials were deployed over spawning coral colonies, concentrating positively buoyant egg-sperm bundles into vials for collection and transport (Hagedorn et al. 2021). Within two hours of spawning, gametes were cross-fertilized in 100-µm filtered ozone-sterilized seawater (FSSW). Fertilized embryos were distributed into 1 L poly-carbonate fat separators and rinsed with FSSW to remove excess sperm. Fertilization for all three species was observed to be between 85-95%. Embryos were dispersed at an approximate density of 1 larva/mL into 110 L containers immersed in flow-through raceway tank systems, with raceways maintained at ~28°C (Fig. 1.1c). Partial water changes (~75%) were conducted at first daily, and then every other day to reduce microbial load and maintain a stable pH in the rearing environment throughout development (Vermeij 2006; Hagedorn et al. 2021).

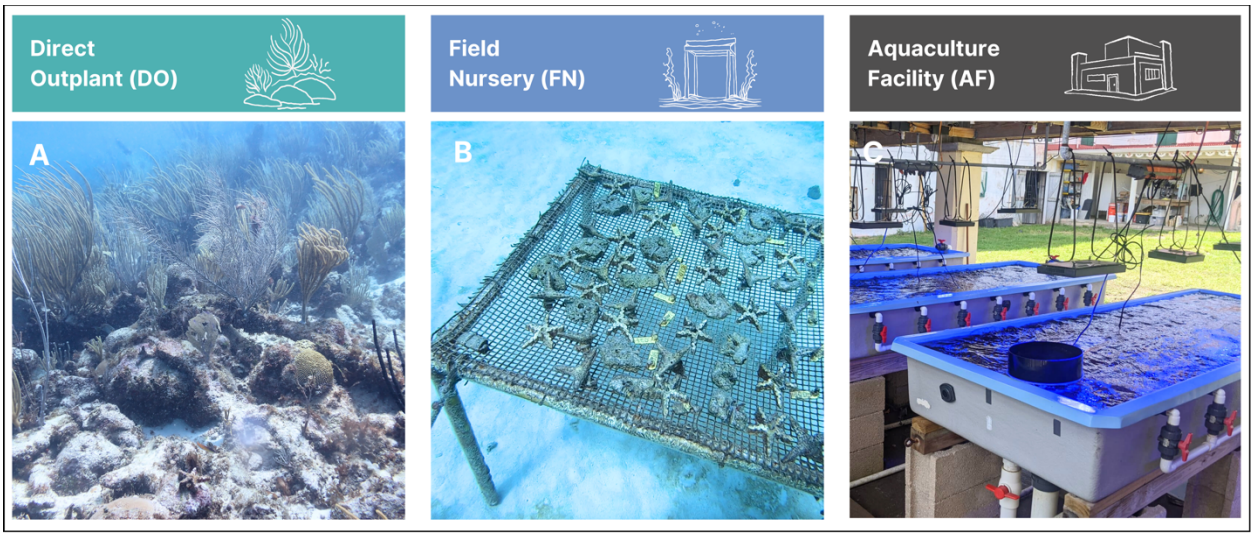


Figure 1.1 Coral recruit rearing investment treatments. (A) Direct outplant to reef (DO, low investment), (B) In situ field nursery table (FN, medium investment), (C) Ex situ aquaculture facility (AF, high investment).

Prior to spawning, seeding units (SUs, Fig. S1.2) were conditioned for one month at the St. Croix Yacht Club dock (*D. labyrinthiformis*, 17.76N, -64.60W) or at TNC's Cane Bay nursery (*P. strigosa* and *O. faveolata*, 17.77°N, 64.81°W) to develop a natural biofilm and crustose coralline algae (Fig. S1.1). Ceramic SUs were designed by SECORE International, Inc. in 2020, were approximately 120 cm^2 in surface area (Fig. S1.2), and have previously been found viable as coral seeding units (Randall et al. 2023). Thirty conditioned SUs were introduced to a 110 L larval culture bin for each species upon observing larvae swimming in the water column (2-4 days post-fertilization). Conditioned SUs introduced to *D. labyrinthiformis* for settlement were not cleaned which resulted in poor water quality in larval cultures and notable benthic competition to settled recruits (shading). For this reason, SUs used for *P. strigosa* and *O. faveolata* settlement were scrubbed of macro-fouling organisms prior to larval introduction. Once settlement was observed, freshly isolated Symbiodiniaceae from adult conspecifics were introduced to cultures to inoculate recruits with symbionts (Supplement S1.1). Following observed symbiont uptake, settled recruits were scored and mapped on each SU using a stereo

microscope. Due to the manner in which SUs were affixed to reef substrate, all sides except the bottom surface were scored. Once scored, SUs with ≥ 1 settled recruit were moved to a flow-through raceway for temporary holding until transfer to rearing treatments.

Rearing investment treatment set-up. Three weeks after fertilization (six weeks for *O. faveolata*), SUs were assigned to one of three rearing investment treatments: direct outplant to reef plots (DO), *in situ* field nursery (FN), or *ex situ* aquaculture facility (AF) (Fig. 1.1, 1.2). The thirty SUs for each species were re-scored. SUs with no settlers were removed from the experiment. The remaining SUs (27 for *D. labyrinthiformis*, 27 for *P. strigosa*, and 21 for *O. faveolata*) were ordered by settler density and then randomly assigned to one of the three treatments, ensuring there were no significant differences in initial settler count between treatments (ANOVA $p > 0.05$). SUs assigned to DO were deployed over a 50-m^2 area at Channel Rock reef, with at least 1 m separation between SUs (Fig. 1.1a). Plot depths ranged from 6-8 m for *D. labyrinthiformis* and *O. faveolata* and 4-6 m for *P. strigosa*. Macroalgae was scrubbed from the reef substrate, and SUs were attached to benthos by masonry nail and cable tie (Fig. 1.1a, S1.2b). DO received no further maintenance and was solely revisited for monitoring. SUs assigned to FN were attached by cable-tie to a PVC and plastic mesh nursery table at 7.5 m depth with the table-top approximately 1 m above sand (Fig. 1.1b). Tables were cleaned by SCUBA divers every 1-2 months to remove fleshy macroalgae and cyanobacteria. SUs assigned to AF were maintained in a 650-L outdoor closed system flow-through raceway tank with a whole system exchange rate of 15,000 gallons per hour (Fig. 1.1c). Seawater was ozone sterilized prior to entering the system, and was continuously filtered through a 200 um filter. The raceway received natural sunlight filtered through 95% UV ray-blocking shade cloth. The following seawater parameters were maintained as precisely as possible: temperature: 28°C, pH: 7.8-8.3,

salinity: 33-35 ppt, dissolved oxygen: 6-12 ppm, oxidation reduction potential (ORP): 370-390 mV. The raceway was cleaned biweekly, and SUs were cleaned monthly under a stereoscope with fine paintbrushes and syringe needles until December 2020 when herbivores (*Clibanarius tricolor*, *Paguristes cadenati*, and *Astraea* spp.) were introduced. Large pieces of fleshy macroalgae were removed by hand as needed. Coral recruits were not fed throughout the duration of treatment. A HOBO UA-002-64 pendant temperature and light data logger was deployed at each rearing treatment (Fig. S1.3).

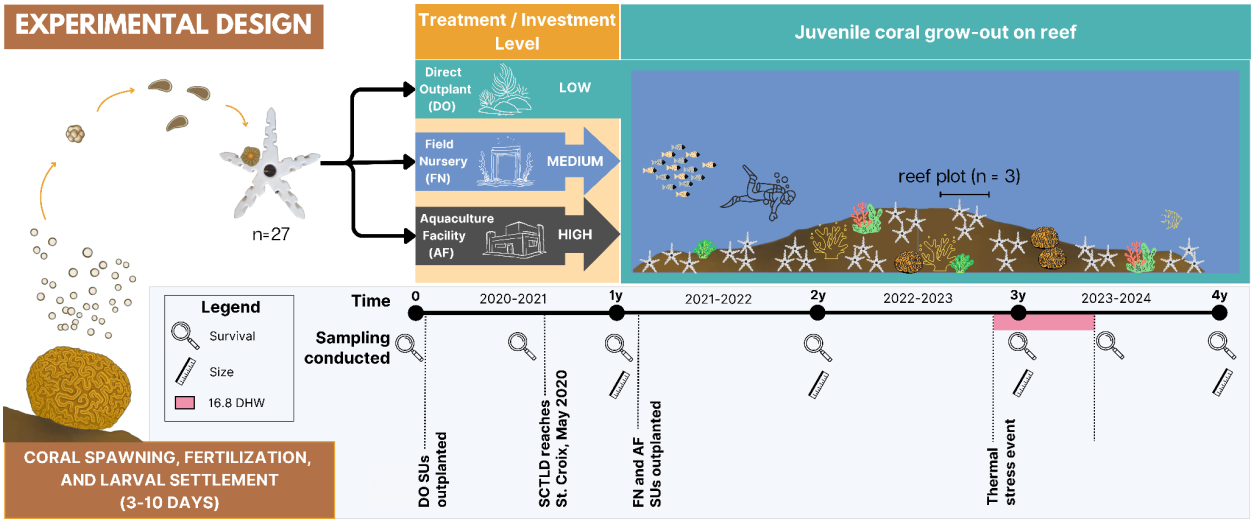


Figure 1. 2 Experimental design of coral recruit rearing investment treatments, outplanting, and subsequent monitoring. Coral larvae were settled onto conditioned SUs, which were scored and distributed into three rearing treatments: direct outplant (DO), in situ field nursery (FN), and ex situ aquaculture facility (AF) (n = 9 SUs per treatment). SUs were scored for survival at six months and for the subsequent three years. Following one year of rearing, corals were outplanted to DO plots (n = 3 per outplant plot, 1 x SU per treatment) at Channel Rock reef. Noteworthy stress events, i.e., the appearance of stony coral tissue loss disease (SCTLD) on St. Croix and the 16.8 degree heating week (DHW) 2023 thermal stress event, have been added to the timeline for reference.

Outplanting and monitoring. After one year of rearing, SUs in the AF and FN treatments harboring ≥1 surviving juvenile were outplanted to Channel Rock reef in proximity (<30cm) of directly outplanted SUs (Fig. 1.2). Each original outplant plot then had one SU from each investment treatment (n = 3 per plot). Survival was scored by divers on SCUBA at 6, 12, 24, 36,

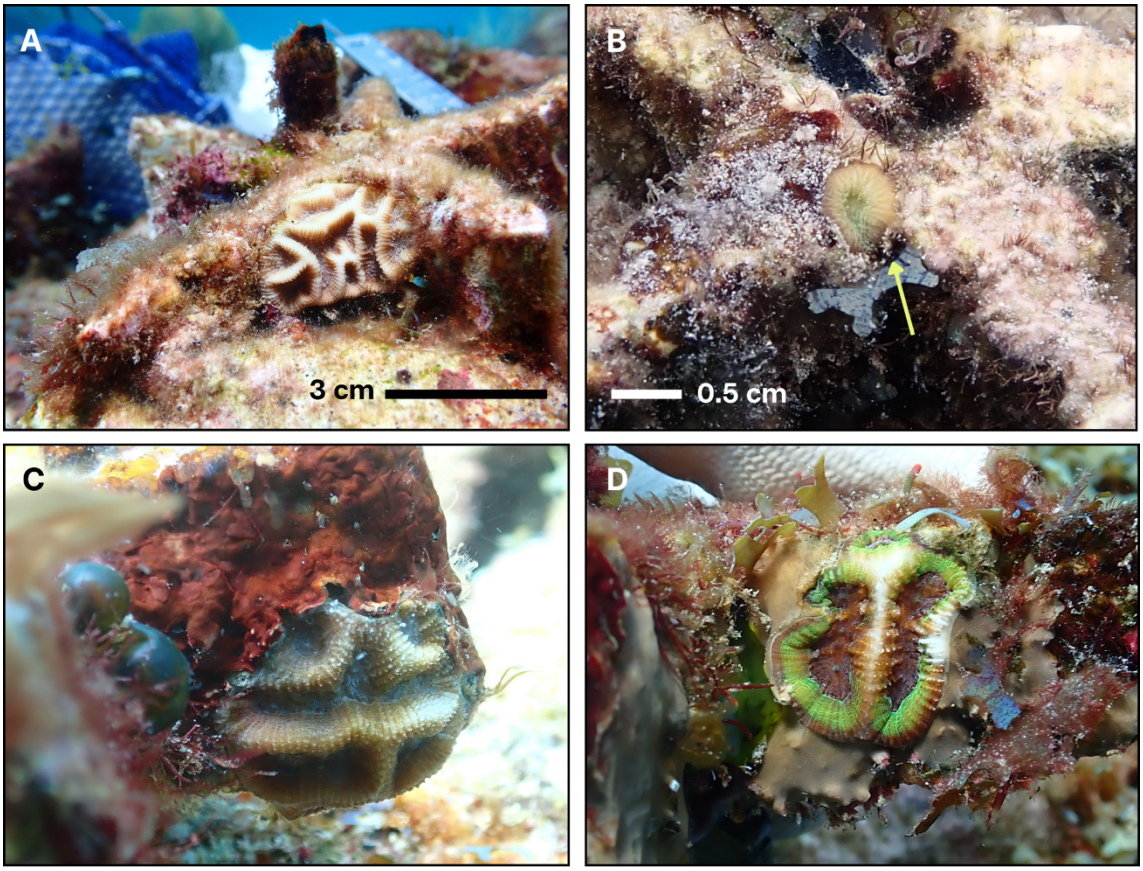


Figure 1.3 Photos depicting three- and four-year-old juvenile corals. (A) Juvenile *P. strigosa* (B) Juvenile *D. labyrinthiformis* (C) *Ramicrusta* encroaching on juvenile *P. strigosa* (D) *Millepora* encroaching on juvenile *P. strigosa*.

and 48 months (± 3 months depending on species) using blue light underwater flashlights (Nightsea FL-1 LED), with an additional survival monitoring timepoint added following the 2023 thermal stress event (16.8 degree heating weeks; NOAA Coral Reef Watch) (Fig. 1.2). Maximum colony diameter (mm) of juvenile corals was measured to the nearest 0.1 mm using Vernier calipers at 12, 24, 36, and 48 month timepoints (± 3 months) (Fig. 1.2, 1.3). Two

touching polyps were considered a chimera when a distinct skeletal line was present and were counted and measured individually.

Statistical analyses. Statistical tests were performed in R (v2023.03.0+386 Rstudio, Inc; https://github.com/enixon96/SPIkids) and conducted at a significance level of alpha = 0.05. To test for differences in juvenile coral survival, investment treatments were compared using a Kaplan-Meier's survival analysis (Kaplan and Meier 1958) followed by a Log-Rank post-hoc pairwise comparison. Differences in individual coral size, proxied by maximum colony diameter (mm), were compared between treatments at each timepoint using Welch's t-test followed by a Games-Howell post-hoc (Games & Howell 1976). Due to discrepancies in survival scoring, if a SU received a higher score than in the previous timepoint it was standardized to the lowest of the two scores. Therefore, n values for survival and colony size may differ slightly, and reported survival is conservative. SU yield, defined as the proportion of SUs in a treatment retaining at minimum one coral recruit, was compared at 12, 24, 36, and 48 months via a Fisher Exact test with the Freeman-Halton extension followed by a pairwise Fisher Exact test post-hoc comparison (Freeman & Halton 1951). SUs that were absent or not located at a timepoint were removed prior to analysis.

Cost analysis. SU yield is a metric commonly used by restoration practitioners to quantify sexual coral restoration success and associated financial costs (e.g., Chamberland et al. 2015; Randall et al. 2023). We defined SU yield as the proportion of seeding units within the restoration site that harbored at least one living coral. Retention (i.e. whether a SU remained secured to the reef plot) was not considered as a metric in SU yield calculations because it is independent of rearing technique.

Restoration costs were calculated using the methods outlined in Edwards et al. (2010) and used in other coral restoration cost-efficiency studies (e.g., Chamberland et al. 2015). Costs were divided into six stages (preparation, gamete collection, hatchery, rearing, outplanting, and monitoring) and seven cost categories (equipment, consumables, labor, dive gear, SCUBA tanks, boat use, and other). The following tasks were included in each cost category: 1) preparation - creation of a local spawning calendar, one spawning site survey, conditioning and manual scrubbing of SUs; 2) gamete collection - three nights of spawning observation and/or gamete collection dives; 3) hatchery - one night of gamete fertilization, one week of larval rearing, one month of recruit rearing; 4) rearing - nursery setup and transfer to nursery (FN only), nursery or aquaria maintenance and SU care; 5) outplanting - transfer to outplant site; deployment of SUs to reef; and 6) monitoring - initial scoring of 27 SUs in the laboratory; annual survival scoring of 27 SUs and measurement of surviving juveniles (Table S1.2).

Costs to establish the aquaculture facility were not included in the cost analysis because this was a one-time expense, and the analysis was based on running costs. Running costs of the aquaculture facility included utilities, operational costs (e.g., maintenance, repair, and replacement of system parts), consumables for maintenance and monitoring of water tables, and labor. The installation costs for the *in situ* nursery were included since nursery structures are not considered permanent and typically need to be replaced after three to five years of use. Maintenance costs included dive gear, SCUBA tanks, and boat upkeep, as well as marina fees, insurance, and fuel for boat use. Equipment, including dive gear, was estimated to have a lifespan of three to five years, and SCUBA tanks and boats were estimated to have a lifespan of 10 years. Per-use costs were calculated by dividing purchase price by years of use and number of uses per year. Labor was divided into two categories (subject matter expert and local

practitioner), and hourly wages were based on median wages for equivalent categories within TNC.

Costs were calculated assuming 4,000 SUs based on the capacity of the aquaculture facility. SU yield was modeled by applying the percent yield of the SUs monitored in the experiment to 4,000 SUs. Cost per SU was calculated at one-, two-, three-, and four-year timepoints by dividing the total restoration cost by SU yield.

Results

Coral recruit survival. Mean settlement of *D. labyrinthiformis* was 37.81 (SD = 18.96) recruits per SU with a range of 8-79 recruits per SU, and an approximate settlement rate of 3.4%. In total, 368 recruits were assigned to DO, 336 to FN, and 317 to AF. Early mortality was high (63-69% at six months) across all treatments (Fig. 1.4a). Survival continued to decline across all treatments throughout the study; however, corals initially reared in FN displayed significantly higher survivorship compared to AF or DO, while AF and DO did not significantly differ in survival (K-M: $X^2 = 23.1$, $p < 0.01$; FN vs AF, FN vs DO $p = 0.04$, AF vs DO $p = 0.86$). At one year, the FN treatment (19.6% survival; n = 66 corals) conferred 2 times and 6 times higher survival than DO (9.5% survival; n = 35 corals) and AF (3.2% survival; n = 10 corals) respectively (Fig. 1.4a). This trend continued through the 24 and 36 month timepoints with FN survival 2 times and 8 times higher than in DO and AF respectively (Fig. 1.4a). By 48 months, survival dropped to 1.8% (n = 6 corals) for FN, 0.54% (n = 2 corals) for DO and 0% for AF (Fig. 1.4a).

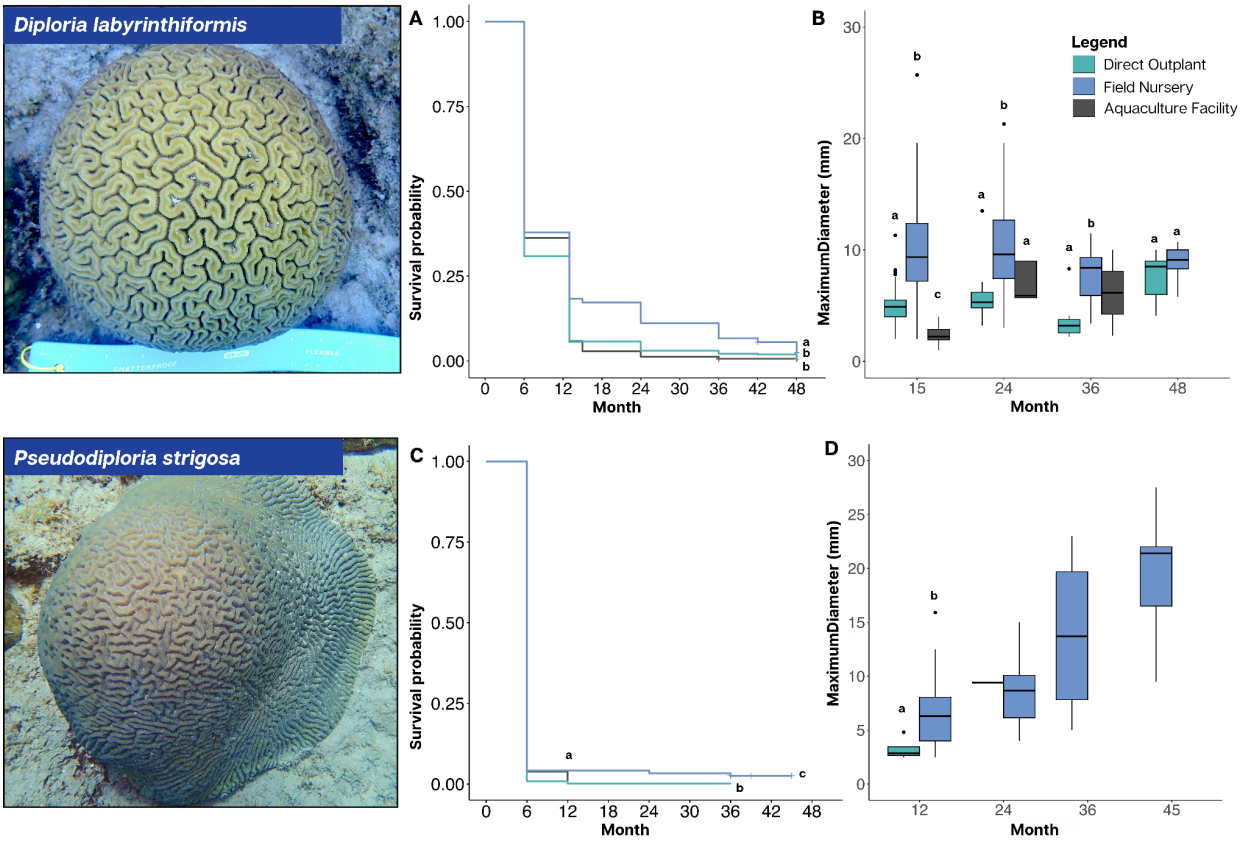


Figure 1.4 Survival and maximum diameter of sexually propagated *D. labyrinthiformis* and *P. strigosa* reared at three levels of investment: direct outplant (DO, teal), in situ field nursery (FN, blue), ex situ aquaculture facility (AF, black). Kaplan-Meier survival analysis for (A) D. labyrinthiformis and (C) *P. strigosa*. Letters next to lines indicate statistically different survival ($p < 0.05$) between treatments determined via Log-Rank post-hoc pairwise comparison. Boxplots of juvenile colony size, maximum diameter (mm) by investment treatment at each monitoring timepoint for (B) *D. labyrinthiformis* and (D) *P. strigosa*. Letters indicate statistically different ($p < 0.05$) colony sizes between treatment groups and were determined by Welch's t-test with post-hoc comparison.

Mean settlement of *P. strigosa* recruits was 69.44 (SD = 38.1) recruits per SU and ranged from 9-142 recruits per SU, and a settlement rate of approximately 3.75%. In total, 611 recruits were assigned to DO, 620 to FN, and 644 to AF. *P. strigosa* recruits displayed substantially higher initial mortality than *D. labyrinthiformis*, with 96-99% mortality seen across treatments in the first six months and no AF recruits surviving to one year (Fig. 1.4c). Survival was significantly different among all investment treatments (K-M: $X^2 = 32.1$, $p < 0.01$; FN vs DO $p < 0.01$, FN vs AF $p < 0.01$, DO vs AF $p < 0.01$). Similar to *D. labyrinthiformis* recruits, *P. strigosa*

recruits initially reared in the FN treatment exhibited significantly higher survival than AF and DO corals (Fig. 1.4c). After one year of investment, FN recruits (6.5% survival; n = 40 corals) were 10 times more likely to survive than DO (0.65% survival; n = 4 corals) (Fig. 1.4c). Following the investment period, *P. strigosa* survival declined by approximately 50% annually in both FN and DO, diminishing to 0% for DO and 0.65% (n = 4 corals) for FN at 45 months (Fig 1.4c).

Initial scores of *O. faveolata* recruits ranged from 1-78 recruits per SU with an average of 23.22 (SD = 18.0), and an approximate settlement rate of 2.51%. In total, 205 recruits were assigned to DO, 221 to FN, and 201 to AF. *O. faveolata* SUs were scored at one year, at which time only five coral recruits remained. Three of these corals were reared in FN and two were DO, representing 1.4% and 1.0% survival respectively, with no *O. faveolata* recruits surviving in the AF treatment (Table S1.3). While FN corals were not outplanted and monitored further, full mortality was observed in DO by 24 months (Table S1.3).

Recruit Diameter. At 15 months, *D. labyrinthiformis* mean maximum colony diameter (mm) differed significantly among all treatments (Welch's t-test with pairwise post-hoc comparison, $p < 0.01$). FN corals reached a mean maximum diameter of 10.0 mm (n = 66 corals, SD = 4.43), whereas DO- and AF-reared corals averaged at 5.0 mm (n = 35 corals, SD = 1.93) and 2.3 mm (n = 10 corals, SD = 0.866) respectively (Fig. 1.4b). Following outplanting to reef plots, only AF juveniles displayed an increase in mean colony size from 15 to 24 months (Fig. 1.4b). At this timepoint, FN juvenile corals maintained a significantly higher mean maximum colony diameter of 10.26 mm (n = 34 corals, SD = 4.48) in comparison to DO and AF corals, 5.97 mm (n = 11 corals, SD = 2.71) and 7.06 mm (n = 5 corals, SD = 1.78) respectively (DO vs FN $p < 0.01$, DO vs AF $p = 0.6$, FN vs AF $p = 0.03$). Interestingly, at 36 months of age (two

years following outplanting of FN and AF), mean maximum diameter decreased across all treatments, though FN juvenile corals mean maximum diameter remained significantly higher than DO corals (DO vs FN $p < 0.01$) (Fig. 1.4b). This trend reversed at 48 months, with FN and DO mean colony size increasing to 8.78 mm (n = 5 corals, SD = 1.90) and 7.52 mm (n = 5 corals, SD = 2.41) respectively (DO vs FN $p = 0.4$).

After one year of investment treatments, *P. strigosa* recruit colony size differed significantly between FN and DO corals ($p < 0.01$), with FN corals reaching a mean maximum diameter of 6.66 mm (n = 40 corals, SD = 2.99) in comparison to DO corals which grew to 3.25 mm (n = 4 corals, SD = 1.05) (Fig. 1.4d). FN juvenile mean colony size continued to increase throughout the entirety of the study, measuring 8.58 mm (n = 22 corals, SD = 2.85) and 13.7 mm (n = 12 corals, SD = 6.99) at 24 months and 36 months respectively (Fig. 1.4d). Notably, at 36 months half of surviving *P. strigosa* juvenile cohort were on average triple the colony size of the other half, measuring a mean maximum diameter of 20.07 mm (n = 6 corals, SD = 2.26) and 7.3 mm (n = 6 corals, SD = 2.24) respectively (Fig. 1.4d). At 45 months, mean maximum diameter reached 19.38 mm (n = 5 corals, SD = 6.76) (Fig. 1.4d).

The three surviving FN *O. faveolata* reached a mean maximum diameter of 2.53mm (SD = 0.289) at one year, while the two surviving DO measured at 1.9 and 3.0 mm.

Seeding unit yield and cost analysis. Restoration costs of directly outplanting to reef (DO) were the least expensive, totaling $31,741.52 for 4,000 SUs. Restoration costs incorporating a one-year rearing phase totaled $55,115.58 and $80,105.03 for *in situ* nursery (FN) and aquaculture facility (AF) investment treatments, respectively (Fig. 1.5a, c). Costs of preparation, gamete collection, hatchery, outplanting, and monitoring stages were nearly identical across rearing methods (Table S1.2). Thus, differences in costs among treatments are

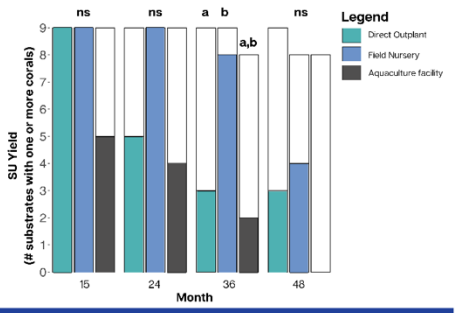
due to costs associated with the rearing phase. Rearing was the most expensive stage for FN and AF, representing 42% and 61% of total costs, respectively (Table S1.2). Labor was the most expensive cost category for all treatments, making up 63% (DO), 65% (FN), and 75% (AF) of costs.

Diploria labyrinthiformis

A

Rearing method	Restoration cost of 4,000 SUs (USD)	1 Year		2 Years		3 Years		4 Years	
		SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)
Direct outplant	31,741.52	100	7.94	56	14.28	33	23.81	33	23.81
In situ nursery	55,115.58	100	13.78	100	13.78	89	15.50	50	27.56
Aquaculture facility	80,105.03	56	36.05	44	45.06	25	80.11	0	-

B



Pseudodiploria strigosa

C

Rearing method	Restoration cost of 4,000 SUs (USD)	1 Year		2 Years		3 Years		4 Years	
		SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)
Direct outplant	31,741.52	22	35.71	11	71.42	0	-	0	-
In situ nursery	55,115.58	89	15.50	78	17.72	63	22.05	57	24.11
Aquaculture facility	80,105.03	0	-	0	-	0	-	0	-

D

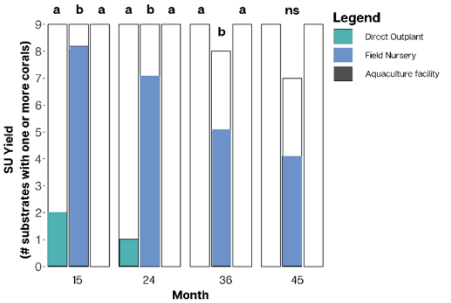


Figure 1.5 Cost table and SU Yield of *P. strigosa* and *D. labyrinthiformis*. (A, C) Total cost, SU yield (%), and cost per SU for each rearing method at one, two, three, and four years of age. Assumes 4,000 SUs produced. All costs in US dollars. (B, D) SU Yield (# substrates) of each investment treatment over the course of the study. Statistical differences were determined by Fisher Exact test with pairwise post-hoc analysis and are letter denoted.

At one year, nine DO and FN SUs retained a minimum of one *D. labyrinthiformis* coral (100% yield), while only five AF SUs retained at least one living coral (56% yield), but this difference was not significant (Fig. 1.5b; Fisher's exact test with pairwise post-hoc comparison, $p > 0.25$). DO was the least expensive method ($7.94 per SU) at this timepoint (Fig. 1.5a), but the most cost-effective method changed over time as yield dropped in all treatments. FN had the highest yield and therefore lowest cost per SU in years two (100% yield; $13.78) and three (89% yield; $15.50) (Fig. 1.5a, b). By year four, yield remained highest in FN (50%) but DO had a

narrowly lower cost per SU ($23.81 for DO vs $27.56 for FN). Differences in yield were only significant between FN and AF treatments in year three (Fig. 1.5b, FN vs AF $p < 0.05$). AF SUs had the highest cost at all timepoints.

With the exclusion of the final 45-month timepoint ($p = 0.06$), yield was significantly higher in the FN treatment than the DO and AF treatments for *P. strigosa* (Fig. 1.5d). AF had 0% yield in year one, while DO decreased to 0% yield by year three (Fig. 1.5d; DO vs FN and FN vs AF $p < 0.01$). FN was the most economical method throughout the study, retaining 57% yield in year four with a cost of $24.11 per SU (Fig. 1.5c).

SU yield for *O. faveolata* was low across treatments. In year one, DO yield was 22%, FN yield was 33%, and AF yield was 0%. DO had the lowest cost per SU at $35.71 but yield fell to zero by 24 months (Table S1.3).

Discussion

Determining the restoration potential of coral propagation techniques is vital for effective implementation. The scale at which commonplace practices (i.e., asexual fragmentation and outplanting) act is insufficient to match the scale of the current climate change crisis (Bellwood et al. 2019; Bruno et al. 2019). Given the limitations of conventional asexual propagation techniques (limited scale and genetic diversity), coral sexual propagation has been suggested as a technique with the potential to overcome these limitations, though a scarcity of information exists on long-term viability in the Caribbean (Chamberland et al. 2015; Chamberland et al. 2017; Henry et al. 2021). To our knowledge, this is the longest study (four years) to examine the ecologic and economic effectiveness of sexually propagated corals in the Caribbean and highlights the benefits of a post-settlement *in situ* nursery rearing phase for two brain coral species, *D. labyrinthiformis and P. strigosa*.

In situ nursery confers survival and growth advantages to sexually propagated corals. While brain corals *D. labyrinthiformis* and *P. strigosa* retained juvenile survival throughout the study, a post-settlement bottleneck was observed across treatments at six months (63-69% and 96-99%, respectively). This aligns with previous sexual propagation studies, with high post-settlement mortality occurring in the first year (reviewed in Omori et al. 2018). Despite initial mortality, sexually propagated brain corals assigned to one year of *in situ* nursery rearing displayed survival benefits that persisted in the three years post-outplanting, and were found to be significantly larger in size when compared to directly outplanted and aquaculture facility-reared recruits. We hypothesize that the *in situ* nursery provides protection from stressors found on algae-dominated reefs, while conferring benefits of *in situ* rearing. *In situ* nursery tables protect juvenile corals from antagonistic features that characterize degraded reefs such as benthic competition (Arnold and Steneck 2011), sedimentation (Speare et al. 2019), predation (Gallagher and Doropoulos 2017), hypoxia (Mallon et al. 2023), and microbialization (Vermeij et al. 2009), while maintaining natural temperature, pH, light, flow, nutrients, and access to symbionts. Although no microbial, physical, or chemical parameters were recorded, water-column restoration structures have been found to hold lower microbial load and to be more impervious to low-oxygen diel swings than nearby degraded reefs (Baer et al. 2024). For these reasons, an intermediate nursery rearing phase for sexually propagated corals prior to outplanting is increasingly supported as an essential practice (e.g., Guest et al. 2014; Ligson et al. 2019; Humanes et al. 2021).

Regardless of initial investment, fleshy macroalgae, turf algae, *Ramicrusta*, fire coral, and sponges were observed to play a direct role in slowing and even decreasing juvenile coral colony growth once outplanted onto the reef site. Interestingly, while one year of *in situ* rearing

conferred increased survival and colony size in both brain corals, performance differed once outplanted to the reef. At four years, 1.8% (n = 6 corals) of *D. labyrinthiformis* juveniles remained, with no increases in colony size post-outplanting. Notably, while only 0.65% (n = 4 corals) of *P. strigosa* juveniles remained, mean colony size continued to increase following outplanting. Growth rates of these two species are reportedly similar (Moorsel 1988; Chamberland et al. 2017), thus, we hypothesize this difference in growth rates to indicate that *P. strigosa* overcame a size-dependent mortality threshold, a life history trait that is well documented in juvenile corals (Raymundo & Maypa 2004; Vermeij & Sandin, 2008; Ferrari et al. 2012). *O. faveolata*, which faced 100% recruit mortality by year two, proved to be an ineffective species for sexual restoration in this study. While it is recognized that results are inevitably biased by cohort-effects, this mortality rate (99-100%) is commonly observed in *O. faveolata* sexual propagation efforts (Alvarado-Chacón et al. 2020; Nixon & Gutting pers. obs.) and is concurrent with low natural recruitment rates (Hughes and Tanner 2000). Results highlight the importance of considering species-specific life-history traits when strategizing restoration. Based on this report, we recommend maintaining sexually propagated brain corals for one year or longer on *in situ* nursery structures. Further research is required to determine species-specific size-to-escape thresholds and the most effective nursery grow-out time periods.

In situ nursery efforts and brain coral species are the most cost-effective multi-year investment. Understanding the economic investment needed to restore habitat is crucial to successfully planning and implementing restoration projects. SU yield calculations and cost analyses provide applicable insights for restoration practitioners that are essential for scalable implementation. The most cost-effective method for *D. labyrinthiformis* changed over the course of the multi-year study, highlighting the importance of long-term monitoring. For *D.*

labyrinthiformis, *in situ* nursery rearing resulted in a higher yield but similar cost to direct outplanting; for *P. strigosa, in situ* nursery rearing was the only method that yielded surviving juveniles by the end of the study. These results suggest that a greater initial investment may yield greater long-term return. *O. faveolata* had the highest cost of the three species at one year, ranging between $34 and $42 per SU. While these values were not considerably different from the ranges seen in the brain corals at one year, the 100% mortality of directly outplanted *O. faveolata* seen at two years rendered propagation of this species to be all cost with no return. SU cost was comparable to other studies, which have found SUs retaining at least one coral to be $5.30-325 USD at 1-3 years of age (Chamberland et al. 2015; reviewed in Omori 2018). The most effective way to reduce this cost is by improving SU yield via increased survival during the early recruit stage (<1 year). Husbandry techniques such as feeding, co-rearing, and symbiont acquisition stand to greatly increase survival and thus cost-effectiveness (reviewed in Omori et al. 2018). Cost-effectiveness can also be improved by identifying and reducing the most expensive cost categories. In this study, labor was the highest cost category across all treatments; therefore, reducing restoration steps and manual labor holds potential to decrease cost per SU.

High operational costs and eventual complete mortality rendered the aquaculture facility rearing an extremely expensive investment (>$80,000 USD) with no return. These results do not suggest that land-based facilities hold no potential for successfully scaling coral sexual propagation but do represent realistic obstacles to establishing controlled aquaculture facilities on small islands with limited resources. Facilities utilized in this study were established in 2020, only months before the start of this study. During the investment period the facility faced multitudinous operational issues, including insufficient operating equipment, island-wide power failures, and staffing shortages. Diel temperature generally fluctuated by 1.5°C, rising at peak

daylight hours, with a maximum recorded swing of 4.4°C in December 2020. Colder months elicited substantial temperature fluctuations (± 2.8°C) as systems were equipped with chillers but no heaters and were unable to maintain temperatures at night. Salinity was also highly variable as staff were managing a newly running closed system. Temperature and salinity are known to affect coral recruit survival (Vermeij et al. 2006; Randall & Szmant 2009), and despite monthly SU cleaning, these variable physical (light, temperature) and chemical (salinity, pH) conditions inevitably contributed to the low to no survival observed across species. Additionally, survival rates could likely have been improved and costs reduced by feeding recruits (Conlan et al. 2017) and introducing herbivores to the tanks earlier in the study and in greater quantities. While survival rates may be increased with improved facilities control and additional husbandry, the high cost of operating an aquaculture facility requires greatly increased survival rates and yield to make it an economically viable technique, with Chamberland et al. (2015) reporting 2.5-year-old SUs to be over 20 times more costly when reared in an aquaculture facility as compared to directly outplanted on reef.

These findings highlight the importance of multi-year monitoring for restoration research. Future cost scenarios should examine how to further decrease cost per SU by either increasing recruit survival and thereby SU yield, or reducing costs associated with the most expensive restoration stages and cost categories. Highest cost categories will vary by program and region, and program-specific cost-effectiveness analyses are recommended to identify where reductions can be most beneficial on an individual program basis. Additionally, standardizing methods of cost evaluations across programs is recommended to allow for cross-programmatic comparisons.

Additive stressors have largely shifted Caribbean reefs to algae-dominated systems, characterized by increased hypoxia (Altieri et al. 2017; Candy et al. 2023), microbialization, and

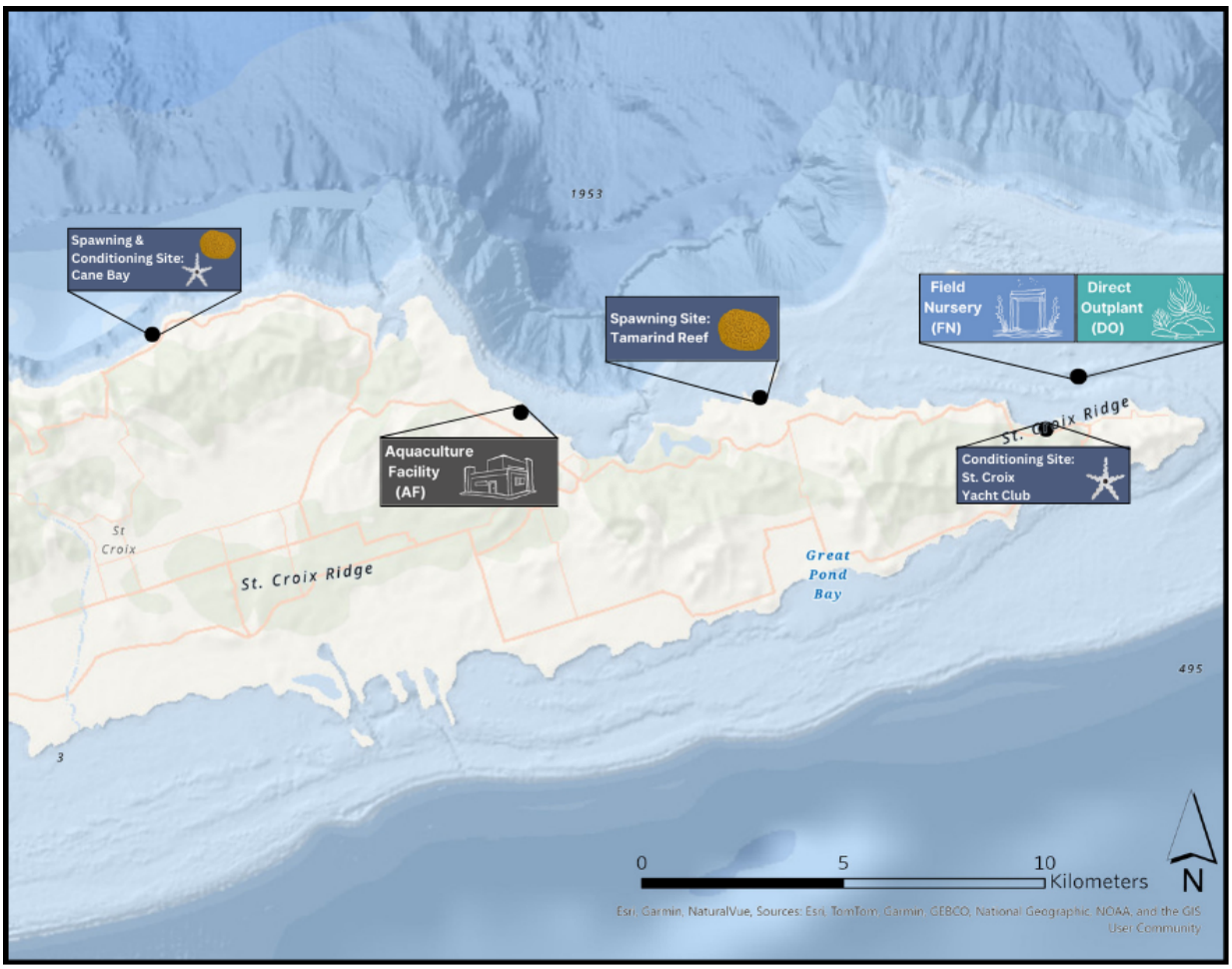
disease (Vega Thurber et al., 2008; Haas et al. 2016; Becker et al. 2024), all of which are known detriments to coral recruit survival (Vermeij et al. 2009; Williamson et al. 2022; Mallon et al. 2023). Ultimately, it is important to recognize that sexual propagation alone is not a viable restoration strategy for restoring algae-dominated reefs to calcifying scleractinian coral-dominated systems. Nonetheless, substrate unit yield and cost effectiveness of four-year-old juveniles suggests that outplanting of sexually propagated corals is a viable addition to the coral restoration toolkit, and that implementing an *in situ* nursery rearing stage should be considered when scaling up sexual propagation efforts for future reef restoration.

Acknowledgements

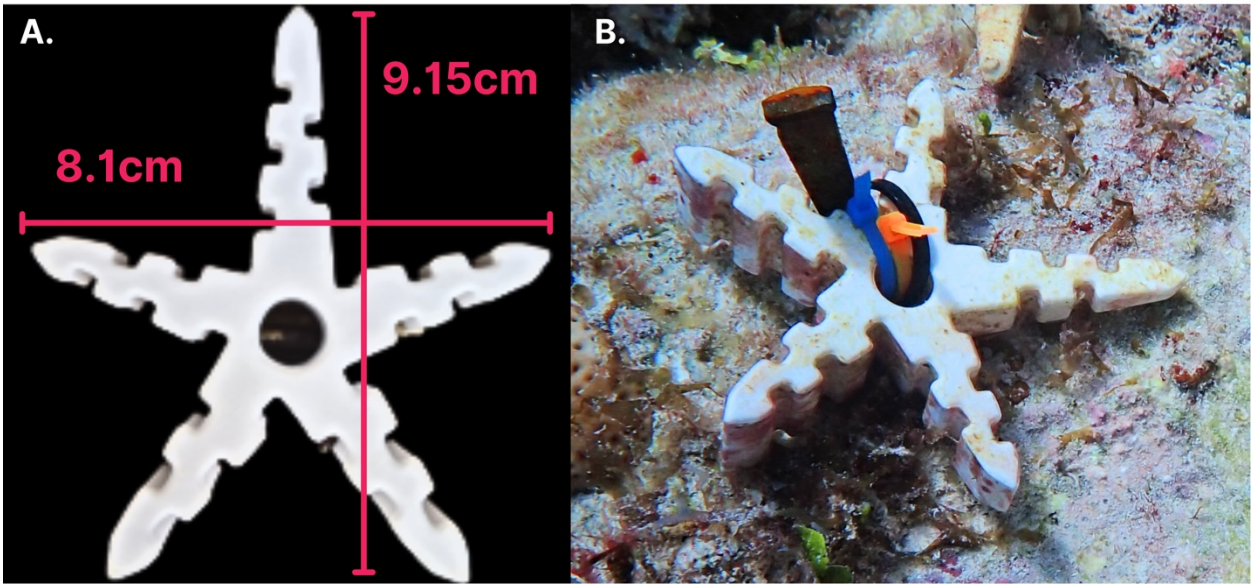
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Chapter 1, in full, is a reprint of the material as it appears in *Restoration Ecology*, 2025. Nixon, E. N., Gutting, A. N., Cook, S., Wegley Kelly, L., & Lillis, A. (2025). “Multi-year evaluation of rearing techniques for three sexually propagated Caribbean corals in a restoration setting”. The dissertation author was a co-primary researcher and author of this paper.

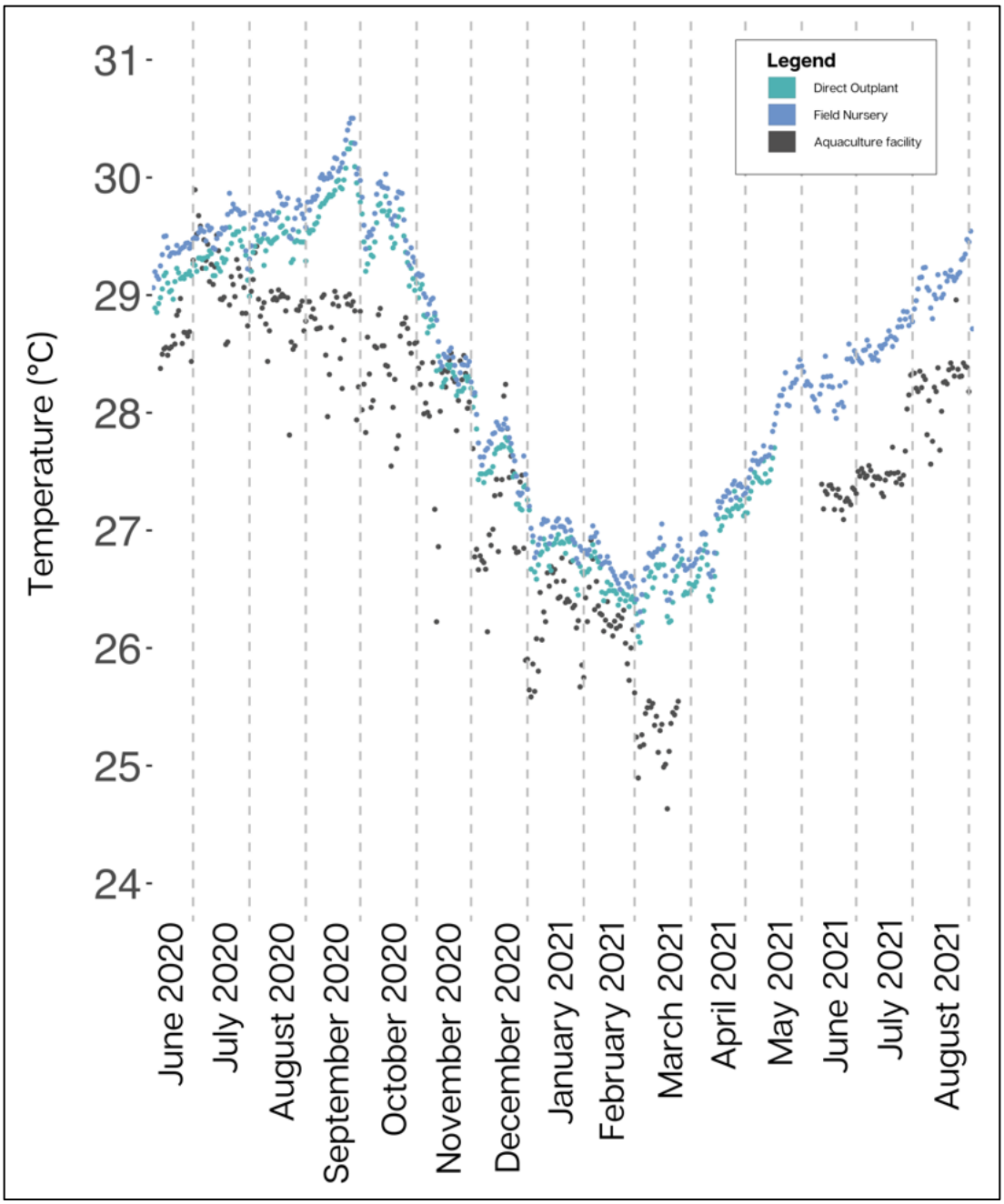
Supplemental Figures



Supplemental Figure 1.1 Map of St. Croix, USVI. Substrate conditioning, spawning, and treatment sites.



Supplemental Figure 1.2 Substrate unit diagram and described outplanting technique. (a) Fabrication diagram of SECORE substrate unit (Yixing Gear 2020). Units were manufactured of 95% alumina ceramic and produced via injection molding. Arms of unit ranged between 4.0-5.75 cm in length and the center hole was 0.108 cm in diameter. Surface area of the entire unit (b) Photograph of outplanted substrate unit attached to reef by nail and cable-tie.



Supplemental Figure 1.3 Treatment temperature data. Mean monthly temperature for FN and DO (MMT) during the one year of rearing treatment ranged from 26.7°C in March 2021 to 29.7°C in August 2020. Minimum temperature experienced for FN and DO was 25.9°C in March 2021 to 29.7°C in August 2020. Minimum temperature experienced for FN and DO was 25.9°C in March 2021 and maximum temperature experienced was 30.9°C in September 2020. MMT in the AF was consistently lower than the temperatures in the field and remained within 1 degree of the target temperature (28°C) for 9 of 13 months monitored, where in July 2020 MMT increased to 29.2°C and from January to March 2021 lowered to 25.3-26.3°C.

Supplemental Tables

Supplemental Table 1.1 Spawning metadata table. Spawn collection and scoring data for the three focal species, *Diploria labyrinthiformis, Pseudodiploria strigosa, and Orbicella faveolata.*

Species	Collection Site	Collection Date	# of Parent Colonies	SU per Treatment	1-year Score	Outplant Date	2-year Score	3-year Score	4-year Score
Diploria labyrinthiformis	Cane Bay	5/18/2020	14	9	9/3/2021	9/3/2021	6/9/2022	7/6/2023	6/11/2024
Pseudodiploria strigosa	Tamarind Reef	8/10/2020	9	9	9/3/2021	9/3/2021	8/12/2022	9/21/2023	6/11/2024
Orbicella faveolata	Tamarind Reef	9/8/2020	12	7	10/14/2021	-	8/12/2022	-	-

Supplemental Table 1.2 Specific cost breakdowns per coral recruit investment treatment Costs of materials and labor modelled using substrate unit yield data and scaled to 4,000 SU.

Direct Outplant

	Equipment	Consumables	Labor	Dive gear	SCUBA tanks	Boats	Other	All categories	% Total cost
Preparation	$8.76	$8.00	$1,926.42	$189.87	$101.77	$0.00	$0.00	$2,234.82	7.04%
Gamete collection	$765.81	$16.00	$2,056.53	$189.87	$101.77	$0.00	$0.00	$3,129.99	9.86%
Hatchery work	$545.77	$5,722.17	$8,211.65	$0.00	$0.00	$0.00	$306.12	$14,785.70	46.58%
Rearing	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	0.00%
Outplanting	$10.20	$50.00	$4,727.27	$253.16	$271.40	$990.30	$0.00	$6,302.34	19.86%
Monitoring	$772.64	$43.00	$3,120.45	$126.58	$135.70	$1,090.30	$0.00	$5,288.67	16.66%
All phases	$2,103.17	$5,839.17	$20,042.33	$759.49	$610.64	$2,080.61	$306.12	**$31,741.52**	
% Total cost	6.63%	18.40%	63.14%	2.39%	1.92%	6.55%	0.96%		

Supplemental Table 1.2 (continued) Specific cost breakdowns per coral recruit investment treatment. Costs of materials and labor modelled using substrate unit yield data and scaled to 4,000 SU.

In situ Field Nursery

	Equipment	Consumables	Labor	Dive gear	SCUBA tanks	Boats	Other	All categories	% Total cost
Preparation	$8.76	$8.00	$1,926.42	$189.87	$101.77	$0.00	$0.00	$2,234.82	4.05%
Gamete collection	$765.81	$16.00	$2,056.53	$189.87	$101.77	$0.00	$0.00	$3,129.99	5.68%
Hatchery work	$545.77	$5,722.17	$8,211.65	$0.00	$0.00	$0.00	$306.12	$14,785.70	26.83%
Rearing	$1,196.27	$163.50	$15,570.45	$791.14	$746.34	$4,906.36	$0.00	$23,374.06	42.41%
Outplanting	$10.20	$50.00	$4,727.27	$253.16	$271.40	$990.30	$0.00	$6,302.34	11.43%
Monitoring	$772.64	$43.00	$3,120.45	$126.58	$135.70	$1,090.30	$0.00	$5,288.67	9.60%
All phases	$3,299.43	$6,002.67	$35,612.78	$1,550.63	$1,356.98	$6,986.97	$306.12	**$55,115.58**	
% Total cost	5.99%	10.89%	64.61%	2.81%	2.46%	12.68%	0.56%		

Aquaculture Facility

	Equipment	Consumables	Labor	Dive gear	SCUBA tanks	Boats	Other	All categories	% Total cost
Preparation	$8.76	$8.00	$1,926.42	$189.87	$101.77	$0.00	$0.00	$2,234.82	2.79%
Gamete collection	$765.81	$16.00	$2,056.53	$189.87	$101.77	$0.00	$0.00	$3,129.99	3.91%
Hatchery work	$545.77	$5,722.17	$8,211.65	$0.00	$0.00	$0.00	$306.12	$14,785.70	18.46%
Rearing	$3,858.42	$1,563.34	$40,380.61	$0.00	$0.00	$0.00	$3,367.35	$49,169.72	61.38%
Outplanting	$10.20	$50.00	$4,727.27	$253.16	$271.40	$990.30	$0.00	$6,302.34	7.87%
Monitoring	$772.64	$43.00	$2,652.39	$94.94	$101.77	$817.73	$0.00	$4,482.46	5.60%
All phases	$5,961.59	$7,402.51	$59,954.87	$727.85	$576.72	$1,808.03	$3,673.47	**$80,105.03**	
% Total cost	7.44%	9.24%	74.85%	0.91%	0.72%	2.26%	4.59%		

Supplemental Table 1.3 *Orbicella faveolata* cost table. Total cost, SU yield (%), and cost per SU for each rearing method at one, two, three, and four years of age. Assumes 4,000 SUs produced. All costs in US dollars.

Rearing method	Restoration cost of 4,000 SUs (USD)	1 Year		2 Years		3 Years		4 Years	
		SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)	SU yield (%)	SU cost (USD)
Direct outplant	31,741.52	22	35.71	0	-	0	-	0	-
***In situ* nursery**	55,115.58	33	41.75	Not scored	-	Not scored	-	Not scored	-
Aquaculture facility	80,105.03	0	-	0	-	0	-	0	-

Supplemental Materials

Supplemental Material 1.1 Symbiodiniaceae algal cell isolation

Tissue was removed from an adult coral fragment using a hand-held paint sprayer and collected in a zip-loc bag. The slurry was then decanted into a 50 mL conical tube (Falcon), and then emulsified using a Tissue-Tearor (BioSpec Products) to break open coral cells and break up mucus. Algal cells were separated from the emulsified homogenate via sequential low-speed centrifugation. The homogenate was spun down for five minutes, the top layer of coral tissue and water was removed with a plastic transfer pipette, and the algal pellet was resuspended in seawater. This rinsing process was repeated until all coral tissue was rinsed (approximately three rinses). The algal isolate was resuspended in rinse water, resuspended, and 100 µL was aliquoted to a haemocytometer for cell counts. Once density was quantified, the isolate was then distributed into settlement bins.

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Chapter 2 COMPOSITE SUBSTRATES FOR CORAL LARVAL SETTLEMENT AND REEF RESTORATION BASED ON NATURAL HYDRAULIC LIME AND INORGANIC STRONTIUM AND MAGNESIUM COMPOUNDS

Abstract

Coral reefs face unprecedented threats from climate change and human activities, making reef restoration increasingly important for the preservation of marine biodiversity and the sustainability of coastal communities. One promising restoration method relies on coral breeding and larval settlement, but this approach requires further innovation to achieve high rates of settlement and survival. In this study, we built on our previous work engineering lime mortar-based coral settlement substrates by investigating three different compositions of a natural hydraulic lime (NHL) base material as well as composite NHL substrates containing alkaline earth metals. These materials were tested with larvae of three reef-building Caribbean coral species: *Orbicella faveolata* (Mountainous star coral), *Diploria labyrinthiformis* (Grooved brain coral), and *Colpophyllia natans* (Boulder brain coral). We found that the base material composition as well as the addition of the inorganic additives strontium carbonate ($SrCO_3$), magnesium carbonate ($MgCO_3$), and magnesium sulfate ($MgSO_4$) enhance coral larval settlement rates and swimming behaviors. Overall, NHL formulations with lower concentrations of silicate and higher concentrations of strontium and magnesium carbonates significantly increased coral settlement. Further, when dissolved ions of magnesium and strontium were added to seawater, both had a significant effect on larval motility, with magnesium promoting settlement and metamorphosis in *C. natans* larvae, supporting the observation that these additives are bioactive when incorporated into substrates. Our results demonstrate the potential benefits of incorporating specific inorganic ion additives such as Mg^{2+} and Sr^{2+} into substrates to facilitate early coral life history processes including settlement and metamorphosis. Further, our results highlight the importance of optimizing multiple aspects of coral substrate design,

including material composition, to promote settlement and survival in coral propagation and reef restoration.

Introduction

Coral reefs are one of the most diverse ecosystems on the planet, providing habitat for a wide range of marine species and supporting the livelihoods of millions of people worldwide (El-Naggar, 2020; Hoegh-Guldberg et al., 2017). These ecosystems are highly sensitive to climate change, pollution, overfishing, and coastal development, resulting in the ongoing loss of coral reefs globally (Hoegh-Guldberg et al., 2017; Huang, 2012; T. P. Hughes, 1994; T. P. Hughes et al., 2003). In response to this decline, a concerted effort has emerged among marine conservation organizations and scientists to develop more effective and scalable tools for restoring these ecosystems. One reef restoration strategy involves the fabrication and deployment of artificial structures(Lee et al., 2018) created using techniques such as molding or slip-casting. On an ecosystem-scale, the final goal of these artificial reef structures is to develop into self-sustaining natural reefs by offering suitable surfaces for coral larval recruitment and facilitating their growth into mature colonies, thus accelerating the permanent establishment of a robust reef ecosystem. On a smaller scale, settlement substrates are being developed and optimized to support the recruitment, growth, and survival of juvenile corals, which remains a major challenge in coral larval propagation and settlement.

Innovative approaches to support coral recruitment and promote settlement involve using biological cues such as bacterial isolates (Tebben et al., 2011), bacterial biofilms (Negri et al., 2001), crustose coralline algae (CCA) (Gómez-Lemos et al., 2018) and more recently inorganic cues such as Ca^{2+} ions (Yang et al., 2022). Calcium is a known component of coral skeleton but is presumed to initiate settlement in part due to the role of Ca^{2+}-binding proteins in various cell

signal pathways related but not limited to invertebrate settlement and metamorphosis(Carafoli et al., 2001; Yang et al., 2022). Additional alkaline ions, namely elemental strontium and magnesium, are found in coral skeletons beginning at very early life stages and it is presumed that these elements play an important role in coral skeletal calcification (Akiva et al., 2018; Mass et al., 2017; Neder et al., 2019; Sun et al., 2015). In fact, the Sr:Ca ratio in the skeleton of corals and coral fossils is widely used to estimate the ancient composition and temperature of seawater (Hughes et al., 2019; Sayani et al., 2022; Walter et al., 2023). Further, our previous work demonstrated increased coral settlement in response to strontium carbonate when incorporated into a lime mortar base ($SrCO_3$; (Levenstein, Marhaver, et al., 2022). This work also demonstrated a settlement response in the presence of silica (SiO_2), but not in the presence of dolomite ($CaMg(CO_3)_2$, an insoluble form of magnesium). Here, we continue to investigate the role of inorganic elements and ions in novel coral settlement substrate compositions. We hypothesized that the presence of elemental and ionic strontium and magnesium would increase coral larval swimming and settlement as would the silica content of the base material. In general, materials capable of incorporating these potential settlement cues and even facilitating their release, are of high interest in coral propagation and reef restoration (Banaszak et al., 2023).

Currently, materials such as Portland cement are widely used in artificial reef construction and coral propagation (Banaszak et al., 2023; Chamberland et al., 2017; Paxton et al., 2020). However, these materials are often highly alkaline and common compositions can leach harmful chemicals into the water, causing detrimental effects on coral growth and survival. Alternative materials gaining interest in restoration include lime and natural hydraulic lime (NHL) mortars (Diaz-Basteris et al., 2022; Levenstein, Marhaver, et al., 2022). Our recent research demonstrated that lime mortars can be useful in coral reef restoration providing a stable,

calcium carbonate-based substrate for coral larvae to attach and grow (Levenstein, Marhaver, et al., 2022). Calcium carbonate is the primary component of the coral skeleton, thus carbonated NHL mortars mimic certain aspects of the natural calcium carbonate composition of coral reefs. Incorporating calcium carbonate is hypothesized to promote coral attachment and growth in restoration materials (Spieler et al., 2001). Moreover, carbonated NHL has several advantages over conventional materials, such as ceramic or Portland cement, that are currently used in coral reef restoration. It has a higher microporosity than fired ceramics (Silva et al., 2008)and polymers (Mayhoub et al., 2022), allowing for better water perfusion and nutrient exchange, which are essential for coral growth (Yanovski & Abelson, 2019). Before setting, the NHL paste is malleable, allowing it to conform to irregular surfaces giving it the potential to mimic more natural reef structures and features. This material is also non-toxic and biocompatible; thus, it does not leach harmful chemicals into the water and is not considered to pose any risks to marine life. Additionally, the preparation of lime mortar substrates allows for the incorporation of inorganic and organic additives that may further enhance early coral settlement and growth. To further build upon previous research, here we expand our investigation from non-hydraulic lime mortars to natural hydraulic lime (NHL) mortars.

Importantly, NHL mortars can be tailored to meet species-specific needs and incorporate a variety of features such as structure, texture, and micro-niches. Producing substrates with a variety of these features provides a test bed to determine optimal parameters for coral settlement and growth. For this purpose, it is essential to understand the chemistry occurring during the hydration and carbonation processes when NHL mortars are employed. Here, we examined the effects of NHL composition and several inorganic additives on the swimming behavior and settlement of coral larvae. Two experiments investigated the impact of ion additives and base

material formulation on the early life history of corals: (1) a behavioral experiment examined the impacts of dissolved Mg^{2+} and Sr^{2+} ions on the survival, swimming behavior, settlement and metamorphosis of *Colpophyllia natans* (Boulder brain coral) larvae; and (2) a larval settlement study examined the effects of NHL-based substrates with and without ionic additives on the settlement rates of *Orbicella faveolata* (Mountainous star coral), *Diploria labyrinthiformis* (Grooved brain coral), and *C. natans* larvae.

Materials and Methods

Preparation of coral settlement substrates using natural hydraulic lime (NHL) base material. Three different commercial formulations of natural hydraulic lime mortar (NHL, Lime Works, USA) were used with increasing concentrations of calcium silicate (C2S); NHL2, NHL3.5, and NHL5 contained 17, 35, and 43 wt % C2S, respectively (values provided by the supplier, Lime Works, USA). NHL powder was mechanically homogenized with deionized water to yield pastes with a water:lime ratio of 0.7 (w:w). The NHL3.5 mortar was also then used as a base material to produce various settlement substrate compositions containing a range of inorganic additives, including strontium carbonate ($SrCO_3$), magnesium carbonate ($MgCO_3$), and magnesium sulfate ($MgSO_4$, all additives sourced from Sigma-Aldrich). For these NHL substrates with inorganic additives (Fig. 2.1a), NHL3.5 was dry-mixed with the selected inorganic additives using a shaker (Tornado II Paint Shaker 51000, Blair, Michigan, USA) prior to blending and homogenizing the NHL paste. NHL3.5 was used for these composite materials (instead of NHL2 and NHL5) due to its intermediate silicate content and following preliminary, small-scale trials demonstrating its ability to induce coral settlement without any biological or inorganic chemical additives.

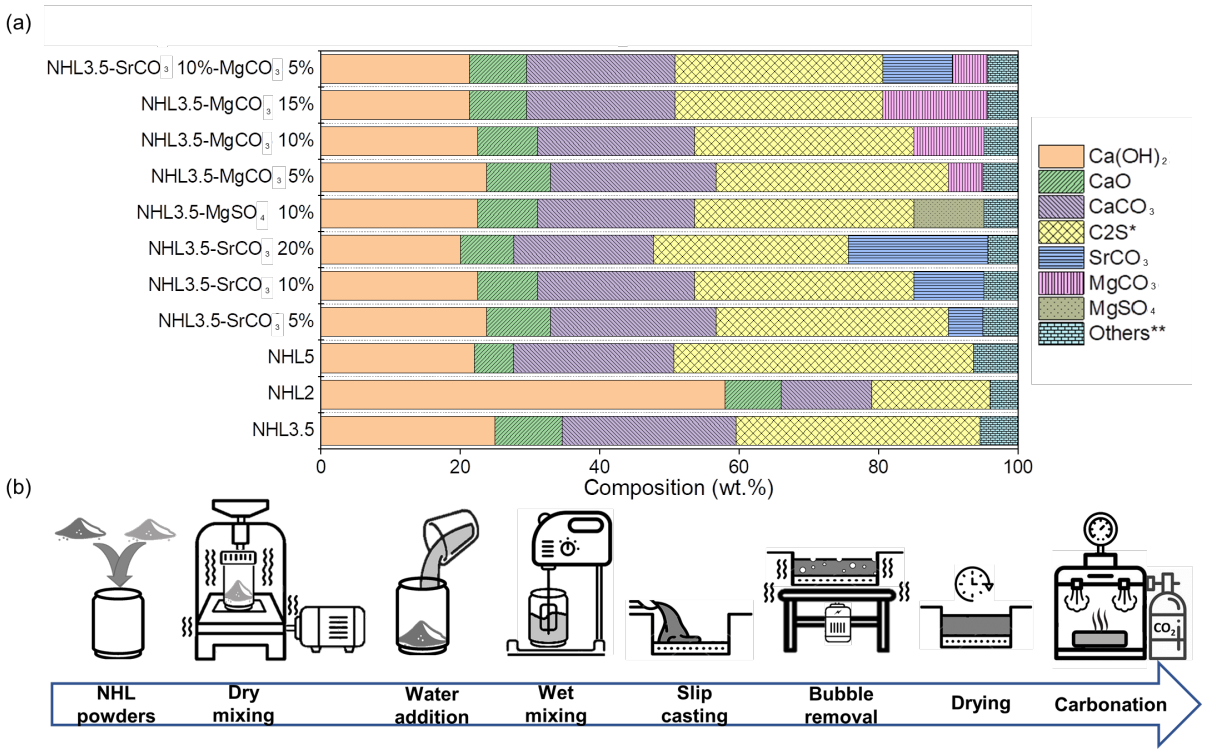


Figure 2.1 a) List of coral settlement substrate names and compositions. Substrate composition is shown as wt % of each component (both base material components and additives) in the mixture of dry NHL powder with additives, prior to the addition of water. *Dicalcium Silicate. **"Others" includes the trace components tricalcium aluminate, dicalcium alumina silicate, brownmillerite, and calcium sulfate. b) Schematic of the protocol used for manufacturing NHL-based coral settlement substrates.

The paste was then cast into round, food-grade, flexible silicone molds (2.54 cm diameter, 8 mm height) which were placed on a modified vibrating table to remove air bubbles. These disk-shaped substrates were left in the mold to dry overnight (16-24 h). Dry substrates were removed from the molds and placed into a pressurized, sealed chamber (Central Pneumatic, USA) with a CO_2 atmosphere of 15psi (1bar), thus accelerating carbonation speed relative to ambient conditions (i.e., accelerating the conversion of $Ca(OH)_2$ to $CaCO_3$). The 9.5L chamber held up to 120 substrates. This pressurized carbonation process led to full carbonation of calcium hydroxide in the substrates, as inferred from Fourier transform infrared spectroscopy (FTIR), described below.

NHL mortars contain a mixture of calcium hydroxide, calcium carbonate, and calcium silicates. These mortars are set by hydration, which refers to the effect of water binder ratio on the early hydration of NHL. After hydration, the carbonation of NHL leads to the transformation of the remaining calcium hydroxide into calcium carbonate (Ball et al., 2011; Figueiredo et al., 2016). This carbonation process is partly driven by the gaseous diffusion of CO_2 within the NHL microstructure. It is, therefore, a multi-decadal process under ambient conditions depending on the properties of the sample (Auroy et al., 2018). Thus, an alternative approach to accelerate carbonation is utilized to speed up this process. Using a modified pressurized CO_2 chamber, up to 95% carbonation can be achieved in 6h, with the added advantage that the equilibrium pH of the final material decreases from 12.4 to 8.4, ensuring that there is no calcium hydroxide left that could alter the pH of the experimental system or ecosystem upon deployment.

Characterization of substrate composition, carbonation, and hardness. The chemical composition of the three commercial NHL powders under investigation was confirmed by powder X-ray diffraction (XRD) using a D8 Advance X-ray diffractometer (Bruker, USA) equipped with a Cu source, TRIO optics, and a 2D Eiger2 R 500K detector (Dectris, USA). Hydrated and carbonated NHL substrates were ground into a fine powder with a mortar and pestle prior to analysis.

The carbonation rate of the substrates in the CO_2 chamber depended not only on the time of CO_2 exposure but also on the position inside the chamber. To determine the influence of the location on carbonation, and to ensure that all substrates achieved full carbonation during production, the carbonation chamber was fully loaded with NHL2 substrates (26 per rack, 4 stacked racks) and substrates were sub-sampled through time.

The presence of vertical gradients in CO_2 concentration would result in variations in the carbonation extent of substrates in each rack. To examine potential carbonation gradients in the radial direction, each rack was subdivided into quarters. To determine the time required in the chamber until full carbonation of calcium hydroxide in the substrates, one substrate was randomly selected from each rack and position in the carbonation chamber after 3, 16, and 23 h. Substrates were ground into a fine powder prior to determining the presence of calcium hydroxide by FTIR (Frontier FT-IR, PerkinElmer, USA).

Because substrate durability undoubtedly influences the success of coral restoration efforts, the hardness of each substrate formulation was determined by Vickers micro-indentation using an automated Vickers hardness device (Shimadzu Model HMV-M3 Micro Hardness Tester), with an applied load of 200 g. Vickers hardness (VHN) was calculated using the following equation (eq. 2):

$$VHN = 1854.4(M/d^2) \quad \text{(eq. 2)}$$

where M is the load (g) and d is the mean diagonal of the indentation (mm).

To further investigate the long-term stability and durability of NHL substrates when placed underwater, we out-planted substrates of 4 compositions (NHL3.5, NHL3.5-$MgCO_3$ 10%; NHL3.5-$SrCO_3$ 10%; and NHL3.5-$SrCO_3$ 10%-$MgCO_3$ 5%) at a common location on the coral reef in front of CARMABI Research Station (12°07’19.4”N 68°58’09.7”W). Substrates were imaged monthly for three months (underwater) and then again at 20 months (in lab) and visually inspected. At the 20-month endpoint, the substrate composition was analyzed by XRD (D8 Advance X-ray diffractometer, Bruker, USA) to measure any changes in chemical composition that had occurred. Substrates were ground into a fine powder prior to determining the crystallography of the substrates.

Collection and fertilization of coral gametes. Coral gamete bundles were collected during a succession of natural spawning events in August, September, and October 2021. Gametes were collected on the coral reefs off the Southern Caribbean island nation of Curaçao at the Zakitó dive site (also known as Water Factory; 12°06'29.7"N 68°57'12.6"W). Following methods described in previous works, egg-sperm bundles were collected from spawning coral colonies using weighted nylon mesh nets attached to plastic funnels and 50 mL conical polypropylene tubes (BD Falcon), thus concentrating the buoyant gamete bundles into a small volume for collection and transport(Hagedorn et al. 2021; Levenstein et al. 2022). *O. faveolata* gametes were collected from parent coral colonies on the night of September 26th; *C. natans* were collected on August 30th and September 27th, and *D. labyrinthiformis* were collected on October 3rd. Once ~5 mL of egg-sperm bundles had accumulated in each conical tube, the tube was removed from the funnel and capped. Gametes were returned to the lab directly after the spawning event, cross-fertilized by mixing in 1 L polycarbonate fat separators (Norpro), and incubated for at least 1 h. After fertilization, embryos were rinsed with 0.5 μm filtered seawater (FSW; serial spun polypropylene sediment filters with pore size 50, 20, 5, and 0.5 μm; H2O Distributors). Fertilized gametes were then distributed into polystyrene clamshell food containers (Dart Container Corporation) containing 1 L of FSW at the approximate density of 1 larva/mL (~1,000 larvae per bin). Water changes were performed daily to reduce microbial load and maintain a stable pH in the rearing environment throughout development (Chamberland et al. 2017; Hagedorn et al. 2021; Vermeij 2006).

All experiments were run in a temperature-controlled laboratory at CARMABI with a thermostat set to the water temperature of the local reefs (27.5 ± 1.5 °C). All unsettled larvae were released into the sea at the end of each experiment. Settled coral recruits were either

outplanted onto a restoration structure, moved to aquaria for long-term monitoring, or preserved for microscopic analysis.

Larval survival and swimming behavior experiment using dissolved magnesium and strontium ions. One advantage of using NHL as a coral settlement substrate is the ability to incorporate diffusible additives that can be slowly released into the surrounding seawater, supporting initial settlement or longer-term survival and growth. To test for survival and swimming behavior effects of ion additives, we conducted a laboratory experiment to mimic the environment that coral larvae would experience when in proximity to NHL substrates that release magnesium and strontium ions. To mimic these conditions, $MgSO_4$ and $SrCO_3$ stock solutions were prepared based on the observed 3-day dissolution rates of Sr^{2+} from the NHL composite substrate (Fig S2.1). The effect of dissolved inorganic additives on larval survival, swimming behavior, and settlement was then monitored to understand how the proposed additives may affect coral larvae during dispersal and settlement. Each experimental block consisted of one six-well plate, using the four outside wells, with one well per treatment in each plate. Wells within a block were assigned a treatment using a random number generator. In total, there were four treatments: each inorganic additive individually (Mg^{2+} and Sr^{2+}), the inorganic additives synergistically (Mg^{2+} and Sr^{2+}), and a control (FSW). For Sr^{2+} treatments, a stock solution with $SrCO_3$ was prepared by dissolving 0.336g of $SrCO_3$ (Sigma-Aldrich, purity ≥98%) into 200 mL of FSW and stirring on low heat (40-50 ºC) for 24 h. 2 mL of the stock solution was then diluted in 498 mL FSW to obtain a final ion concentration of 12 ppm of Sr^{2+} (150% with respect to ambient seawater). For Mg^{2+} treatments, 1.6g of $MgSO_4$ anhydrous (Sigma Aldrich, ≥99.5%) was dissolved directly into 500 mL of FSW to obtain a final concentration of 1950 ppm of Mg^{2+} (150% with respect to ambient seawater).

Two days after fertilization, 800 swimming planula larvae were pooled from rearing bins using a 70 μm nylon cell strainer (Fisher Scientific) and rinsed in FSW. Larvae were then evenly allocated to 20 replicate wells per treatment, 10 larvae per well. Larval behavior was scored at 09:00h daily for six consecutive days. Behaviors and survival were scored using four distinct categories: actively motile or swimming, motionless, settled and metamorphosed, or dead. Dead larvae lose their rounded bilateral symmetry and appear frayed or become completely absent (Marhaver et al., 2013) and are thus distinguishable from motionless larvae. Settled and metamorphosed coral larvae have successfully adhered to the well and developed a polyp mouth. The proportion of motile larvae was calculated using the total number of larvae observed alive in each well. A linear mixed-effects model was fit to compare the effect of factors Mg^{2+} and Sr^{2+} on the proportion of both motile and alive larvae in a given well using days 1-6 as the repeated measures response variable. Well location and plate number were included in the linear regression as random factors. Due to non-normality, a non-parametric permuted ANOVA was performed to compare the effects of ion additives on the proportion of larvae that underwent successful settlement and metamorphosis on the final day of the experiment. Originally, a two-way permuted ANOVA was run with Mg^{2+} and Sr^{2+} as factors. Due to no significant interaction, the model was simplified to two one-way permuted ANOVAs with well number included in the model as a random factor. An additional permuted ANOVA was performed to compare the effects of Mg^{2+} and Sr^{2+} on survival. Well number was included in the model as a random factor.

Larval settlement experiment using NHL base materials and inorganic additives In the field of larval propagation, settlement is often promoted by adding known positive settlement cues such as CCA (Heyward & Negri, 1999a; Morse et al., 1988; Tebben et al., 2015). Here, all settlement studies were conducted without such cues to best elucidate larval response to NHL-

based substrates and the aforementioned inorganic additives of interest. Prior to beginning the settlement experiments, substrates in the lab were conditioned in FSW for 3 days. Substrates were turned over every 12 hours to release any air bubbles that had escaped from the substrate pores and accumulated on the substrate surfaces. Substrates were then each placed in a 200 mL polystyrene cup with 150 mL of FSW to evaluate the effects of NHL composition, inorganic additive type, and additive concentration on larval settlement. 100 (±5) larvae were pipetted into each cup, for a total of eight replicate cups per substrate type. All larvae were healthy, motile, and between 2-3 days old at the start of the experiments. Larval settlement on the top and bottom of each substrate was recorded following 13 days of exposure.

The total number of settlers on each substrate was used for statistical analysis. Due to non-normality, settlement data were analyzed via two separate one-way permuted ANOVAs (# permutations = 10,000) to compare the effect of NHL composition and inorganic additive type and weight % (Fig 2.1) on larval settlement for each of the three coral species. A series of Wilcoxon rank-sum pairwise comparisons were performed to distinguish differences between NHL and inorganic additives compositions separately.

Results

Characterization of substrate composition of NHL base materials and composite materials. The kinetics of accelerated carbonation of NHL mortars was studied by combining XRD and FTIR spectroscopy. The extent of carbonation was determined by FTIR. A peak at ~3650 cm^{-1} indicates the presence of calcium hydroxide, signifying incomplete carbonation (Fig 2.2a). The XRD data for NHL3.5 before and after accelerated carbonation confirmed that the pressurized CO_2 chamber achieved full carbonation of the material (Fig 2.2c). The dry NHL powder contained 25% calcite ($CaCO_3$), demonstrated by the diffraction peak at 29.6°. The

characteristic peaks of portlandite ($Ca(OH)_2$) occurred at 18° and 34.2°. After carbonation, those peaks disappeared, while the intensity of the calcite peaks increased.

Further, the presence of the $SrCO_3$ additive was confirmed by peaks at 25.3° and 26.0°, indicating its stability after the fabrication process (Fig 2.2c). In contrast, the addition of $MgSO_4$ provoked a reaction and formation of both gypsum, illustrated by peaks at 11.7° and 20.8° in cyan, and of dolomite, illustrated by a peak at 31°. It is also noteworthy that the addition of amorphous $MgCO_3$ did not shift any peaks; although this material is invisible in XRD, we confirmed that it did not modify either the intensities nor the positions of peaks of other crystalline phases that would occur if the carbonate reacted with the other materials present. This result suggests a high stability of the $MgCO_3$ during the production process.

The composition of three different NHL-based substrates (NHL2, NHL3.5, and NHL5) was qualitatively determined from the ratio of the silicate peak at 27.3° and the carbonate peak at 29.4° (Fig 2.2b). As expected, NHL2 had the highest portlandite content (peak at 18.1°) and lowest silicate content (peaks at 26.2°, 32.1°, 32.7° and 41.1°). NHL5 had the highest silicate content and the lowest amount of calcium hydroxide. NHL3.5 had intermediate levels of silicate and calcium hydroxide. This material was chosen as the base substrate material for testing the inorganic additives ($SrCO_3$, $MgCO_3$, and $CaSO_4$) at different concentrations. All compositions are listed in Fig 2.1a.

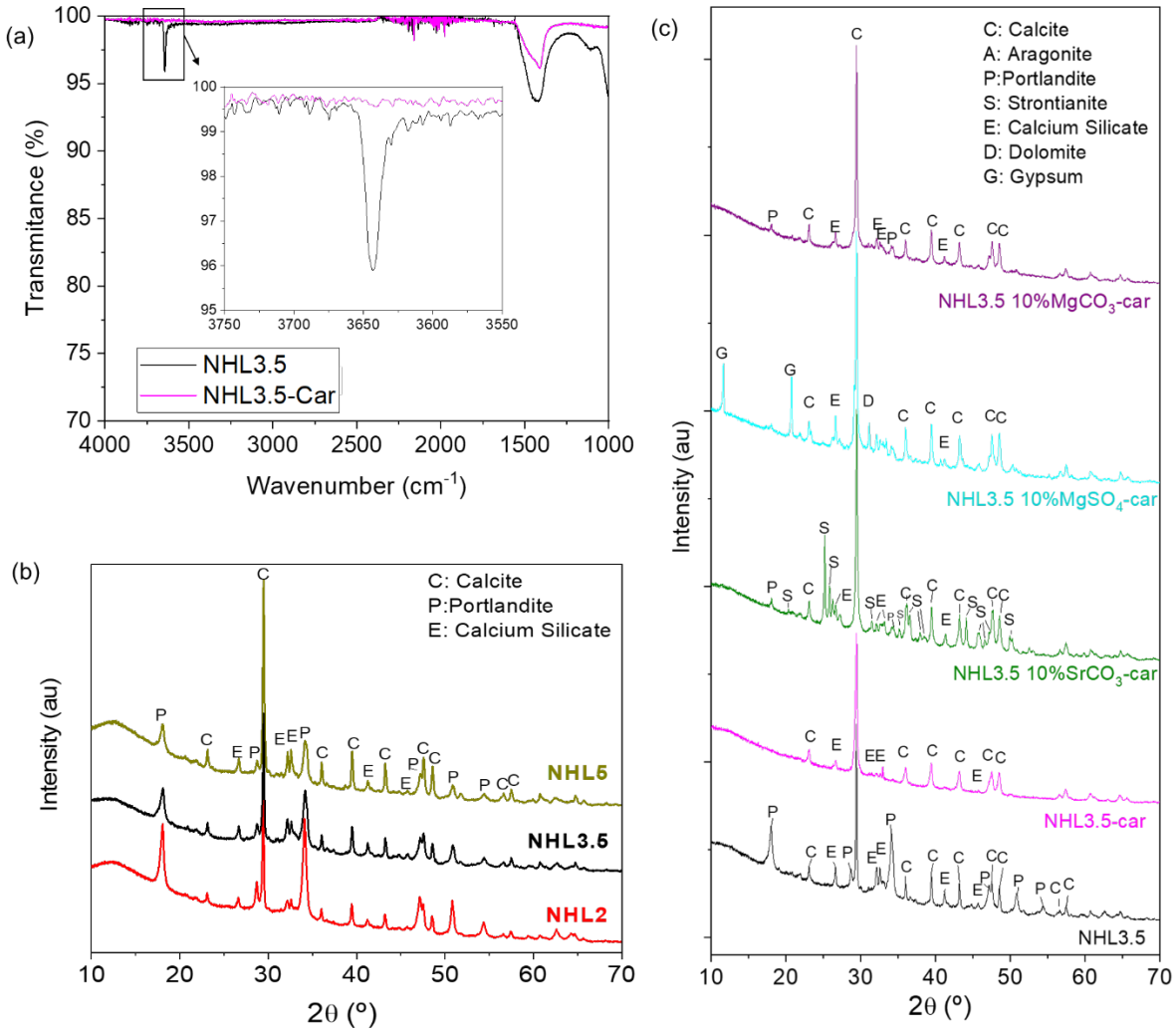


Figure 2.2 (a) Infrared spectra of NHL3.5 substrates before and after carbonation obtained via Fourier transform infrared spectroscopy (FTIR). A peak at 3650 cm^{-1} reveals the presence of $Ca(OH)_2$, indicating incomplete carbonation. (b) X-ray diffractogram comparing the different commercial NHL powders used in this study. The NHL powders were subject to analysis before carbonation. (c) X-ray diffractogram of unmodified NHL3.5 substrates before and after carbonation, and of the post-carbonated ("-car") NHL3.5 composite substrates containing 10wt.% $MgCO_3$, 10wt.% $SrCO_3$, and 10wt.% $MgSO_4$.

Carbonation of NHL substrates in a pressurized CO_2 chamber. To ensure that all substrates were fully carbonated in the production process, we investigated the degree of carbonation within the pressure chamber and determined whether substrate location affected the rate of carbonation results, specifically whether carbonation was faster for substrates located on the lower shelves (Figs 2.3a and 2.3b). Figs 2.3a and 2.3b show the vertical (racks 1 to 4) and

quarter (1 to 4) divisions used to determine if there were gradients during the carbonation reaction (eq. 2).

$$Ca(OH)_{2(s)} + CO_{2(g)} \rightarrow CaCO_{3(s)} + H_2O_{(g)} + \Delta \qquad \text{(eq2.)}$$

Due to the higher density of the gaseous CO_2 compared to the water vapor, the released H_2O (g) rises, creating a concentration gradient of CO_2 and potentially slowing the carbonation of the substrates located on the upper racks. Overall, carbonation was faster for substrates located on the lower shelves (Fig 2.3c). No radial gradient was observed. FTIR spectra are provided in the supplemental information (Fig S2.2).

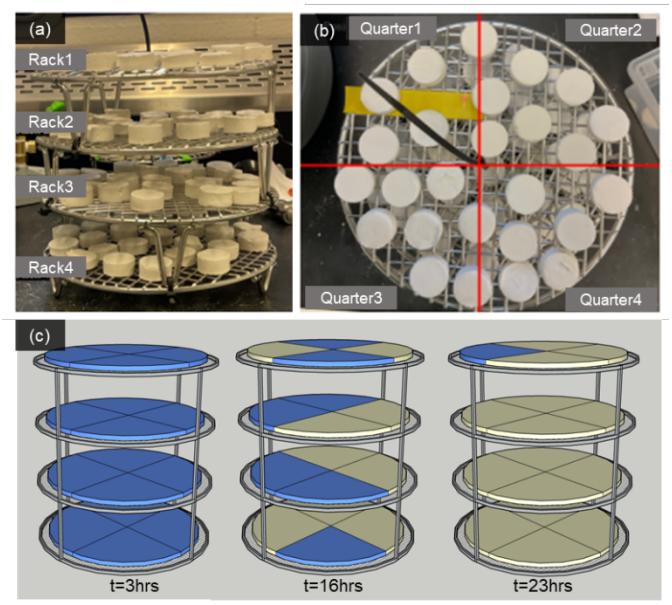


Figure 2.3 Carbonation chamber shelves and inner structure. Photos of the (a) vertical division into 4 racks and (b) sectorial division in 4 quarters. (c) Schematic showing the degree of carbonation after 3h, 16h, and 23h. Light yellow: fully carbonated, blue: not completely carbonated.

Material durability as measured by indentation. The hardness of substrates intended for use in coral reef restoration is an important material property. We use hardness as a simple proxy to indicate handleability and stability in the marine environment. Substrate stability is important for settlement whereas an unstable surface will hinder settlement and stable attachment, reducing

the success of restoration efforts. The hardness of the substrate can also affect its ability to withstand environmental stresses such as wave action and storm surge. A soft material may be more vulnerable to damage or displacement during high-energy events, which could undermine the stability of newly established corals.

Vickers micro-indentation tests were used to measure the hardness of all NHL base materials and composites (Fig. 2.4)**.** The sample composition with the highest Vickers hardness was NHL5 followed by NHL3.5, NHL3.5- $MgSO_4$ 10%, NHL3.5- $SrCO_3$ 10%, NHL2, NHL3.5 - $SrCO_3$ 10%- 5%, NHL3.5- $MgCO_3$ 10%. The strength of NHL mortars is mainly associated with the hydration of C2S. The calcium silicate hydrate (C-S-H) contributes to the self-setting property and spontaneously increases the strength of the material (Liu et al., 2019). Based on the C2S content of NHL (Fig. 2.1a), the expected results from the hardness tests agree with the observation of increasing strength with increasing C2S (i.e., NHL5 > NHL3.5 > NHL2). In addition, the addition of any additive is expected to decrease hardness, which we observed in the results from the NHL3.5 base material used in a variety of composites. $MgSO_4$ and $SrCO_3$ additives reduced the substrate hardness while $MgCO_3$ additives further decreased hardness even below that of NHL2 alone. This is explained by the chemical bonding and crystal structures of the two minerals, i.e., $MgCO_3$ has a less dense and more open crystal structure. The weaker ionic bonding between magnesium cations (Mg^{2+}) and carbonate anions (CO_3^{2-}) results in a less rigid structure, leading to lower strength compared to $SrCO_3$. Furthermore, from simply handling the substrates, it was evident that the addition of $MgCO_3$ greatly reduced the strength of the mortar.

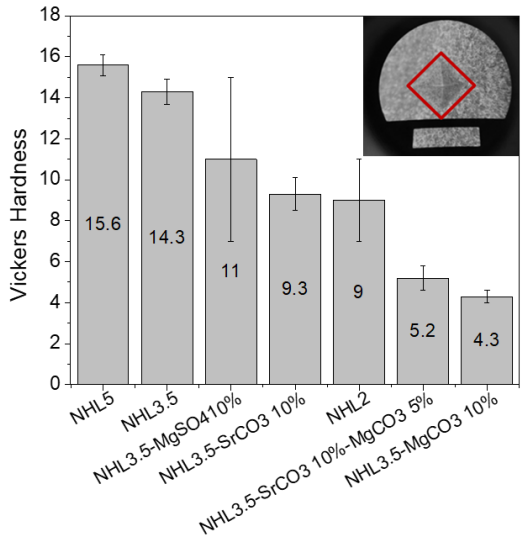


Figure 2.4 Substrate hardness of NHL base materials and composites. Average Vickers Hardness for the seven substrate compositions of interest (% referred to as wt.% of the additive mixed with dry NHL3.5). Inset: image of one indentation created during hardness testing. n = 3 replicate measurements per substrate type. Error bars represent SD.

In addition to measuring the hardness of newly-produced substrates, we investigated the long-term stability, durability, and fouling of four of the NHL3.5 substrate compositions by deploying replicate substrates underwater and monitoring them by imaging monthly for three months and then at one final timepoint after 20 months (Fig 2.5)**.** We observed that all four substrate compositions were vulnerable to fouling by mixed, turfing macroalgae and cyanobacteria, two benthic coral reef fouling groups known to inhibit coral settlement and survival (Birrell et al., 2005). However, we also observed the presence of settlement promoting crustose coralline algae (CCA), identifiable as small, calcified pink areas on the substrates (Fig 2.5, marked with small arrows in the image). Observations of the substrate surfaces also provided evidence of erosion and grazing by marine organisms. This was visible at months 2, 3, and 20 as dozens of white scrape marks where all fouling organisms had been removed and the bare, underlying material had been exposed (Fig 2.5). This is attributable to grazing organisms such as mollusks and parrotfish feeding on the proliferating turf algae. Parrotfish grazing can facilitate coral recruitment by removing macroalgae, leaving clean spaces for coral larval

settlement (Mumby, 2009). Overall, results also suggest that these substrates hold potential as viable options for promoting coral recruitment and growth since CCA appears to grow on them and this is an established coral settlement cue. Furthermore, while the proliferation of turfing algae is a potential deterrent to coral settlement and survival on these substrates, the relatively high density of grazing marks is an important indicator that these substrates can be effectively

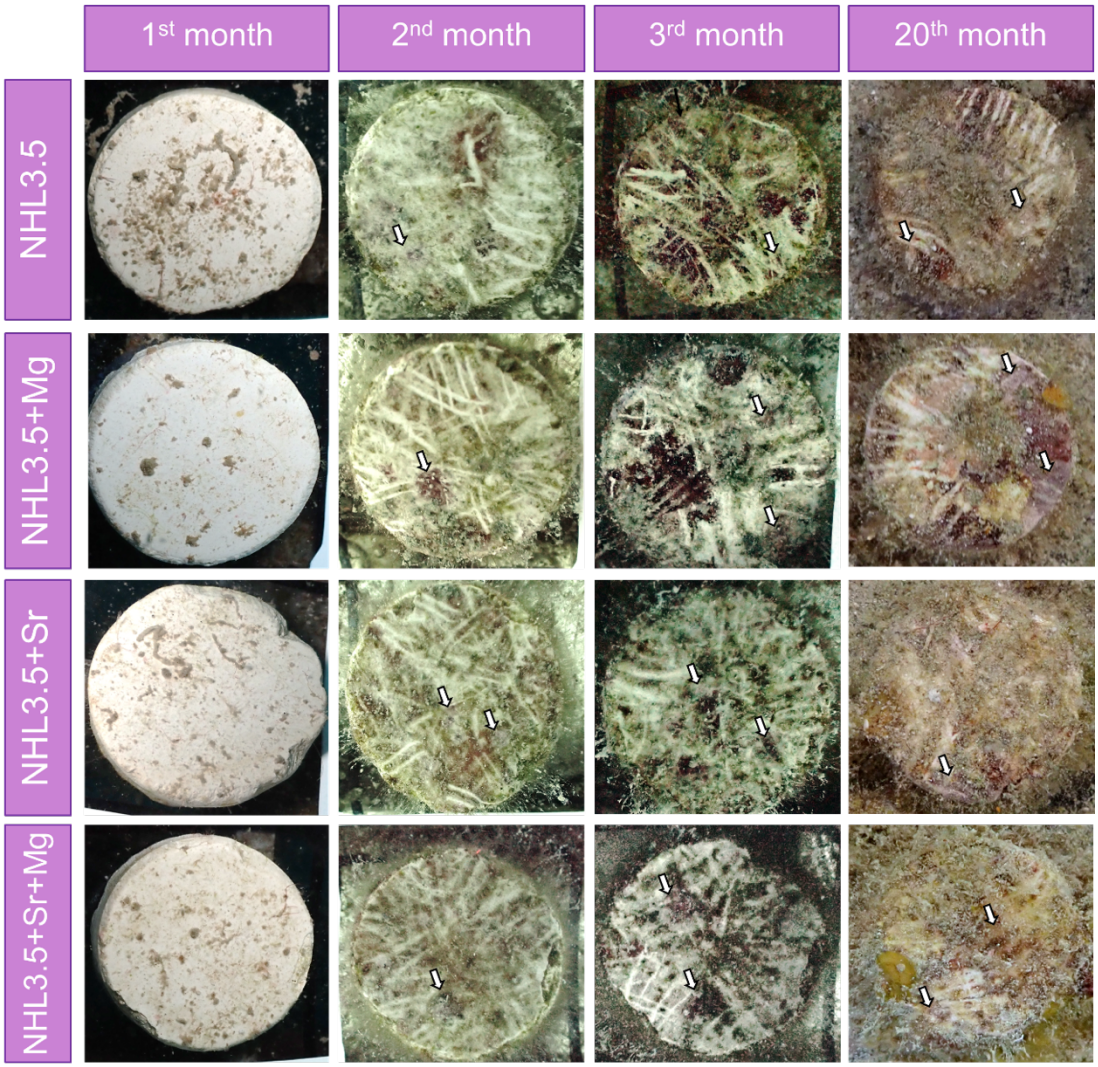


Figure 2.5 Growth and grazing of marine fouling organisms on NHL-based substrates deployed underwater for 20 months. Images show the progression of fouling by turfing macroalgae and the grazing (scraping) activity by marine organisms over time. Substrates were out planted on the reef in front of CARMABI Research Station in Curacao at 12 m depth. Black and white arrows mark areas where crustose coralline algae (CCA) was observed growing on the substrates. All substrates are approximately 2.54 cm in diameter.

grazed back to bare substrate, creating repeated opportunities for coral settlement and growth. This stands in sharp contrast to harder and sharper restoration materials such as ceramic and Portland cement, which typically become heavily fouled upon deployment and are rarely if ever grazed back to the bare substrate. Further investigations are needed to gain a more comprehensive understanding of the dynamics of fouling and colonization of these novel substrate compositions and to identify formulations that further promote the growth of CCA over turfing algae.

The substrate composition was further investigated by XRD after 20 months on the reef, confirming that out-planting over this period did not induce significant compositional changes. X-ray diffractograms of NHL3.5, NHL3.5-$MgCO_3$ 10%, and NHL3.5-$SrCO_3$ 10%-$MgCO_3$ 5% are presented in Fig. S2.3 of the supplemental information.

Exposure to inorganic ions in solution stimulates larval swimming behavior. To understand the potential effects of inorganic ion dissolution from composite substrates, we investigated the survival and behavior of coral larvae during the planktonic stage when exposed to ions in solution. Survival, swimming behavior, and settlement rates were measured in response to inorganic additives at 150% ambient seawater concentrations and in the absence of a settlement substrate. Survivorship remained high throughout the study, with the mean survival remaining above 97% for all treatments (Fig. S2.5). Mg^{2+} and Sr^{2+} had no significant effect on larval survival throughout the duration of the experiment ($p > 0.05$, Fig. S2.5). Time had a significant effect on survivorship ($p < 0.01$, Fig. S2.5), with an overall trend of decreased survivorship over time.

Dissolved Mg^{2+} elicited the strongest effect on coral larval motility ($p < 0.01$, Fig. 2.6a). This was most evident in the first three days of the experiment. On day 1, Mg^{2+} treatments had a

mean larval motility of 94% and 87% respectively. In comparison, to a mean larval motility in the FSW control of 62%. This treatment effect persisted through day 3, at which time mean larval motility in Mg^{2+} treatments had a mean larval motility of 59% and 52% respectively, in

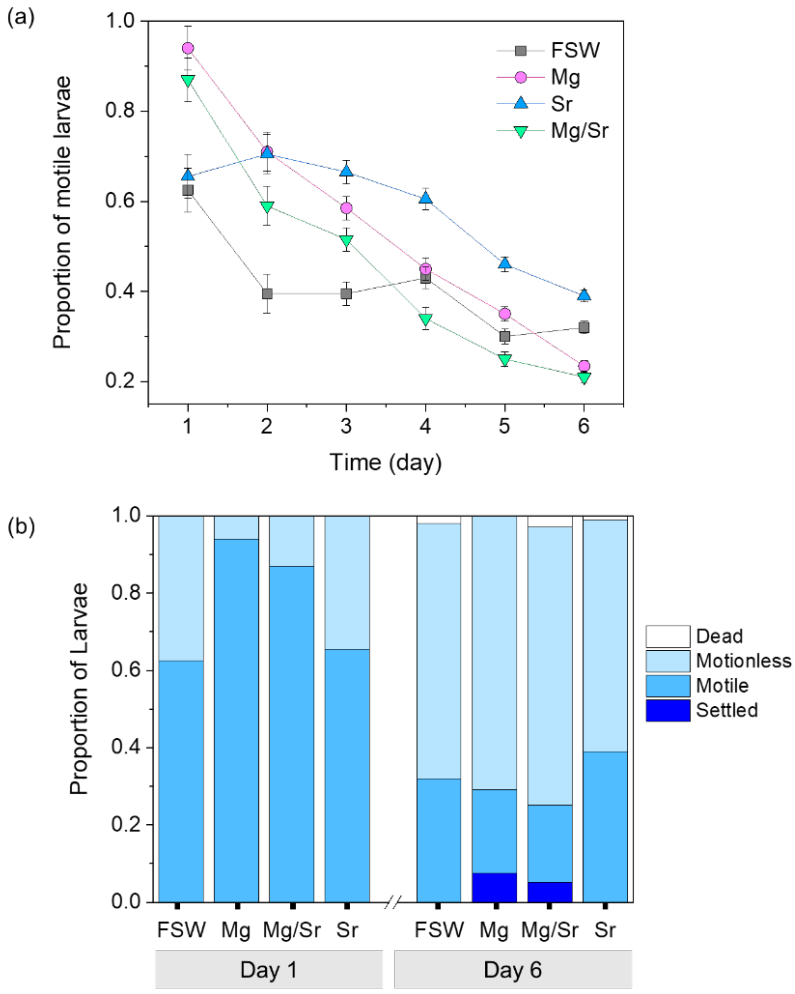


Figure 2.6 Swimming behavior and settlement of *C. natans* coral larvae when exposed to solutions of two inorganic ion additives used in composite settlement substrates**.** Larvae were exposed to elevated concentrations (150% with respect to ambient seawater) of Mg^{2+} (dots), Sr^{2+} (triangles), both Mg^{2+} and Sr^{2+} (inverted triangles), or a filtered seawater (FSW, squares) control absent of inorganic additives (black squares). Behavioral data were collected at 0900h daily for six consecutive days (n = 20 replicates/treatment). (a) Values reported are the treatment means for the mean proportion of motile larvae in each replicate well. Error bars represent +/-1 standard deviation from the mean. (b) Stacked bar plot comparison of *C. natans* survival and swimming behavior by treatment: dead, motionless, motile, and settled. Data are shown for day 1 and day 6 of the experiment.

comparison to 40% in the FSW control. This effect diminished by day 6, at which time Mg^{2+} treatments had a mean larval motility of 23% and 21% respectively, in comparison to 32% in the FSW control. Dissolved Sr^{2+} also caused a significant increase in larval motility ($p < 0.01$, Fig. 2.6a). Independent of Mg^{2+}, the effect of Sr^{2+} became apparent on day 2, with a mean larval motility of 71%. Larval motility remained higher in this treatment than in the control through day 5, at which time the Sr^{2+} treatment had a mean motility of 46%, in comparison to 30% in the FSW control. Similar to the dissolved Mg^{2+}, this effect of Sr^{2+} diminished by day 6, at which point the mean larval motility in this treatment decreased to 39%. Increased larval motility often signifies an increased search for suitable settlement substrate. This is supported by Mg^{2+} treatments significant effect on larval settlement and metamorphosis, with 5.5% and 8% mean settlement in Mg^{2+} and $Mg^{2+}Sr^{2+}$ respectively (permuted one-way ANOVA, $p = 0.04$, Fig. 2.6b). Notably, no settlement was observed in FSW control or Sr^{2+} treatment ($p > 0.05$, Fig. 2.6b).

Larval settlement onto NHL base materials and composite substrates. To examine the potential utility of NHL substrates and inorganic additives in coral propagation and reef restoration, we measured coral larval settlement preferences by scoring the number of coral larvae settled on each substrate in each replicate experimental cup. Box-and-whisker plots show settlement counts for *D. labyrinthiformis*, *O. faveolata*, and *C. natans* respectively (Fig. 2.7). In general, larvae preferred the bottoms of the substrates due to the protected, enclosed habitats created between the substrate and the cup. For statistical analysis, the sum of settlers on each substrate was considered whether on the top, bottom, or sides of the substrate. Both *D. labyrinthiformis* and *O. faveolata* larvae settled in higher proportions onNHL2 substrates compared to NHL3.5 and NHL5 ($p < 0.05$, Fig. 2.7a), while *C. natans* also settled on NHL2 to a slightly higher degree, there was no significant settlement preference ($p > 0.05$, Fig. 2.7a). NHL2

contains a smaller amount of silicates and more portlandite ($Ca(OH)_2$) that transforms into calcite after carbonation, so higher $CaCO_3$ content in NHL2 attracts slightly more larvae for settlement.

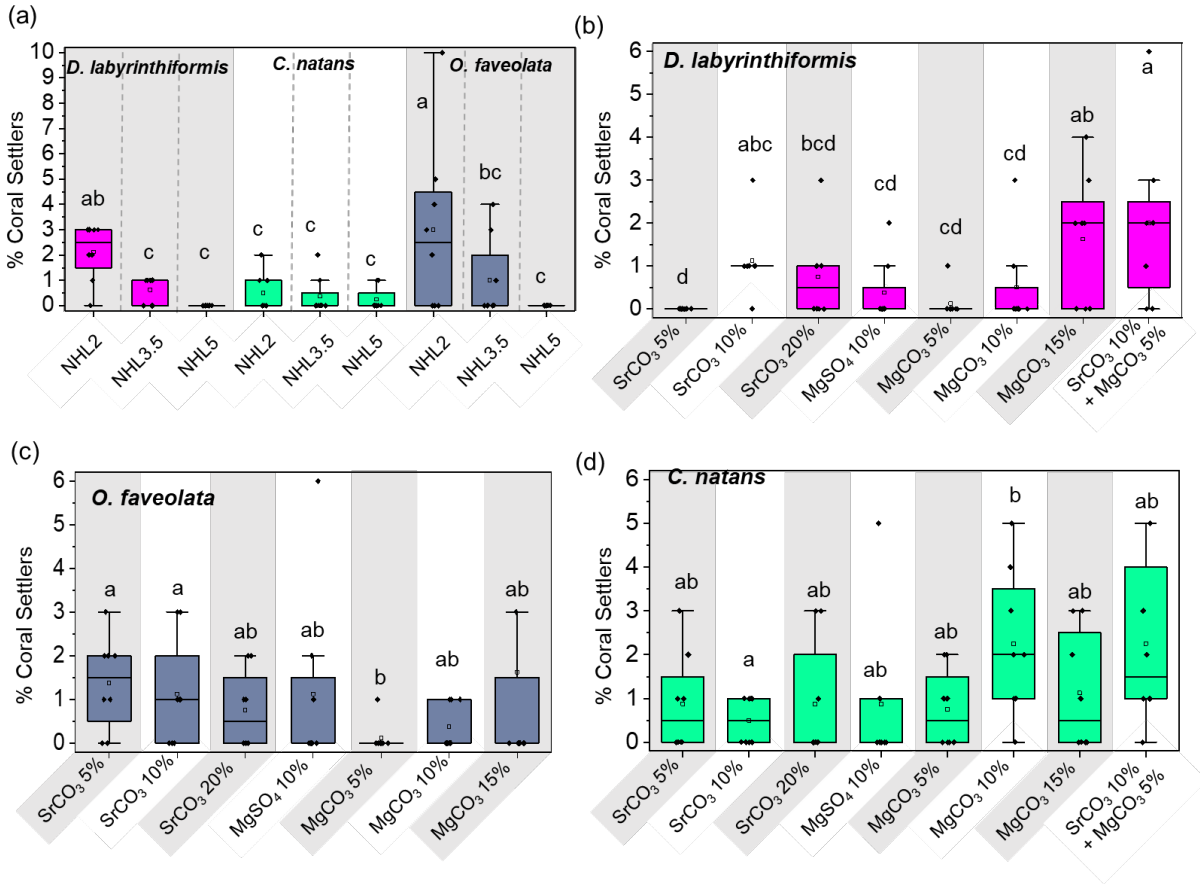


Figure 2.7 Settlers of *D. labyrinthiformis*, *O. faveolata*, and *C. natans* coral larvae onto NHL substrates and composites. Box-and-whisker plots show the percentage of larvae that settled onto each substrate type by day 13 of the experiment. (a) Settlement onto substrates composed of NHL2, NHL3.5, and NHL5 base materials only, with no additives. (b-d) Settlement of (b) *D. labyrinthiformis*, (c) *O. faveolata,* and (d) *C. natans* larvae onto NHL3.5 substrates with the X% of inorganic additives (referred to as wt.% of the additive mixed with dry NHL3.5). n = 8 replicates per experiment. Different letters indicate statistically significant differences among the groups ($p < 0.05$).

When comparing the various NHL3.5 substrates prepared with inorganic additives, *D. labyrinthiformis* larvae exhibited a statistically significant preference for substrates that contained greater than 10 wt.% of both $MgCO_3$ and $SrCO_3$ compared to substrates that contained $MgCO_3$ 5 wt.% and $SrCO_3$ 5 wt.% ($p < 0.05$, Fig. 2.7b). *O. faveolata* larvae avoided substrates

with $MgCO_3$ 5 wt.% settling in greater numbers on $SrCO_3$ 5 wt.% and $SrCO_3$ 10wt.% ($p < 0.05$, Fig. 2.7c). No significant settlement preference was observed between other substrates for this species. Similar to *D. labyrinthiformis*, *C. natans* larvae were significantly more attracted to the combination of $SrCO_3$ and $MgCO_3$ but also settled to a lesser degree on substrates with 10 wt.% of $MgCO_3$ ($p < 0.5$, Fig. 2.7d). However, for this species, no other significant differences were found among the different substrates besides the preference for NHL3.5 with 10wt% of $MgCO_3$.

Discussion

Material characterization and underwater durability of NHL-based coral settlement substrates. The results of this study highlight the influence of substrate composition and inorganic additives on coral larval behavior and settlement. We developed new coral-settlement substrate materials employing a pressurized CO_2 chamber to accelerate the carbonation of NHL-based substrates. The carbonation process transformed portlandite ($Ca(OH)_2$) into calcite ($CaCO_3$), changing substrate composition and increasing hardness. As expected, NHL5 exhibited the highest hardness, followed by NHL3.5, and NHL2, which correlated with the C2S content of NHL. The incorporation of inorganic additives, especially $MgCO_3$, decreased the substrate hardness (Fig. 2.1). However, this represents a potential trade-off in this material given that this additive may support coral skeleton growth; Mg^{2+} influences shape, morphology, and polymorphism of bioproduced $CaCO_3$, all of which influence in vivo skeletal growth in coral (Zaquin et al., 2022). Overall, based on our results, we recommend incorporating between 5 and 10 wt.% $MgCO_3$ into the substrates. With any more than 10 wt.% $MgCO_3$, substrates become fragile. This fragility was not observed in substrates with $SrCO_3$. Notably, $SrCO_3$ incorporation of more than 10 wt.% demonstrates no significant enhancement in Sr^{2+} release over short periods of time (Fig. S2.1). However, it will be released for longer. Therefore, incorporating more of this

carbonate is of potential significance, particularly if it promotes skeletal growth or positively modulates other local environmental factors pertinent to the sustained health of coral ecosystems over extended durations.

Substrates immersed in the ocean for 20 months showed no significant changes in material composition, confirming their general stability and durability on a relevant timescale to coral propagation and outplanting (Fig. S2.3). Substrates were subject to moderate fouling by turfing macroalgae and light fouling by CCA. Substrates also exhibited clear signs of grazing by marine organisms (Fig. 2.5), which creates newly available surfaces and grooves that can facilitate coral settlement. The presence of various CCA species is well established as a coral settlement cue (Abdul Wahab et al., 2023; Heyward & Negri, 1999b; Morse et al., 1988) and, in fact, CCA is almost always present within healthy reefs (Gómez-Lemos et al., 2018; Tebben et al., 2015; Yang et al., 2022). The fact that our NHL-based substrates fostered CCA growth and facilitated successful grazing by herbivorous animals demonstrates their potential viability as a material for promoting coral recruitment in restoration programs. Nevertheless, it is also important to consider the potential challenges associated with algal fouling and longer-term erosion when using NHL-based substrates for coral reef restoration. Further development and optimization of substrate compositions will play a key role in their overall effectiveness and utility in coral reef restoration.

Coral larval behavior in response to dissolved strontium and magnesium. Most coral larvae are lecithotrophic, meaning they have fixed energetic reserves on which they rely during planktonic dispersal and settlement. Thus, pre-settlement and settlement swimming and searching behaviors and the ultimate choice of settlement location represent particularly crucial aspects of their life history. While coral larvae of some species have been found to swim for up

to hundreds of days (Graham et al., 2008), swimming is energetically expensive (Bennett & Marshall, 2005) and any perturbation to larval baseline metabolism can have long-lasting, if not lethal consequences (Hartmann et al., 2015; Kwok & Ang, 2013; Vermeij, 2006). Therefore, chemical effects on larval swimming and attraction to the substrate, settlement success and survival, toxicity, etc. are important to consider when engineering restoration materials.

Our results show that in solution, Mg^{2+} and Sr^{2+} stimulated significant increases in larval motility throughout day three and day four of the study respectively (Fig. 2.6a). Ecologically, larval swimming signifies the ability to actively search for a final place of settlement (Marhaver et al., 2013; Vermeij et al., 2010). Thus we conclude that Mg^{2+} and Sr^{2+} ions promote the search for suitable settlement habitats. This is supported by the increased settlement we observed on substrates containing $MgCO_3$ and $SrCO_3$ (Fig. 2.7a), as well as the settlement and metamorphosis seen on day six in the treatments containing dissolved Mg^{2+} (Fig. 2.6b). Alternatively, this initial increase in motility may indicate a stress response (Vermeij, 2006), though toxicological studies predominantly find sublethal or deleterious effects to be demonstrated by a reduction in larval swimming (Faimali et al., 2006; Wallace & Estephan, 2004), with few studies reporting hyperactivity (Roast et al., 2000). Coral larvae exhibit decreased motility without settlement in response to a multitude of stressors (Antonio-Martínez et al., 2020; Faimali et al., 2006; Hartmann et al., 2015; Kwok & Ang, 2013). Although on the final day of the study over 50% of larvae were motionless, this did not significantly differ across treatments and is thus not likely an effect of the inorganic ion additives (Fig. 2.6b) and was more likely an artifact of poor water quality or due to lack of suitable substrate.

Importantly, no significant differences in survival were found between treatments over the six-day exposure period. This finding indicates that the proposed additives, Mg^{2+} and Sr^{2+}, at

150% relative to ambient ocean concentrations are not immediately perilous to *C. natans* larvae (Fig. S2.5). Lethality may be of concern at higher concentrations, which has been found with the settlement inducer Ca^{2+} (Yang et al., 2022) as well as with environmental pollutants (Kwok & Ang, 2013), though this threshold remains to be determined for Mg^{2+} and Sr^{2+}. Therefore, we conclude Mg^{2+} and Sr^{2+} at 150% ambient concentrations display no deleterious effect on larval survival, swimming, and settlement, with both ions increasing larval swimming in the initial 4 days of exposure, and Mg^{2+} promoting successful settlement and metamorphosis. Future studies will examine the actual biochemical pathways underlying treatment differences to fully elucidate the behavioral response to Mg^{2+} and Sr^{2+} ions observed in this study.

Coral larval settlement choices are influenced by inorganic substrate composition. The NHL base material composition, which influenced both the initial $Ca(OH)_2$ content and the final $CaCO_3$ content, played an important role in larval settlement in this study. The NHL base composition and the addition of specific inorganic additives had significant effects on coral larval settlement. *D. labyrinthiformis* and *O. faveolata* larvae exhibited a significant preference for NHL2 compared to NHL3.5 and NHL5. We attribute this preference to the final content of $CaCO_3$; after carbonation, the $CaCO_3$ weight concentration of NHL2 substrates was 20%, higher than NHL3.5 and 30% higher than NHL5. Consistent with our previous findings (Levenstein, Gysbers, et al., 2022), our results suggest that a higher $CaCO_3$ content in the substrates increased the number of coral settlers, emphasizing the potential role of $CaCO_3$ in promoting coral larval settlement. On the other hand, *C. natans* larvae did not display a significant preference for any of the three NHL base materials. Thus, the attraction reported for *D. labyrinthiformis* and *O. faveolata* is not generalizable to all coral species. Future experiments will aim to customize substrate designs by target species, out-planting habitat, ocean region, etc.

For the three coral species studied here, settlement preferences also varied based on the type and number of inorganic additives incorporated into the NHL3.5 base material. *D. labyrinthiformis* larvae preferred substrates with 10 and 15wt.% $MgCO_3$ and 10 or 20 wt.% $SrCO_3$. *O. faveolata* larvae avoided substrates with only 5 wt.% of $MgCO_3$. *C. natans* larvae were attracted to substrates with high concentrations (>5 wt.%) of either $SrCO_3$ or $MgCO_3$ (Fig. 2.7). In general, for *D. labyrinthiformis* and *C. natans* the incorporation of both carbonates together leads to a higher number of recruits compared to the plain NHL3.5. In the case of *O. faveolata,* the evaluation of this combination was precluded by the insufficient availability of samples for testing. Given these findings, future studies will investigate substrates comprised of NHL2 (e.g., substrates with a higher content of $CaCO_3$) with added $SrCO_3$ and $MgCO_3$. Overall, we found that coral settlement preference depends on the compositions of the NHL base material as well as the inorganic additives incorporated into the substrates. Importantly, we have shown that we can influence coral larval settlement and thus facilitate the coral recruitment processes using inorganic materials alone.

Conclusion

Building on our previous research (Levenstein, Marhaver, et al., 2022), we demonstrated the influence of NHL substrate composition and inorganic additives (strontium and magnesium) on coral larval survivorship, swimming behavior, and settlement. The carbonation process of NHL-based materials led to the transformation of portlandite into calcite, resulting in changes in substrate composition. The NHL base materials itself, particularly NHL2, promoted coral larval settlement in the absence of any additives or biological cues. In tests of dissolved Mg^{2+} and Sr^{2+} ions in seawater, both ions increased larval motility, with Mg^{2+} inducing settlement and

metamorphosis. These tests confirmed that these potential additives could help promote settlement when incorporated into substrates.

Furthermore, coral larval settlement was enhanced via the addition of inorganic additives into the NHL-based substrates. In particular, the combination of strontium and magnesium carbonates enhanced settlement. The long-term stability and durability of NHL substrates were confirmed through observations of substrates out-planted underwater for 20 months. Turf algal fouling was observed on all substrates; however, this was partially offset by light surface erosion attributed to grazing animals. Such grazing behavior is not detrimental to coral growth as it removes fouling organisms, leaving a bare surface underneath for coral growth or additional coral settlement. Materials that can be effectively reset to bare substrate by grazing herbivorous animals are much needed in reef restoration, which until now has largely relied on materials that are too sharp and hard for successful grazing. In addition, the growth of CCA, a known biological cue that promotes coral settlement was also observed growing on the NHL substrates. Overall, our findings contribute to a better understanding of the interactions between substrate composition, inorganic additives, and coral larval swimming and settlement, highlighting the potential of NHL-based substrates in future coral reef restoration applications.

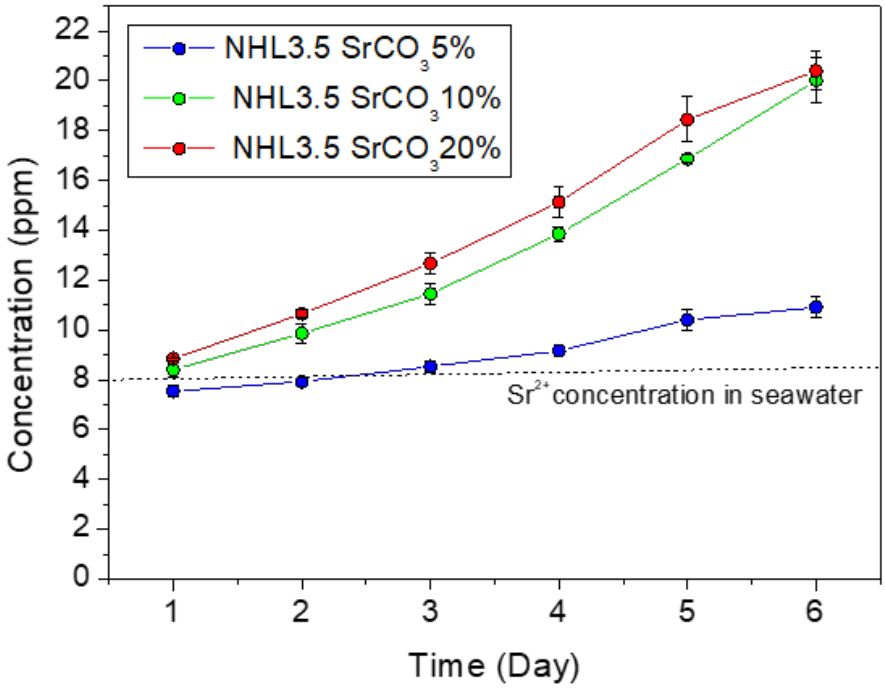
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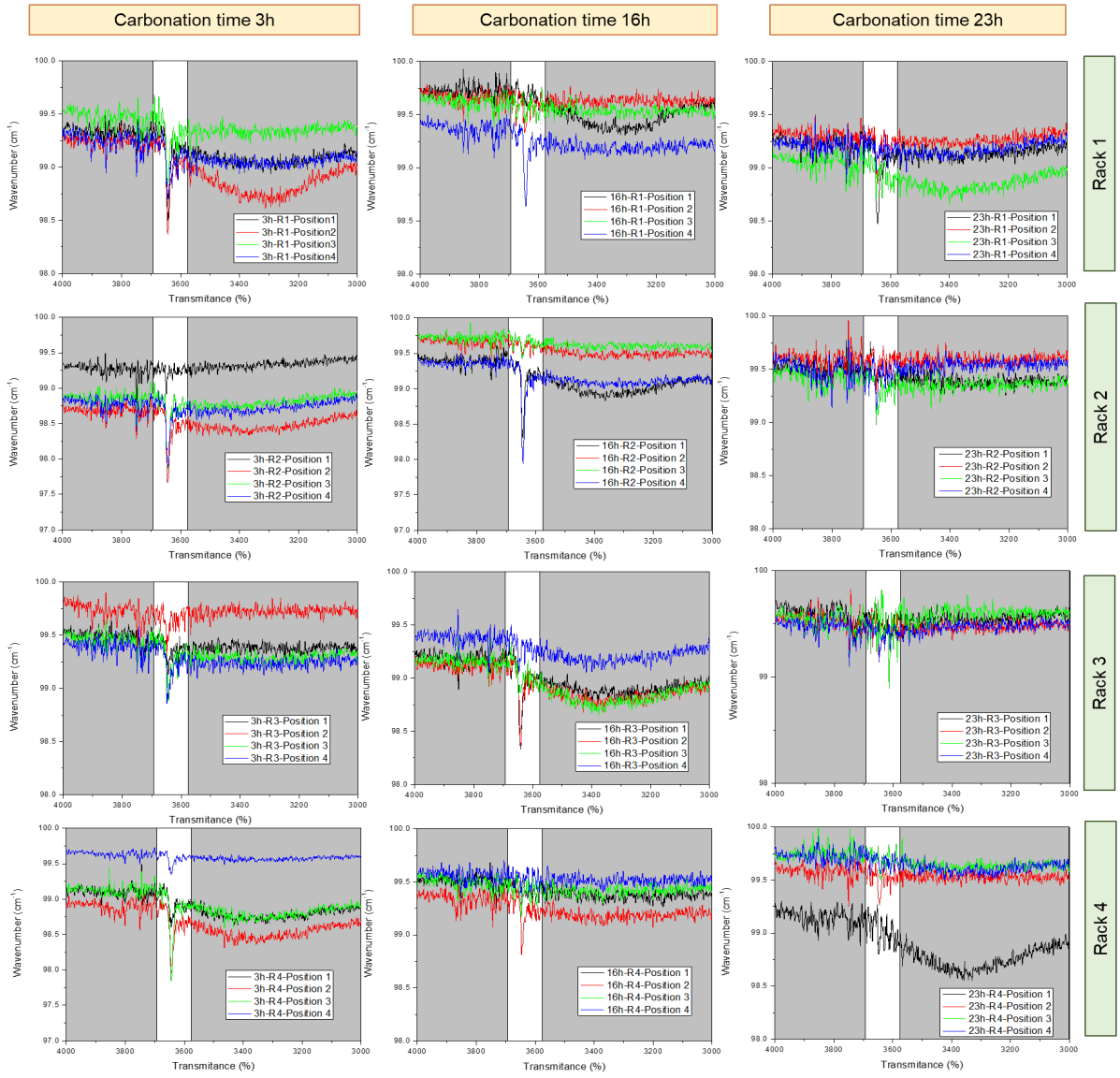
care of coral larvae. We are grateful to Carmen Mata for her kind help in designing some figures. We also thank the many students, interns, and volunteers at CARMABI during the 2021 coral spawning season, and the staff of The Diveshop Curaçao.

Chapter 2, in full, is a reprint of the material as it appears in *Ecological Engineering*, 2024. Yus, J., Nixon, E.N., Li, J., Noriega Gimenez, J., Bennett, M.-J., Flores, D., Marhaver, K. L., Wegley Kelly, L., Espinosa-Marzal, R. M., & Wagoner Johnson, A. J. "Composite substrates for coral larval settlement and reef restoration based on natural hydraulic lime and inorganic strontium and magnesium compounds". The dissertation author was a co-primary researcher and author of this paper.

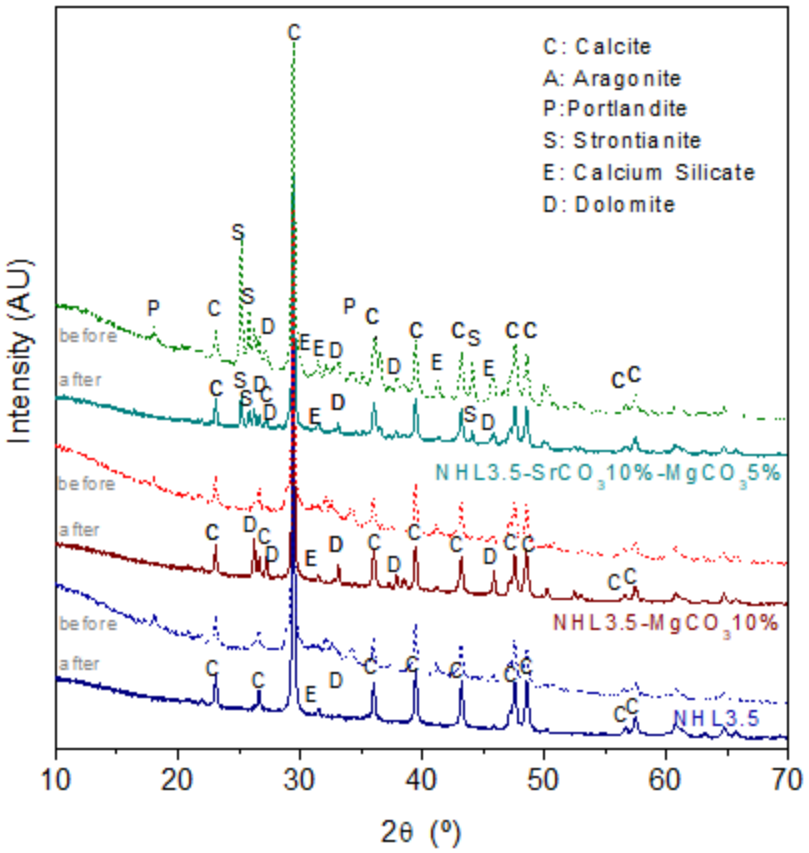
Supplemental Figures



Supplemental Figure 2.1 Concentration of Sr^{2+} in artificial seawater dissolved from NHL3.5 substrates prepared with different $SrCO_3$ contents. The concentration was measured by ICP-MS. The dissolution rate is 2 ppm per day for the treatments with at least 10wt.% of $SrCO_3$ in their composition. Thus, after four days, when settlement happens, the concentration reaches 12 ppm, 150% the initial concentration of Sr^{2+} in seawater. A concentration of 12 ppm was chosen for the larvae behavior experiments.



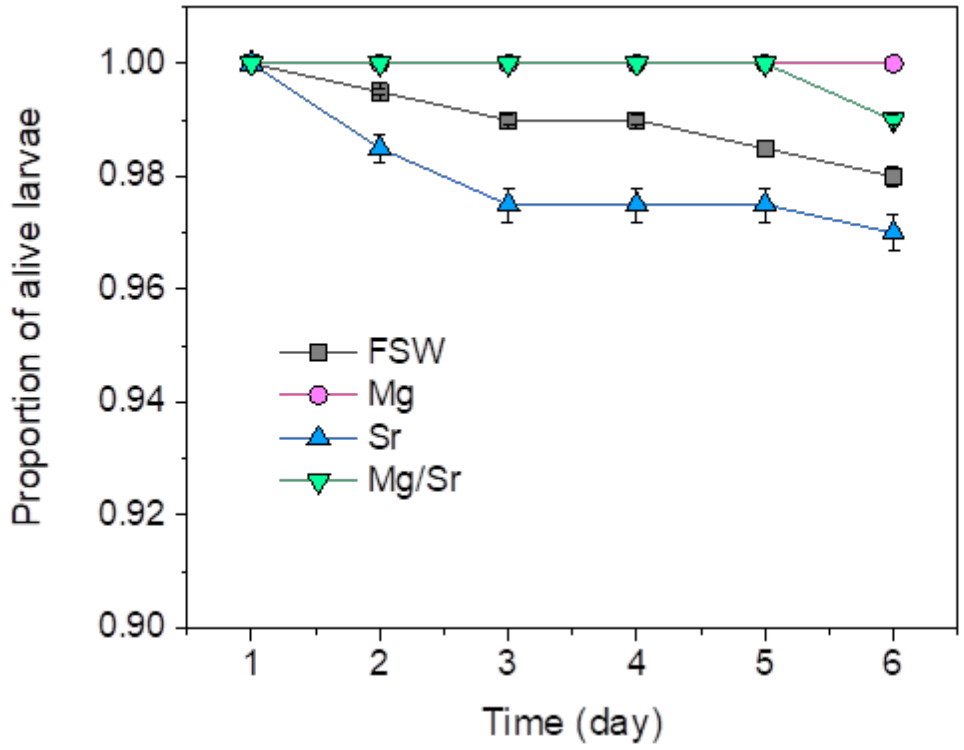
Supplemental Figure 2.2 FTIR spectroscopy of the substrates after different carbonation times, in function of the vertical and radial position in the chamber (racks . The peak at 3640 cm^{-1} gives XXX and hence, it was employed to determine the degree of carbonation, whether complete or incomplete, in the analyzed specimens.



Supplemental Figure 3.3 Xray diffractograms of NHL3.5, NHL3.5-$MgCO_3$10%, and NHL3.5-$SrCO_3$10%-$MgCO_3$5% to examine long-term stability of substrate composition. The plot shows diffractograms before (dashed lines) and after (full lines) 18 months immersed in the ocean in Curacao. The diffractograms demonstrate that the substrate composition remains similar over a period of 18 months. $SrCO_3$ and $MgCO_3$ are identified by the peaks labeled with the letter *S* and *D,* respectively.

	Proportion motile		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	0.59	0.49 – 0.68	**<0.001**
Mg^{2+}	0.43	0.30 – 0.56	**<0.001**
Sr^{2+}	0.20	0.07 – 0.33	**0.002**
Day	-0.05	-0.07 – -0.03	**<0.001**
$Mg^{2+} \times Sr^{2+}$	-0.31	-0.50 – -0.13	**0.001**
$Mg^{2+} \times$ Day	-0.08	-0.12 – -0.05	**<0.001**
$Sr^{2+} \times$ Day	-0.01	-0.04 – 0.02	0.526
$(Mg^{2+} \times Sr^{2+}) \times$ Day	0.02	-0.03 – 0.06	0.441
Random Effects			
σ^2	0.04		
$\tau_{00\ \text{well ID}}$	0.01		
τ_{00} plate	0.00		
ICC	0.17		
$N_{\text{well ID}}$	80		
N_{plate}	20		
Observations	480		
Marginal R^2 / Conditional R^2	0.404 / 0.504		

Supplemental Figure 2.4 Repeated measures linear mixed effects model testing effects of inorganic additives Mg^{2+} and Sr^{2+} on larval motility. Larvae were exposed to elevated concentrations (150% with respect to ambient seawater) of Mg^{2+}, Sr^{2+}, both Mg^{2+} and Sr^{2+}, or a filtered seawater control absent of inorganic additives. Data were collected at 0900h daily for six consecutive days (n = 20 replicates/treatment). Day as a factor and as the repeated measure, with well and six-well plate included in the model to account for random effects.



Supplemental Figure 2.5 Survival of *C. natans* coral larvae when exposed to solutions of two inorganic ion additives used in composite settlement substrates**.** Larvae were exposed to elevated concentrations (150% with respect to ambient seawater) of Mg^{2+} (pink dots), Sr^{2+} (blue triangles), both Mg^{2+} and Sr^{2+} (green inverted triangles), or a filtered seawater (FSW, grey squares) control absent of inorganic additives. Data were collected at 0900h daily for six consecutive days (n = 20 replicates/treatment). Values reported are the treatment means for the mean proportion of larvae alive in each replicate well. Error bars represent +/-1 standard deviation from the mean.

	Proportion alive		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	1.00	0.99 – 1.02	**<0.001**
Mg^{2+}	-0.00	-0.02 – 0.01	0.743
Sr^{2+}	0.00	-0.02 – 0.02	0.971
Day	-0.00	-0.01 – -0.00	**<0.001**
$Mg^{2+} \times Sr^{2+}$	-0.00	-0.03 – 0.02	0.857
$Mg^{2+} \times$ Day	0.00	0.00 – 0.01	**0.005**
$Sr^{2+} \times$ Day	0.00	-0.00 – 0.00	0.085
$(Mg^{2+} \times Sr^{2+}) \times$ Day	-0.01	-0.01 – -0.00	**<0.001**
Random Effects			
σ^2	0.00		
$\tau_{00\ well}$	0.00		
$\tau_{00\ plate}$	0.00		
N_{well}	80		
N_{plate}	20		
Observations	480		
Marginal R^2 / Conditional R^2	0.234 / NA		

Supplemental Figure 2.6 Repeated measures linear mixed effects model testing effects of inorganic additives Mg^{2+} and Sr^{2+} on larval survival. Larvae were exposed to elevated concentrations (150% with respect to ambient seawater) of Mg^{2+}, Sr^{2+}, both Mg^{2+} and Sr^{2+}, or a filtered seawater control absent of inorganic additives. Data were collected at 0900h daily for six consecutive days (n = 20 replicates/treatment). Day as a factor and as the repeated measure, with well and six-well plate included in the model to account for random effects.

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Chapter 3 IDENTIFYING CORAL MICROBIOME NEEDLES IN THE MICROBIAL HAYSTACK: SAMPLING AND ANALYSIS CONSIDERATIONS FOR PROKARYA IN PLANULAE AND POLYPS

Abstract

Bacteria are fundamental to coral health and resilience, though a dearth of knowledge exists on the formation of the coral microbiome in early life history stages. Although this initial microbial consortium is thought to hold key roles throughout development, its sparsity is easily obscured by environmental background microbiota. This opacity renders accurate studies of the early coral microbiome difficult, and limits application in breeding and restoration techniques. Therefore, accounting for the presence of non-host-associated bacteria in the background environment is imperative for the accurate identification of early coral-bacteria associations. Here we outline a workflow that both minimizes and accounts for the environmental background microbiome (EBM) in sampling, downstream data cleaning, and statistical analysis. Utilizing this workflow, we examined the bacterial communities associated with planktonic coral larvae and primary polyps of two scleractinian corals, *Orbicella faveolata* and *Colpophyllia natans*, with consideration for two EBMs, sampling rinse water and the settlement substrate biofilm. 16S rRNA amplicon sequencing revealed substantial mixing of coral-associated bacterial communities with those of the underlying rinse water and biofilms. Nonetheless, the coral larval microbiome could be statistically delineated from the rinse water background, which displayed species specificity as early as four days after fertilization. Paired-sample statistical analysis yielded similar results, with several putative coral primary polyp-associated bacteria found to be distinct from the biofilm background microbiome. These results suggest that (a) the presence of non-host-associated bacteria in early life history coral samples is inevitable and necessitates careful consideration when this life stage, and (b) the coral microbiome signal can be distinguished from the EBM with strategic sampling and subsequent downstream analysis.

Introduction

Corals host a dense and diverse suite of microbial life, including zooxanthellate mutualists and symbiotic bacteria, archaea, viruses, and endolithic fungi and algae (Rohwer et al., 2002; reviewed in Bourne et al., 2016). Including the coral animal host, this community is known collectively as the coral holobiont: a dynamic and collaborative consortium of life that together forms one functioning unit (Knowlton and Rohwer, 2003). Microbiota are integral in coral holobiont processes such as nitrogen fixation, nutrient (re)cycling, immune and oxidative stress response (Lesser et al., 2007; Wegley et al., 2007; Siboni et al., 2008; Matthews et al., 2020), as well as in mechanisms of environmental resilience (Baker et al., 2004; Parker et al., 2020; Levy et al., 2024). While widely studied, much of this multi-partite relationship remains poorly understood. Specifically, little is known about initial microbiome formation, such as in the planula larva and primary polyp life stages. The uptake of microbiota in each new generation occurs through horizontal (environmental), vertical (parental), or mixed-mode transmission (Baird et al., 2009). Regardless of transmission strategy, this initial bacterial community is found to be sparse (Apprill et al., 2009, 2012; Sharp et al., 2010), with most microbial associates acquired from the surrounding marine environment (Baird et al., 2009; Damjanovic et al., 2020b,c).

Despite this initial low-density microbiome, early life history corals are found to host distinct bacterial communities throughout development and show differential uptake across bacterial strains (Apprill et al., 2009, 2012). Elucidating these associations is important for understanding the specificity and succession of the microbiome during this period, where the immediate microbial needs of a coral larva or primary polyp differ greatly from adult coral colonies (Bernasconi et al., 2019). This is most apparent in the coral recruitment process, where

microbiota are imperative in inducing settlement and metamorphosis (Negri et al., 2001; Webster et al., 2004) during a time where settled polyps are simultaneously more sensitive to microbe-induced mortality (Smith et al., 2008; Vermeij et al., 2009). Additionally, identifying these early and few coral-prokaryote associations may shed light on the "core" microbiome, that is microbiota found ubiquitously across life history, species, and even regions (Rohwer et al., 2002; Hernandez-Agreda et al., 2018).

Certain challenges must be acknowledged when studying the coral microbiome at early life history stages. Accurate sampling of planktonic and post-settlement corals is of key concern. The presence of non-host-associated microbiota, by virtue of either the environmental background microbiome (EBM) or artefactual microbiomes resulting from sequencing methodologies (Goffau et al., 2018; Fierer et al., 2025), is nearly inevitable in sampling low-biomass specimens hosting low-density microbiomes. As a result, the resolution of the early coral microbiome stands to be grossly inaccurate without proper sampling practices and rigorous downstream statistical analyses. While both coral and environment microbiomes are comprised of diverse microbiota, including fungi and viruses, in this study we exclusively examine the archaeal and bacterial component.

To date, rinsing low-biomass coral specimens in sterile saline solution is standard practice prior to preservation (16 studies further described in Table S3.1). While this approach is effective in removing the microbiota found in rearing water, the extremely low microbiome density of planktonic coral life stages remain at high risk of obscuration by the rinse water EBM as well as by sequencing artefacts. Therefore, sequencing and accounting for the rinse water EBM in downstream analysis is a necessary quality control measure. A second concern specific to sampling newly settled coral primary polyps arises from the microbial biofilm on the

settlement substrate. This surface-bound bacteria, or biofilm, is considered a prerequisite for most coral larval settlement processes (Webster et al., 2004; Vermeij et al., 2009). Although it is largely unquantified in the literature, post-settlement primary polyps likely host on the order of hundreds to thousands of bacterial cells per polyp, while marine biofilms host an estimated 10^6-10^9 cells/cm^2 (Duan et al., 2021; Briand et al., 2022). Despite this difference in density, the biofilm surrounding a newly settled coral primary polyp remains widely unaccounted for in studies of the coral juvenile microbiome.

Here we examine the microbiome of planktonic larvae and primary polyps of *Orbicella faveolata* (Mountainous star coral; Ellis and Solander, 1786) and *Colpophyllia natans* (Boulder brain coral; Houttuyn, 1772) with consideration for two EBMs, sampling rinse water and adjacent substrate biofilms. We present an overview of recommended field collection and larval rearing practices for research application and provide a workflow for aseptic sampling techniques that account for EBMs in downstream statistical analysis. The methods developed here aim to increase the accuracy with which we can define the coral-associated bacteria during early life history stages.

Materials and methodology

Field collection and larval rearing. Gamete collection and larval rearing, including successful fertilization and propagation, is a critical precursor to early life history coral studies. While minimizing microbial load during propagation is essential to protect embryo and larval development, it is also an important component for coral microbiome resolution. In addition to affecting larval survival, bacteria prevalent in the rearing culture water EBM stand to obscure the coral microbiome signal. For these reasons, here we recommend propagation techniques for larval rearing stages preceding sampling.

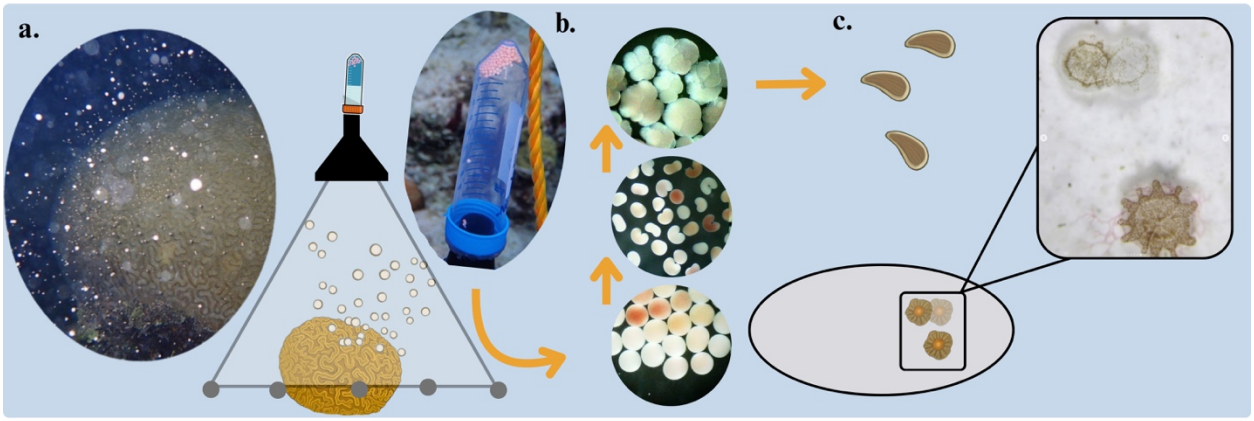


Figure 3.1 Workflow for collection and rearing of broadcast spawning corals. (a.) Egg-sperm bundles of broadcast spawning corals are accumulated using gamete collection nets. Inset displays positively buoyant gametes concentrated in a conical tube. (b.) Gametes are returned to lab for batch fertilization (c.) Larvae are reared at low density and settled on substrates for experimentation and outplanting.

Collection and fertilization of coral gametes. In this study, all coral propagation and sampling was conducted at the CARMABI Marine Research Station in September 2021, 2022, and 2023. Gamete bundles were collected during natural broadcast spawning events occurring on the coral reefs off the Southern Caribbean island nation of Curaçao at the Zakitó dive site (also known as Water Factory; 12°06'29.7"N 68°57'12.6"W). Both *C. natans* and *O. faveolata* are hermaphroditic broadcast spawners, releasing egg-sperm gamete bundles into the water column during seasonal mass spawning events.

Following methods described in previous work, gametes were collected from spawning coral colonies and returned to the lab for fertilization (Hagedorn et al. 2021; Yus et al. 2024). In brief, gametes were collected from parent colonies using weighted nylon mesh nets attached to plastic funnels and 50 mL conical polypropylene tubes (BD Falcon), with positively buoyant gamete bundles concentrated in tubes for transport (Fig. 3.1a). Gametes were returned to lab, cross-fertilized by mixing in 1 L polycarbonate fat separators (Norpro) and incubated for at least 1 hour. After fertilization (Fig. 3.1b), embryos were rinsed with 0.5 µm filtered seawater (FSW;

seawater pumped from 10m depth offshore seawater from ocean intake pipe sequentially filtered through serial spun polypropylene sediment filters with pore size 50, 20, 5, and 0.5 µm; H2O Distributors). Using sterile petri dishes, fertilized gametes were gently scooped and distributed into new, polystyrene clamshell food containers (Dart Container Corporation) containing approximately 750 mL of FSW at an approximate density of 1 larva/mL. All reusable field, lab fertilization, and propagation equipment was sterilized using a solution of approximately 100 ppm bleach (6% NaHCl2, in water). Bleach was neutralized by rinsing items in a solution of approximately 100 ppm sodium thiosulfate pentahydrate (Fisher Scientific; Na2O3S2*5H2O). Items were then rinsed in fresh tap water and dried prior to use. Local tap water is produced by ocean desalination and obtained from a standard groundwater spigot using a potable drinking water hose.

Ex situ *larval rearing.* Following successful fertilization and embryonic development, planulae larvae were reared in the lab for sampling and settlement (Fig. 3.1c). While coral larval rearing may be conducted in either open- or closed-systems, here we describe closed-system rearing techniques (see Chamberland et al. 2017; Hagedorn et al. 2021; Levenstein et al. 2022). Larval cultures were maintained in rearing containers with approximately 750 mL of 0.5 µm FSW at the approximate density of 1 larva/mL. Water changes were performed daily by hand-pipetting larvae (9-inch Pasteur pipettes, Fischer Scientific) into new containers to reduce microbial load and maintain a stable pH in the rearing environment throughout development (Vermeij et al., 2006). Larval cultures were maintained in a temperature-controlled laboratory with experimental water temperatures kept consistent with that of the local reefs (27.5 ± 1.5 ˚C). Following sampling, all remaining larvae were released into the sea and settled corals were outplanted onto a restoration structure or preserved for molecular analysis.

Sampling methodologies and EBM considerations. While some coral larvae inherit microbial symbionts from their parents (Sharp et al., 2010; Epstein et al., 2019; Damjanovic et al., 2020c), Caribbean broadcast spawning coral larvae are generally found to be devoid of internal bacteria until several days after fertilization and even post-metamorphosis (Sharp et al., 2010). Thus, it is imperative to both minimize and account for EBM communities throughout the sampling process. Here we evaluate specimen rinse water sterility and outline sampling techniques for both planktonic larvae and coral primary polyps.

Considerations for planktonic larvae: exploring the role of rinse water on the sequenced larval microbiome. When sampling coral microbiomes at early stages, i.e. low-biomass samples with low microbial density, minimizing transfer of culture water EBM is a key concern. For this reason, it is standard practice to implement a sterile rinse prior to preservation to prevent the rearing culture water EBM from obscuring the coral larvae microbiome signal (Table S3.1). Low-biomass sample signal may also be increased by specimen pooling, where one sample replicate comprises multiple biological individuals. The inherent risk in specimen pooling for microbiome analysis, however, lies in the potential for increased volume of the EBM in question. Here we compare four rinse water solutions, three specimen pooling densities, and assess the effects of each on 16S rDNA amplicon sequenced microbiomes of *O. faveolata* larvae.

Several sterile saline solutions may be suitable for removing environmental bacterioplankton from early life history coral samples (Table S3.1). In the present study, four rinse waters were tested for EBM sequencing effects. FSW was collected from the wet lab filtration system at CARMABI Marine Research Station and was either used directly (0.5-FSW; 0.5 μm FSW run through pump lines with no filter) or further filtered through a peristaltic pump with either a 0.22 μm filter (0.22-FSW; Millipore Sterivex-PES Pressure Filter Unit), or a 0.02

µm filter (0.02-FSW; Whatman Anotop, 25 mm). A fourth rinse solution of 35 ppm artificial seawater was created by dissolving approximately 35 g of aquarium salt (API Aquarium Salt) in 1 L of MilliQ water and run through two 0.22 µm Sterivex filters (AFSW). All rinse water treatment solutions were stored in acid-washed glass bottles. Following filtration, 5 mL of each rinse water treatment were fixed in 80 µL of PFA (paraformaldehyde 32% solution, EM-grade, Electron Microscopy Sciences), except for the 0.5-FSW treatment in which 1 mL of rinse water was fixed in 16 µL of PFA (Supplemental S3.1).

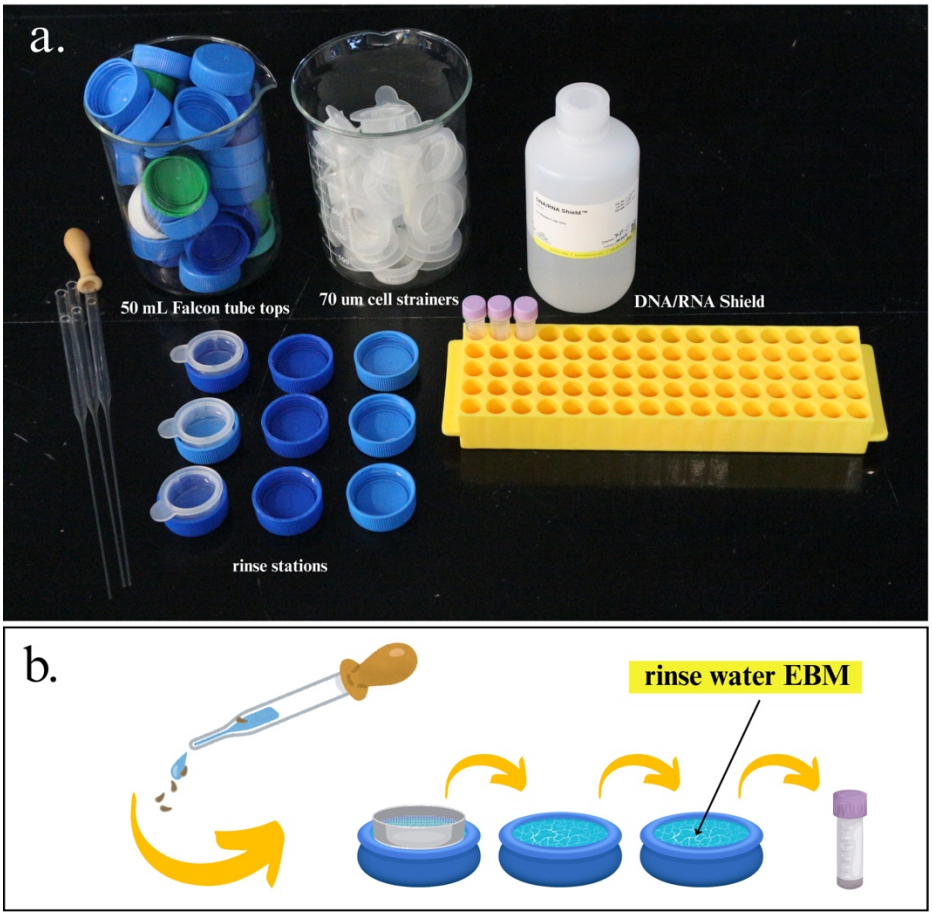


Figure 3.2 Materials and method for rinsing coral larvae samples. (a.) Photograph of lab bench with materials for sampling planktonic coral larvae. (b.) Larvae were deposited onto a 70 µm nylon mesh cell strainer and sequentially triple-rinsed in sterile saline solution. Specimens were then pipetted into 2.0 mL Eppendorf tubes containing DNAShield and stored at -80°C. Three 250 µL aliquots of rinse water were sampled from each rinse water treatment to account for rinse water EBM.

Larvae were pipetted from rearing culture containers using a new 9-inch Pasteur pipette (Fischer Scientific), deposited onto a 70 μm nylon mesh cell strainer (Falcon), and triple-rinsed via serial submergence of the cell strainer in three pools of treatment rinse water (50 mL conical tube tops; Fig. 3.2a,b). Larvae were then pipetted from the cell strainer in the final rinse pool, with minimal water transfer, and stored in 2.0 mL Eppendorf tubes containing 500 μL of DNAShield (Zymo Research) at -80°C (Fig. 3.2a,b). To examine the effects of sample pooling on the sequenced larval microbiome, specimens were pooled in groups of one, three, or ten individuals per replicate tube, with five replicates of each pooling density per treatment. Three replicate 250 μL control aliquots of each rinse water treatment were sampled and stored identically to larval samples to characterize EBM communities in downstream analysis.

Additionally, we compared *C. natans* and *O. faveolata* larvae microbiomes sampled over the course of three spawning seasons: 2021, 2022, and 2023. In 2021, larvae were rinsed in 0.5-FSW, while in 2022 and 2023, larvae were rinsed in AFSW. All replicates were sampled four days after fertilization as previously described and were pooled in groups of five larvae per tube. The effects of rinse water treatment, specimen pooling density, and species specificity on the sequenced larvae microbiome were tested separately via permutational analysis of variance (PERMANOVA, adonis2 function in the vegan package) with a pairwise post hoc test (pairwise.adonis2 function in the vegan package).

Considerations for settled primary polyps: controlling for surface biofilm EBM in sampling techniques and downstream analysis. While coral microbiome density increases throughout early development, the microbial density of the surrounding environment remains several orders of magnitude higher. Despite the disparity in microbiome density between coral primary polyps and marine biofilms, to our knowledge no study to date accounts for the

underlying biofilm microbiome when characterizing the coral primary polyp microbiome. Although care is taken during sampling to minimize uptake of the adjacent biofilm, accounting for this dense EBM in downstream analysis is vital. Here we examine the *C. natans* primary polyp microbiome in respect to its underlying biofilm EBM.

To most accurately identify coral-associated bacteria, each coral primary polyp sample was paired with an adjacent biofilm sample (Fig. 3.3a,b). Coral larvae were reared as previously described before being introduced to conditioned natural hydraulic lime substrates for settlement (Yus et al., 2024). Substrates were conditioned for six weeks in aquaria with flow-through seawater from the CARMABI wet lab intake system. One-week-old primary polyps and their respective adjacent biofilm were then sampled for microbiome analysis (Fig. 3.3b). First, substrates with settled corals were transferred to a clean container of FSW and visible biofilm on the perimeter of the coral specimen was carefully scraped away using a syringe with a 16G needle. Using the syringe, FSW was then gently propelled over the scraped area to remove remaining debris. The primary polyp was then sampled by carefully needle scraping the specimen. Beginning at the perimeter and scraping to center using the sharp end of the needle, the skeleton, tissue, and mucus was consolidated and detached from the substrate. The specimen was transferred to an AFSW rinse using a new 9-inch glass Pasteur pipette (Fischer Scientific, capillary stem opening diameter ~ 1.5 mm), then transferred to a sample tube pre-filled with 500 µL of DNAShield at -80°C (Fig. 3.3c). Water for each transfer was limited to one droplet, or approximately 200 µL. Immediately following coral sampling, the substrate surface directly neighboring (approximately 0.5 cm distance) the primary polyp was scraped with a sterile needle-syringe, rinsed with AFSW and preserved in 500 µL of DNAShield at -80°C (Fig. 3.3b,c). The needle was cleaned with ethanol and dried with a lint-free laboratory wipe (Kim-

wipe) between each replicate sample. Each sample pair was pooled in triplicate, with three corals per tube and three respective biofilm scrapings per tube.

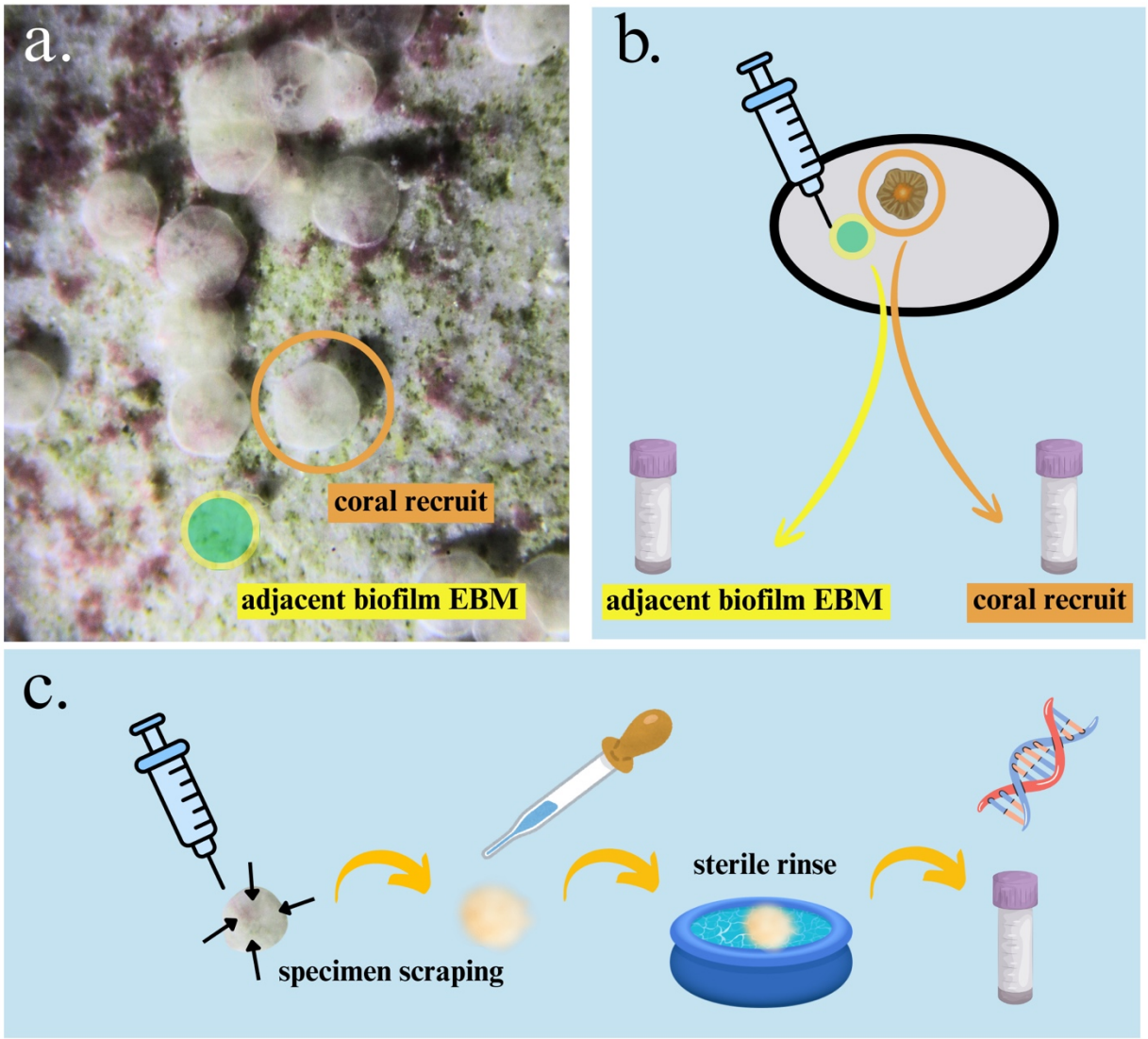


Figure 3.3 Paired sampling method for the coral primary polyp and adjacent biofilm. (a.) Stereoscope view of primary polyp and adjacent biofilm EBM (b.) Paired sampling approach for coral primary polyps and adjacent biofilm EBM, with three corals and three respective biofilm specimens sampled per tube pairing (c.) Polyps were scraped from the outer perimeter towards the center of the polyp using a sterile needle. The scraping was rinsed in AFSW, then pipette transferred to a 2.0mL Eppendorf tube containing DNAShield and stored at -80°C. Following this same technique, a scraping of adjacent biofilm EBM neighboring each coral specimen was rinsed and preserved for downstream analysis.

DNA extraction and 16S rRNA amplicon sequencing. All samples were subject to DNA extraction with the Zymobiomics miniDNA kit (Zymo Research) following kit protocol with

further optimizations made for low-biomass low-density microbiome samples (Supplemental S3.2). Purified DNA was sent to The University of Hawaii at Mānoa Integrative Center for Environmental Microbiomes and Human Health Microbial Genetics and Analytical Laboratory (ICEMHH MGAL) for 16S rDNA amplicon library preparation and sequencing. The V4 region of the small subunit (16S) rRNA gene was amplified via polymerase chain reaction with primers 515F-Parada: GTGYCAGCMGCCGCGGTAA and 806R-Apprill: GGACTACNVGGGTWTCTAAT (Walters 2016) following Earth Microbiome Project protocols (Caporaso et al., 2018) except triplicate reactions were not pooled and 1 μL of genomic template was used consistently. Amplicons were cleaned and normalized to equal concentrations (SequelPrep kit, Thermo Fisher Scientific) and subsequently pooled and sequenced on the Illumina MiSeq platform (600 cycle V3 chemistry). Sequences were demultiplexed using a custom script (Frøslev et al., 2017): sequences were assembled, denoised, and quality trimmed using DADA2 (Callahan et al., 2016). The resulting sequence data was globally aligned with mothur (Schloss et al., 2009) to the SILVA (v. 132) global SSU rRNA alignment database. Sequence data were then Bayesian consensus classified (70%) to the Genus level (Wang et al., 2007) and then clustered into operational taxonomic units (OTUs) at the 97% average sequence identity level using vsearch (Rognes et al., 2016), with LULU used to refine and reduce intragenomic taxonomic splitting errors (Frøslev et al., 2017). Sequence data that could not be classified at the Domain level or were classified a chloroplast or mitochondrial DNA were removed from further analysis. All bioinformatic processing was completed on the C-MAIKI gateway (Cleveland et al., 2022) at the University of Hawai‘i at Mānoa by the Center for Microbial Oceanography: Research and Education.

Following sequencing, datasets were subsampled to account for variability in sequencing

effort. The larvae rinse water dataset and 2022 and 2023 species specificity samples were subsampled to 1,000 reads. 2021 species specificity samples and paired coral primary polyp biofilm samples were subsampled to 5,000 reads. Samples for this study were pulled from several projects and as such were on several sequencing runs (Table S3.2). Only samples on the same run were ever compared for statistical analysis. Samples with fewer than 1,000 or 5,000 reads respectively were discarded from further analysis. While higher sequencing thresholds are an efficient tool to cull poorly sequenced samples from a dataset, in this study we intentionally set a low threshold to ensure maximal inclusion of the rinse water samples in the analysis.

OTUs were determined to be rare or contaminant and removed from analysis if not exceeding a maximum relative abundance of 0.5% or if not found to be above 0.2% in 3 or more samples. Within each sample set, a second maximum abundance threshold of 0.5% was set to filter for ecologically relevant OTUs.

Downstream data processing and analysis: identifying coral microbiome signals in the face of EBM. Given the low microbial density of early life history corals, it is important to consider EBM sources when characterizing the microbiome of planktonic and early post-settlement stages. Here we outline a downstream statistical method to more accurately, albeit more conservatively, identify putative coral-associated bacteria.

In this study, we define putative coral-associated bacteria as those significantly enhanced in coral samples in comparison to their respective EBM control sample (rinse water or biofilm). For each OTU, we calculated a ratio of its relative abundance in a coral sample (coral) to its relative abundance in a respective EBM control sample (EBM: rinse water or biofilm). The relative abundance ratio was then log base 2 transformed (log2(coral/EBM), transformed ratios hereafter referred to as log2 fold change or L2FC). L2FC analyses are commonly used in ratio

analyses, and center values symmetrically around zero. For larval samples (n = 10-15 per treatment), EBM denominator values were calculated using the average relative abundance value found in its respective rinse water control samples (n = 3 aliquots per treatment). For coral primary polyps, EBM denominator values stemmed from paired substrate biofilm EBM samples specific to each coral sample (n = 9 coral-biofilm pairs). Biofilm was sampled comparably to primary polyps and thus accounted for both sampling rinse water and biofilm EBM (Fig. 3.3c). For each OTU, L2FC ratios were subjected to a one-sided, one-sample *t* test to identify OTUs significantly enhanced in coral samples that held mean L2FC values significantly greater than 0.5 (where an L2FC = 0.5 represents an OTU relative abundance in coral ~1.5x that in the EBM sample), which in this study we define as our threshold for coral-associated bacteria. Corrections for multiple comparisons were carried out using the Benjamini and Hochberg (BH) correction (Benjamini and Hochberg 1995).

Results

Cell counts of rinse water treatments reveal varying levels of sterility. Bacterial cell counts in rinse water were found to vary between rinse water treatments (Fig. S3.1). 0.22-FSW and 0.02-FSW samples both yielded zero detectable bacterial cells across all replicate filters (n = 3 filters, Anodisc). Average bacterial cell counts in AFSW samples (2x 0.22 μm filtered artificial seawater) was found to be 218 cells/mL, whereas 0.5-FSW samples on average hosted 230,000 cells/mL (Fig. S3.1).

Rinse water background microbiome drives significant differences in sequenced coral larval microbiome community. Overall, we found rinse water strongly influenced the sequenced microbiome obtained from coral larvae, with larvae rinsed in each of four different water treatments displaying distinct microbiome signatures ($p < 0.01$, Fig. 3.4a,b). The sequenced

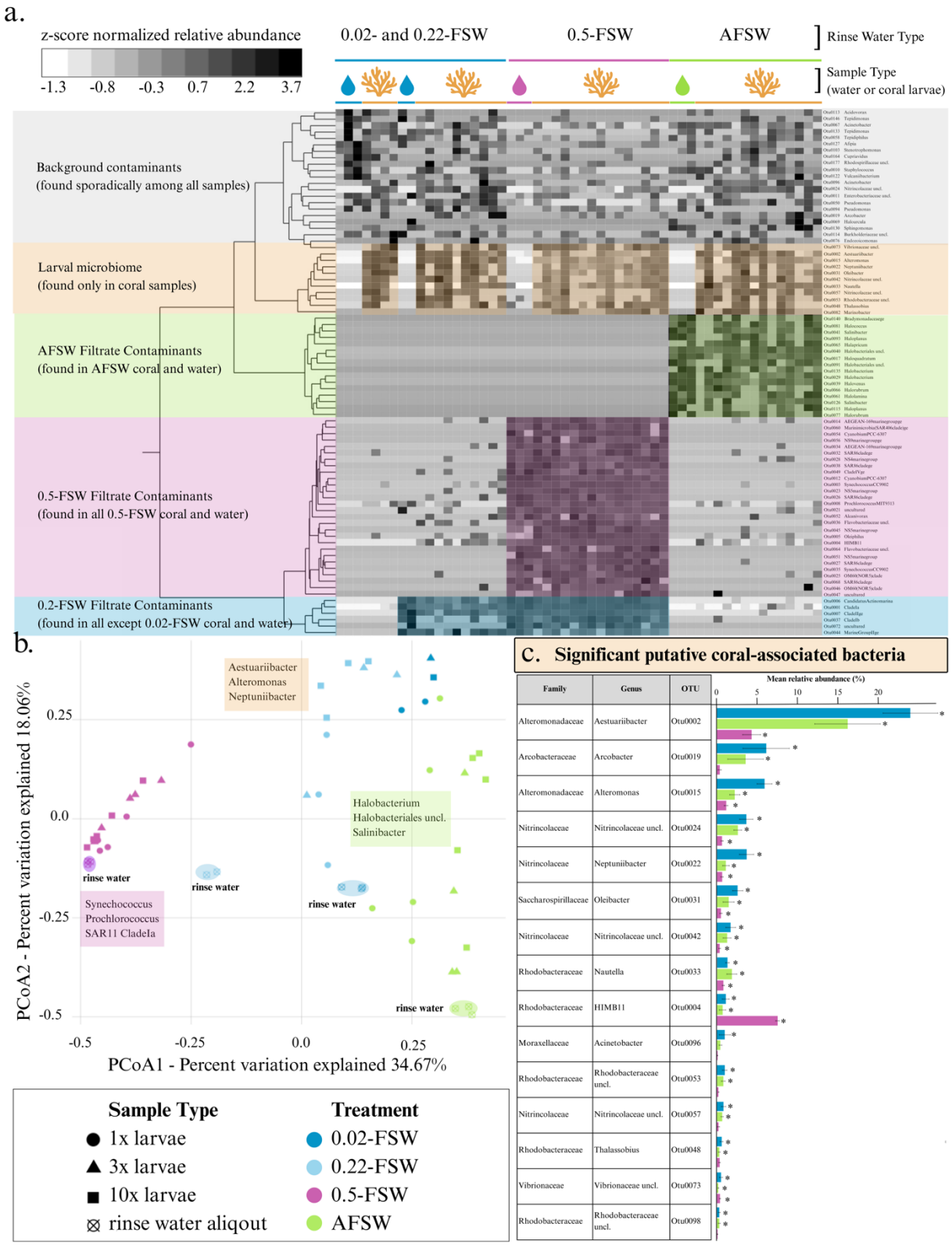
microbial communities from larvae rinsed in 0.02-FSW and 0.22-FSW were similar to one another ($p = 0.08$) but differed significantly from the sequenced microbiomes of larvae rinsed in AFSW ($p < 0.003$) and 0.5-FSW ($p < 0.002$) (Fig. 3.4b). Principle coordinates analysis (PCoA) visualization of Bray-curtis diversity supports these differences in treatment microbiomes, which are depicted by three distinct clusters in community space (Fig. 3.4b). Larvae rinsed in sterile 0.22- and 0.02-FSW were found to cluster together and were characterized by OTUs that were nearly absent from all rinse water controls (Fig. 3.4a, Table S3.3). 0.22-FSW and 0.02-FSW (hereafter referred to as sterile rinse water) were grouped for further analysis due to similarity in microbiome community composition (PERMANOVA, $p > 0.05$, Fig. 3.4b) and limited sample size in the 0.02-FSW treatment ($n = 4$). Contrary to the sterile rinse water larvae, the sequenced microbiome of larvae rinsed in AFSW and 0.5-FSW were influenced by OTUs stemming from several halophilic families and a diverse collection of common reef water families respectively, which were found to dominate the community composition of rinse water EBM controls (Fig. 3.4a, Table S3.3).

While a small significant difference was found between pools of 1x and 10x larvae in the 0.22-FSW treatment ($p = 0.03$, Table S3.4), specimen pooling density did not affect the sequenced larvae microbiome community composition in 0.5-FSW ($p = 0.45$), AFSW ($p = 0.12$), or 0.02-FSW treatments ($p = 0.17$). Due to this near absence in effect, sequence data obtained from larval pooling densities were combined for subsequent analysis.

Putative coral larvae-associated bacteria identified despite rinse water EBM. A one-way one-sample *t* test (meanL2FC > 0.5, $p < 0.05$) of the L2FC values for each OTU was conducted for each of the four rinse water treatments, resulting in four lists of significant OTUs, that is OTUs enhanced in coral larvae in comparison to their respective rinse water EBM controls

(Table S3.5). Within the sterile rinse water treatments (0.22- and 0.02-FSW), a total of 15 OTUs were revealed to be significantly higher in relative abundance in coral larvae samples in comparison to their respective rinse water, 14 and 9 of which were found to be significantly enhanced in AFSW and 0.5-FSW rinsed larvae respectively (Fig. 3.4c). These taxa together comprised 54% of the coral larvae microbiome in the sterile rinse water treatment. The most abundant OTUs were in the families Alteromonadaceae (genera *Aestuariibacter* (24.1%) and *Alteromonas* (6.0%)), Arcobacteraceae (6.2%), Nitrincolaceae (*Neptuniibacter* and three unclassified genera (cumulatively 9.8%)), and Rhodobacteraceae (*Nautella, HIMB11, Thalassobius*, and two unclassified genera (cumulatively 4.1%)). Additionally, OTUs belonging to the genera Oleibacter (2.4%), Acinetobacter (1.0%), and an unclassified Vibrionaceae (0.5%) were found to be significantly enriched in larvae rinsed with sterile rinse water. With the exception of Acinetobacter, these aforementioned OTUs were all significantly enhanced in AFSW rinsed larvae, while 9 were found to be significant in 0.5-FSW rinsed larvae (Table S3.5). Due to their statistical significance and prevalence across all treatments, we define these 15 OTUs as strong putative coral-associated bacteria (Fig. 3.4c).

Figure 3.4 Effects of rinse water EBM on coral larvae microbiome community (a.) Heatmap of z-score normalized relative abundance values. Family and OTUs of relevant taxa are represented on the right y-axis with treatment rinse water contaminants outlined on the left y-axis and their respective EBMs highlighted. Rinse water filtrate type and sample type is denoted on the x-axis. (b.) Ordination of coral larvae and rinse water microbiomes using PCoA, distance = Bray-Curtis Dissimilarity. Color denotes rinse water treatment: 0.22- 0.02-FSW (medium and light blue respectively), 0.5-FSW (purple), AFSW (green), and putative coral-associated bacteria (orange) with the taxa defining each cluster denoted. (c.) Mean relative abundance bar plot for the 15 OTUs found to be significantly enriched in coral larvae rinsed in sterile water, with bar color denoting rinse water treatment. Error bars denote standard error, and asterisks denote OTUs found to be significantly enriched in a given treatment (*t* test, $p < 0.05$).



Family	Genus	OTU
Alteromonadaceae	Aestuariibacter	Otu0002
Arcobacteraceae	Arcobacter	Otu0019
Alteromonadaceae	Alteromonas	Otu0015
Nitrincolaceae	Nitrincolaceae uncl.	Otu0024
Nitrincolaceae	Neptuniibacter	Otu0022
Saccharospirillaceae	Oleibacter	Otu0031
Nitrincolaceae	Nitrincolaceae uncl.	Otu0042
Rhodobacteraceae	Nautella	Otu0033
Rhodobacteraceae	HIMB11	Otu0004
Moraxellaceae	Acinetobacter	Otu0096
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0053
Nitrincolaceae	Nitrincolaceae uncl.	Otu0057
Rhodobacteraceae	Thalassobius	Otu0048
Vibrionaceae	Vibrionaceae uncl.	Otu0073
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0098

AFSW rinse reveals species specificity in sequenced larval microbiome despite presence of EBM. To test the hypothesis that proper sampling and rinse water impacts microbiome resolution, we compared species specificity in the sequenced microbiomes of *O. faveolata* (OFAV) and *C. natans* (CNAT) larvae sampled across the 2021-2023 spawning seasons. In 2021, larvae were triple rinsed in 0.5-FSW, whereas in 2022 and 2023 samples were triple rinsed in AFSW (following methodologies outlined in Fig. 3.3). Distance centroids were calculated and differences between both coral species as well as rinse water samples within each year were tested via PERMANOVA. In 2021, the sequenced microbial community composition of *O. faveolata* and *C. natans* coral larvae were not significantly different from one another (Fig. S3.2a; CNAT vs OFAV $p = 0.1$). In 2022, both *C. natans* and *O. faveolata* sequenced coral larvae microbiomes were found to be distinct from one another (Fig. S3.2b; CNAT vs OFAV $p = 0.001$). These significant species-level distinctions were also found in 2023 (Fig. S3.2c; CNAT vs OFAV $p = 0.002$).

Paired sampling and downstream analysis distinguish coral primary polyp-associated bacteria from biofilm EBM. A major challenge in describing the microbiome of coral primary polyps is accounting for the underlying biofilm EBM. While genomic sequencing methods have inherent limitations in distinguishing the source location of microbiota, i.e. differentiating between coral and biofilm microbiome members, to our knowledge this study serves as the first to account for biofilm background microbiome signals in downstream data analysis. Overall, across all coral primary polyp and biofilm samples, our dataset was comprised of 307 OTUs, with only 90 found to have mean L2FC values higher than zero (where, L2FC = 0 signifies a 1:1 relative abundance ratio between coral and adjacent substrate biofilm; Fig. 3.5). Of these 90 OTUs, a one-way one-sample *t* test ($mu = 0.5$, $p < 0.05$) revealed 18 OTUs which we define as

putative coral bacterial symbionts (Fig. 3.5; Table S3.6). On average, these 18 OTUs comprised 17.3% of the coral primary polyp microbiome. In the present study, bacterial family groups most strongly associated with coral samples included Alteromonadaceae (2 OTUs, mean relative abundance = 4.2%), Rhodobacteraceae (3.2%), Nitrincolaceae (2 OTUs, 1.2%), Kordiimonadaceae (1.6%), Sphingomonadaceae (2 OTUs, 1.2%), and Vibrionaceae (1.0%) (Fig. 3.5; Table S3.6).

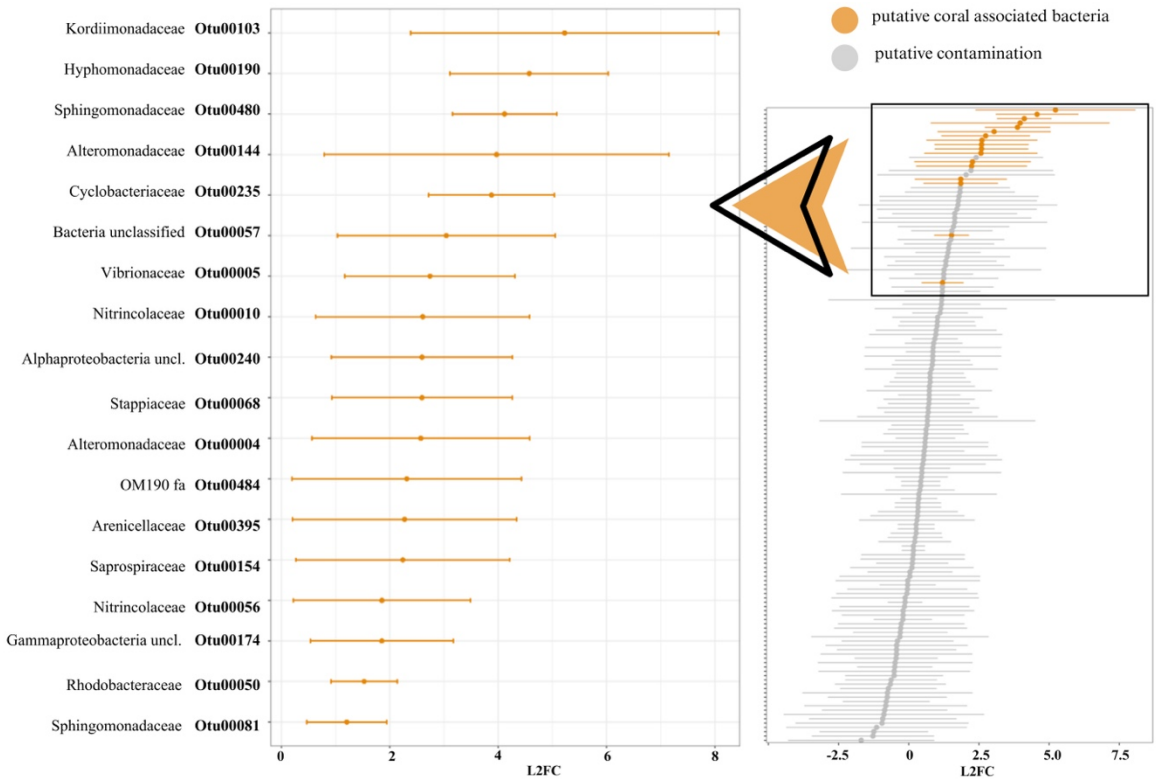


Figure 3.5 Mean log-2-fold-change (L2FC) plot of *C. natans* coral primary polyp bacterial associates in comparison to background biofilm at the OTU level (n = 9 paired samples). Putative coral bacterial associates (orange) were determined by a one-sample one-way t test and are listed on the y-axis by Family and OTU ($p < 0.05$). Error bars represent standard deviation.

Discussion

Sterility of rinse water plays consequential role in sequenced larval microbiome.

Notably, we found that even rinse water treatments nearly absent of detectable cells affected the sequenced coral larvae microbiome. This was most starkly displayed by the presence of rinse

water control EBM in larval samples despite the near absence of countable cells in the AFSW treatment (Fig. 3.4a, Fig. S3.1). Both AFSW and 0.5-FSW rinsed larvae microbiome communities were significantly affected by rinse water microbiomes, manifesting as a suite of covarying taxa enriched in both the larvae and rinse water controls associated with each of the two rinse treatments (Fig. 3.4a, Table S3.3). Contrastingly, 0.22- and 0.02-FSW sequenced larvae microbiomes were enriched in bacteria nearly exclusively found in coral larvae, which we classify as putative coral-associated bacteria (Fig. 3.4b,c). Further, while specimen pooling lacked effect in most rinse water treatments, a significant effect was found between 1x and 10x larvae in the 0.22-FSW rinse treatment (PERMANOVA, $p = 0.03$). Interestingly, increased pooling resulted in an increased relative abundance signal for many of our defined putative coral-associated bacteria (Table S3.4). Therefore, we conclude that pooling ten larvae or more is beneficial to microbiome resolution provided adequate sterility in rinse water.

In our comparisons between larvae of the two coral species *O. faveolata* and *C. natans*, we detected statistically significant species-level differences in the sequenced microbiomes rinsed in AFSW (PERMANOVA, $p < 0.01$), though notably the rinse water EBM remained present in the sequenced coral larvae microbiome (Table S3.7). We hypothesize that this EBM, as well as the EBM described above in section 4.2, were a result of artifactual free DNA occurring in the samples from the production of aquarium salts used to make AFSW rinse water. We hypothesize that this EBM may be remediated with a post-extraction DNAse cleaning step for sample purification or subsequent downstream analysis.

Overall, the strongest coral larvae microbiome signals were seen in the sterile seawater treatments (0.22- and 0.02-FSW). Interestingly, 14 of the 15 OTUs deemed putative coral-associated bacteria in these treatments were also found to be significant in AFSW, though often

with diminished relative abundance values (Fig. 3.4c, Table S3.5). These coral-associated OTU signals were further diminished in 0.5-FSW, with 9 of the 15 OTUs deemed significantly enriched in the coral larvae relative to the respective rinse water control (Fig. 3.4c, Table S3.5). While downstream ratio-based statistical analysis performed well across rinse water treatments, of the rinse waters tested we recommend using 0.02 or 0.22 μm FSW in future studies. Importantly, despite reported sterility, several OTUs from the EBM or sequencing artefacts remained in the sequenced microbiome of 0.22-FSW rinsed coral larvae (see '0.22-FSW Filtrate Contaminants and Background contaminants' in Fig. 3.4a). Therefore, we find water sample controls to be a necessary measure regardless of rinse water sterility.

Importance and limitations of paired coral-biofilm sampling and downstream analysis. Coral reef surface biofilms are ubiquitous, rapidly changing, and highly dependent on both local environmental conditions and the underlying substrate. Thus, the challenges of identifying and separating out the biofilm background microbiome will vary based on the study. For example, research comparing between species or rearing treatment may control for biofilm background microbiome by way of study design (e.g. rearing corals in the same aquaria or on similarly conditioned substrates, Damjanovic et al., 2019, 2020d). Additionally, biofilm development is certainly impacted by substrate type, with substrate surface composition found to affect both biofilm density and microbial community composition (Thomason et al., 2002). While autoclaved settlement substrates are commonly employed in larval settlement research, and this does indeed reset surface succession, biofilm growth rates act on the order of hours and days and thus the biofilm EBM is an important consideration regardless of substrate conditioning treatment or substrate age (Sweet et al., 2011). Tropical marine biofilms can become distinct from their surrounding water column source in as early as 30 minutes to two hours post-

exposure, with significant shifts occurring between colonizing species, one-day- and one-week-old biofilms (Sweet et al., 2011). Further, microbial members of coral reefs are often found to be pervasive, with known coral microbiome associated bacteria reported in substrate biofilm (Sweet et al., 2011), as well as in the surrounding water column (Nelson et al., 2023).

While this study was deliberately designed to be rigorous in sampling procedures and downstream statistical analysis, several caveats exist in our identification of early life history coral microbiome members. Regarding sampling procedures, we recommend several amendments to increase resolution. This includes using sterilized transfer pipettes and using a new sampling needle for each sample instead of sterilization in between samples. Alternatively, if used repeatedly the needle may be sterilized by a more effective agent, such as hydrogen peroxide or sodium hypochlorite. While care was taken to scrape neighboring biofilm away from the polyp prior to sampling, this pre-sampling precaution may have exposed the polyp to further cross-contamination from the biofilm via scraping adherence or bacteria released into the sampling water. We chose this step based on our sampling method and settlement substrate composition, though future studies may want to consider sampling without this step. Further, due to the fragile nature of the early skeleton, there is no feasible way to remove one-week old polyps without compromising the integrity of the sample. This in combination with the rinse step may have resulted in the loss of microbiome members in the final sample.

DNA extraction, amplification, and sequencing methodologies are an additional consideration in accurately identifying coral-associated bacteria, and inherently bias microbiome results (Fouhy et al. 2016). For example, gram-positive bacteria feature robust cell walls that can withstand standard DNA extraction mechanical lysis and are then underreported in the literature (Starke et al. 2019). In efforts to recover both gram-positive and gram-negative bacteria samples

were subjected to bead beating lysis followed by an additional proteinase K incubation (Supplemental S3.2). Here, gram-positive bacterial DNA sequences were successfully recovered (Table S3.7). While not highly abundant, with mean relative abundance <1% across sample types, maximum relative abundances ranged from 1.14-13.4% (Supplemental S3.7). While low fractions may signify inefficient lysis, maximum values suggest that gram-positive bacteria were successfully lysed and that these clades may not comprise a significant fraction of this respective sample set. Further, PCR based library preparations introduce amplification biases. DNA with high GC content is more difficult to denature and therefore amplify (McDowell et al. 1998). Additionally, with amplicon based sequencing there exists primer-based biases, where the use of different primers results in differences in taxa abundance and detection (Suzuki et al. 1996). However, we have previously demonstrated in coastal marine systems (like those considered in this manuscript) how these primer and amplification decisions generally only subtly bias specific taxonomic group relative abundances while having little effect on overall community patterns (Wear et al. 2018).

Statistically, many coral-associated bacterial taxa were inevitably not identified due to the conservative nature of this workflow. Despite there being 90 OTUs with L2FC values above zero, i.e. bacteria in higher relative abundance in coral primary polyps than their paired biofilm EBM controls, only 18 OTUs were defined as significant coral-associated bacteria by our statistical threshold (one-way one-sample *t* test, mu = 0.5). While for this study we intentionally filtered for a more refined list of putative coral associates, future studies may consider a lower threshold based on data distribution for a broader scope of putative microbiome members (e.g. mu = 0). Furthermore, even with a lower statistical threshold, it was nearly impossible for OTUs present in both coral primary polyp and the adjacent biofilm to be defined coral-associated per

our determined thresholds and ratio test. Therefore, OTUs found in both coral and biofilm remain unassigned and designating their identities as coral-associated or not is outside the scope of this 16S-based analysis. To better identify true coral-bacteria associations, we recommend incorporating fluorescent microscopy with taxa-specific probes to better verify bacterial associates from the EBM (e.g. Sharp et al., 2010, 2012; Apprill 2012).

Conclusion

With the current steep decline in coral reefs, understanding healthy holobiont associations and drivers of these associations may be informative for both current and future reef management. While much of the attention devoted to coral microbiomes has focused on identifying and understanding the role of antagonistic coral reef microbiota, it is equally valuable to identify beneficial associations that assist in coral holobiont resilience (e.g. Santoro et al., 2021; reviewed in van Oppen et al., 2026). With larval propagation increasing in popularity, identifying beneficial symbionts for microbially informed solutions extends to early life history corals (Damjanovic et al., 2019; Matsuda et al., 2022). The methods outlined in this study serve to increase our understanding of early coral-microbe associations and better support the development of new molecular tools to understand and assist coral resilience in a changing world. While conservative, we believe this sampling and analysis approach is a necessary step for accurately identifying early coral microbiome needles amidst the immense diversity of the marine microbial haystack.

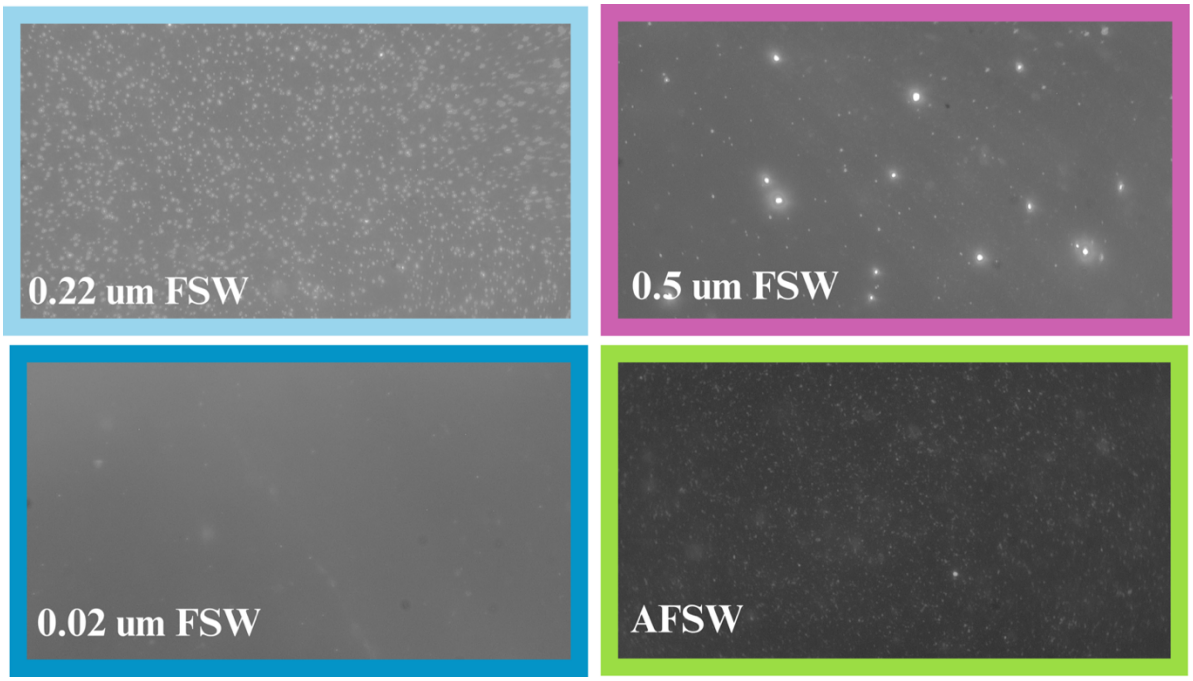
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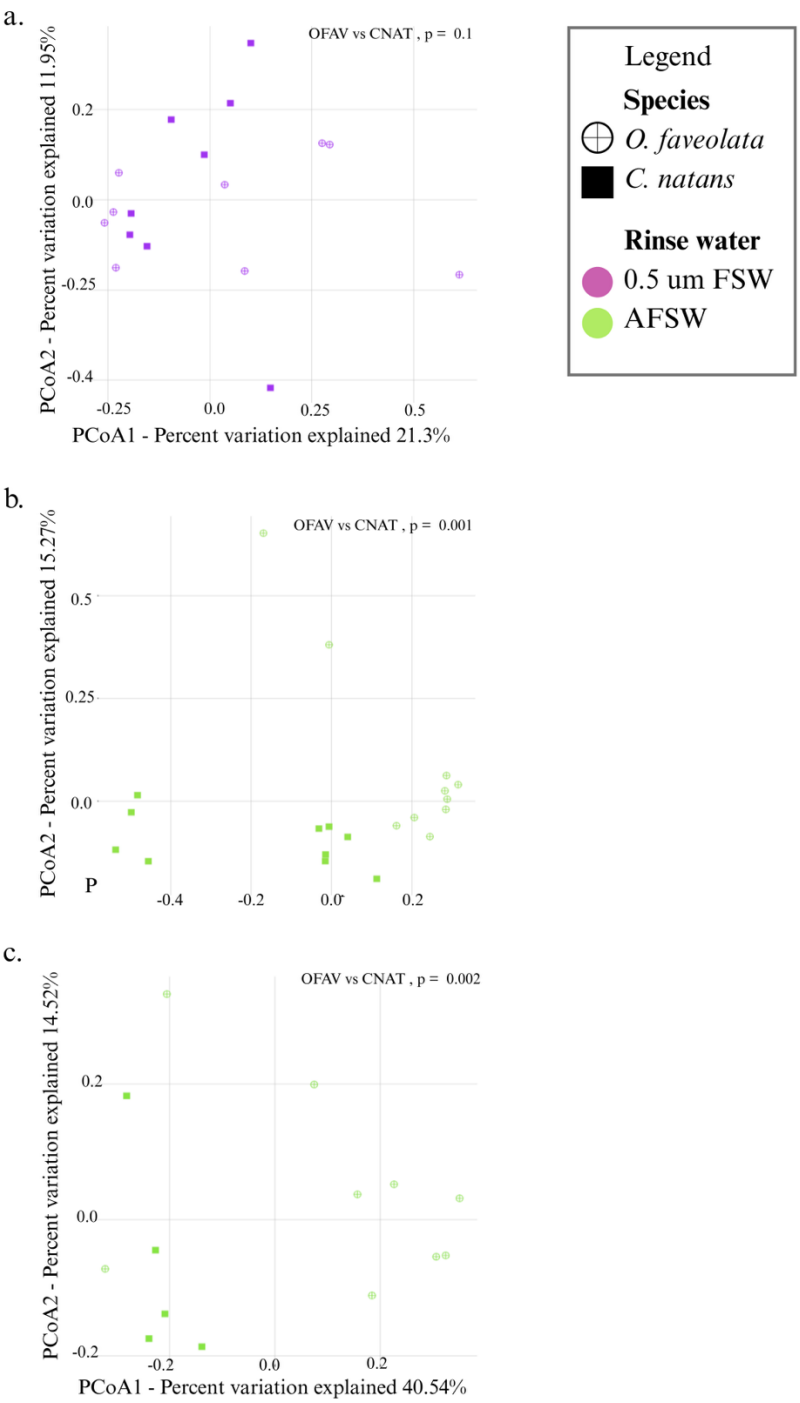
and lab assistance throughout the period of this study, with to special thanks M. Vermeij, M.J. Bennett, D. van Bendegom, C. Lippens (CARMABI), and V. Chamberland, K. Latijnhouwers, and J. Schneider (SECORE International) for their logistical support and assistance with the collection and care of coral larvae. Big thanks to S. Zumo, I. Rojas, and J. Kendall-Bar for the much needed final leg of technological support and to S. Cliffs generally. This project was funded by the US National Science Foundation under award CBET-2133675 (to L.W.K. A.J.W.J., R.M.E.-M., and K.L.M.). Field research during the 2023 coral spawning season was further supported by funding from Global Coral R&D Accelerator Platform (CORDAP) under award CAP 2022-1193 (to K.L.M.).

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Supplemental Figures



Supplemental Figure 3.1 Micrographs of DAPI-stained 0.02 µm Anodisc filters representative of 0.22-FSW, 0.02-FSW, 0.5-FSW, and AFSW (2x 0.22 µm filtered artificial rinse water). Larger particles seen in the 0.5 µm FSW rinse water represents bacterial cells.



Supplemental Figure 3.2 PCoA ordination comparing C. natans and O. faveolata coral larvae microbiomes sampled using different rinse waters, distance = Bray-Curtis Dissimilarity. Color denotes rinse water used 0.5-FSW (purple), AFSW (green), and symbols represent coral species: O. faveolata (circles with cross) and C. natans (squares). Coral were rinsed in 0.5-FSW (purple) in (a) 2021 and AFSW (green) in (b) 2022 and (c) 2023.

Supplemental Tables

Supplemental Table 3.1 Literature review of early life history coral studies, including species, life stages sampled, and the respective rinse water used during sampling.

Publication Year	Author	Title	Species	Sample type	Rinse water
2009	Apprill et al.	The onset of microbial associations in the coral Pocillopora meandrina	Pocillopora meandrina	Larvae	?
2012	Apprill et al.	Specificity of associations between bacteria and the coral Pocillopora meandrina during early development	Pocillopora meandrina	Larvae	Millipore Pellicon 2 mini-TFF unit equipped with a 30-kDa-cutoff regenerated-cellulose filter cassette (Millipore Corp., Billerica, MA)
2019	Bernasconi et al.	Establishment of coral-bacteria symbioses reveal changes in the core bacterial community with host ontogeny	Acropora digitifera	Egg bundles, larvae, spat, 6mo recruit	0.22 µm filtered seawater
2020	Damjanovic et al.	Mixed-mode bacterial transmission in the common brooding coral Pocillopora acuta	Pocillopora acuta	Larvae, 4do recruits	0.22 lm FSW
2020	Damjanovic et al.	Bacterial and algal symbiont dynamics in early recruits exposed to two adult coral species	Pocillopora acuta	1wo coral recruit, 9wo recruit	0.22 lm FSW
2020	Damjanovic et al.	Early life stages of a common broadcast spawning coral associate with specific bacterial communities despite lack of internalized bacteria	Acropora tenuis	Egg-sperm bundle, embyros, larvae	0.22 µmFSW
2019	Epstein et al.	Parental and early life stage environments drive establishment of bacterial and dinoflagellate communities in a common coral	Pocillopora damicornis	1wo recruits	sterile PBS
2017	Leite et al.	Broadcast spawning coral Mussismilia hispida can vertically transfer its associated bacterial core	Mussismilia hispida	Egg-sperm bundles, larvae	sterile NaCl (3%)
2014	Lema et al.	Onset and establishment of diazotrophs and other bacterial associates in the early life history stages of the coral Acropora millepora	Acropora millepora	larvae, 2wo, 3mo, 6mo, 12mo recruit	autoclaved artificial sea water (ASW)
2009	Littman et al.	Bacterial communities of juvenile corals infected with different Symbiodinium (dinoflagellate) clades	Acropora tenuis	9mo recruits	artificial seawater
2020	Quigley et al.	Co-dynamics of Symbiodiniaceae and bacterial populations during the first year of symbiosis with Acropora tenuis juveniles	Acropora tenuis	40do, 71do, 111do, 129do, 167do, 194do, 316do,	0.1-µm filtered seawater
2010	Sharp et al.	Bacterial Acquisition in Juveniles of Several Broadcast Spawning Coral Species	Orbicella annularis, Orbicella franksi, Orbicella faveolata, Acropora palmata, Acropora cervicornis, and Pseudodiploria strigosa	Egg-sperm bundles, larvae, spat	0.22 µmFSW
2012	Sharp et al.	Diversity and dynamics of bacterial communities in early life stages of the Caribbean coral Porites astreoides	Porites astreoides	Larvae, spat	0.22 µm FSW
2017	Zhou et al.	Microbiome dynamics in early life stages of the scleractinian coral Acropora gemmifera in response to elevated pCO2	Acropora gemmifera	Egg-sperm bundles, larvae, 6do, 31 do recruits	autoclaved artificial seawater
2019	Damjanovic et al.	Experimental inoculation of coral recruits with marine bacteria indicates scope for microbiome manipulation in Acropora tenuis and Platygyra daedalea	Acropora tenuis and Platygyra daedalea	3wo recruits	0.22 µm FSW
2025	Pérez-Trejo et al.	Differentiation of the bacterial communities associated with Orbicella faveolata across different growth conditions and life-cycle stages	Orbicella faveolata	various aged recruits	no rinse

Supplemental Table 3.2 Sequencing run metadata for experiments used in this study.

Sample set	Year	Sequencing run	Subsample threshold
Species specificity	2021	1	5,000
Species specificity	2022	2	1,000
Species specificity	2023	2	1,000
Coral-biofilm	2023	3	5,000
Larvae rinse water	2024	4	1,000

Supplemental Table 3.3 List of top 20 OTUs with highest mean relative abundance (ra) in sequenced larval microbiome for each rinse water treatment. Table cells are color coded by rinse water treatment.

Family	Genus	OTU	Treatment	Coral ra	Water ra
Alteromonadaceae	Aestuariibacter	Otu0002	0.22um	0.241	0.000
Arcobacteraceae	Arcobacter	Otu0019	0.22um	0.062	0.000
Alteromonadaceae	Alteromonas	Otu0015	0.22um	0.060	0.000
Clade_I	Clade_Ia	Otu0001	0.22um	0.042	0.148
Nitrincolaceae	Nitrincolaceae uncl.	Otu0024	0.22um	0.036	0.000
Nitrincolaceae	Neptuniibacter	Otu0022	0.22um	0.035	0.000
Saccharospirillaceae	Oleibacter	Otu0031	0.22um	0.025	0.000
Pseudomonadaceae	Pseudomonas	Otu0050	0.22um	0.022	0.010
Clade_II	Clade_II_ge	Otu0007	0.22um	0.021	0.109
Nitrincolaceae	Nitrincolaceae uncl.	Otu0042	0.22um	0.018	0.000
Alteromonadaceae	Rheinheimera	Otu0099	0.22um	0.014	0.000
Endozoicomonadaceae	Endozoicomonas	Otu0076	0.22um	0.014	0.000
Actinomarinaceae	Candidatus_Actinomarina	Otu0006	0.22um	0.013	0.045
Marine_Group_II_fa	Marine_Group_II_ge	Otu0044	0.22um	0.013	0.019
Rhodobacteraceae	Nautella	Otu0033	0.22um	0.012	0.000
Hydrogenophilaceae	Tepidiphilus	Otu0058	0.22um	0.012	0.041
Moraxellaceae	Acinetobacter	Otu0067	0.22um	0.011	0.011
Rhodobacteraceae	HIMB11	Otu0004	0.22um	0.011	0.000
Moraxellaceae	Acinetobacter	Otu0096	0.22um	0.010	0.000
Pseudomonadaceae	Pseudomonas	Otu0094	0.22um	0.010	0.003
Clade_I	Clade_Ia	Otu0001	0.5um	0.120	0.159
Cyanobiaceae	Synechococcus_CC9902	Otu0003	0.5um	0.090	0.123
Rhodobacteraceae	HIMB11	Otu0004	0.5um	0.075	0.039
Oleiphilaceae	Oleiphilus	Otu0005	0.5um	0.072	0.001
Alteromonadaceae	Aestuariibacter	Otu0002	0.5um	0.044	0.001
Actinomarinaceae	Candidatus_Actinomarina	Otu0006	0.5um	0.039	0.058
Clade_II	Clade_II_ge	Otu0007	0.5um	0.037	0.047
Cyanobiaceae	Prochlorococcus_MIT9313	Otu0008	0.5um	0.037	0.061
Cyanobiaceae	Cyanobium_PCC-6307	Otu0012	0.5um	0.026	0.049
AEGEAN-169_marine_group	AEGEAN-169_marine_group_ge	Otu0014	0.5um	0.025	0.024
Halieaceae	OM60(NOR5)_clade	Otu0025	0.5um	0.015	0.007
Cryomorphaceae	uncultured	Otu0021	0.5um	0.014	0.014
Flavobacteriaceae	NS4_marine_group	Otu0028	0.5um	0.014	0.012
Alteromonadaceae	Alteromonas	Otu0015	0.5um	0.012	0.000
Flavobacteriaceae	NS5_marine_group	Otu0023	0.5um	0.012	0.013
SAR86_clade_fa	SAR86_clade_ge	Otu0026	0.5um	0.012	0.022
SAR86_clade_fa	SAR86_clade_ge	Otu0027	0.5um	0.011	0.011

Supplemental Table 3.3 (continued) List of top 20 OTUs with highest mean relative abundance (ra) in sequenced larval microbiome for each rinse water treatment. Table cells are color coded by rinse water treatment.

Family	Genus	OTU	Treatment	Coral ra	Water ra
SAR86_clade_fa	SAR86_clade_ge	Otu0032	0.5um	0.010	0.009
Cyanobiaceae	Synechococcus_CC9902	Otu0035	0.5um	0.009	0.013
Flavobacteriaceae	Flavobacteriaceae uncl.	Otu0036	0.5um	0.009	0.003
Alteromonadaceae	Aestuariibacter	Otu0002	AFSW	0.163	0.000
Haloferacaceae	Haloquadratum	Otu0017	AFSW	0.089	0.145
Halobacteriaceae	Halobacterium	Otu0029	AFSW	0.044	0.109
Halobacteriales uncl.	Halobacteriales uncl.	Otu0040	AFSW	0.040	0.046
Arcobacteraceae	Arcobacter	Otu0019	AFSW	0.036	0.000
Haloferacaceae	Halovenus	Otu0039	AFSW	0.028	0.081
Nitrincolaceae	Nitrincolaceae uncl.	Otu0024	AFSW	0.025	0.000
Rhodothermaceae	Salinibacter	Otu0041	AFSW	0.025	0.083
Alteromonadaceae	Alteromonas	Otu0015	AFSW	0.023	0.000
Halomicrobiaceae	Halapricum	Otu0065	AFSW	0.018	0.007
Rhodobacteraceae	Nautella	Otu0033	AFSW	0.017	0.000
Pseudomonadaceae	Pseudomonas	Otu0050	AFSW	0.017	0.011
Haloferacaceae	Halorubrum	Otu0066	AFSW	0.016	0.040
Haloferacaceae	Halolamina	Otu0061	AFSW	0.016	0.029
Saccharospirillaceae	Oleibacter	Otu0031	AFSW	0.014	0.000
Halomicrobiaceae	Haloarcula	Otu0069	AFSW	0.013	0.007
Nitrincolaceae	Nitrincolaceae uncl.	Otu0042	AFSW	0.013	0.000
Halococcaceae	Halococcus	Otu0081	AFSW	0.012	0.025
Nitrincolaceae	Neptuniibacter	Otu0022	AFSW	0.011	0.000
Halobacteriales uncl.	Halobacteriales uncl.	Otu0091	AFSW	0.010	0.024

Supplemental Table 3.4 Top 20 OTUs displaying the largest mean difference in relative abundance (ra) between 1x larvae and 10x pooled larvae sequenced microbiomes. Putative coral-associated bacteria are bolded and highlighted (orange).

Family	Genus	OTU	1x Larvae ra	10x Larvae ra	Difference (10x ra - 1x ra)
Alteromonadaceae	**Aestuariibacter**	**Otu0002**	**0.1576**	**0.2842**	**0.1266**
Arcobacteraceae	**Arcobacter**	**Otu0019**	**0**	**0.0978**	**0.0978**
Nitrincolaceae	**Neptuniibacter**	**Otu0022**	**0.0114**	**0.0528**	**0.0414**
Saccharospirillaceae	**Oleibacter**	**Otu0031**	**0.0096**	**0.0372**	**0.0276**
Clade_I	Clade_Ia	Otu0001	0.026	0.053	0.027
Clade_II	Clade_II_ge	Otu0007	0.0146	0.0378	0.0232
Lactobacillaceae	Lactobacillus	Otu0151	0	0.0216	0.0216
Alteromonadaceae	**Alteromonas**	**Otu0015**	**0.0454**	**0.0602**	**0.0148**
Rhodobacteraceae	**Rhodobacteraceae uncl.**	**Otu0053**	**0.0028**	**0.016**	**0.0132**
Rhodobacteraceae	**Nautella**	**Otu0033**	**0.005**	**0.0152**	**0.0102**
Actinomarinaceae	Candidatus_Actinomarina	Otu0006	0.0082	0.0168	0.0086
Nitrincolaceae	**Nitrincolaceae uncl.**	**Otu0057**	**0.0044**	**0.0124**	**0.008**
Nitrincolaceae	**Nitrincolaceae uncl.**	**Otu0042**	**0.0066**	**0.0122**	**0.0056**
Family_XI	Anaerococcus	Otu0282	0.0032	0.0084	0.0052
Marine_Group_II_fa	Marine_Group_II_ge	Otu0086	0	0.0052	0.0052
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0085	0	0.0042	0.0042
Halobacteriales uncl.	Halobacteriales uncl.	Otu0562	0	0.004	0.004
Flavobacteriaceae	Flavobacteriaceae uncl.	Otu0036	0	0.0034	0.0034
Flavobacteriaceae	NS5_marine_group	Otu0023	0	0.003	0.003
Alphaproteobacteria uncl.	Alphaproteobacteria uncl.	Otu0198	0	0.0028	0.0028

Supplemental Table 3.5 List of OTUs found to be significantly enriched in coral larvae samples in respect to rinse water EBM control samples for each rinse water treatment (one-way one-sample *t* test, mu = 0.5) in sequenced larval microbiome for each rinse water treatment. Table cells are color coded by rinse water treatment and report mean log-2-fold-change (L2FC) and relative abundance (ra).

Family	Genus	OTU	Treatment	L2FC	Coral ra	Water ra
Alteromonadaceae	Aestuariibacter	Otu0002	0.02um	9.285	0.241	0
Alteromonadaceae	Alteromonas	Otu0015	0.02um	6.969	0.060	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0024	0.02um	5.876	0.036	0
Nitrincolaceae	Neptuniibacter	Otu0022	0.02um	5.323	0.035	0
Saccharospirillaceae	Oleibacter	Otu0031	0.02um	5.014	0.025	0
Rhodobacteraceae	Nautella	Otu0033	0.02um	4.402	0.012	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0042	0.02um	4.288	0.018	0
Arcobacteraceae	Arcobacter	Otu0019	0.02um	3.621	0.062	0
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0053	0.02um	3.350	0.009	0
Rhodobacteraceae	HIMB11	Otu0004	0.02um	3.171	0.011	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0057	0.02um	3.170	0.009	0
Rhodobacteraceae	Thalassobius	Otu0048	0.02um	2.939	0.006	0
Vibrionaceae	Vibrionaceae uncl.	Otu0073	0.02um	2.613	0.005	0
Moraxellaceae	Acinetobacter	Otu0096	0.02um	2.471	0.010	0
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0098	0.02um	2.228	0.003	0
Alteromonadaceae	Aestuariibacter	Otu0002	AFSW	7.431	0.163	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0024	AFSW	5.617	0.025	0
Alteromonadaceae	Alteromonas	Otu0015	AFSW	5.368	0.023	0
Rhodobacteraceae	Nautella	Otu0033	AFSW	4.989	0.017	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0057	AFSW	3.648	0.007	0
Arcobacteraceae	Arcobacter	Otu0019	AFSW	3.530	0.036	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0042	AFSW	3.467	0.013	0
Saccharospirillaceae	Oleibacter	Otu0031	AFSW	3.189	0.014	0
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0053	AFSW	3.104	0.008	0
Nitrincolaceae	Neptuniibacter	Otu0022	AFSW	3.100	0.011	0
Rhodobacteraceae	HIMB11	Otu0004	AFSW	2.820	0.007	0
Clade_I	Clade_Ia	Otu0001	AFSW	2.717	0.010	0
Marinobacteraceae	Marinobacter	Otu0082	AFSW	2.438	0.006	0
Halomicrobiaceae	Halapricum	Otu0155	AFSW	2.214	0.003	0
Rhodobacteraceae	Thalassobius	Otu0048	AFSW	2.084	0.003	0
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu0098	AFSW	2.080	0.003	0
Bdellovibrionaceae	OM27_clade	Otu0104	AFSW	1.886	0.005	0
Endozoicomonadaceae	Endozoicomonas	Otu0076	AFSW	1.872	0.004	0
Halobacteriales uncl.	Halobacteriales uncl.	Otu0207	AFSW	1.823	0.008	0
Micrococcaceae	Kocuria	Otu0211	AFSW	1.816	0.005	0
Xanthomonadaceae	Vulcaniibacterium	Otu0122	AFSW	1.808	0.007	0

Supplemental Table 3.5 (continued) List of OTUs found to be significantly enriched in coral larvae samples in respect to rinse water EBM control samples for each rinse water treatment (one-way one-sample *t* test, mu = 0.5) in sequenced larval microbiome for each rinse water treatment. Table cells are color coded by rinse water treatment and report mean log-2-fold-change (L2FC) and relative abundance (ra).

Family	Genus	OTU	Treatment	L2FC	Coral ra	Water ra
Halomonadaceae	Oceanospirillum	Otu0144	AFSW	1.736	0.002	0
Cyanobiaceae	Prochlorococcus_MIT9313	Otu0008	AFSW	1.696	0.002	0
Vibrionaceae	Vibrionaceae uncl.	Otu0073	AFSW	1.494	0.002	0
Oleiphilaceae	Oleiphilus	Otu0005	0.5um	6.716	0.072	0.00067
Alteromonadaceae	Aestuariibacter	Otu0002	0.5um	5.188	0.044	0.00067
Alteromonadaceae	Alteromonas	Otu0015	0.5um	4.844	0.012	0.00033
Nitrincolaceae	Nitrincolaceae uncl.	Otu0024	0.5um	3.724	0.006	0
Nitrincolaceae	Neptuniibacter	Otu0022	0.5um	3.431	0.006	0
Alcanivoracaceae	Alcanivorax	Otu0055	0.5um	3.201	0.004	0
Flavobacteriaceae	NS5_marine_group	Otu0045	0.5um	3.140	0.006	0.00067
Saccharospirillaceae	Oleibacter	Otu0031	0.5um	3.111	0.005	0
Vibrionaceae	Vibrionaceae uncl.	Otu0073	0.5um	3.050	0.004	0
Nitrincolaceae	Nitrincolaceae uncl.	Otu0042	0.5um	2.370	0.004	0
SAR116_clade	SAR116_clade_ge	Otu0087	0.5um	1.894	0.002	0.00033
Rhodobacteraceae	Nautella	Otu0033	0.5um	1.822	0.007	0.00167
Moraxellaceae	Acinetobacter	Otu0067	0.5um	1.707	0.002	0
Marinobacteraceae	Marinobacter	Otu0082	0.5um	1.696	0.002	0
Bdellovibrionaceae	OM27_clade	Otu0104	0.5um	1.432	0.001	0
Enterobacteriaceae	Enterobacteriaceae uncl.	Otu0011	0.5um	1.418	0.002	0.00033
Flavobacteriaceae	Flavobacteriaceae uncl.	Otu0036	0.5um	1.363	0.009	0.00333
Flavobacteriaceae	uncultured	Otu0047	0.5um	1.300	0.005	0.00133
Gammaproteobacteria uncl.	Gammaproteobacteria uncl.	Otu0123	0.5um	1.261	0.001	0
NS9_marine_group	NS9_marine_group_ge	Otu0059	0.5um	1.055	0.005	0.00167
Rhodobacteraceae	HIMB11	Otu0004	0.5um	0.942	0.075	0.03867

Supplemental Table 3.6 Table of the 18 OTUs found to be significantly enhanced in coral primary polyp samples in comparison to paired biofilm samples (one-way one-sample *t* test, mu = 0.5). Table is ordered by mean log-2-fold-change ratio values (L2FC) and mean relative abundance (ra) values for coral primary polyp and adjacent biofilm samples.

Family	Genus	OTU	L2FC	Coral ra	Biofilm ra
Kordiimonadaceae	Kordiimonas	Otu00103	5.226	0.019	0.000
Hyphomonadaceae	uncultured	Otu00190	4.573	0.007	0.000
Sphingomonadaceae	Erythrobacter	Otu00480	4.116	0.004	0.000
Alteromonadaceae	Alteromonadaceae uncl.	Otu00144	3.970	0.010	0.000
Cyclobacteriaceae	Marinoscillum	Otu00235	3.877	0.004	0.000
Bacteria uncl.	Bacteria uncl.	Otu00057	3.042	0.003	0.000
Vibrionaceae	Vibrionaceae uncl.	Otu00005	2.741	0.012	0.001
Nitrincolaceae	Neptuniibacter	Otu00010	2.608	0.006	0.002
Alphaproteobacteria uncl.	Alphaproteobacteria uncl.	Otu00240	2.594	0.004	0.001
Stappiaceae	Labrenzia	Otu00068	2.593	0.008	0.002
Alteromonadaceae	Alteromonas	Otu00004	2.572	0.034	0.004
OM190_fa	OM190_ge	Otu00484	2.312	0.003	0.000
Arenicellaceae	Arenicella	Otu00395	2.272	0.002	0.000
Saprospiraceae	uncultured	Otu00154	2.240	0.003	0.000
Nitrincolaceae	Nitrincolaceae uncl.	Otu00056	1.854	0.006	0.002
Gammaproteobacteria uncl.	Gammaproteobacteria uncl.	Otu00174	1.853	0.009	0.003
Rhodobacteraceae	Rhodobacteraceae uncl.	Otu00050	1.526	0.031	0.011
Sphingomonadaceae	Erythrobacter	Otu00081	1.206	0.008	0.003

Supplemental Table 3.7 Table of the gram-positive bacteria organized by phyla, sample type, and EBM study.

EBM study	Sample type	Phyla	min ra (%)	mean ra (%)	max ra (%)
rinse water	250 uL aliquot	Actinobacteria	0	0.22	13.4
rinse water	coral larvae	Actinobacteria	0	0.16	5.9
biofilm	coral primary polyp	Actinobacteria	0	0.07	2.36
biofilm	biofilm scraping	Actinobacteria	0	0.12	1.68
rinse water	250 uL aliquot	Firmicutes	0	0.13	4.5
rinse water	coral larvae	Firmicutes	0	0.11	10.8
biofilm	coral primary polyp	Firmicutes	0	0.03	1.14
biofilm	biofilm scraping	Firmicutes	0	0.07	2.24

Supplemental Material

Supplemental Material 3.1 *Determination of microbial cell abundances in rinse water.* Following filtration, three technical replicates of each rinse water treatment were fixed in 32% paraformaldehyde (PFA). 5 mL of each rinse water treatment were fixed in 80 µL of PFA, apart from 0.5-FSW in which 1 mL of rinse water was fixed in 16 µL of PFA. All fixed samples were incubated in the dark for one hour. Following fixation, each 5 mL replicate was filtered onto a 0.02 µm filter (Anotop), dried, and frozen at -20°C. For 0.5-FSW, 500 µL of fixed rinse water was diluted into 4.5 mL of sterile lab grade water and subsequently filtered. Discrepancies in dilution factors between 0.5-FSW and other respective rinse water treatments were due to the 0.5 µm pore size being larger than most bacterial cells, resulting in the 0.5 µm FSW rinse water treatment hosting more bacterial cells than the other rinse solutions. Cell counts were obtained using fluorescent microscopy, where Anotop filters were DAPI stained, mounted on microscopy slides, and imaged for counts. 10 images were taken for each technical replicate filter.

Supplemental Material 3.2 *Low-biomass coral DNA extraction Zymobiomics DNA miniprep kit base protocol with amendments*

*Samples should be stored in Lysis Buffer, DNA/RNA shield, or ethanol

Note: For samples stored in ethanol, spin down at 10,000 g for 1 minute. Decant the ethanol and add molecular grade water. Resuspend sample in water, spin down and decant. Repeat this water rinse for a total of two rinses. Add lysis buffer after second rinse and resuspend.

1. Add ½ of beads from the alotted **ZR BashingBead Lysis Tubes** (0.1 & 0.5 mm) directly to sample. Shake beads in tube before splitting to ensure both bead sizes are distributed to each sample (this is not a perfect process).

2. Aliqout 50 µL of proK (20mg/mL) into each sample. If stored in <750 µL of buffer, add the appropriate amount to the tube (for a total of ~750 µL solution) and cap tightly.

3. Secure in bead beater with 2mL tube holder assembly and process according to Zymo DNA miniprep appendix instructions for your device (Appendix D). At SIO this equates to 3 cycles of 1 min beating at 6 m/s with 5 minute rest intervals.

4. Spin down samples at 10,000 g for 2 minutes.

5. Invert gently several times and incubate at ~60°C for 1.5 to 2 hours. Invert tubes every 30 to 40 minutes.

6. Spin down samples at 10,000 g for 1 minute.

7. Transfer all supernatant (~750 µL) to the **Zymo-Spin III-F Filter** in a Collection Tube and centrifuge at 8,000 *g* for 1 minute. Discard the **Zymo-SpinTM III-F Filter**.

8. Transfer 750 µL of supernatant to a >5 mL tube with 2,400 µL **ZymoBIOMICS DNA Binding Buffer** to the filtrate in the Collection Tube from Step 4. Pipette gently up and down to mix well.

9. Transfer 800 µL of the mixture from Step 8 to a **Zymo-Spin IICR Column** in a Collection Tube and centrifuge at 10,000 *g* for 1 minute.

10. Discard the flow through from the Collection Tube and repeat Step 6 until all supernatant has been filtered through the IICR column.

11. Add 400 µL **ZymoBIOMICS DNA Wash Buffer 1** to the **Zymo- Spin IICR Column** and centrifuge at 10,000 *g* for 1 minute. Discard the flow-through.

12. Add 700 µL **ZymoBIOMICS DNA Wash Buffer 2** to the **Zymo- Spin IICR Column** in a Collection Tube and centrifuge at 10,000 *g* for 1 minute. Discard the flow-through.

13. Add 200 µL **ZymoBIOMICS DNA Wash Buffer 2** to the **Zymo- SpinTM IICR Column** in a Collection Tube and centrifuge at 10,000 *g* for 1 minute.

14. Transfer the **Zymo-SpinTM IICR Column** to a clean 1.5 ml microcentrifuge tube.

15. Perform a series of micro-elutions, adding ~70°C DNase/RNase free water directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 g for 1 minute. Repeat this elution twice more for a total 60 µL stock solution.

Note: Due to the micro nature of this elution step, it is very important that the 20 µL lands directly on the filter column matrix. If the droplet gets stuck on the wall of the tube tap it or move it with pipette tip to ensure that it reaches the column filter matrix.
Note: We have excluded the final purification step with the **Zymo-SpinTM III-HRC Filter** from the amended protocol. The main purpose of this step is to remove PCR inhibitors commonly associated with plant and soil samples. Due to the relatively aposymbiotic nature of coral larvae and recruits, we have confirmed with PCR and qBit that this step is unnecessary for these sample types.

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Chapter 4 BENTHIC BIOFILMS INFLUENCE CORAL LARVAL SETTLEMENT AND MICROBIOME DYNAMICS

Abstract

Corals host a dense and diverse microbial community of endolithic fungi and algae, dinoflagellate endosymbionts, archaea, viruses, and bacteria that collectively perform as a "holobiont". One driver of this microbial consortium is the surrounding biogeochemical environment, where neighboring benthic reef organisms influence water and biofilm microbiomes, the latter of which importantly act as a settlement cue for coral larvae. While horizontal transmission of microbiota from the surrounding environment is considered the dominant form of initial acquisition, a dearth of knowledge exists on the exact external drivers of early coral microbiome development. This study examines the benthic "neighbor effect" at the settlement scale, investigating the effect of neighboring biofilms on early life history coral microbiome development. Settlement substrates were conditioned for six weeks in flow-through aquaria with crustose coralline algae (CCA), the macroalga *Dictyota*, or ambient seawater to promote distinct biofilms. Larvae were introduced to these conditioned substrates and settlement was recorded after one week. 16S rRNA gene amplicon sequencing revealed distinct biofilm communities, and notably CCA and *Dictyota*-promoted biofilms incurred high settlement, with *Dictyota* hosting the highest settlement per substrate. Larval microbiomes were largely conserved, with several taxa in the families Alteromonadaceae, Rhodobacteraceae, and Nitrincolaceae likely originating prior to biofilm exposure. Despite that nearly half (22 of 46) of larval-associated bacteria were shared across treatments, biofilm treatments displayed influence on initial microbiome development. Notably, larvae exposed to seawater-promoted biofilms were inundated by a single bacterial stain in the family Vibrionaceae. Similarly, coral settler microbiomes were influenced by biofilm treatment: settlers on CCA-promoted biofilm were

enriched by taxa in Kordiimonadaceae, Francisellaceae, and Cyclobacteraceae, while corals on *Dictyota*-promoted biofilms were enriched in Nitrincolaceae and Methylophagaceae. While very few corals settled in the seawater-promoted biofilm treatment, these coral microbiomes were enriched in Sphingomonadaceae, and single OTUs in the families Alteromonadaceae and Kordiimonadaceae. Although reported results are conservative, findings provide evidence that the microbial biofilm environment influences early life history coral microbiome dynamics within the scope of a laboratory setting.

Introduction

Coral reefs exist as microbially-driven systems, with bacteria underpinning the processes of primary production, photosynthate and nutrient recycling, rapid detritus decomposition, as well as facilitating net accretion (Nelson et al. 2023). This microbiome community is dynamic, structured by both biotic and abiotic features (e.g. Quinlan et al. 2019; Nelson et al. 2013; Wegley Kelly et al. 2019). One biotic feature that influences this community is benthic composition, where neighboring reef organisms influence the surrounding microbiota via suspension feeding (McNally et al. 2017) and the release of organic exometabolites (Quinlan et al. 2019; Nelson et al. 2013, 2023). These exometabolites, hereafter referred to as exudates, also drive microbial biofilm succession, with benthic reef organisms shown to significantly structure marine biofilm succession and community composition (Remple et al. 2021).

Biofilms are integral components of aquatic ecosystems, functioning as a well-synchronized unit, enzymatically digesting and releasing exudates into the water column (Battin et al. 2003; reviewed in Qian et al. 2022). Distinct from the bacterioplankton communities in the water column, the biofilm extracellular polymeric matrix and microbial metabolism make these surface-bound communities a reservoir for nutrients, antimicrobial compounds, and importantly

a source of chemical cues for marine invertebrate settlement and metamorphosis (reviewed in Qian et al. 2022). On coral reefs, surface-bound bacteria and their respective exudates are a vital component of the recruitment process (Webster et al. 2004; Tran & Hadfield 2011). These chemical cues are responsible for attracting a larva to specific microhabitats on the reef and even facilitate aspects of development, namely larval attraction, attachment, and metamorphosis (Heyward & Negri 1999; Negri et al. 2001; Webster et al. 2004; Sneed et al. 2014; Tebben et al. 2011). Importantly, coral larvae display chemotaxis to specific chemical cues and biofilms. For example, coral display habitat specificity, with naïve larvae from deep-reef and shallow-reef taxa preferentially settling on deep-reef and shallow-reef biofilms respectively (O'Brien et al. 2025).

Another example of this selectivity is seen in the chemotaxis of coral larvae towards specific species of crustose coralline algae, CCA, a calcifying algae known to be associated with healthy reef systems (Heyward & Negri 1999; Tebben et al. 2015; Sneed et al. 2014). Both the algae and its episymbiotic biofilm are implicated in settlement induction (Tebben et al. 2015; Gómez-Lemos et al. 2018). While difficult to discern the role of the microbial community from that of its algae host, metabolites from surface-attached bacteria are certainly key attractants (Sneed et al. 2014; Siboni et al. 2020). One microbial metabolite involved in this process is tetrabromopyrrole, or TBP, a chemical compound produced by a strain of *Pseudoalteromonas* isolated from the surface of CCA (Tebben et al. 2011; Sneed et al. 2014). Notably, TBP triggers metamorphosis pathways in coral larvae, though not necessarily attachment (Tebben et al. 2011; Sneed et al. 2024). Untargeted metabolomics studies reveal a suite of CCA holobiont exudates incur strong larval chemotaxis and settlement (Quinlan et al. 2023; Deshpande et al. 2025). This evidence supports that a suite of algae host and associate microbiota metabolites, rather than a

"smoking gun" or single metabolite, drive successful settlement processes (i.e., sequentially: chemotaxis, attachment, settlement, and metamorphosis).

Although phylogeny plays a role in coral holobiont community composition, an adult coral's microbiome is also influenced by the "neighborhood", that is its biogeochemical environment (Littman et al. 2009; Hernandez-Agreda et al. 2018). This may also hold true for coral larvae and settlers, though on a finer scale. Since coral larvae and settlers exist at the millimeter-scale, the biofilm that attracts larvae may also hold a role in facilitating endosymbiont acquisition. The biofilm environment may serve as a promoter for horizontal transmission, i.e. the acquisition of microbial symbionts from a coral's surrounding environment (Apprill 2012). This transmission may occur at the larval stage via swimming or surface probing, where larvae up-take bacteria while crawling on or probing a substrate during the settlement process (Apprill 2012). A second mechanism that may occur pre- and/or post-settlement is suspension feeding of biofilm-promoted bacteria, via the detachment of biofilm cells, or indirectly via bacterioplankton enriched by biofilm exudate release (McNally et al. 2017; Sharp et al. 2010). While the role of microbial biofilms in successful coral settlement is highly supported, its potential role in early coral microbiome development remains unresolved.

Here we examine the benthic "neighbor effect" at the settler- or millimeter-scale, evaluating the capacity of the biofilm to influence coral larval settlement and early life history microbiome formation. Biofilms were produced by conditioning settlement substrates (natural hydraulic lime; Yus et al. 2024) for six weeks in flow-through seawater tanks each with one of three common Caribbean reef benthic taxa (crustose coralline algae or CCA, the brown macroalgae *Dictyota*, and an ambient seawater control). After biofilm conditioning, substrate were introduced by treatment to *Colpophyllia natans* (Grooved brain coral) larvae for settlement.

Settlement in each treatment was recorded after one week, and two-week-old swimming coral larvae and metamorphosed coral settlers were sampled for microbiome analysis. Unlike adult conspecifics, coral larvae begin life on the reef nearly aposymbiotic, with little known on how the microbiome is selected or established. We hypothesized one potential reservoir for this establishment to be the biofilm environment on which a coral settles. More specifically, we proposed that biofilm community composition would be distinct to its respective benthic taxa treatment (Remple et al. 2021), and that these distinct biofilm communities would differentially influence both settlement and initial microbiome development of larvae and post-settlement corals.

Methods

Field collection and biofilm conditioning. Benthic treatment organisms, CCA and *Dictyota,* were collected on SCUBA on the coral reef slope adjacent to CARMABI Marine Research Station off the southern coastline of the Caribbean island nation of Curaçao (12°12’02.07”N, -68°97’02.45”W). *Dictyota* (48.3±0.58 g wet weight/aquaria) and CCA-encrusted coral rubble (372.6±28.1 cm^2 /aquaria) were distributed into three replicate aquaria each (n = 3 replicate aquaria per treatment, Fig. 4.1). Three additional replicate aquaria with no benthic organism served as a seawater conditioning control treatment (SW).

Experimental aquaria (n = 9) were set up in a shaded outdoor facility and consisted of 10 L rectangular polycarbonate storage containers that were acid-washed, rinsed and dried before filling. Aquaria were each independent flow-through systems, receiving unfiltered seawater retained at approximately three-hour residency time, with water mixed in each aquarium via a recirculating pump. Natural hydraulic lime, or NHL, settlement substrates were prepared at University of Illinois Champagne-Urbana facilities as described by Yus et al. (2024). Substrates

were placed within experimental aquaria on tables constructed of polystyrene "egg crate" lighting panel grids, approximately 10 cm above the respective benthic organism treatment. Settlement substrates (n = 22 in each aquaria) were conditioned for six weeks in flow-through aquaria with either CCA, *Dictyota*, or unfiltered seawater (SW, control) to generate biofilms representative of a "calcifying reef", "fleshy algae reef", and control respectively (Fig. 4.1). Due to gradual decay in the *Dictyota* treatment, all benthic organisms were replaced with fresh specimens halfway through the conditioning period.

Gamete collection and larval rearing. Seven days after the full moon in October 2023, gametes were collected from three adult *C. natans* colonies during a synchronized broadcast spawning event. Gametes were collected by SCUBA divers on the reef slope off Seaquarium (12°08'29.43"N, -68°89'62.85"W). Following methods described in previous works (Chamberland et al. 2017; Hagedorn et al. 2021), gametes were collected using conical tents attached to plastic funnels and 50 mL conical propylene tubes (BD Falcon) to capture positively buoyant egg-sperm bundles. Gametes were returned to lab for cross-fertilization and subsequently distributed into polystyrene clamshell food containers (Dart Container Corporation) containing 750 mL of 0.5 um filtered seawater (FSW) at the approximate density of 1 larva/mL. Water changes were performed daily to reduce microbial load and maintain a stable pH in the rearing environment throughout development (Vermeij 2006; Chamberland et al. 2017; Hagedorn et al. 2021). All experiments were run in a temperature-controlled laboratory at CARMABI with experimental water temperatures kept consistent with that of the local reefs (27.5 ± 1.5 °C).

Settlement experiment and microbial sampling. Following the conditioning period, substrates were removed from conditioning aquaria. Macro-successional organisms were gently

removed from substrates using a silicon mascara wand, and substrates from each replicate treatment aquarium were distributed into two replicate clamshells containing 750 mL of FSW (n = 18 clamshells, each with 11 randomized substrates, Fig. 4.1). Conditioned substrates remained separated by treatment and conditioning aquaria to account for treatment and aquaria effects in downstream statistical analysis.

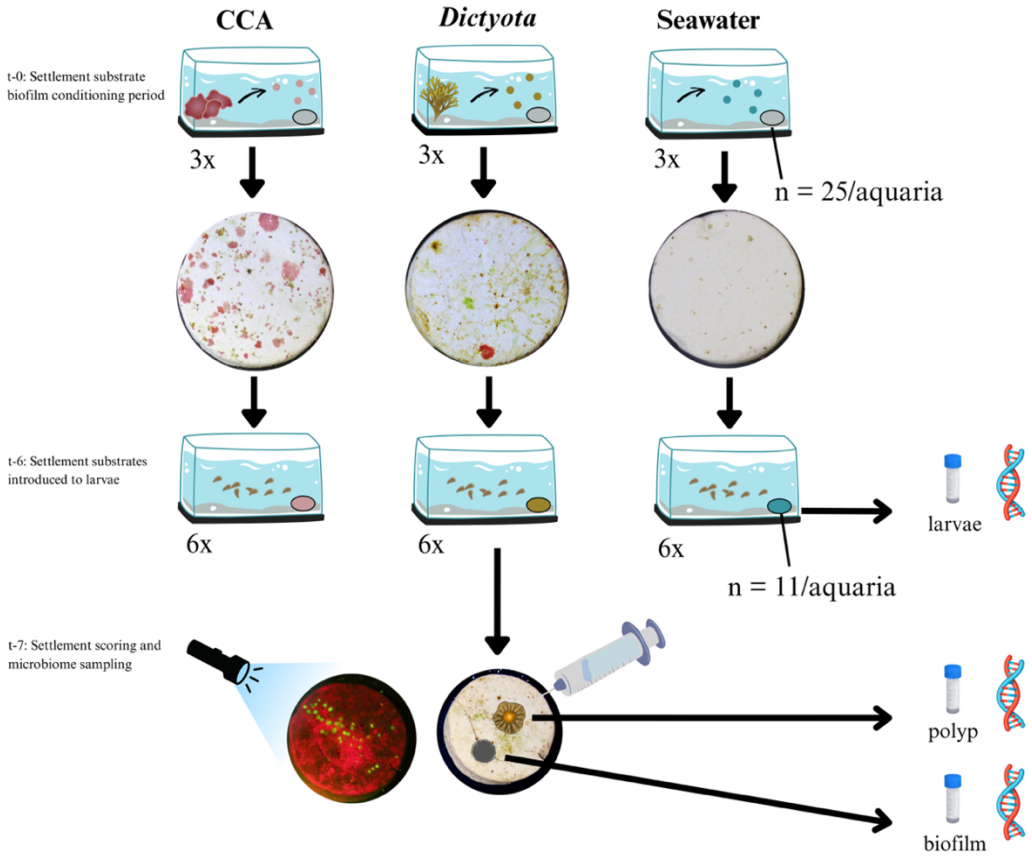


Figure 4.1 Diagram of experimental setup and design for settlement substrate conditioning, larval settlement, and microbiome sampling. Settlement substrates (n = 22 substrates/aquaria) were distributed into 9 aquaria (10 liters), which contained either freshly collected crustose coralline algae, *Dictyota*, or seawater with no benthic taxa (n = 3 replicates per treatment). Aquaria were kept on an outdoor shaded bench and received flow-through raw seawater with a residence time of approximately three hours. After six weeks, settlement substrates were introduced to *Colpophyllia natans* larvae for settlement (n = 6 settlement containers per treatment, 2 per conditioning aquaria). Settlement was recorded after two days using a blue light. Two-weeks following fertilization, substrate-paired biofilm and coral settlers were sampled from settlement containers. To increase downstream DNA yield, coral settlers and substrate-paired biofilms were pooled (n = 3 specimens per replicate) and sampled using a sterile needle, rinsed in 2x 0.22 µm artificial filtered seawater, and stored at -80°C in DNAshield for downstream analysis.

3-day-old swimming *C. natans* larvae were evenly distributed among replicate settlement clamshells at the approximate density of 2 larva/mL (2,000 larvae per bin). The majority of settlement occurred after two days, at which time substrates were photographed for settlement analysis. After one week of uninhibited biofilm exposure, larvae were sampled (11-day-old), with ten larvae from each settlement bin pooled for microbiome analysis. Water changes resumed every other day, and metamorphosed coral settlers (15-day-old) were sampled for microbiome analysis. To better understand larval microbiome development absent of substrate, 2-day-old and 5-day-old larvae were sampled as previously described. Coral larvae and settlers were rinsed prior to preservation using methods described in Nixon et al. 2026. Briefly, coral settlers were scraped off settlement substrate using a sterile 16G needle, with each replicate sample containing three coral settlers, each from a unique settlement substrate. Paired biofilm scrapings were sampled in complement to coral samples to account for the biofilm environmental background microbiome (EBM) in downstream statistical analysis, with an adjacent biofilm sampled for each coral settler (Fig. 4.1). All samples were rinsed in 2x 0.22 μm artificial seawater (AFSW), preserved in DNA Shield (Zymobiomics), and stored at -80°C. Sterivex used to prepare AFSW were frozen for downstream characterization of the rinse water EBM.

DNA extraction and 16S rRNA amplicon sequencing. All sample DNA was extracted with the Zymobiomics miniDNA kit (Zymo Research) following kit protocol with further optimizations made for low-biomass low-density microbiome samples (Supplemental S4.2). Purified DNA was sent to the University of Hawaii at Mānoa Integrative Center for Environmental Microbiomes and Human Health Microbial Genetics and Analytical Laboratory (ICEMHH MGAL) for 16S rDNA v4 amplicon library preparation (Caporaso et al. 2012;

Walters et al. 2012) and sequencing as described in Nixon et al. 2026. Sequences were demultiplexed and all bioinformatics were pipelined using a custom script written in NextFlow (Arisdakessian et al. 2020). Sequences were assembled, denoised, and quality trimmed using DADA2 (Callahan et al., 2016). The resulting sequence data was globally aligned with mothur (Schloss et al., 2009) to the SILVA (v. 132) global SSU rRNA alignment database. Sequence data were then Bayesian consensus classified (70%) to the genus level (Wang et al., 2007) and then clustered into operational taxonomic units (OTUs; 97% average sequence identity level) using vsearch (Rognes et al., 2016), with LULU used to minimize intragenomic taxonomic splitting errors (Frøslev et al., 2017). Sequence data that could not be classified at the domain level or were classified as chloroplast or mitochondrial DNA were removed from further analysis. All bioinformatic processing was completed on the C-MAIKI gateway (Cleveland et al., 2022) at the University of Hawai‘i at Mānoa by the Center for Microbial Oceanography: Research and Education. To account for variability in sequencing depth effort, we randomly subsampled the dataset to 5,000 reads. Samples with fewer than 5,000 reads were discarded from further analysis. Finally, OTUs were removed from analysis if the relative abundance was not 0.2% or greater in 3 or more samples, or if the maximum relative abundance below 0.5%. A second maximum abundance threshold of 0.5% was set within each statistical test subset to filter for ecologically relevant OTUs. In all, our dataset comprised of 2 AFSW water filters, 26 larval pools (n=10 each), 36 coral settlers and 36 paired adjacent biofilm samples, 5 mock communities, and 3 extraction blanks.

Statistical Analysis. Settlement differences between biofilm treatments were tested using a generalized linear mixed model (lme4 package, family = poisson), including settlement container as a random effect. All 16S statistical analysis was performed at the OTU level, where

taxa are defined by 97% sequence similarity. For each sample type (larvae, settler, biofilm), alpha diversity and species richness were compared between treatments. Alpha diversity was calculated using the Shannon Index (Shannon 1948) and evenness calculated using Pielou Evenness (Pielou 1966). Species richness was calculated following subsampling and the removal of rare OTUs. Shannon diversity, evenness, and richness means were compared between treatments through analysis of variance (ANOVA) followed by a Tukey HSD test. For coral larvae, settlers, and biofilm, Bray-Curtis distance matrices were calculated and visualized using principal coordinates analysis (PCoA, Vegan package). For coral larvae, differences in microbial community composition between biofilm treatment (CCA, *Dictyota*, Seawater) were tested via a permutational analysis of variance (PERMANOVA, adonis2 function in the vegan package) and pairwise post-hoc testing (pairwiseAdonis package). Differences in microbial community composition between sample type (coral settler and adjacent biofilm) and biofilm treatment, as well as the interaction between the two factors were tested via a two-factor PERMANOVA. Due to an interaction in the seawater-promoted biofilm treatment, the PERMANOVA was simplified to two one-factor tests to confirm distinction between sequenced coral and biofilm microbiomes as well as treatment effects.

To more finely distinguish the OTUs differing among treatments, and as a measure of controlling for the EBM inevitably present in low-biomass samples, we ratioed the relative abundance of a given OTU in a coral sample (larvae or settler) against that in its respective EBM sample (EBM: rinse water or biofilm respectively, see Nixon et al 2026). For larval samples, EBM denominator values were calculated using the average relative abundance values in rinse water control samples (n = 2 Sterivex), while for coral settlers, EBM denominator values stemmed from paired substrate biofilm samples (n = 36 coral-biofilm pairs). The relative

abundance ratio was then log-base 2 transformed (log2(coral/EBM), transformed ratios hereafter referred to as log2 fold change or L2FC). L2FC analyses are commonly used in ratio analyses and center values symmetrically around zero, where zero signifies a 1:1 ratio of an OTU in a coral sample and its respective EBM. For both life stages separately, we performed a one-sided, one-sample *t*-test on L2FC values within each treatment to identify OTUs with mean L2FC values significantly greater than 0.5, representing an OTU relative abundance in coral ~1.5x that in the EBM sample, which here we define as our threshold for defining an OTU as *coral-associated*. Corrections for multiple comparisons were carried out using the Benjamini and Hochberg (BH) correction (Benjamini and Hochberg 1995). These tests resulted in three lists of coral-associated bacteria for each life stage. Relative abundance values of these select taxa were then arc sine square root transformed and tested for significant treatment differences via an ANOVA test.

Results

Algae-promoted biofilms increase coral settlement via variation in taxonomic composition. Treatment substrates hosted distinct microbial communities (Fig. 4.2a,c; PERMANOVA $p < 0.01$; pairwise tests each $p < 0.01$). CCA-promoted biofilms (CCA) were enriched by taxa in the orders Rhodovibrionales, while *Dictyota*-promoted biofilms (DICT) were enriched by several OTUs in the orders Rhodobacterales and Oceanospirillales. Seawater-promoted biofilms (SW) were enriched by several OTUs in the class Gammaproteobacteria (Table S4.1). Species richness was similar between DICT and SW biofilms, which comprised on average 204 and 184 OTUs respectively. CCA biofilms were richer, encompassing on average 231 OTUs in total, though treatments were not significantly different (ANOVA, $p > 0.05$). The mean richness of DICT, SW, and CCA biofilms were 204, 184, and 231 respectively, none of

which were significantly different (ANOVA, $p > 0.05$). When incorporating evenness, shannon diversity of biofilms was similar across biofilm conditioning treatments, with no significant differences between Shannon Index values (ANOVA, $p = 0.26$), which were 5.19, 4.96, and 5.18, for CCA, DICT, and SW biofilms respectively.

After two days of exposure to treatment substrates, mean *C. natans* larval settlement (# of coral settlers/substrate) differed significantly in response to biofilm treatments (general linearized model, $p < 0.01$; Fig. 4.2a,b). CCA and DICT biofilms both displayed similar settlement (pairwise $p = 0.97$) while both were significantly higher than that of SW (pairwise $p < 0.05$). DICT biofilms averaged the highest settlement with 12 settlers per substrate, while CCA biofilm settlement averaged 7 settlers per substrate. Opposite to the algae-promoted biofilms, SW biofilms attracted few settlers with an average of 1 settler per substrate.

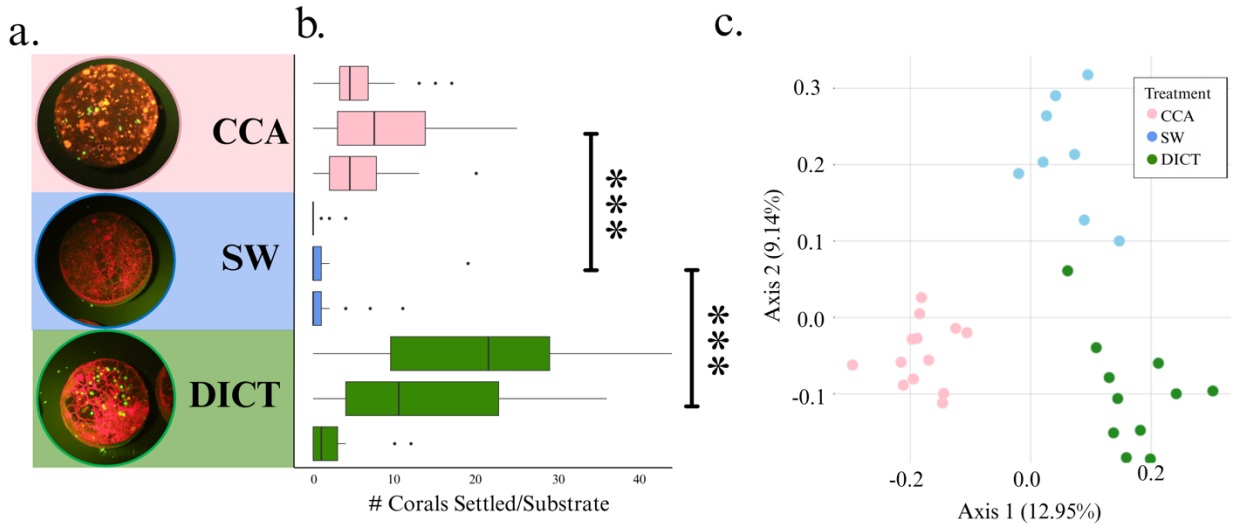


Figure 4.2 Effects of substrate conditioning treatments on larval settlement and coral settler microbiome. (a) Representative photographs of settlement observed in each conditioning treatment: CCA (pink), *Dictyota* (DICT, green), and seawater (SW, blue), with green fluorescence representing newly settled corals imaged using a yellow filter lens and blue light. (b) Mean settlement/substrate from each conditioning aquaria ($n = 3$ / treatment). Asterisks indicate significance ($p < 0.1$, GLM). (c) Ordination of biofilm community composition for the three conditioning treatments using PCoA, distance = Bray-Curtis Dissimilarity. Color denotes conditioning treatment of settlement substrate. CCA, crustose coralline algae; DICT, *Dictyota*, SW, seawater.

The coral larval microbiome is influenced by biofilm environment but largely conserved. After eight days of both development and exposure to treatment biofilm environments, the microbiome community composition of 11-day-old planula larvae displayed a significant shift from that of two- and six-day-old larvae pre-exposure (Fig. 4.3a,b, PERMANOVA with pairwise comparison, $p < 0.01$). Interestingly, the microbiome community composition of 11-day-old larvae did not significantly differ between algae-promoted treatments (PERMANOVA, CCA vs DICT $p = 0.14$), but both were distinct from larvae exposed to seawater-promoted biofilm (PERMANOVA, CCA vs SW $p = 0.01$, DICT vs SW $p = 0.02$).

Larval microbiomes are consistently dominated by a handful of core genera. To determine bacteria enriched in11-day-old larvae, a one-way one-sample *t* test (mean L2FC > 0.5, $p < 0.05$) of the L2FC values for each OTU was conducted for each of the three biofilm treatments, resulting in three lists of significant OTUs, that is OTUs enhanced in coral larvae in comparison to a rinse water control sample. A total of 32 and 37 OTUs were determined to be significantly enriched in coral larvae samples in CCA and DICT treatments respectively (Fig. 4.3a,b; Fig. 4.4c). 33 OTUs were enriched in larvae exposed to SW-promoted biofilm (Fig. 4c). Combined, these lists held 46 unique taxa, which comprised on average 75% of the sequenced larval microbiome across biofilm treatments. Roughly half (22) of these 46 OTUs were significantly associated with larvae across all treatments (Fig. 4.4c) comprising on average 54% of the larvae microbiome, including the genera *Aestuariibacter* (mean relative abundance = 11.7%), *Alteromonas* (4.7%), *Oleibacter* (4.0%), *Oleiphilus* (1.0%), and 8 unclassified OTUs from the family Rhodobacteraceae (12.2%). Of these 22, approximately half were also both abundant and prevalent in two- and five-day-old larvae, which were dominated by Alteromonadaceae, Rhodobacteraceae, and Nitrincolaceae (Fig. 4.3b).

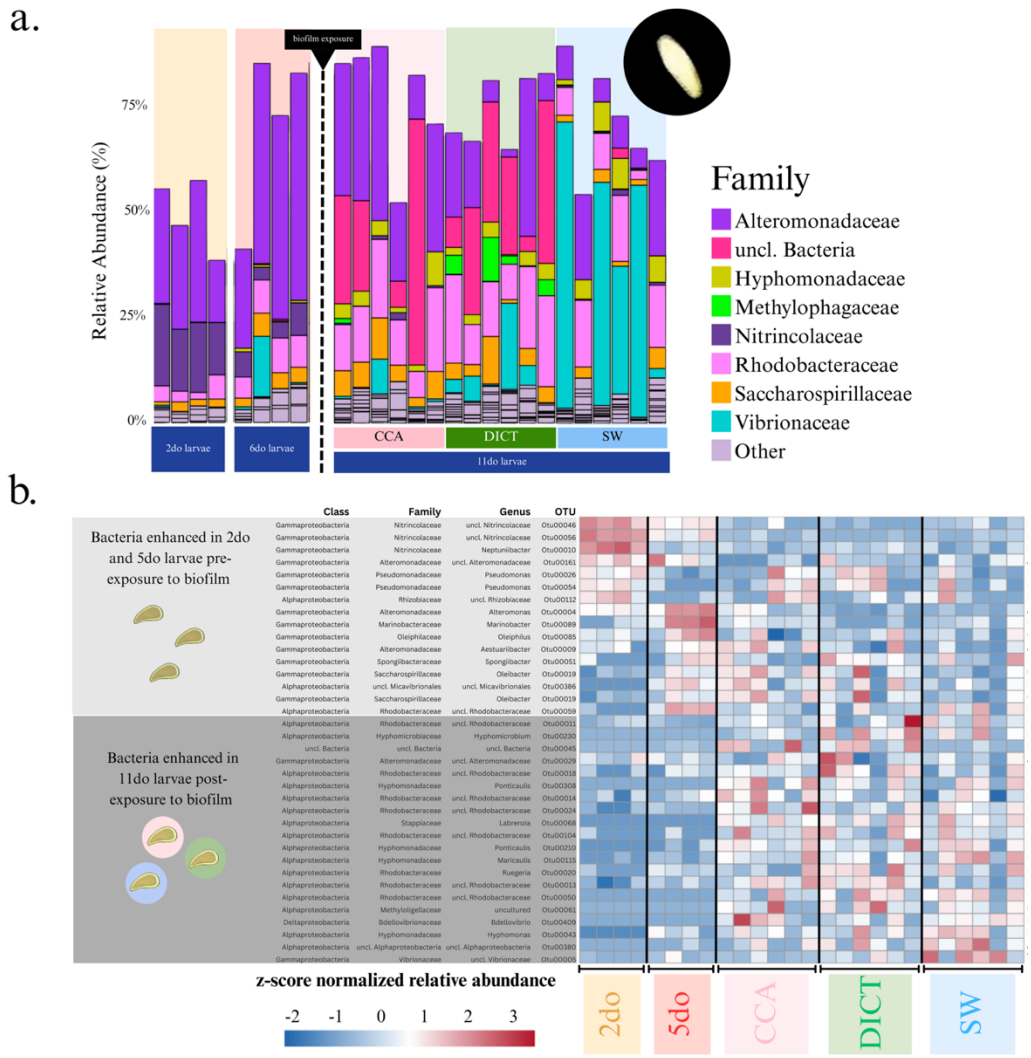


Figure 4.3 Larvae-associated bacteria by life stage and biofilm treatment. (a) Barplot of the 8 most abundant larvae-associated bacterial families in two-day-old (2do, orange), five-day-old (5do, red), and 11-day-old larvae post-exposure to biofilm. (b) Heatmap of z-score standardized relative abundance values. Class, Family, Genus, and OTUs of relevant taxa are represented on the left y-axis with life stage outlined. Life stage and biofilm treatment of 11-day-old larvae is denoted on the x-axis. Color denotes life stage: 2-day-old (orange), 5-day-old (red), and treatment: CCA (pink), DICT (green), SW, (blue).

Despite many larvae-associated OTUs being found in two- and five-day old larvae prior to treatment exposure, age and biofilm treatment in concert displayed influence on overall microbiome community composition (Fig. 4.3a,b; Fig. 4.4a; PERMANOVA with pairwise comparison 2do vs 5do vs 11do, $p < 0.01$).

At 11 days after differential exposure to biofilm substrates, larvae exposed to CCA and DICT-promoted biofilms were enriched with similar taxa relative to the seawater control. In the CCA treatment group this included 3 OTUs in the family Hyphomonadaceae (2.1%), and single OTUs in the families Stappiaceae (1.0%), Bdellovibrionaceae (0.8%), Methyloligellaceae (0.4%), and Saccharospirillaceae (0.4%). Taxa enriched in the DICT treatment group included many overlapping taxa, including 3 OTUs in Hyphomonadaceae (1.6%), 2 OTUs in Rhodobacteraceae (0.9%), 2 OTUs in Bdellovibrionaceae (0.5%), and single OTUs in families Stappiaceae (1.1%), Hyphomicrobiaceae (0.7%), and Methyloligellaceae (0.5%). Similarly, the microbiome of larvae exposed to SW biofilms were enriched in 3 OTUs in Hyphomonadaceae (1.9%), and single OTUs in Stappiaceae (1.1%) and Bdellovibrionaceae (0.4%) (Fig. 4.3a,b).

Algal biofilms enrich specific taxa in larval microbiomes. Significant differences between biofilm treatments were defined by only eight of these 44 OTUs (ANOVA with Tukey post-hoc). Larvae exposed to CCA biofilm were significantly more enriched in *Alteromonas* (8.1%) than DICT (2.3%), but not SW (3.6%). CCA treatment larvae were also significantly enriched in Marinobacter (0.4%) and an unclassified Spongiibacter (0.2%) in comparison to DICT and SW respectively (Fig. 4.3a,b). DICT and CCA treatment larvae shared several taxa in significantly higher relative abundance in comparison to the SW larvae. Most notably, an unclassified bacteria OTU was in both CCA (1.3%) and DICT (5.5%) larval microbiomes, while absent from SW larvae (0.0%) (Fig. 4.3a,b). Larvae reared in algae-promoted biofilms were also significantly enriched in an unclassified Rhodobacteraceae OTU, with CCA and DICT biofilm larvae had a mean relative abundance of 3.5%, almost triple that of the SW biofilm larvae (1.3%). Larvae exposed to SW biofilms were dominated by a single OTU in the family Vibrionaceae (35%),

which was substantially higher in relative abundance than in larvae exposed to CCA (1.7%) and DICT (5.6%; Fig. 4.3a,b).

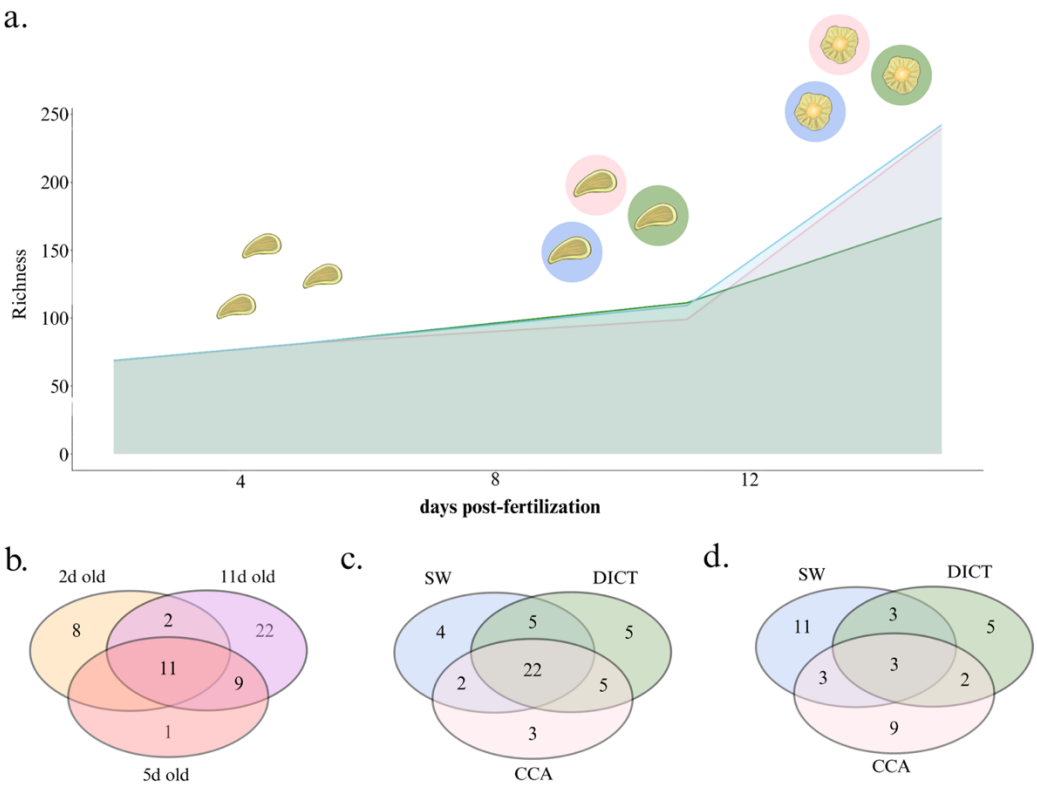


Figure 4.4 Microbiome richness and coral-associated bacteria across life history stages. (a) Mean OTU richness over time for each treatment. Larvae were exposed to biofilms beginning at 2 days post-fertilization and sampled at 11 days post-fertilization. Separately, larvae were reared in the absence of biofilm substrate and sampled at 2 and 6 days post-fertilization. Settlers were sampled 15 days post-fertilization. No significant differences in richness were found between treatment groups at each life stage. Venn diagram of significant coral-associated bacteria per the L2FC one-sample t-test for (b) each larval stage (c) 11-day-old larvae (d) 15-day-old metamorphosed settler. Colors denote larval life stage: 2-day-old (orange), 5-day-old (red), 11-day-old (purple), and treatment group: CCA (pink), SW (blue), DICT (green).

Biofilm environment promotes distinct bacteria in the coral settler microbiome.

Microbiome community composition analyses revealed significant differences between coral settler and biofilm microbiomes (within treatment PERMANOVA, coral vs. biofilm $p < 0.01$), though both displayed strong conditioning treatment effects (single-factor ANOVA coral: CCA vs DICT vs SW, $p < 0.01$, biofilm: CCA vs DICT vs SW, $p < 0.01$).

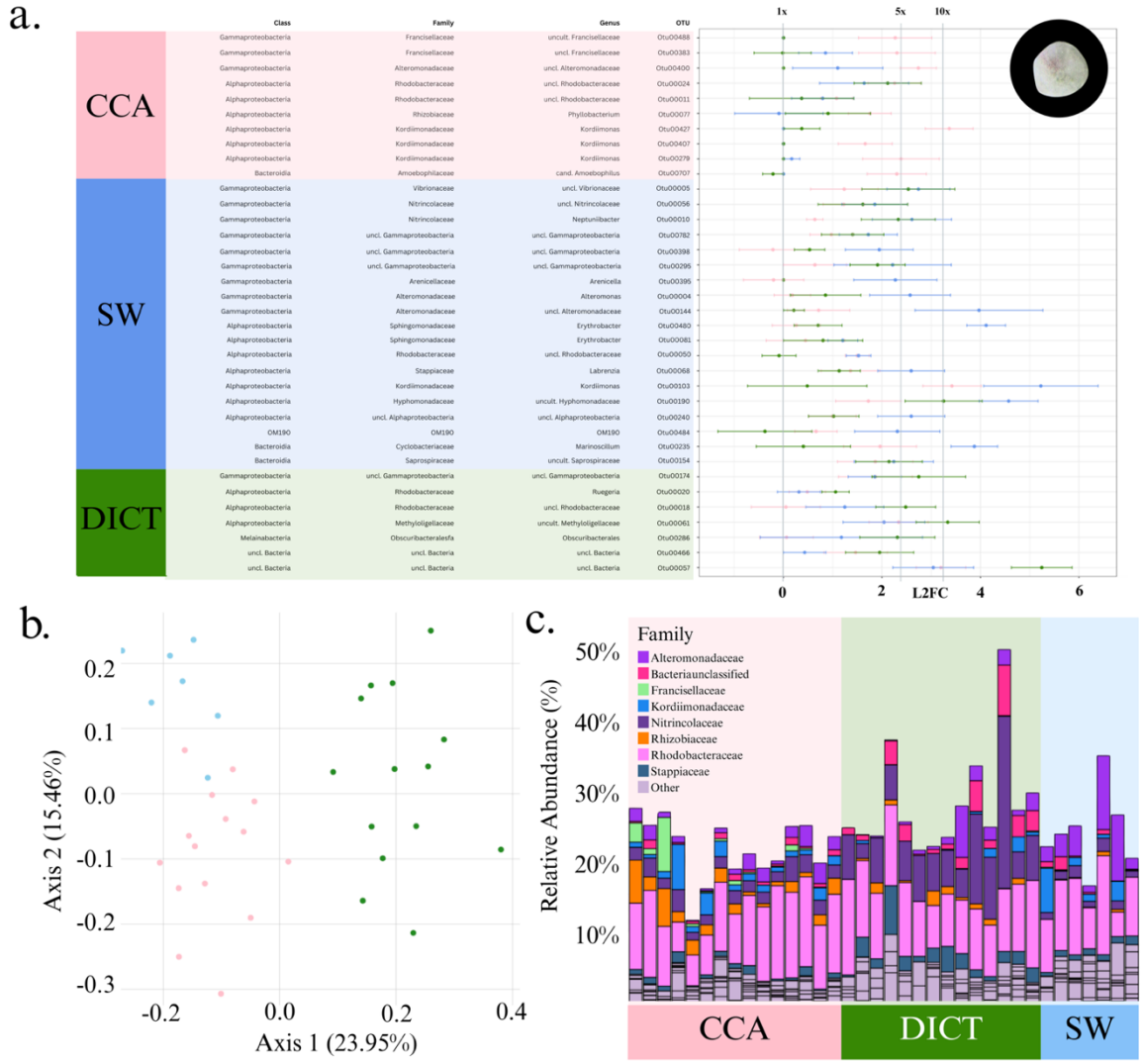


Figure 4.5 *C. natans* settler-associated bacteria. (a) Mean log2 fold-change (L2FC) values of coral-associated taxa were organized by treatment: crustose coralline algae-promoted biofilm, CCA, pink; seawater-promoted biofilm, SW, blue, and *Dictyota*-promoted biofilm, DICT, green. When a given OTU was defined as a coral associate in more than one treatment it was assigned as enriched in the treatment with the highest mean L2FC. (b) Ordination of microbiome community composition for coral settlers reared and settled in the three biofilm treatments using PCoA, distance = Bray-Curtis Dissimilarity. (c) Barplot of the 8 most abundant families present in 15-day-old settlers post-exposure to biofilm. Color denotes settlement substrate biofilm type as described above.

To conservatively examine biofilm microbiome influence, putative settler-associated bacteria were defined as high relative abundance OTUs in coral sample microbiomes in comparison to directly paired background biofilm microbiomes. This was determined, similarly

to larvae, via a one-way one-sample t test of the L2FC values for each OTU (meanL2FC > 0.5, $p < 0.05$). Tests were conducted within each of the three biofilm treatments, resulting in three lists of OTUs significantly enhanced in the coral settler in comparison to its respective paired biofilm sample. These tests resulted in 18, 13, and 18 significantly enriched OTUs, for corals settled on CCA, DICT, and SW biofilm respectively (Fig. 4.4d; Fig. 4.5a). In total, these lists summed to 34 OTUs comprising ~20% of the sequenced settler microbiome. Differences in community composition of the resulting coral-associated subset (n = 34 OTUs) were visualized by PCoA and statistically tested again via a one-way PERMANOVA for biofilm treatment (Fig. 4.5b). While 8 of the 34 OTUs were defined as coral-associated bacteria in more than one conditioning treatment, this refined coral microbiome was significantly different between all biofilm treatments (PERMANOVA with pairwise comparison, $p < 0.01$).

Microbiomes of corals settled on CCA biofilm substrates were significantly enriched in coral-associated OTUs belonging to the families Rhizobiaceae (1.9%), Kordiimonadaceae (0.7%), and Francisellaceae (0.9%). Additional taxa characterizing CCA settlers included 2 OTUs in the family Sphingomonadaceae (1.2%), 2 OTUs in the family Alteromonadaceae (4.4%), 2 OTUs in the family Nitrincolaceae (1.2%), and single OTUs in families Rhodobacteraceae (3.1%), Kordiimonadaceae (total 1.9%), Vibrionaceae (1.2%), and Stappiaceae (0.8%) (Fig. 4.5a,c; Fig. 4.6a). Corals settled on *Dictyota*-promoted biofilm were significantly enriched by two OTUs in the family Rhodobacteraceae (5.9%), two OTUs in the families Nitrincolaceae (7.0%), Hyphomonadaceae (0.6%), and Rhizobiales (2.0%). OTUs in the

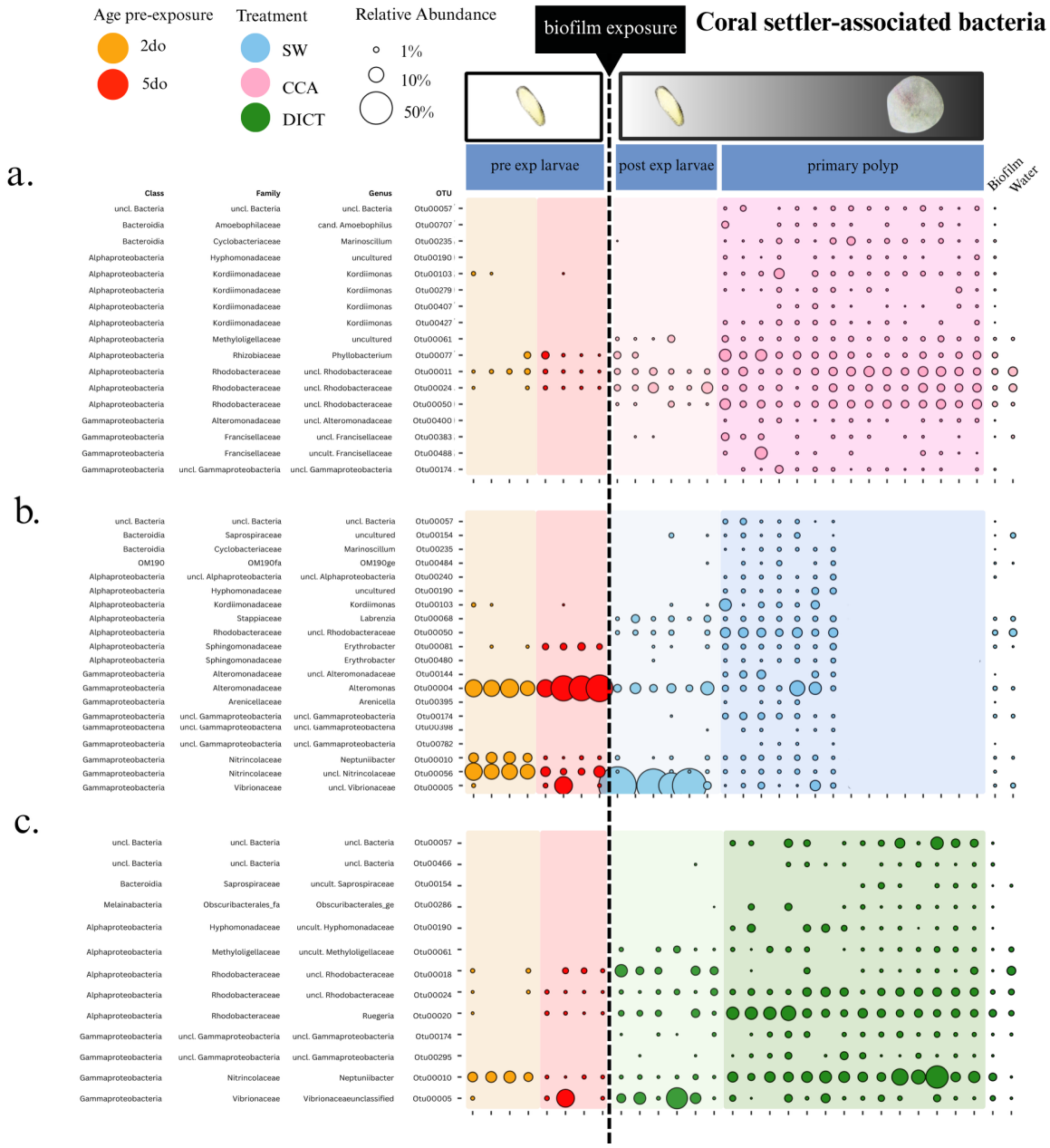


Figure 4.6 Presence and abundance of significant *C. natans* settler-associated bacteria across early life history stages. Bubble plot denoting the relative abundance and prevalence of significant settler-associated bacteria in each treatment by sample. The black dotted line represents the introduction of biofilm treatments to larvae, with two-day-old (2do, orange) and five-day-old (5do, red) larvae sampled prior to biofilm exposure, and subsequently 11-day-old larvae and 15-day-old metamorphosed settlers from each treatment (a, crustose coralline algae-promoted, CCA, pink; b, seawater-promoted, SW, blue; c, *Dictyota*-promoted, DICT, green) sampled following one week of exposure. Plot includes presence and mean relative abundance of settler-associated bacteria in biofilm and rearing water for each treatment.

family Rhodobacteraceae (total 6.4%), Vibrionaceae (0.8%), Methyloligellaceae (0.6%), and an unclassified Obscuriibacterales (0.5%) also characterized coral settled on DICT biofilms. (Fig. 4.5a,c; Fig. 4.6c) OTUs significantly enriched in corals settled on seawater-conditioned substrates were comprised of an unclassified Alteromonadaceae (1.1%), a single OTU in the family Kordiimonadaceae (1.6%), Sphingomonadaceae (1.2%), and an unclassified Rhodobacteraceae (3.2%). Additional larvae-associated taxa in this treatment include OTUs in the family Alteromonadaceae (total 4.4%), 2 OTUs in Nitrincolaceae (1.2%), Vibrionaceae (1.2%), Stappiaceae (0.8%), and Hyphomonadaceae (0.7%) (Fig. 4.5a,c; Fig. 4.6b).

Interestingly, within each treatment several OTUs were significantly associated with both coral larvae and metamorphosed settlers. These OTUs were both in two- and five-day-old larvae and unique to larvae reared in biofilm environments (Fig. S4.1).

Discussion

Coral larval settlement is specific to algae-promoted biofilms. In the present study, *C. natans* larvae displayed high settlement in response to both algae-promoted biofilms, CCA and DICT (Fig. 4.2b). These findings corroborate the role of biofilms (Webster et al. 2004; Hadfield et al. 2011) and CCA (Heyward & Negri 1999; Tebben et al. 2015; Sneed et al. 2014) as key settlement inductors. Broadly, the presence of bacteria is considered compulsory in the coral larval settlement process (Vermeij et al. 2009; Webster et al. 2004). In respect to CCA, several species are renowned settlement inducers (Siboni et al. 2020; Ritson-Williams et al. 2014), with both host and microbiome responsible for attracting larvae and triggering metamorphosis (Tebben et al. 2015; Gómez-Lemos et al. 2018). Notably, the CCA surface biofilm and its respective metabolites are known to hold a significant role in inducing coral settlement and metamorphosis (Sneed et al. 2014; Sneed et al. 2024). Substrates exposed to CCA were

established with CCA spores that grew into thin CCA crusts throughout the conditioning period, and therefore both the algae and the algae-promoted biofilm may be influencing settlement results.

Fleshy macroalgae on reefs is associated with lower recruitment and juvenile survival (Vermeij et al. 2009; Webster et al. 2015). However, its role in larval settlement induction remains unclear. Interestingly, several studies have found non-calcifying macroalgae to induce settlement in coral larvae. Xiao et al. (2024) found the presence of red alga *Hypnea pannosa*, green alga *Caulerpa serrulata*, and brown algae *Turbinaria gracilis* and *Sargassum polycystum* to induce 60-90% settlement in *P. damicornis* larvae. Exudates of various fleshy algae have also been found to differently influence coral settlement. Birrell et al (2008) found *Lobophora variegata* exudates to induce high settlement in *A. millipora*, while algae *Chlorodesmis fastigiate* and *Padina sp*. delayed and reduced settlement respectively. In a contrasting study, both *Sargassum* and *Padina* increased settlement in *A. muricata* (Denis et al. 2014). While the specific attractants of algae remain largely unknown, the algal microbiome is thought to play a role (Yang et al. 2020; Xiao et al. 2024). Interestingly, *Dictyota sp.* negatively impacted settlement and survival of the Caribbean coral *P. astreoides* (Kuffner et al. 2006; Paul et al. 2011; Olsen et al. 2014), where in the present study *Dictyota*-promoted biofilm held the opposite effect (Fig. 4.2b). These differences may be explained in the one-degree of separation between the algae and coral larvae, where larvae were exposed to the biofilm that *Dictyota* exudates promoted, not the alga or the exudates itself.

Unlike the physical and waterborne influence of non-calcifying macroalgae, the influence of macroalgae-promoted biofilms on coral settlement remains largely understudied. It is generally understood that benthic reef taxa, including algae, release distinct exudates into the

water column (Wegley Kelly et al. 2022) that subsequently drive distinct biofilm communities (Remple et al. 2021). This biochemical seeding is recapitulated in the present study, where presence and taxa of algae engendered significantly distinct biofilm communities (Fig. 4.2c). Concurring with previous trends in algae-related settlement studies, CCA and *Dictyota*-promoted biofilms incurred significantly higher settlement in *C. natans* larvae in comparison to the seawater-promoted biofilms (Fig. 4.2b). These settlement inducing biofilms hosted several taxa in higher relative abundance in comparison to the seawater-promoted biofilm control (Table S4.1). Notably, this included genera *Neptuniibacter* and *Ruegeria*, both of which are positively correlated with larval settlement (Siboni et al. 2020; O'brien et al. 2025). Previous studies have associated *Ruegeria* with coral health (reviewed in Voolstra et al. 2024; Wallace et al. 2025), sulphur cycling (Wirth et al. 2020), and protection against pathogens (Miura et al. 2019; Xu et al. 2024). CCA surface biofilms were additionally enriched in several OTUs within the order Kiloniellales, which although not implicated in settlement is a known coral symbiont (mutualist) in both early (Quigley et al. 2020; Pérez-Trejo et al. 2025) and adult (Pollock et al. 2018) life stages. Despite the limitations of lab-based studies, these findings are the first to provide evidence that a distinct microbial biofilm promoted by *Dictyota*, a non-calcifying macroalgae, can induce high settlement in coral larvae.

Biofilm environment and development drive larval microbiome. The planktonic coral larval environment is known to play a defining role in early microbiome formation (Epstein et al. 2019), and in several broadcast spawning species marks the earliest stage of observed uptake (Sharp et al. 2012; Apprill et al. 2009; Damjanovic et al. 2020). In the present study, larvae hosted microbiomes as early as two days post-fertilization. This microbiome increased in both alpha and beta diversity at five days, which included an increase in diversity and relative

abundance within the dominant families Alteromonadaceae and Rhodobacteraceae (Fig. 4.3a,b). Following exposure to biofilm treatments, 11-day-old larvae continued this trend of increasing microbial diversity with age, insinuating a continuous acquisition with development (Fig. 4.3a,b). Despite this increased diversity, larvae-associated bacteria as defined by our refined analysis were largely ubiquitous across treatments, suggesting development rather than environment as the strongest driver of microbiome formation in the planktonic life stage. 22 of our 46 defined larvae-associated OTUs were significantly associated with 11-day-old old larvae across the three biofilm treatments, comprising 54% of the sequenced larval microbiome (Fig. 4.3a,b). While this study is the first to characterize the *C. natans* larval microbiome, several of these conserved taxa have been associated with coral larvae in previous studies (Fig. 4.3a,b). Most prominent are bacteria within the families Alteromonadaceae, Rhodobacteraceae, and Nitrincolaceae (of the order Oceanospirillales), all of which are associated globally with early life history corals across the literature. Bacteria from these taxa were vertically transmitted in the coral *P. acuta* (Damjanovic et al. 2020c), the earliest associates in both broadcast (Damjanovic et al. 2020b) and brooding coral larvae (Sharp et al. 2015), and were prominent taxa in both larvae and settlers of the broadcast spawning corals *A. millipora* (Lema et al. 2014) and *A. digitifera* (Bernasconi et al. 2019). Several OTUs from the order Caulobacterales were associated with larvae across biofilm treatments. Caulobacterales and Rhodobacteraceae are heterotrophic Alphaproteobacteria and are important carbon-cycling bacteria in both juvenile (Pérez-Trejo et al. 2025; Guo et al. 2025b) and adult corals (Sun et al. 2024). Additionally, Caulobacterales are well-established in the biofilm literature for surface adhesion and monolayer formation (reviewed in Goley et al. 2007) and are associated with coral reef biofilms (e.g. Remple et al. 2021) as well as settlement induction of *P. damicornis* coral larvae (Guo 2025a).

The prevalence and abundance of these taxonomic groups across species and life stages signifies their importance in the early life history coral microbiome and suggests that the establishment of these early associations is not passive but rather controlled by developmental processes. Despite this uniformity in the larval microbiome, exposure to biofilm environment during the planktonic stage displayed a small but distinguishable effect (Fig. 4.3a; Fig. 4.4d). Several OTU-level differences existed between treatments, suggesting that the biofilm environment (biofilm and biofilm-promoted water community) influenced the coral larval microbiome. Not being exposed to algae-promoted biofilm environments stood to be detrimental to microbiome development, with larvae exposed to seawater-promoted biofilm substrates laden with a single Vibrionaceae OTU making up on average 34.6% of the larval microbiome in this treatment (Fig. 4.3a; Fig. 4.5b). While Vibrio is a ubiquitous member of the global coral microbiome (Huggett & Apprill 2018), this taxonomic group and its proliferation within corals under stress has been directly associated with bleaching and disease (reviewed in Tout et al. 2015). Interestingly, this Vibrio dominance occurred in four separate settlement bins with substrates from two of the three SW aquaria, with larvae exposed to the substrates from the third SW aquaria lacked Vibrio almost entirely (mean relative abundance ~1%; Fig. 4.3a), suggesting an aquaria conditioning biofilm effect rather than a settlement bin water effect. Most notably, larvae reared in CCA-promoted biofilms were significantly enriched in the Alteromonadaceae genus *Alteromonas* (8.1%), while larvae reared in DICT-promoted biofilms were significantly enriched in an OTU from the family Methylophagaceae (3.5%; Fig. 4.3a). While results suggest developmental stage to be the primary determinant of the *C. natans* larval microbiome, this study serves as the first evidence that the neighboring biofilm environment has the capacity to influence the larval microbiome.

Broadcast spawning corals were shown to be devoid of internal microbial associates in planktonic early life history stages (e.g. Apprill et al. 2009, but see Leite et al. 2017). Though largely unstudied in the Caribbean, evaluation of coral gametes and larvae via fluorescent *in situ* hybridization (FISH) found no evidence of bacterial transmission from parent to offspring in seven broadcast spawning species (Sharp et al. 2010). Further, even in cases of observed vertical transmission the early coral microbiome is dominantly acquired from the surrounding environment (Damjanovic et al. 2020c; Epstein et al. 2019). Thus, we assume horizontal transmission to be the primary mode of microbiome acquisition in *C. natans*. Nearly all identified larvae-associated bacteria in this study were present in either the biofilm or the water environment, and very often present in both (Fig. S4.1). Therefore, within the limitations of 16S amplicon sequencing, sourcing the exact reservoirs of these bacteria was not possible within the scope of this study. We do, however, postulate both environments to be reservoirs.

Here we propose two mechanisms for the observed horizontal transmission. The first mechanism is that larvae may acquire bacteria from the water column, with the water column being a well-supported reservoir (Apprill et al. 2012). A second mechanism of uptake may be adherence of bacteria resulting from contact with the biofilm during the probing phase of the settlement process, an interchange that remains largely unconsidered in the literature (but see Apprill 2012). While ingestion followed by phagocytosis is considered the primary mechanism of symbiont acquisition in corals, this is not supported in FISH microscopy studies, which have determined the onset of bacteria in broadcast spawning coral larvae *P. meandrina* and brooder *P. astreoides* to be localized in the ectoderm (Apprill et al. 2009, 2012; Sharp et al. 2012).

Interestingly, even following the development of a mouth and gut, Apprill et al. (2012) found bacterial cells were most concentrated in the ectoderm at the aboral pole of the larva.

Consequently, the aboral pole of coral larva is enriched in neuroreceptors and mucus cells that are responsible for its attraction to settlement cues and its subsequent adhesion (Ramon-Mateu et al. 2025) and thus serves as a direct point of contact between a coral larva and its biofilm attractants. Further, Apprill et al. (2012) found larvae to selectively uptake bacteria, including a strain of *Pseudoalteromonas*, which is a widely reported settlement inducer. Here we present preliminary support of this second mechanism, with a collective of larvae-associated bacteria present solely in the biofilm and absent in the water (Fig. S4.1). While variation existed between treatments, this collective included OTUs in the genera *Pseudomonas, Oleiphilus,* and *Ruegeria*. Future studies utilizing targeted FISH microscopy are necessary to resolve the relationship between larvae, settlement-inducing bacteria, and their role in the initial coral microbiome.

Biofilm environment drives distinct settler-associated bacterial communities. Unlike the larval stage, the post-settlement coral microbiome community significantly differed based on treatment. Corals in the three treatments displayed microbiome communities that were strongly driven by their biofilm environment (Fig. 4.5b) but remained distinct from their respective background biofilm microbiomes. This significance held on a broad level, with the sequenced microbiome, as well as when examining the coral settler microbiome with a more refined lens through paired statistical testing (Fig. 4.5a). Therefore, we conclude that the biofilm environment significantly influences the establishment of symbionts post-settlement. We propose this to be uptake either from biofilm cell detachment, or from biofilm exudates promoting specific water column bacteria. In a refined analysis accounting for the background biofilm community, we identified 25% of the sequenced settler microbiome to be coral-associated bacteria (Fig. 4.5c). Within this select subset, both significant community-level and OTU-level differences existed between treatments (Fig. 4.5b,c; Fig. 4.6). Settlers settled on DICT biofilms were enriched in

taxa including families Rhodobacteraceae and Nitrincolaceae, while those settled on CCA biofilms were enriched in taxa including families Kordiimonadaceae and Franciscellaceae. Additionally, DICT and CCA corals were each enriched in Rhizobiales genera *Labrenzia* and *Phyllobacterium* respectively (Fig. 4.5b,c; Fig. 4.6). Rhizobiales are nitrogen-fixing bacteria and are frequently reported in the early coral microbiome (e.g. Sharp et al. 2012; Lema et al. 2014) as well as adult microbiomes (Lema et al. 2012; Ainsworth et al. 2015).

Comparison between the microbiome at larval and settler stages suggest that only a subset of the identified settler-associated bacteria were present in the microbiome at the larval stage. This includes several OTUs in the family Rhodobacteraceae and Nitrincolaceae, including genus groups *Ruegeria and Neptuniibacteria* respectively (Fig. 4.6). The prevalence seen in Rhodobacteraceae across early life stages and globally in adult colonies articulates the importance of this family in the coral holobiont (Luo et al. 2021; Huggett & Apprill 2019). Specifically, Rhodobacteraceae are highlighted in the coral holobiont for their abilities in DMSP degradation (Sweet et al. 2021), sulfur oxidation (Luo et al. 2021), and nitrogen cycling (Xiang et al. 2022). The presence of *Ruegeria* and *Neptuniibacter* in coral larvae, settlers, and in settlement inducing biofilms provides evidence of potential biofilm-coral attraction, horizontal transmission, and warrants further investigation. *Alteromonas* was also maintained as a coral-associate across life stages, a genus that has similarly been implicated as a protectant against pathogens (Ushijima et al. 2023) and more relevantly as a larval beneficiary and settlement inducer (Friere et al. 2019; Ceh et al. 2013). The majority absence of settler-associated bacteria in the larval life stages suggests treatment differences in microbiome developed post-settlement and metamorphosis. This finding is supported in the literature, where coral-associated bacteria were not observed in Caribbean broadcast spawning coral until following metamorphosis (Sharp

et al. 2010). Further, several studies denote an increase in microbiome diversity between larvae and post-settlement stages, suggesting acquisition controls shift with developmental stage (Lema et al. 2014, Sharp et al. 2012). Results provide evidence that the biogeochemical "neighborhood effect" found in adult coral microbiomes may apply on the settler-scale. Notably, biofilms that attract and induce coral settlement may also serve as a microbial reservoir that stands to influence the early coral microbiome and potentially microbiome trajectories.

Similarly to larvae, the role of development and the exact reservoir for microbiome formation in early life history corals remains unclear due to the constraints of this study. Coral reef microbiota are pervasive, with known coral-associated bacteria found in biofilm (Sweet et al. 2011) as well as the surrounding water column (Nelson et al. 2023). While this study was careful in design, many coral-associated bacteria remain unidentified due to the conservative nature of this workflow. Most notably, it was nearly impossible for OTUs with similar relative abundance in both coral settler and the neighboring biofilm, a hypothesized reservoir, to be defined as coral-associated per our determined threshold and ratio test. Therefore, OTUs belonging to both coral and biofilm microbiomes remain unassigned. This is highlighted in our coral larvae microbiomes, where nearly all larvae-associated bacteria were found in the settler microbiome but could not be assigned as settler-associated due to its presence in the biofilm. Within the scope of 16S sequencing, we cannot confirm these larvae-associates are retained in the settler microbiome, though it is likely. Further, we cannot confirm direct horizontal transmission of biofilm or biofilm-promoted bacteria with the coral settler. For example, known coral symbionts such as Kiloniellales (Pollock et al. 2018), which have been found in early life history corals (Quigley et al. 2020; Pérez-Trejo et al. 2025), could not be defined as settler-associated within the constraints of this study due to its similar presence in both the CCA biofilm

(8.2%) and *C. natans* settler (8.0%). Inevitably, this study underreports the coral settler microbiome as well as the influence of the biofilm environment. To better elucidate the role of biofilm in shaping the early life history coral microbiome, future studies should incorporate fluorescent microscopy with taxa-specific probes to verify coral-bacteria associations from that of the environmental background microbiome (e.g. Sharp et al. 2010; Apprill et al. 2012).

Conclusion

Successful recruitment is crucial for maintaining coral populations and recovering from episodic disturbance (Linares et al. 2011; Doropoulos et al. 2015). With the role of biofilm in this process well-recognized, its potential role in early microbiome seeding and development remains a black box. In an evolutionary context, larvae may be attracted to cues promoted by healthy reef space microbiota (Harrington et al. 2004; Price 2010) and or taxa-specific reef habitat (O'Brien et al. 2025). For example, coral larvae display chemotaxis to sugars specific to the algal symbiont Symbiodiniaceae (Hagedorn et al. 2015). This chemotaxis of larvae towards chemical indicators of symbiotic or non-antagonistic neighbors may then directly or indirectly facilitate the uptake and formation of endosymbiont associations. Further, Levy et al. (2024) found biofilms from a healthy reef to bolster coral fragment health even when transferred to a degraded reef. Future studies should investigate the relationship between biofilm community composition, early coral microbiome dynamics, and post-settlement survival. Overall, this study is the first to characterize the *C. natans* microbiome in early life history stages and most notably is one of the first to provide evidence that the biofilm microbial settlement environment plays a role in shaping the early coral microbiome, at least in lab setting.

While aquaria conditioning was beneficial for the purpose of this specific study, isolating and concentrating singular benthic taxa effects, it is important to recognize the described

biofilms were produced in low-flow aquaria and may not be representative of natural algae-driven biofilm succession. Additionally, closed-system settlement containers likely experienced salinity, pH, and broad chemical shifts that are not characteristic of natural reef processes. We maintain that this laboratory study displays the capacity for algae-promoted biofilms to induce coral settlement and provides novel evidence for the role of biofilm in shaping the early life history coral microbiome. However, physical and chemical reef parameters, such as water flow, stands to dampen this effect. Further, the capacity for a coral to selectively uptake bacteria in both larval (Apprill et al. 2012) and settler (Hoadley et al. 2021; McNally et al. 2017) life stages may dampen this effect when exposed to a more diverse and expansive microbial environment. Future studies should seek to corroborate these findings in a natural reef setting to more accurately understand algae-coral bacterial interactions *in situ*.

Coral reefs are in steep decline, with this trend being especially stark in the Caribbean. A large contributor to this decline is recruitment failure (Hughes and Tanner 2000; Vermeij et al. 2011). While widely agreed upon, much remains unknown as to what stage (i.e. pre- or post-settlement, age post-settlement) this failure occurs. When considering coral health, it is important to include the holistic health of the coral holobiont, though little is known on the acquisition of the early coral microbiome or its role in early coral survival. With increasing use of larval propagation in reef restoration strategies, understanding the early coral holobiont is increasingly vital. For example, we show here that the environment in which settlement substrates are conditioned will define their respective biofilm microbiome, which then holds the capacity to influence early coral microbiome dynamics. While the *in situ* implications of biofilm on holobiont health and post-settlement survival remain unstudied, considering the holobiont and its drivers at this life stage may provide insights to further understanding coral success. Broadly,

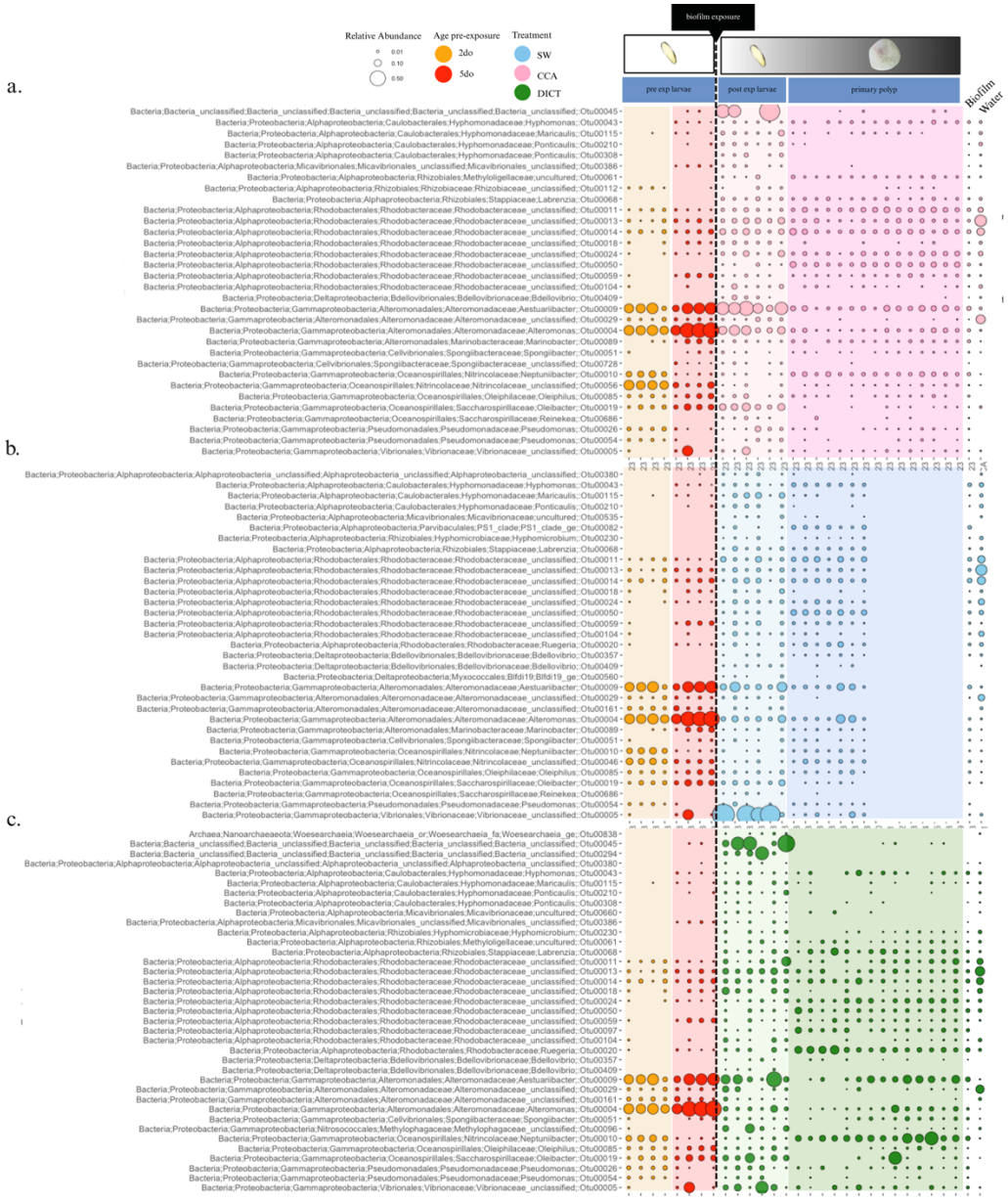
results from this study serve to increase our understanding of early coral-bacteria associations and provide a more holistic holobiont perspective towards restoration strategy.

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Chapter 4, in full, is currently being prepared for submission for publication of the material. Nixon, Emily N.; Romero, Sarah L.; Flores, Daisy M.; ter Horst, Lars J.V.; Yus, Joaquin; Marhaver, Kristen L.; Wagoner Johnson, Amy J.; Espinosa-Marzal, Rosa M.; Nelson, Craig E.; Wegley Kelly, Linda. “Benthic biofilms influence coral larval settlement and microbiome dynamics.” The dissertation author is the primary researcher of this paper.

Supplemental Figures



Supplemental Figure 4.1 Presence and abundance of significant *C. natans* larvae-associated bacteria across early life history stages. Bubble plot denoting the relative abundance and prevalence of significant larvae-associated bacteria in each treatment by sample. The black dotted line represents the introduction of biofilm treatments to larvae, with two-day-old (2do, orange) and five-day-old (5do, red) larvae sampled prior to biofilm exposure, and subsequently 11-day-old larvae and settlers from each treatment (a, crustose coralline algae-promoted, CCA, pink; b, seawater-promoted, SW, blue; c, *Dictyota*-promoted, DICT, green) sampled following one week of exposure. Plot includes presence and mean relative abundance of settler-associated bacteria in biofilm and rearing water for each treatment.

Supplemental Tables

Supplemental Table 4.1 List of the top 50 OTUs found in biofilm across treatments. Columns CCA, SW, and DICT denote mean relative abundance of a given OTU for the respective treatment.

Class	Family	Genus	OTU	CCA	SW	DICT
Gammaproteobacteria	Woeseiaceae	Woeseia	Otu00036	0.025	0.017	0.033
Gammaproteobacteria	Alteromonadaceae	Aestuariibacter	Otu00009	0.020	0.015	0.026
Alphaproteobacteria	Rhodobacteraceae	Rhodobacteraceae_unclassified	Otu00014	0.017	0.024	0.017
Alphaproteobacteria	Clade_I	Clade_Ia	Otu00002	0.019	0.016	0.015
Alphaproteobacteria	Rhodobacteraceae	Ruegeria	Otu00020	0.017	0.007	0.020
Alphaproteobacteria	PS1_clade	PS1_clade_ge	Otu00082	0.010	0.014	0.014
Gammaproteobacteria	Nitrincolaceae	Neptuniibacter	Otu00010	0.012	0.002	0.023
Alphaproteobacteria	Rhodobacteraceae	Rhodobacteraceae_unclassified	Otu00011	0.013	0.016	0.007
Alphaproteobacteria	Hyphomonadaceae	Hyphomonas	Otu00043	0.008	0.015	0.013
Alphaproteobacteria	Kiloniellaceae	Kiloniellaceae_unclassified	Otu00072	0.034	0.000	0.001
Alphaproteobacteria	Rhodobacteraceae	Rhodobacteraceae_unclassified	Otu00050	0.009	0.009	0.017
Alphaproteobacteria	Uncl. Alphaproteobacteria	Uncl. Alphaproteobacteria	Otu00078	0.009	0.005	0.018
Dadabacteriia	Dadabacteriales	Dadabacteriales	Otu00094	0.009	0.012	0.010
Alphaproteobacteria	uncultured	uncultured	Otu00073	0.025	0.001	0.003
Alphaproteobacteria	Rhodobacteraceae	Uncl. Rhodobacteraceae	Otu00013	0.008	0.009	0.012
Alphaproteobacteria	Kiloniellaceae	uncultured	Otu00091	0.022	0.002	0.004
Gammaproteobacteria	UBA4486	UBA4486	Otu00095	0.015	0.011	0.002
Gammaproteobacteria	BD7-8	BD7-8	Otu00125	0.009	0.013	0.005
Gammaproteobacteria	Marinobacteraceae	Marinobacter	Otu00089	0.010	0.007	0.007
Acidimicrobiia	Actinomarinaceae	Candidatus Actinomarina	Otu00008	0.008	0.008	0.005
Gammaproteobacteria	Alteromonadaceae	Alteromonas	Otu00004	0.009	0.005	0.007
Alphaproteobacteria	Rhodobacteraceae	Uncl. Rhodobacteraceae	Otu00097	0.005	0.005	0.011
Alphaproteobacteria	Clade_II	Clade_II_ge	Otu00007	0.007	0.006	0.008
Gammaproteobacteria	Nitrincolaceae	Uncl. Nitrincolaceae	Otu00046	0.003	0.006	0.011
Planctomycetacia	Pirellulaceae	Rubripirellula	Otu00140	0.005	0.004	0.009
Alphaproteobacteria	Rhodobacteraceae	Uncl. Rhodobacteraceae	Otu00024	0.006	0.006	0.006
Alphaproteobacteria	Kiloniellaceae	uncultured	Otu00109	0.015	0.000	0.001
OM190	OM190_fa	OM190_ge	Otu00205	0.003	0.008	0.004
Gammaproteobacteria	KI89A_clade_fa	KI89A_clade_ge	Otu00158	0.006	0.003	0.006
Alphaproteobacteria	Stappiaceae	Labrenzia	Otu00068	0.003	0.003	0.009
Alphaproteobacteria	Rhizobiaceae	Phyllobacterium	Otu00077	0.010	0.002	0.003
Gammaproteobacteria	Uncl.Gammaproteobacteria	Uncl. Gammaproteobacteria	Otu00111	0.002	0.005	0.007
Gammaproteobacteria	KI89A_clade	KI89A_clade	Otu00107	0.003	0.003	0.008

Supplemental Table 4.1 (continued) List of the top 50 OTUs found in biofilm across treatments. Columns CCA, SW, and DICT denote mean relative abundance of a given OTU for the respective treatment.

Class	Family	Genus	OTU	CCA	SW	DICT
Alphaproteobacteria	Rhodobacteraceae	Uncl. Rhodobacteraceae	Otu00059	0.004	0.005	0.004
Rhodothermia	Rhodothermaceae	uncultured	Otu00198	0.003	0.005	0.004
Alphaproteobacteria	Hyphomicrobiaceae	Filomicrobium	Otu00159	0.003	0.003	0.005
Gammaproteobacteria	Pseudomonadaceae	Pseudomonas	Otu00026	0.002	0.003	0.007
Gammaproteobacteria	Vibrionaceae	Uncl. Vibrionaceae	Otu00005	0.001	0.002	0.009
Oxyphotobacteria	Eurycoccales_Incertae_Sedis	Synechococcus PCC-7336	Otu00172	0.000	0.002	0.009
Gammaproteobacteria	Spongiibacteraceae	Spongiibacter	Otu00051	0.002	0.002	0.007
Bacteroidia	Amoebophilaceae	Candidatus Amoebophilus	Otu00275	0.001	0.000	0.009
Alphaproteobacteria	Rhizobiaceae	Hoeflea	Otu00196	0.001	0.002	0.007
Gammaproteobacteria	Oleiphilaceae	Oleiphilus	Otu00085	0.004	0.006	0.001
Gammaproteobacteria	Woeseiaceae	Woeseia	Otu00201	0.003	0.001	0.007
Oxyphotobacteria	Eurycoccales_Incertae_Sedis	Synechococcus_PCC-7336	Otu00209	0.004	0.003	0.003
Alphaproteobacteria	Terasakiellaceae	Uncl. Terasakiellaceae	Otu00207	0.003	0.004	0.002
Alphaproteobacteria	Parvularculaceae	uncultured	Otu00200	0.002	0.004	0.003
Alphaproteobacteria	Rhodobacteraceae	Uncl. Rhodobacteraceae	Otu00189	0.002	0.006	0.001
Holophagae	Acanthopleuribacteraceae	Acanthopleuribacter	Otu00353	0.000	0.000	0.010

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CONCLUSION

Coral reefs remain in decline due to numerous regional and global anthropogenic stressors, making it more imperative than ever to understand the drivers of ecological recovery and resilience. Assisting coral reproduction and promoting successful outcomes is an essential prospect for reef recovery and restoration, offering both increased larval supply and population genetic diversity. In this body of work, I span research disciplines to elucidate avenues to enhance settlement and post-settlement success of Caribbean corals. I work alongside restoration practitioners to assess rearing technique efficacies, examine both abiotic and biotic settlement cues, and begin my examination of early coral-bacteria associations and mechanisms influencing these relationships.

While broad in focus, my work ultimately concentrates on identifying and influencing coral-bacteria associations in settlement and post-settlement corals. The importance of coral-microbe symbioses is irrefutable in adults and has been implicated in resilience to both anthropogenic and natural stressors (e.g. Silverstein et al. 2015; Parker et al. 2020). Early life history coral-microbe symbioses show similar importance (e.g. Quigley et al. 2023; Olivares-Cordero et al. 2025), though remain understudied. As I have hopefully conveyed, these initial life stages are typified by low-density microbiomes. Much of my focus throughout this period has been directed towards convincingly disentangling coral-associated bacteria from environmental background noise, a necessary precursor to future efforts (Ch. 3). Interestingly, in this study I identified 14 OTUs found prevalently across all *O. faveolata* larvae, insinuating an element of developmental or biological control at this initial stage (Bernasconi et al. 2019). This evidence provides support for a consortium of "core" symbiotic bacteria that may hold key roles in early coral health (Apprill et al. 2009, 2012; Sharp et al. 2012). Corals are equipped with an innate

immune system, which is documented to play an active role in uptake of Symbiodiniaceae cells (Davy et al. 2012). While both the coral and bacteria may play an active role in initial association, this same mechanism of immune recognition likely exists for bacterial cells (see Apprill et al 2012). Future work should seek to further our understanding of these interactions at the molecular level.

Another key aspect of understanding coral holobiont formation is determining the mechanisms in which early life history corals acquire their initial symbionts. While vertical transmission is documented in several species, corals are predicted to acquire most of their microbial associates from the surrounding marine environment via horizontal transmission (Baird et al. 2009; Damjanovic et al. 2020a,b,c). As discussed in my microbial chapters, hypothesized mechanisms by which horizontal transmission occurs include (1) bacterial attachment to the mucus membranes of coral gametes and larvae followed by phagocytosis via the ectoderm (Apprill 2009; outlined in Leite et al. 2017), and (2) suspension feeding of bacterioplankton by coral larvae and recruits (Sharp et al. 2010). In my final chapter, the microbial environment and associated chemical cues that attract a coral to settle and metamorphose appear to influence both larva- and settler-bacteria associations (Fig. 5.1). While biofilms are indefinitely linked to both successful settlement as well as coral health, few studies have examined the capacity of this microbial environment to influence microbiome development throughout this critical life stage. Of note, Apprill (2009) observed initial bacterial associations to be in the posterior ectoderm of *Pocillopora meandrina* larvae, coincidently or not, where chemoreceptors are typically concentrated (Ramon-Mateu et al. 2025). Given the importance of

the coral holobiont and the pertinence of bacterial chemical cues in the settlement process, several promising avenues exist for integrating microbiology with directed restoration strategy.

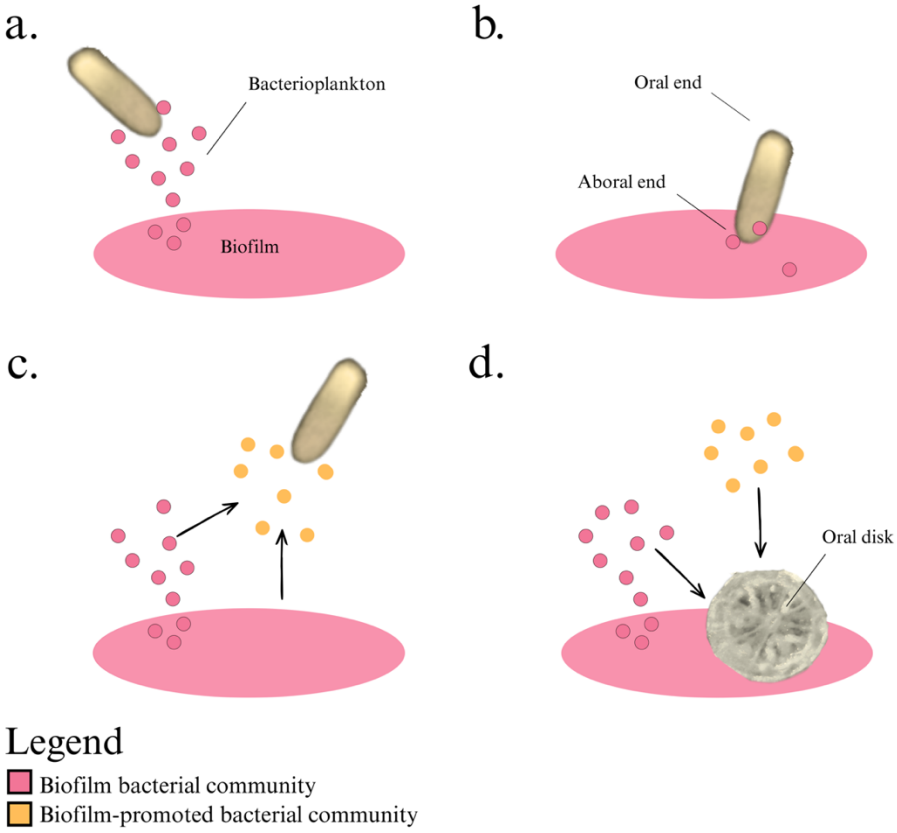


Figure 5.1. Proposed mechanisms of biofilm-influenced microbiome acquisition. a) Passive uptake of biofilm bacterioplankton in water column during swimming phase. This may be potentially linked to chemotaxis towards biofilm. b) Passive uptake of bacteria by larvae from direct contact with biofilm bacterial community during probing and crawling phase. c) Passive uptake of biofilm-promoted bacterioplankton during chemotaxis and swimming in water column. These bacteria may be promoted by biofilm exudates or biofilm bacterioplankton. d) Polyp suspension feeding of biofilm and or biofilm-promoted bacterioplankton.

While this body of work begins to address early coral-bacteria associations, much remains unknown regarding microbiome acquisition and function during these precarious initial stages. Understanding the establishment of these relationships and mechanisms of transmission may reveal powerful insights to advance the field of larval propagation. High alpha diversity of microbes in early life history corals insinuates a degree of symbiont flexibility and environmental influence, providing potential for both broad environmental (see Ch. 4) and

directed (Matsuda et al. 2022; Damjanovic et al. 2020b) inoculations of microbial consortia. Current coral propagation methods poignantly overlook the role of the microbial community on post-settlement survivorship and development. For example, while the benefits of *in situ* rearing nurseries are hypothesized to be tied to biogeochemical parameters (see Baer et al. 2024), no studies to date have examined the surrounding microbiology or the influence of this microbial environment on the coral microbiome in conjunction with corresponding coral success. Due to this continued disconnect between disciplines, the implications of these interventions on the formation and refinement of the coral recruit and juvenile microbiome remain unknown.

The premise that an individual entity relies on its invisible partners to function efficiently is now considered foundational knowledge across biology; the seen relies on the unseen. In this way, microbial communities and intricate symbioses are cornerstones to coral reefs and inevitably hold answers to key issues surrounding coral reef research and restoration. To quote the poet Mary Oliver, "attention is the beginning of devotion," and at the culmination of this work I find my attention has indeed turned to devotion. Studies surrounding coral motes of beings encapsulating further invisible microbial beings bring challenges, heavy thought, and perhaps a backlog of samples. I remain devoted to examining these relationships and the mechanisms by which they originate, for understanding how corals give and receive from the surrounding environment has never been more pertinent. Ultimately, efforts should move away from segregated disciplines and evolve multi-disciplinary work, as shown here, towards an interdisciplinary approach. Resolving the post-settlement bottleneck and beyond will require restoration practitioners, materials engineers, microbiologists, and more, filling knowledge gaps and working towards progress together in these ever-changing seas.

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