Exploring chemical cues for coral settlement and their applicability to coral restoration pipelines

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Final Report

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Management Summary

Our objectives and outcomes of this project have important management implications for coral reefs. The use of tetrabromopyrrole (TBP) to settle corals eliminates the need to pre-condition substrates and has the potential to increase recruit survival and decrease labor time and cost for restoration practitioners. TBP can be used for ex-situ settlement prior to grow-out for subsequent outplanting or to prime larvae for settlement prior to deployment during direct seeding activities. In both cases, larvae can be exposed to TBP for as little as 1 hour and then rinsed to remove the majority of the TBP prior to introducing larvae to a settlement substrate. Understanding the effects, or lack thereof, of TBP treatment on coral microbiomes will allow resource managers to better assess the applicability of this method to sexual propagation pipelines for coral restoration. The use of TBP during settlement could vastly increase propagation success, efficiency and output, but as the use of TBP increases in sexual restoration in corals we want to ensure there are no adverse effects on coral recruits.

Identification of waterborne compounds effective at inducing coral larval settlement will fill an important knowledge gap for coral restoration efforts. These studies will help identify which organisms within the environment facilitate settlement; these organisms could then be incorporated into outplanting efforts. Once the structures of the settlement-inducing compounds are known, they could be chemically synthesized for in situ and ex situ applications. Not only will their discovery provide new tools for inducing settlement during sexual propagation, a challenging life history stage, but it will also inform managers about optimal reef conditions for coral recruitment success.

Executive Summary

Successful larval settlement is one of the most critical and challenging bottlenecks in coral sexual propagation for reef restoration. This study investigated chemical cues that promote settlement and metamorphosis in priority Caribbean coral species.

Larvae of *Colpophyllia natans* and *Pseudodiploria strigosa* were exposed to TBP, a DMSO solvent control, or untreated filtered seawater (FSW) before settling on conditioned or unconditioned ceramic tiles. Notably, after six months, recruits on unconditioned tiles were approximately 30% more likely to survive than those on conditioned tiles, likely due to reduced competition with established organisms on conditioned surfaces. TBP effects on survival could not be assessed for *C. natans* due to low recruitment numbers, however TBP treated *P. strigosa* had final recruit numbers on unconditioned tiles comparable to untreated larvae on conditioned tiles, which represents the current standard operating procedure. Settling corals with TBP will reliably produce settlement levels on unconditioned tiles that will allow for successful restoration, while avoiding the costs and variability associated with substrate conditioning. However, TBP concentration and delivery may need optimization when used in systems with additional waterborne cues. Microbiome data from this experiment are currently being processed.

Additionally, seawater from a diverse coral reef ecosystem and from close proximity to individual reef ecosystem components (*A. cervicornis* and crustose coralline algae) consistently induced settlement, suggesting the critical role of biodiversity in producing effective cues. Filtered seawater (0.2 µm filtration to remove particles and most bacteria) retained its inductive properties for most species, attributing effectiveness to dissolved compounds rather than bacteria or particles. Using solid-phase extraction techniques with C18 resin, inductive compounds were extracted from seawater. These retained compounds proved effective in settlement bioassays. Fractionation over C18 columns indicated that polar fractions induced the highest settlement and metamorphosis. Successive fractionation and HPLC separation identified species-specific responses: a single HPLC fraction induced significant settlement in *C. natans*, while distinct HPLC fractions from a separate subfraction induced significant settlement in *P. strigosa*. These results suggest that multiple compounds with species-specific activity are present in reef aquarium seawater. Continued characterization using liquid chromatography-high resolution mass spectrometry (HRLCMS) is planned.

Together, these findings have direct implications for coral restoration. TBP offers a reliable, low-cost method for inducing settlement on unconditioned substrates, reducing the time, cost, and variability associated with traditional substrate conditioning. The discovery that biodiverse reef aquarium seawater contains dissolved, extractable compounds that induce settlement across multiple coral species underscores the ecological importance of reef biodiversity for supporting natural recruitment. As active compounds are isolated and chemically characterized, they could eventually be applied in both ex situ propagation facilities and in situ restoration efforts to improve settlement success at scale.

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1. INTRODUCTION

1.1. Background

Successful coral settlement and metamorphosis (hereafter referred to as settlement) is key to success in sexual propagation of corals. It is a common bottleneck for both in situ and ex situ sexual propagation methods. Research over the past two decades has demonstrated many ways that coral larvae can be inhibited from settlement, such as presence of macroalgae, cyanobacteria and turf algae (often laden with sediments that can cause hypoxia) (Kuffner et al. 2006, Birrell 2008, Paul et al. 2011, Webster et al. 2015, Ritson-Williams et al. 2020). We know less about positive settlement cues, especially in Florida and the Caribbean, but we do know that the presence of certain species of crustose coralline algae (Ritson-Williams et al. 2016, Randall et al. 2024), bacterial cues (Sneed et al. 2014, 2024, Petersen et al. 2023) and conspecifics can induce settlement for some coral larvae. Over the past two decades, our laboratory has published studies identifying positive cues and inhibitors of coral larval settlement and explored many facets of early recruitment processes (Ritson-Williams et al. 2009, 2014, 2016, 2020, Olsen et al. 2014, Sneed et al. 2014, 2024).

Lack of understanding of coral settlement cues has led to methods that rely on conditioning settlement substrates to facilitate settlement, but this can be an unreliable process that is not well controlled (Miller et al. 2022, Suzuki et al. 2020). Current methods being used by restoration and management practitioners for settlement of sexually propagated corals involve providing larvae with either substrates that have been conditioned in reef environments for weeks to months or the addition of crustose coralline algae (CCA) "dust". These methods can be time and resource consuming. Conditioned substrates often must be cleaned before use and still end up with uneven settlement due to variation in the epibiotic communities. Furthermore, newly settled corals have to compete with already established organisms for space on such substrates. CCA used to dust substrates to induce settlement must be harvested and can outcompete the young recruits over time if it grows on the substrates. Development of a consistent, low-cost settlement cue that could be applied to substrates or to the water column to enhance settlement would greatly increase the effectiveness of restoration via sexual propagation (Randall et al., 2020). The identification of biochemical cues that induce settlement in a wide range of coral species would be invaluable, filling a high-priority knowledge gap for successful coral restoration efforts (Randall et al., 2020).

Tetrabromopyrrole (TBP) is a compound produced by coral reef associated bacteria that reliably induces settlement in Caribbean corals in the absence of any other settlement cue (Sneed et al. 2014, 2024). TBP can be readily synthesized in the laboratory. Using TBP reduces the labor and resource costs associated with other settlement methods. Soaking larvae in TBP treated seawater for as little as 1 hour is sufficient to induce settlement in the absence of any other cue for every species tested to date (Figure 1). These include *Acropora palmata*, *Diploria labyrinthiformis*, *Colpophyllia natans*, *Montastraea cavernosa*, *Orbicella faveolata*, *Pseudodiploria clivosa* and *P. strigosa*. Following the soaking period, the larvae can be rinsed to remove most of the TBP so that minimal, if any, TBP is introduced into the settlement system or into the environment.

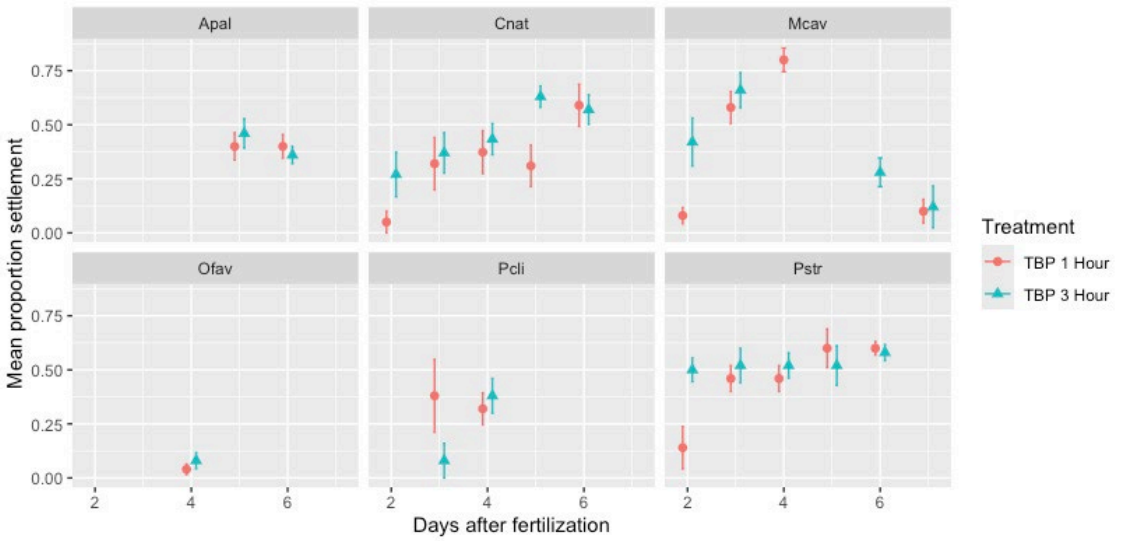

Figure 1. Mean (± standard error) proportion settlement of larvae 48 hours after either 1 hour or 3 hours of soaking in tetrabromopyrrole prior to rinsing and placing in filter-sterilized seawater in sterile 6-well plates. N = 5 replicate wells with 10 larvae per well.

This method used with unconditioned substrates can lead to settlement and survival rates comparable to traditional methods using conditioned substrates, while being more time and labor efficient. Examining the possible implications of treatment with TBP on the resulting recruit microbiome will provide additional understanding of how this method impacts corals and will allow for more educated decision making on its use in coral restoration.

While TBP serves as a promising candidate for the chemical induction of settlement, we continue to explore the presence of other natural settlement cues. This will provide additional information on environmental conditions on reefs that may facilitate settlement in situ and additional methods for inducing larval settlement for restoration. In recent years, we have made the remarkable discovery that filtered seawater from our coral reef aquarium at the Smithsonian Marine Ecosystems Exhibit (SMEE) at the St. Lucie County Aquarium facilitated coral larval settlement without any other cues (Figure 2). This means that waterborne metabolites from an intact coral reef ecosystem can be highly effective as a settlement inducer with over 50% settlement in early trials. Sterile filtering the seawater did not significantly diminish its effectiveness, indicating the settlement was induced by dissolved compounds in the seawater.

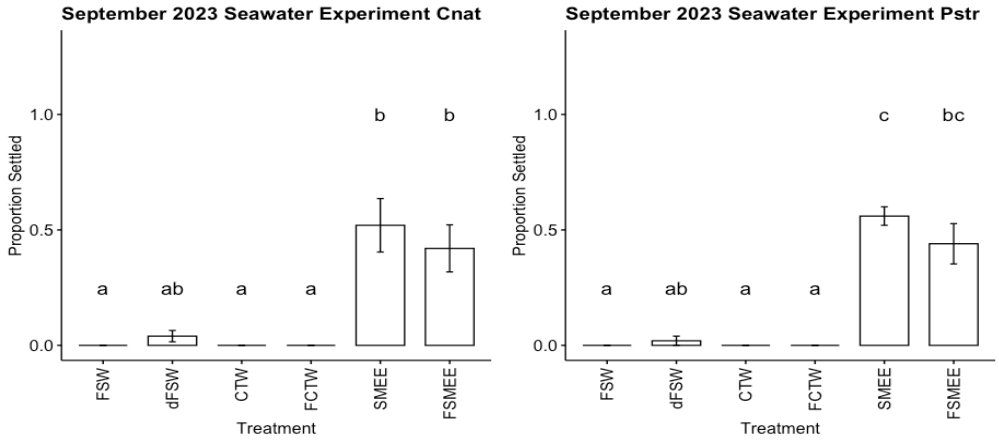

Figure 2. *C. natans* and *P. strigosa* larvae were added to six-well plates containing: filtered offshore seawater (FSW); sterile filtered offshore seawater (dFSW); water from a raceway containing only a few corals (CTW); sterile filtered water from the raceway (FCTW); water from the Smithsonian coral reef ecosystem exhibit (SMEE); sterile filtered water from a coral reef ecosystem exhibit (FSMEE).

During Year 1 of this project, we conducted 36 experiments examining the effects of waterborne cues on coral larval settlement and metamorphosis. We found that waterborne cues from the Smithsonian coral reef ecosystem exhibit at the St. Lucie County Aquarium (SMEE) (as shown in Figure 2) consistently induced metamorphosis in six spawning coral species, *Acropora palmata*, *Colpophyllia natans*, *Montastraea cavernosa*, *Orbicella faveolata*, *Pseudodiploria clivosa* and *P. strigosa*. We also found that water obtained surrounding *Acropora cervicornis* and *A. palmata* in the aquarium induced settlement in most spawning coral species. We compared solid phase extraction methods to retain the inductive compounds from seawater and found that C18 resin worked better and more consistently than HP20 resin and efficiently retained the active compounds, which could be eluted from the resins with methanol. These eluates also induced settlement at natural concentrations, demonstrating that we had developed a method to obtain the inductive compounds from seawater.

We then used solid phase extraction methods with C18 resin to scale up the retention of the inductive compounds from the seawater by passing 3-4 L of the SMEE water over the C18 columns and repeating this multiple times. We also eluted the compounds with different solvents to begin the process of separating what is a complex mixture of compounds into fractions containing fewer and more active compounds. We are confident that further separation methods with larger amounts of seawater processed over columns could lead us to more purified compounds with a goal of characterizing some of the most effective settlement inducing compounds. Furthermore, this demonstrates that seawater alone from various combinations of corals and other reef organisms that are characteristic of a healthy coral reef can facilitate larval settlement. Lack of these natural chemical cues may partially explain the inability of degraded reefs to promote larval settlement and recruitment.

1.2. Project goals

The key goal of this study is to identify chemical cues that increase coral settlement and assess their applicability in reef restoration. To that end, this project aims to not only assess the impacts of using a known settlement cue, tetrabromopyrrole, on the bacterial microbiomes of coral recruits, but also to discover new cues that could be used in the future for restoration purposes.

Objectives for this study include:

1. Do the bacterial microbiomes of recruits that were settled using TBP differ from those of recruits that were settled without TBP?
2. What are other compounds in seawater from coral reef organisms that induce larval settlement and metamorphosis?
3. Are the same isolated chemical cues effective on larvae of different coral species?

2. METHODS

2.1. Task 2: Determining the effects of tetrabromopyrrole use during settlement on coral recruit bacterial microbiomes.

Task 2 used coral larvae of two species, *Colpophyllia natans* and *Pseudodiploria strigosa*, spawned at The Florida Aquarium's Center for Conservation ex-situ spawning facility (TFA). Larvae from both species were exposed to one of three chemical treatments: 1) 1-hour soak in TBP (250 nM in 1 ppt dimethyl sulfoxide (DMSO)), 2) 1-hour soak in seawater treated with a solvent control (1 ppt DMSO), 3) 1-hour soak in untreated filtered seawater (FSW) and then allowed to settle on either conditioned or unconditioned substrates. Conditioned tiles were conditioned in recirculating seawater systems holding broodstock corals at TFA. For each treatment, there were three replicated flow-through settlement bins (n=3) that each contained 15 3 x 3 cm ceramic tiles.

2.1.1. Settlement and Survival Monitoring

For each replicate, approximately 500 larvae were added to two tripour beakers containing 500 mL of FSW with one of the three chemical treatments listed above. Prior to experimental use, larvae were counted and inspected under a microscope to ensure there were no visible deformities. Larvae were left to soak in these tripour beakers for 1 hour before being transferred to a second tripour beaker with 500 mL of FSW for 1 hour to rinse off the chemical treatments. After a 1-hour rinse, larvae were transferred to settlement bins that were placed on a PVC rack in a raceway with recirculating seawater and cultured live rock and corals in the sump to serve as microbiome and symbiont donors.

Larvae were allowed to settle for six to eight days before tiles were removed from the settlement bins for settlement counts. At this time, the number of coral recruits on the top, bottom, and sides of each tile was recorded. Each tile was given an identification tag that

reflected the treatment, replicate (settlement bin), and tile number. One and six months after the experiment with each species started, survival was recorded by inspecting each tile under a microscope and recording the number of recruits remaining on the top, bottom, and sides of the tiles.

2.1.2. Microbiome Sampling

Samples for bacterial microbiome analyses were taken at four timepoints during the experiment, during setup (larvae), one week post set up (recruits), and one and six months post set up. When sampling larvae, five individuals from each replicate tripour beaker were pooled on a sterile work surface and transferred into a microcentrifuge tube containing 1 mL of FSW. This was repeated two more times for a total of three sequential rinses to remove any loosely associated bacteria from the surface of the larvae. When sampling at the one week and one month time points, tiles were removed from the settlement bins or their grow-out raceway and rinsed with 10 mL of FSW using a serological pipette. One recruit was scraped off with a sterile micropipette and transferred to a sterile work surface. This was repeated with four more tiles for a total of five pooled recruits. Six-month juveniles were similarly rinsed and sampled from five different tiles; however, individuals were not pooled. Recruits were scraped from the surface of the tile using a sterile razor blade.

For each time point, individuals were sampled using sterile techniques and taking care to transfer as little water as possible. Samples were placed in in a Qiagen PowerSoil Pro Kit bead beating tube containing 800 µL of solution C1 and stored at 4 °C for up to 48 hours or at –20 °C for longer storage at the Smithsonian Marine Station in Fort Pierce (SMSFP). In addition to this, a 5 µL sample of seawater from each treatment was collected as a negative control for the larval timepoint and a 5 µL sample of seawater from the surface of the tiles as a negative control for other timepoints. DNA was extracted using the Qiagen PowerSoil Pro DNA Extraction Kit. DNA was quantified using a Qubit 4 Fluorometer. Extracted DNA samples were then stored at –20 °C prior to submission for sequencing. Library prep and sequencing was completed by the Michigan State University Genomics Core. The V4 hypervariable region of the 16S rRNA gene was amplified using dual indexed, Illumina compatible primers following the protocol described in Kozich, JJ, et al. (2013). PCR products were batch normalized using the Charm Biotech PCR Normalization and Purification kit and product recovered from the pooled plates. The pool was concentrated and cleaned up using a QIAquick Spin column and AMPure XP magnetic beads. The pool was quality controlled and quantified using a combination of Biotium AccuGreen High Sensitivity dsDNA, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Illumina Library Quantification qPCR assays.

This pool was loaded onto one (1) Element Biosciences AVITI Medium Output flow cell and sequencing was carried out in a 2x250bp paired end format using a Cloudbreak Freestyle 600 cycle reagent kit. Custom sequencing and index primers complementary to the 515f/806r oligomers were added to appropriate wells of the reagent cartridge. Base calling was done by AVITIOS v3.4.2 and the output was demultiplexed and converted to FastQ format using Element Biosciences bases2fastq v2.2.0.

2.1.3. Data Analysis

The mean percent and numbers of recruits in each treatment at one and six months were statistically analyzed using R version 4.5.1. Initial settlement counts were omitted from the datasets for both *C. natans* and *P. strigosa* as there were miscounts leading to seemingly more survivors at one month than there were settlers. Data were analyzed using a generalized linear mixed models using template model builder (glmmTMB). Chemical cue treatment and tile conditioning were included as fixed factors along with the interaction between the two variables with FSW and unconditioned set as reference values (R, glmmTMB package). One-month recruits were analyzed using settlement bin as the unit of replication (n=3) and presented as percent recruitment per bin out of the initial number of larvae added to each bin. Six-month survival was calculated by comparing the number of recruits per tile at 6 months to the number at 1 month with tile as the unit of replication and settlement bin included as a random effect. Planned contrasts comparing chemical cue within tile type and tile type within chemical cue were estimated at the one- and six-month time points using an estimated marginal means (R, emmeans package). Treatments were considered significantly different from the negative controls when $p < 0.05$. All data are presented as mean ± standard error.

2.2. Task 3: Characterizing chemical cues for different species of coral larvae

2.2.1. Aquarium Seawater Collection

Aquarium seawater for testing waterborne cues in coral larval settlement bioassays was collected from multiple locations within the coral reef system at the Smithsonian Marine Ecosystems Exhibit (SMEE) at the St. Lucie County Aquarium (Fort Pierce, Florida). This system contains a diverse assemblage of hard and soft corals, along with a variety of other reef-associated organisms (Table 1). Four seawater types were collected from the main coral reef exhibit, and an additional seawater type was collected from the deep refuge tank. Although not on public display, the deep refuge tank is connected to the exhibit through a shared recirculating seawater system.

All collection containers (1-3 L) were cleaned with reverse osmosis water, air-dried, and rinsed with seawater from the respective aquarium prior to use. A portion of each seawater collection was filter sterilized using 0.2 µm polyethersulfone (PES) membrane filter units (0.5- 1L volume) under vacuum to remove suspended particles, including phytoplankton, bacteria, and sediment.

Table 1. Aquarium seawater identification codes and descriptions. The prefix "F" indicates sterile filtration; the absence of the prefix indicates unfiltered seawater. All seawater types were tested under both conditions.

Seawater ID	Tank	Tank Volume (L)	Collection Method	Collection Location	Hard Coral Contents	Other Organisms

(F)DR	Deep Refuge	568	Submersion	Water column of deep refuge tank	*Orbicella cf. faveolata* *Porites porites* *Porites astreoides* *Porites cf. furcata* *Pseudodiploria strigosa*	*Palythoa grandis* *Zoanthus* sp. *Stichodactyla helianthus* *Pseudocorynactis caribbeorum* *Muricea* sp. *Eunicea flexuosa* *Pseudopterogorgia* sp. *Plexaurella* sp. *Caulerpa* spp. Red Algae Crustose Coralline Algae
(F)Acer	Coral Reef Exhibit	6814	Bulb Pipette	Between branches of *Acropora cervicornis*	*Acropora cervicornis* *Acropora palmata* *Acropora prolifera* *Orbicella faveolata* *Porites astreoides* *Porites divaricata* *Porites porites* *Pseudodiploria strigosa* *Siderastrea siderea* *Orbicella franksi*	Juvenile Reef Fish *Palythoa* spp. Snails Crabs Brittle Stars Sea Stars Shrimp Urchins Anemones Sea Cucumbers Soft Corals Crustose Coralline Algae Other macroalgae
(F)Apal	Coral Reef Exhibit	6814	Bulb Pipette	Adjacent to *Acropora palmata*		
(F)Paly	Coral Reef Exhibit	6814	Bulb Pipette	Adjacent to *Palythoa caribaeorum*		
(F)Ppor	Coral Reef Exhibit	6814	Bulb Pipette	Adjacent to *Porites porites*		

Seawater designated (F)DR was collected by directly submerging collection containers into the deep refuge tank (Table 1). All other seawater types were collected from specific locations within the coral reef exhibit using a 30 mL bulb pipette. For all bulb pipette collections, the bulb pipette was positioned approximately 3–5 cm from the focal organism. Collection locations for each seawater type are summarized in Table 1 and illustrated in Figure 3.

Figure 3. Illustration of seawater collection methods for (F)Acer (left) and (F)Apal (right).

2.2.2. Crustose Coralline Algae Seawater Collection

Crustose coralline algae (CCA), *Hydrolithon boergesenii*, was collected from Big Pine Ledges in the Florida Keys (24.55351, −81.37816). Following collection, the CCA was maintained in FSW under gentle aeration. For experimental use, approximately 95.187 cm² of CCA was incubated in 500–600 mL of FSW for about 48 hours to allow waterborne

cues to accumulate without appreciably affecting salinity. The same CCA pieces were repeatedly used to produce additional seawater every 48 hours. Seawater was collected from this incubation container and filtered to produce FCCA, an additional seawater treatment, for testing in coral larval settlement bioassays.

2.2.3. Coral Larval Settlement Bioassays

Settlement bioassays were conducted in sterile Costar six-well polystyrene plates (Corning 3506) containing 10 mL of seawater per well. Tested species included *Acropora cervicornis*, *Colpophyllia natans*, *Diploria labyrinthiformis*, *Orbicella faveolata*, *Porites astreoides*, and *Pseudodiploria strigosa*. Larvae were maintained in natural filtered seawater until competency and transferred to multi-well plates for settlement bioassays. Microscopes were used to select swimming planula larvae with no visible deformities for all bioassays, and 10 larvae were counted into each well. Treatments were randomized across plates and replicated five times, with each well serving as a single replicate (Figure 4).

For seawater bioassays, treatments included unfiltered aquarium seawater, filter-sterilized aquarium seawater, FCCA, and FSW as a negative control. When available, fragments (~16 mm²) of the CCA *Hydrolithon boergesenii*, a known inducer of settlement in multiple Caribbean coral species (Ritson-Williams et al. 2016), were added to FSW as a positive control to confirm larval competency (Figure 4). The CCA pieces were collected from Big Pine Ledges in the Florida Keys (24.55351, −81.37816) as described above.

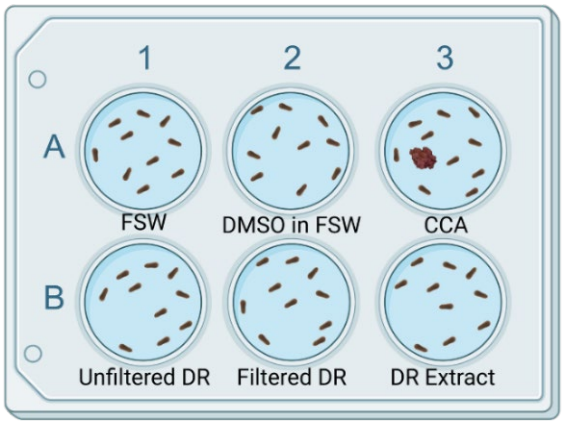

Figure 4. Diagram of an example 6-well plate from a larval settlement bioassay. The DR Extract is an example of an FDR C18 eluate in FSW (see next section). Five replicates were used for each treatment and control.

2.2.4. Extraction of waterborne chemical cues

To extract dissolved organic compounds associated with settlement-inducing activity, filter-sterilized aquarium seawater samples were subjected to chemical extraction using C18 solid-phase extraction columns. Prepacked 10 g Chromabond® C18 columns were conditioned with methanol and rinsed with HPLC-grade water. Aquarium seawater samples (1–3 L) were loaded onto the columns under vacuum, followed by a rinse with

HPLC-grade water to minimize salt retention on the columns. Retained organic compounds were eluted with methanol (Figure 5).

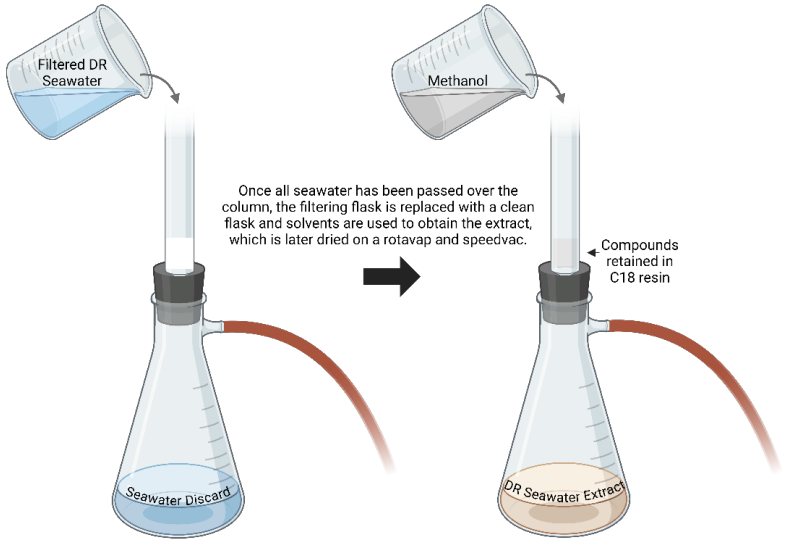

Figure 5. Diagram of FDR seawater being vacuum filtered over a C18 column, and the retained compounds being eluted with methanol under vacuum.

The resulting eluates were first concentrated using a rotary evaporator and transferred in methanol to pre-weighed 7 mL scintillation vials. Samples were further concentrated at 35 °C using a SpeedVac (Thermo Savant SPD121P). Residual water was frozen at -80 °C and removed by lyophilization at -45 °C and 0.15 mbar using a Labconco FreeZone 6 freeze dryer. Dried extracts were weighed and redissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at 1000× the original seawater concentration. For example, if 1 L of seawater was processed, the dried eluate was redissolved in 1 mL DMSO, allowing the extract to be tested at 1× natural concentration. Stock solutions were stored at -20 °C until testing.

To evaluate eluates in coral larval settlement bioassays, 10 µL of the stock solution of the eluate dissolved in DMSO was added to wells containing 10 mL FSW. To account for potential compound loss during solid-phase extraction and reduce false negatives, most eluates were tested at twice their natural concentration. Each experiment included a solvent (DMSO) control consisting of 10 µL DMSO in 10 mL FSW (1‰ DMSO) in addition to the controls described above.

2.2.5. Fractionation of Active Samples

Eluates that induced settlement responses in coral larvae were selected for fractionation and subsequent testing. Selected eluates were separated using C18 chromatography with a series of water and methanol mixtures of increasing methanol concentration. Depending on the sample and fractionation objectives, multiple fractions were collected and concentrated by rotary evaporation, SpeedVac concentration, and lyophilization as described above. Fractions were dissolved in DMSO and tested in coral larval settlement bioassays as described above. Fractions exhibiting settlement-inducing activity were selected for further fractionation and testing. Selected active fractions were further

separated using high-performance liquid chromatography (HPLC). Individual HPLC fractions were collected, concentrated, dissolved in DMSO, and tested in coral larval settlement bioassays. Active HPLC fractions were selected for further chemical characterization.

2.2.6. Data Collection & Statistical Analysis

Larval responses were recorded after 24 and 48 hours and categorized as settled (metamorphosed and attached), metamorphosed but unattached, partially metamorphosed, or swimming (Figure 6).

Figure 6. Examples of swimming, partially metamorphosed, and metamorphosed (attached or unattached) larvae.

Mean proportions of settled larvae (n = 5) per treatment were analyzed in R. Data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Bartlett's tests, respectively. As assumptions of normality were not met, differences among treatments were evaluated using Kruskal-Wallis tests followed by Dunn's post hoc comparisons. Treatments were considered significantly different from their respective negative controls at $p < 0.05$. Aquarium seawater treatments were compared to FSW controls, whereas eluate and fraction treatments were compared to DMSO controls.

3. RESULTS

3.1. Task 2: Determining the effects of tetrabromopyrrole use during settlement on coral recruit bacterial microbiomes.

3.1.1. Colpophyllia natans

At the one-month time point for *C. natans*, larvae that were treated with TBP had 0.5% ± 0.1% and 8% ± 2% recruitment on conditioned and unconditioned tiles respectively (Figure 7). Larvae that were treated with DMSO had 63% ± 6% and 84% ± 5% on unconditioned and conditioned tiles respectively, and larvae that were treated with FSW had 60% ± 9% and 77% ± 7% on unconditioned and conditioned tiles respectively (Figure 7). There was higher recruitment to conditioned tiles than unconditioned tiles for both untreated (FSW) and DMSO treated larvae ($p < 0.001$; emmeans). This pattern was opposite for TBP treated larvae with higher settlement on unconditioned tiles than conditioned tiles ($p < 0.001$; emmeans). Untreated larvae (FSW) and DMSO treated larvae had higher recruitment compared to TBP regardless of tile type ($p < 0.001$;

emmeans). On unconditioned tiles, there was no significant difference in recruitment between untreated (FSW) and DMSO treated larvae (p= 0.330), but DMSO treated larvae had higher recruitment on conditioned tiles compared to untreated larvae (FSW, p < 0.001; emmeans).

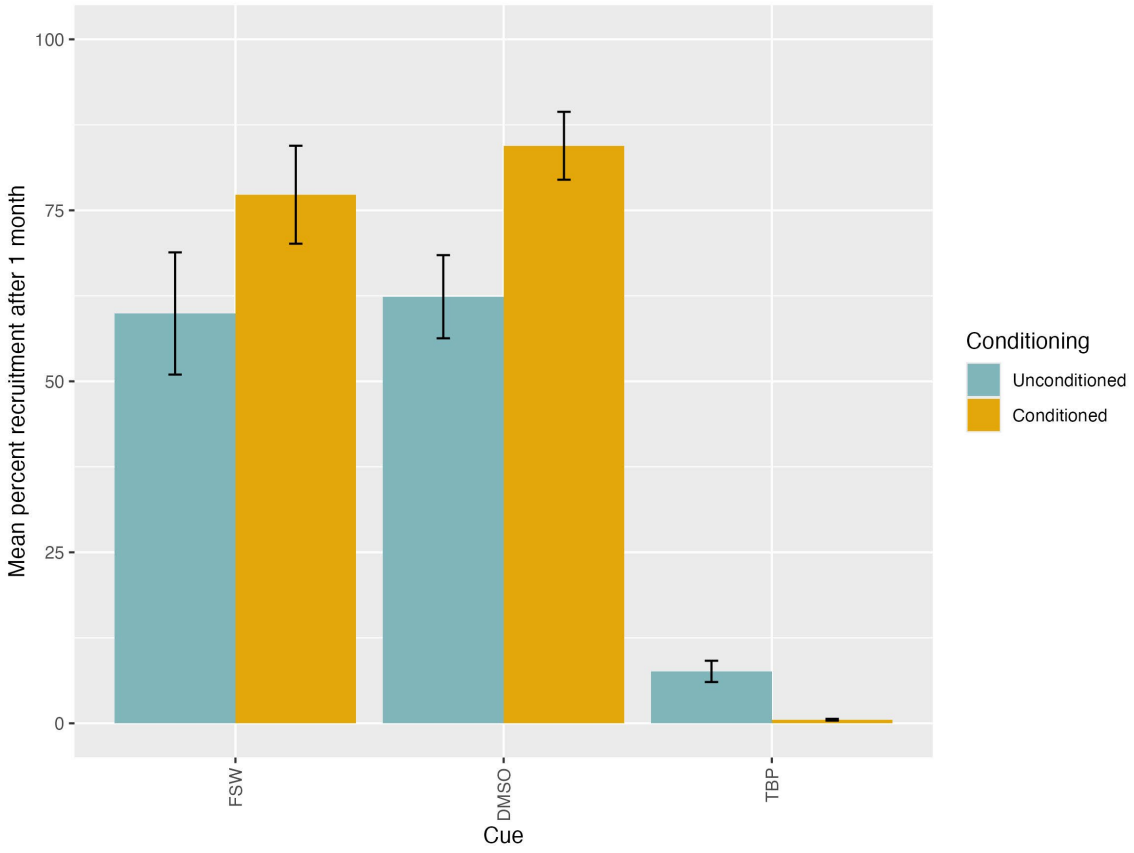

Figure 7. Mean ± standard error percent recruitment of *C. natans* larvae after 1 month. N=3 settlement bins per treatment, ~500 larvae per bin. Larvae were exposed to one of three chemical cue treatments, untreated (FSW), solvent control (DMSO) or 250 nM tetrabromopyrrole (TBP) prior to settlement and given either unconditioned or conditioned tiles as settlement substrate.

At the six-month time point, the treatments that used TBP as a settlement cue were removed from survival analyses because there were too few recruits to get a reliable measure of survival. Percent survival between 1 and 6 months of recruits that had been treated with DMSO was 43% ± 4% and 68% ± 3% on conditioned and unconditioned tiles, respectively (Figure 8). Percent survival of those left untreated (FSW) was 34% ± 4% and 64% ± 4% on conditioned and unconditioned tiles, respectively. There was significantly higher recruit survival on unconditioned tiles for both those that were untreated (FSW) and those treated with DMSO (p < 0.001; emmeans). Recruits that had been treated with DMSO had slightly higher survival compared to untreated (FSW) regardless of tile type (p = 0.039 and 0.007 for unconditioned and conditioned, respectively; emmeans).

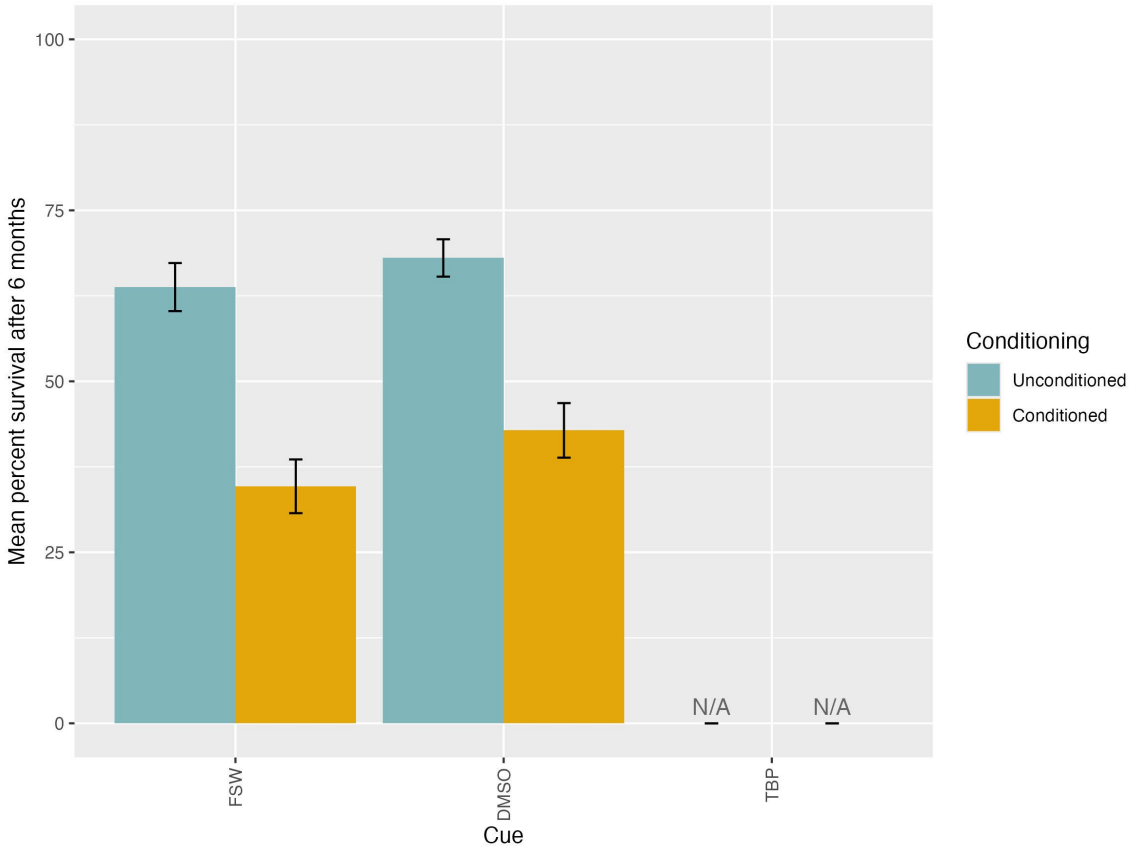

Figure 8. Mean ± standard error percent survival of *C. natans* recruits after 6 months. Larvae were exposed to one of three chemical cue treatments, untreated (FSW) or solvent control (DMSO) prior to settlement and given either unconditioned or conditioned tiles as settlement substrate. TBP treated recruits were excluded from percent survival analysis because of low recruitment rates. N = 43 - 45 tiles per treatment.

In terms of absolute recruit numbers, there was a significantly higher mean number of recruits per tile at 6 months for recruits that had been treated with DMSO on unconditioned versus conditioned tiles ($p = 0.032$, 12.9 ± 1.0 and 9.1 ± 1.5 , respectively; emmeans, Figure 9). The mean number of recruits per tile at 6 months for recruits that were treated without a chemical cue (FSW) was also significantly higher on unconditioned tiles (10.6 ± 1.3) than conditioned tiles (6.1 ± 1.0, $p = 0.007$; emmeans). The mean number of recruits at 6 months was very low for recruits that had been treated with TBP and was not significantly different between tile types ($p = 0.998$, 1.7 ± 0.3 and 0 for unconditioned and conditioned tiles, respectively; emmeans).

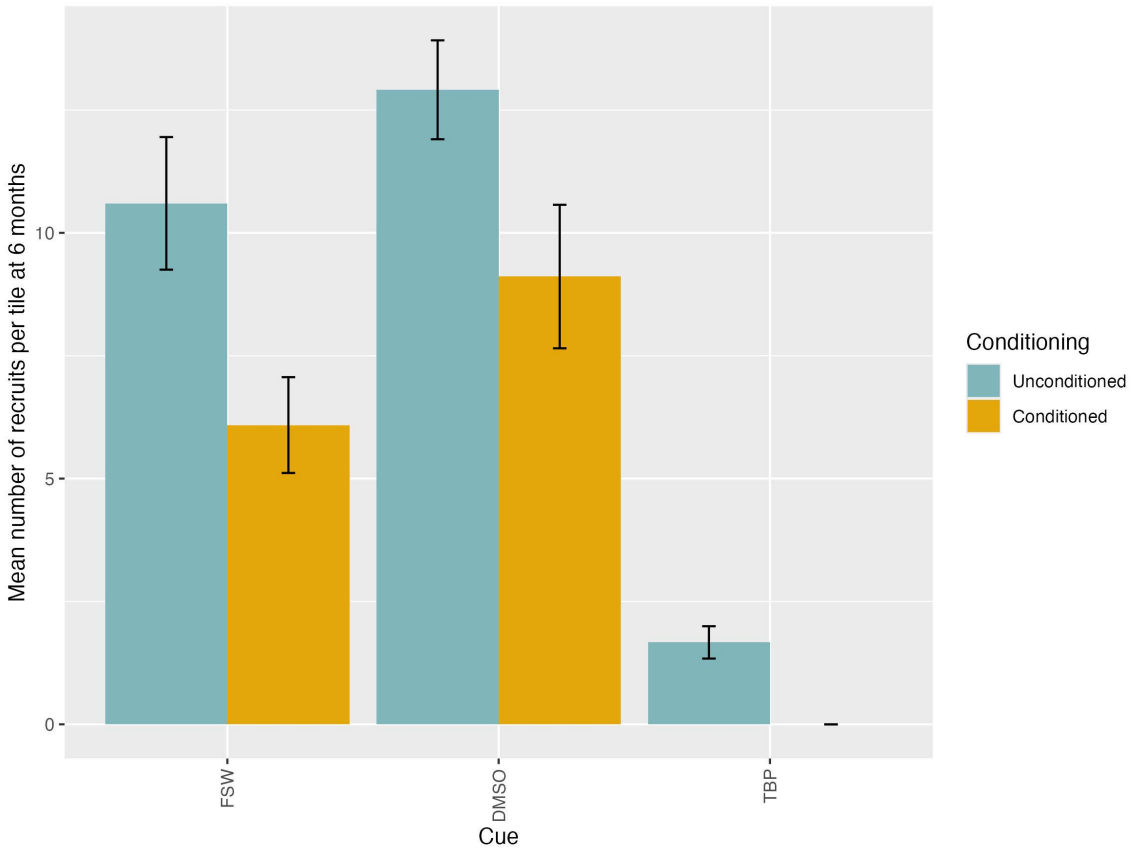

Figure 9. Mean ± standard error number of remaining *C. natans* recruits per tile after 6 months. Larvae were exposed to one of three chemical cue treatments, untreated (FSW), solvent control (DMSO) or 250 nM tetrabromopyrrole (TBP) prior to settlement and given either unconditioned or conditioned tiles as settlement substrate. N = 45 tiles per treatment.

3.1.2. Pseudodiploria strigosa

At the one-month time point for *P. strigosa*, larvae treated with TBP had 12% ± 0.4% and 8% ± 1% recruitment on unconditioned and conditioned tiles respectively, larvae treated with DMSO had 0.2% ± 0.2% and 17% ± 3% recruitment on unconditioned and conditioned tiles respectively, and larvae not treated with any chemical cue (FSW) had 0.9% ± 0.4% and 28% ± 8% recruitment on unconditioned and conditioned tiles respectively (Figure 10). Recruitment was significantly affected by chemical cue, tile conditioning, and the interaction between the two factors. Conditioning significantly increased recruitment on FSW ($p < 0.001$) and DMSO ($p < 0.001$) tiles, but significantly reduced recruitment on TBP tiles ($p = 0.0004$; emmeans). Among conditioned tiles, recruitment was significantly higher on FSW than TBP ($p < 0.001$) and DMSO than TBP ($p < 0.001$), and FSW and DMSO also differed significantly from each other ($p < 0.001$; emmeans). On unconditioned tiles, TBP had significantly higher recruitment than both FSW ($p < 0.001$) and DMSO ($p < 0.001$; emmeans).

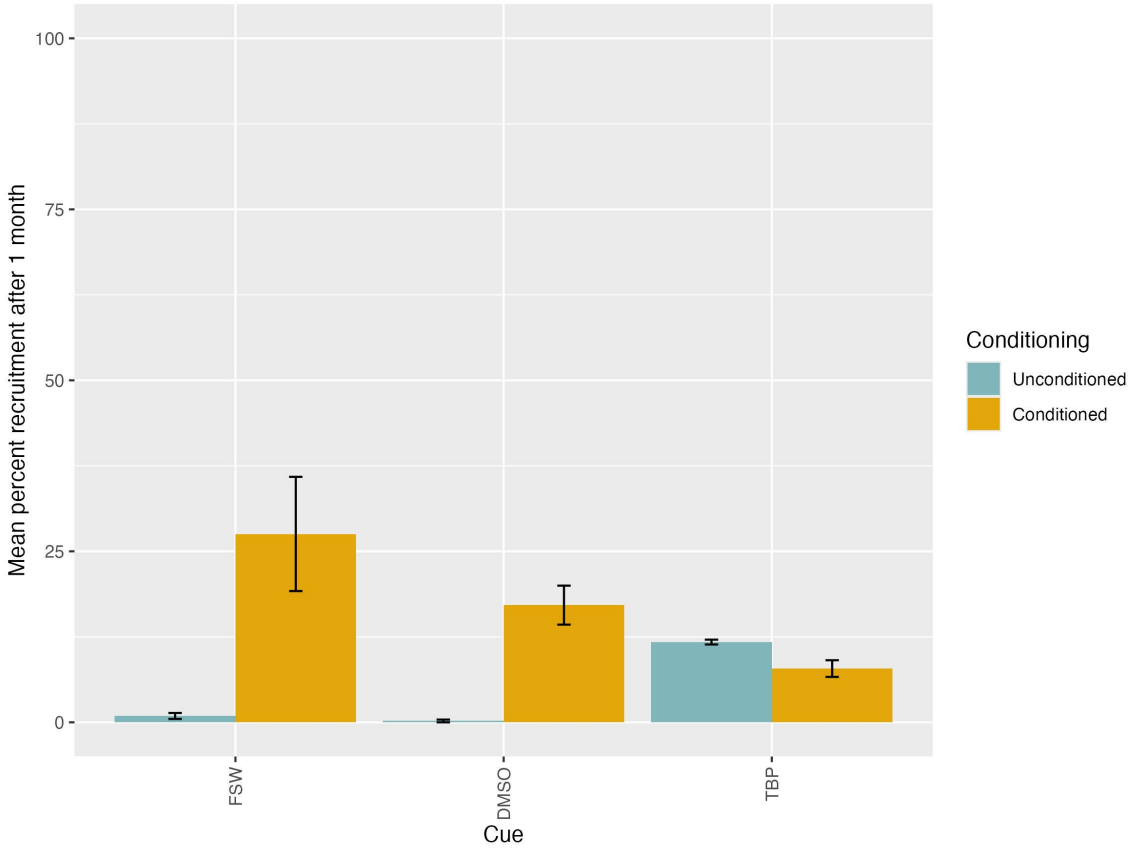

Figure 10. Mean ± standard error percent recruitment of *P. strigosa* larvae after 1 month. N=3 settlement bins per treatment, ~500 larvae per bin. Larvae were exposed to one of three chemical cue treatments, untreated (FSW), solvent control (DMSO) or 250 nM tetrabromopyrrole (TBP) prior to settlement and given either unconditioned or conditioned tiles as settlement substrate.

At the six-month time point, larvae that were settled on unconditioned tiles with DMSO and FSW were removed from all analyses because there was too little recruitment in these treatments to get a clear picture of survival after six months. Percent survival between 1 and 6 months of recruits that had been treated with TBP was significantly higher on unconditioned tiles (77% ± 5%) compared to conditioned tiles (58% ± 6%, p = 0.002; emmeans, Figure 11). Among conditioned tiles, percent survival was higher in recruits that were untreated (FSW, 58% ± 6%) compared to those treated with DMSO (63% ± 5%, p = 0.029), but there were no significant differences between FSW treated and TBP treated (p = 0.392) or TBP treated and DMSO treated (p = 0.669; emmeans,

Figure 11).

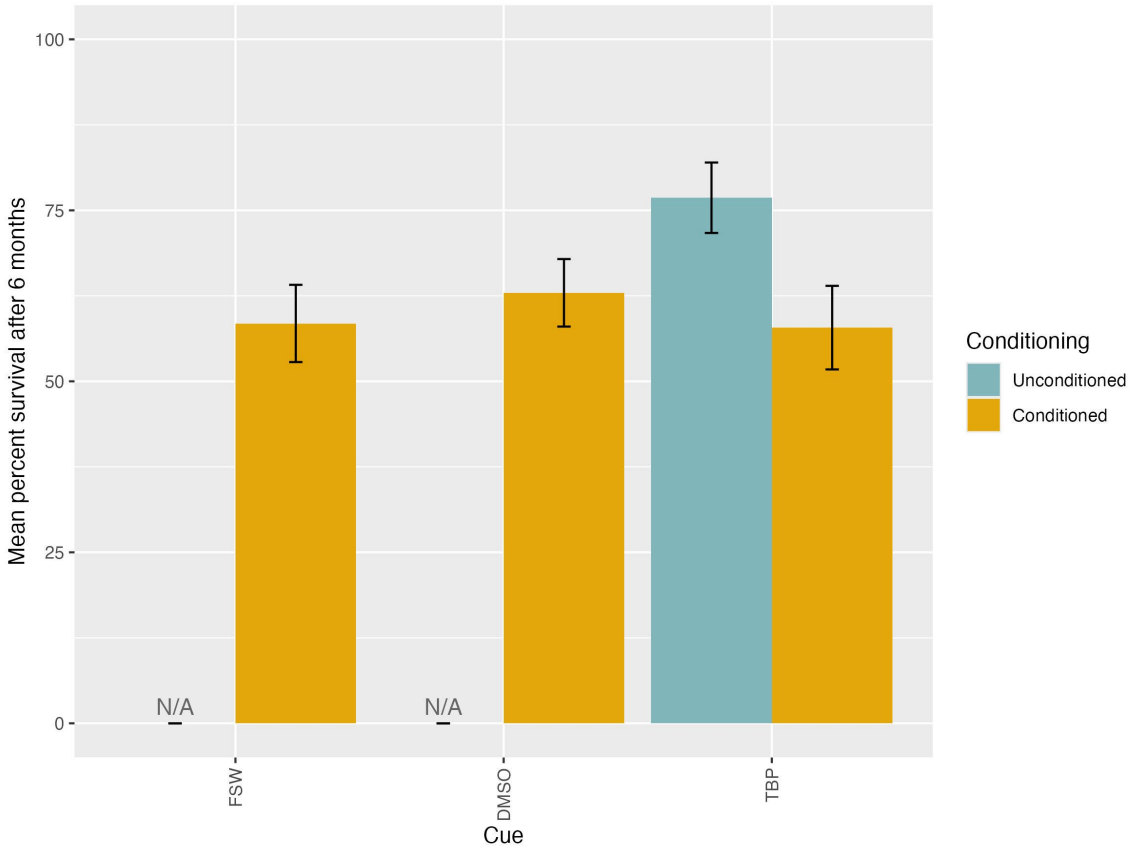

Figure 11. Mean ± standard error percent survival of *P. strigosa* recruits after 6 months. Larvae were exposed to one of three chemical cue treatments, untreated (FSW), solvent control (DMSO) or 250 nM tetrabromopyrrole (TBP) prior to settlement and given either unconditioned or conditioned tiles as settlement substrate. Treatments with unconditioned tiles with FSW and unconditioned tiles with DMSO recruits were excluded from percent survival analysis because of low recruitment rates. N = 35-42 tiles per treatment.

The mean number of recruits per tile after 6 months was negligible for unconditioned tiles with either untreated (FSW, 0.1 ± 0.5 recruits) or DMSO treated (0 recruits) larvae (Figure 12). However, unconditioned tiles with TBP treated larvae had a mean of 2.8 ± 0.4 recruits per tile. This was significantly higher than conditioned tiles with TBP treated larvae (1.3 ± 0.2 recruits per tile, $p = 0.043$) and not significantly different from conditioned tiles with untreated larvae ($p = 0.671$; emmeans).

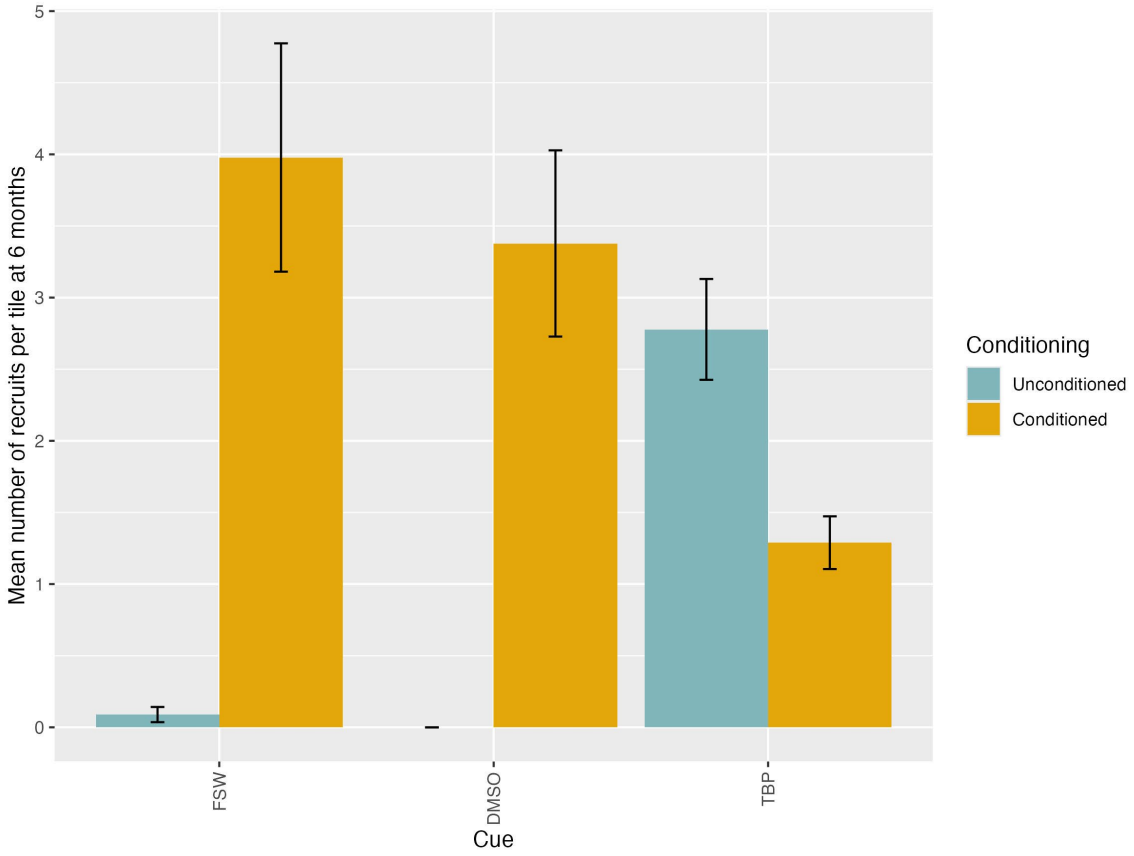

Figure 12. Mean ± standard error number of remaining *P. strigosa* recruits per tile after 6 months. Larvae were exposed to one of three chemical cue treatments, untreated (FSW), solvent control (DMSO) or 250 nM tetrabromopyrrole (TBP) prior to settlement and given either unconditioned or conditioned tiles as settlement substrate. N = 45 tiles per treatment.

3.2. Task 3: Characterizing chemical cues for different species of coral larvae

3.2.1. Aquarium Seawater Bioassays

A total of 37 settlement bioassays were conducted during this reporting period using larvae from 6 coral species. Bioassays evaluated settlement responses to aquarium seawater, seawater eluates, and subsequent fractions and subfractions to support the characterization of coral larval settlement cues. All figures depict settlement data recorded after 48 hours.

Settlement responses of *Colpophyllia natans* and *Pseudodiploria strigosa* larvae were evaluated with seawater collected from multiple locations within the Smithsonian Marine Ecosystems Exhibit (SMEE), including the unfiltered and sterile filtered (F) deep refuge tank (DR/FDR), areas adjacent to *Acropora cervicornis* (Acer/FAcer), *Acropora palmata* (Apal/FApal), *Palythoa caribaeorum* (Paly/FPaly), and *Porites porites* (Ppor/FPpor), as well as seawater collected from dishes containing the crustose coralline alga *Hydrolithon*

boergesenii (FCCA). Settlement responses differed among seawater sources, with several seawater samples inducing settlement in both *C. natans* (Figure 13) and *P. strigosa* larvae (Figure 14). Based on these results and because these seawater types were easily obtained, FDR, FAcer, FApal, and FCCA seawater samples were selected for extraction and further characterization.

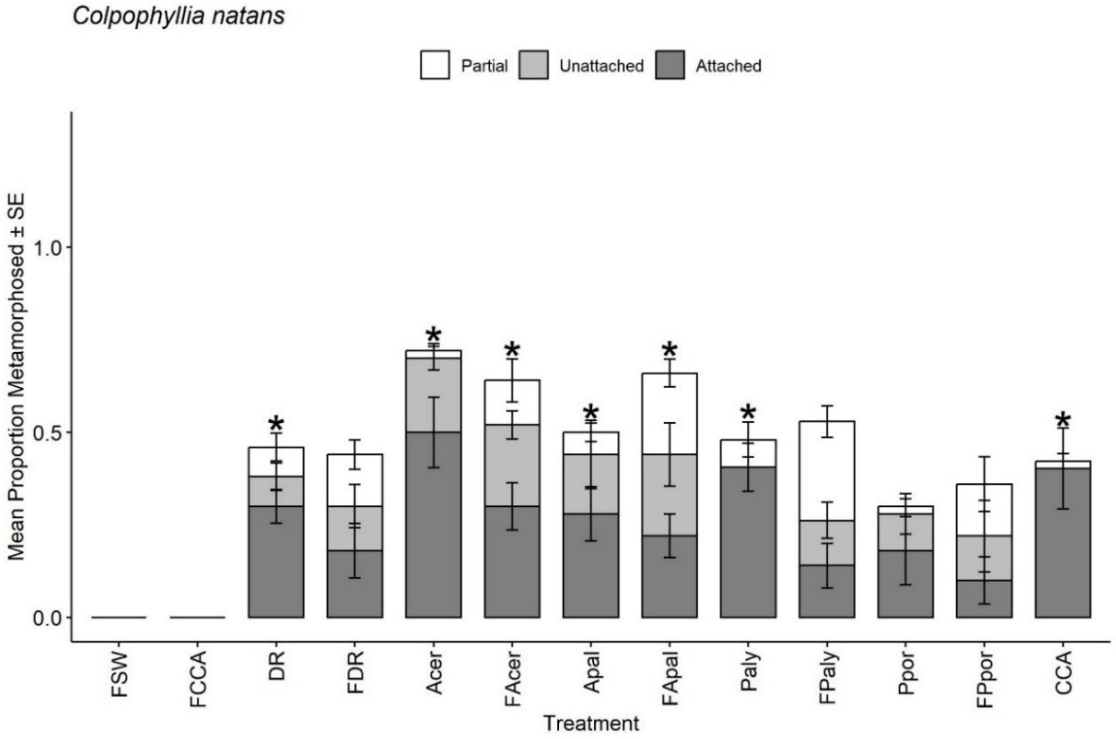

Figure 13. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae in FCCA and aquarium seawater collected from multiple locations within the coral reef exhibit system at SMEE. Unfiltered and sterile filtered (F) seawater of each type was tested. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the FSW negative control ($p < 0.05$).

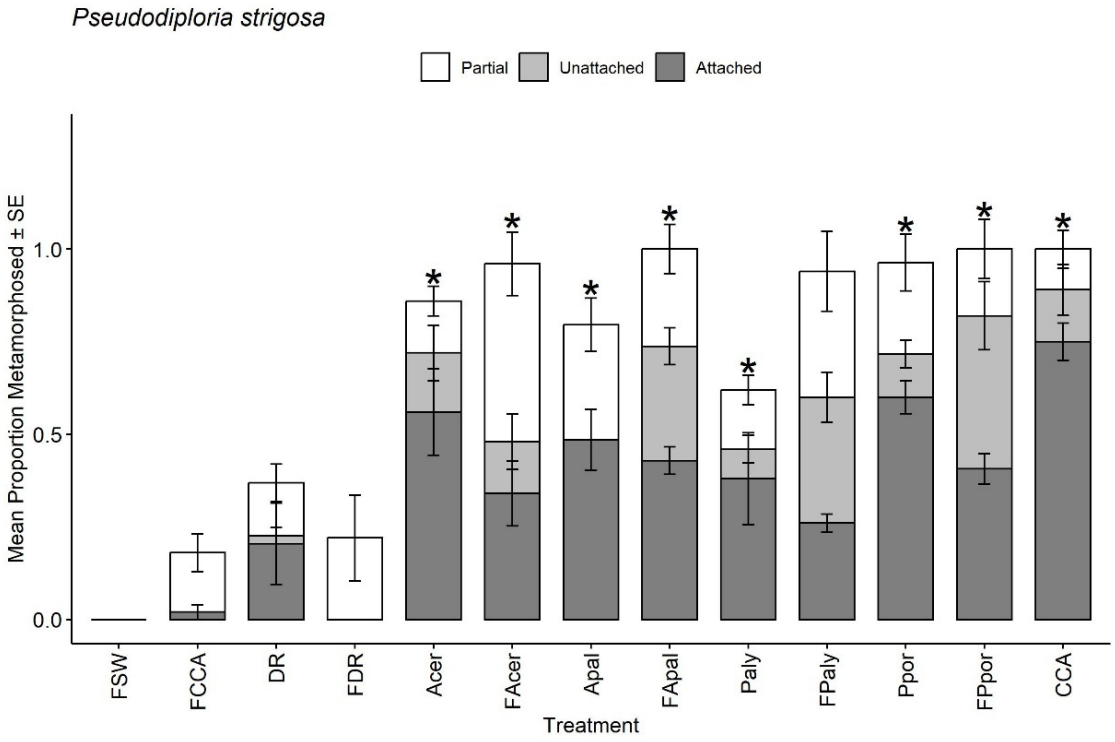

Figure 14. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae in FCCA and aquarium seawater collected from multiple locations within the coral reef exhibit system at SMEE. Both the unfiltered and sterile filtered (F) seawater of each type was tested. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the FSW negative control ($p < 0.05$).

3.2.2. Seawater Eluate Bioassays

Eluates from solid-phase extraction (10g C18 columns) prepared from active seawater samples were subsequently tested with *C. natans* and *P. strigosa* larvae. Several seawater eluates induced settlement and metamorphosis in both species. Among the eluates tested, FDR eluates consistently produced high settlement responses. For *C. natans*, FDR eluate 1CLD33 induced a mean settlement proportion of 0.24 ± 0.07, which was significantly greater than the negative control ($p = 0.002$). FAcer eluate 1CLD34 also produced a mean settlement proportion of 0.18 ± 0.04 in *C. natans*, which was significantly greater than the negative control ($p = 0.005$) (Figure 15). Based on these results, FDR and FAcer eluates were selected for further fractionation and characterization.

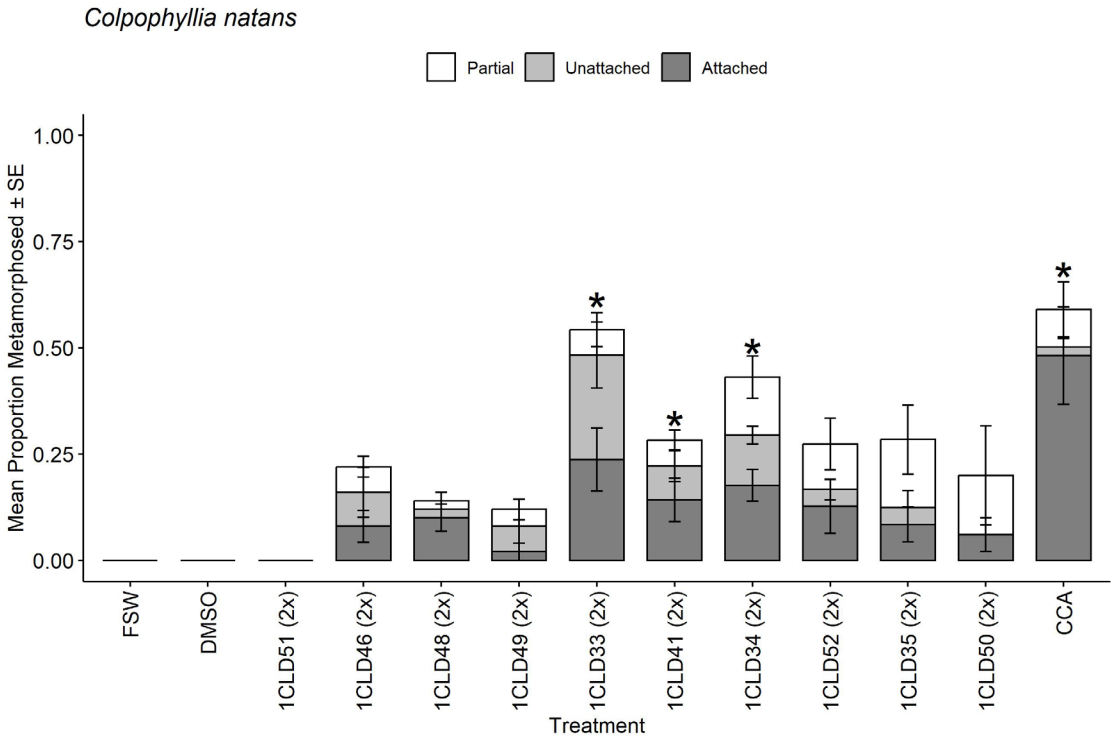

Figure 15. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to seawater eluates. The treatments include sterile filtered seawater (FSW); solvent control (DMSO) 1µg/mL; eluates are coded as follows:1CLD51 is a blank FSW eluate; 1CLD46, 48, and 49 are FCCA eluates; 1CLD33 and 41 are FDR eluates; 1CLD34 and 52 are FAcer eluates; 1CLD35 and 50 are FApal eluates. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

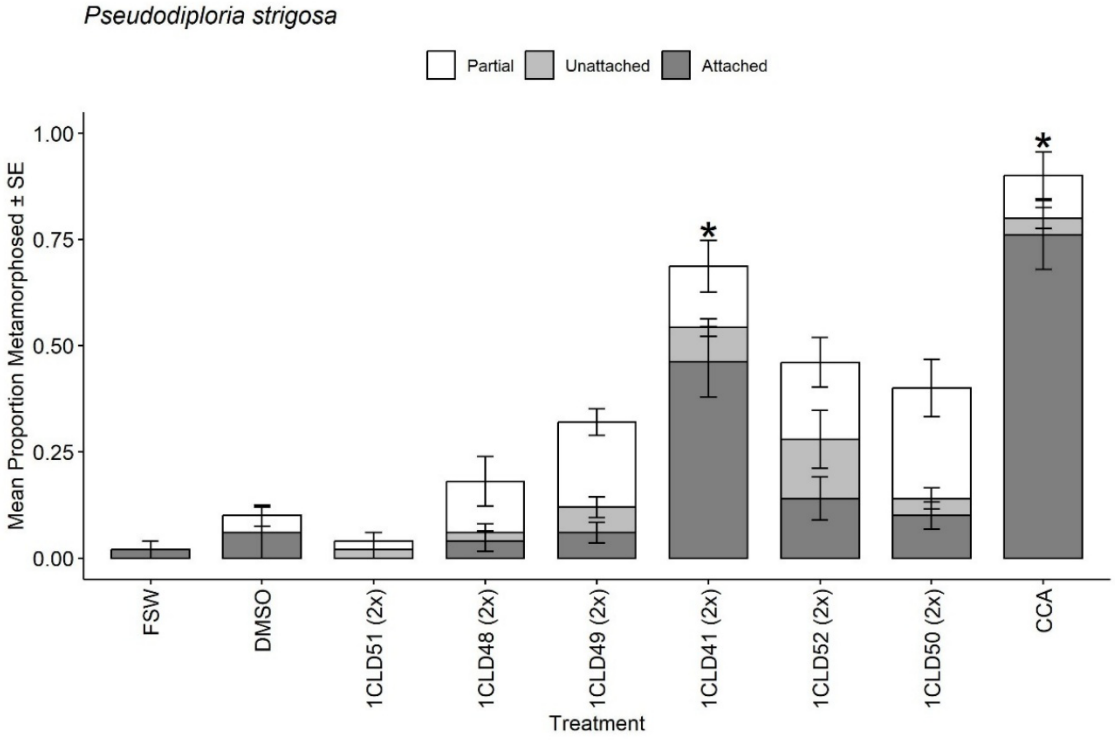

Figure 16. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae exposed to seawater eluates. The treatments include sterile filtered seawater (FSW); solvent control (DMSO) 1µg/mL; eluates are coded as follows:1CLD51 is a blank FSW eluate; 1CLD48 and 49 are FCCA eluates; 1CLD41 is a FDR eluate; 1CLD52 is a FAcer eluate; 1CLD50 is a FApal eluate. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

3.2.3. FDR Fraction Bioassays

Since FDR eluates consistently induced high settlement responses, subsequent characterization efforts focused primarily on FDR. Two independent batches of FDR eluate were fractionated using C18 chromatography to generate five fractions per eluate (1CLD38-1 through 1CLD38-5 and 1CLD43-1 through 1CLD43-5). The 1CLD38 series was prepared from 3.6 L of FDR seawater, whereas the 1CLD43 series was prepared from 10 L of FDR seawater. Fractions from each series were subsequently tested with *C. natans* and *P. strigosa* larvae. The solvent systems used for C18 fractionation of the FDR eluates are summarized in Table 2.

Table 2. Solvent system for 1CLD38 and 1CLD43 fractionation.

Fraction #	1CLD38 (3.6L) Solvent	1CLD43 (10L) Solvent
1	75% H_2O - 25% MeOH	80% H_2O - 20% MeOH
2	50% H_2O - 50% MeOH	60% H_2O - 40% MeOH
3	25% H_2O - 75% MeOH	40% H_2O - 60% MeOH
4	100% MeOH	20% H_2O - 80% MeOH
5	100% EtOAc	100% MeOH

Similar patterns were observed in both fractionation series, with settlement activity concentrated within fractions 1 through 3 and highest in fraction 2 (Figures 17 and 18). For *C. natans*, significant settlement responses were observed in fractions 1CLD38-2 (0.26 ± 0.098, $p = 0.03$), 1CLD43-1 (0.19 ± 0.09, $p = 0.04$), and 1CLD43-2 (0.28 ± 0.07, $p = 0.02$).

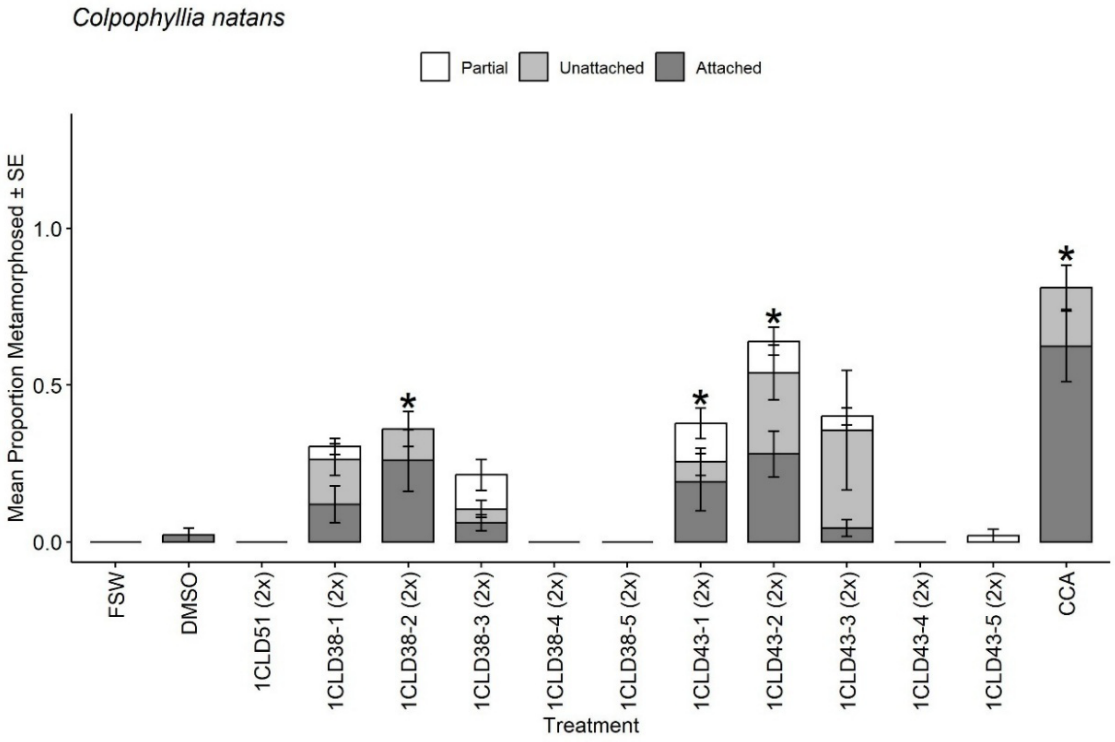

Figure 17. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to fractions derived from two independent FDR eluate preparations. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

Similarly, *P. strigosa* exhibited significant settlement responses to fractions 1CLD38-2 (0.64 ± 0.08, $p = 0.0004$), 1CLD38-3 (0.36 ± 0.04, $p = 0.009$), 1CLD43-2 (0.53 ± 0.08, $p = 0.001$), and 1CLD43-3 (0.36 ± 0.098, $p = 0.01$). Interestingly, fraction 3 was more active for *P. strigosa* larvae than for *C. natans* larvae. The consistent activity observed across two separately prepared FDR eluates supported continued characterization through additional fractionation.

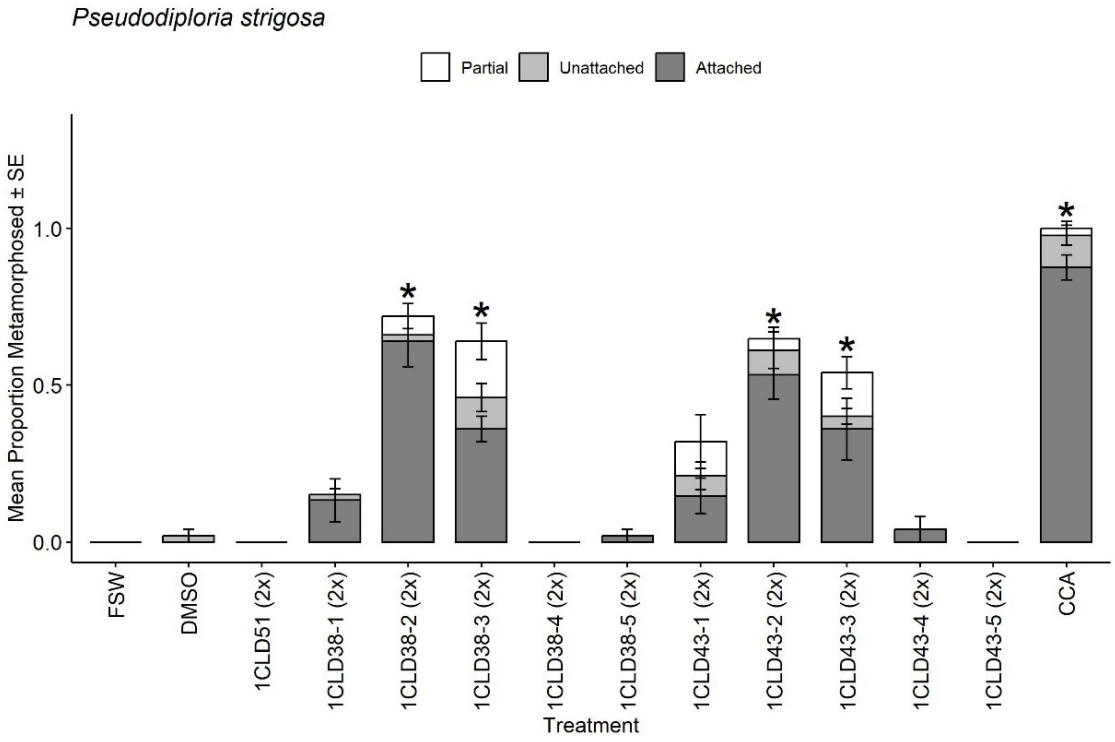

Figure 18. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae exposed to fractions derived from two independent FDR eluate preparations. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

3.2.4. FDR Subfraction Bioassays

Active parent fraction 1 from the 1CLD43 FDR fractionation series above was further separated into five subfractions and evaluated in settlement bioassays (Table 4).

Table 3. Solvent system for 1CLD43-1 subfractions.

Fraction ID	Solvent
1CLD43-1-1	80% H_2O - 20% MeOH
1CLD43-1-2	60% H_2O - 40% MeOH
1CLD43-1-3	40% H_2O - 60% MeOH
1CLD43-1-4	20% H_2O - 80% MeOH
1CLD43-1-5	100% MeOH

Settlement responses of *C. natans* larvae were concentrated within subfractions 1CLD43-1-1 and 1CLD43-1-2, whereas responses to the remaining subfractions were comparatively low (Figure 19). In contrast, settlement responses of *P. strigosa* larvae were low across all subfractions from fraction 1 (Figure 20), but fraction 1 was not very active for *P. strigosa* larvae (Figure 18). Subfractions 1CLD43-1-1 and 1CLD43-1-2 were selected for further characterization by HPLC fractionation.

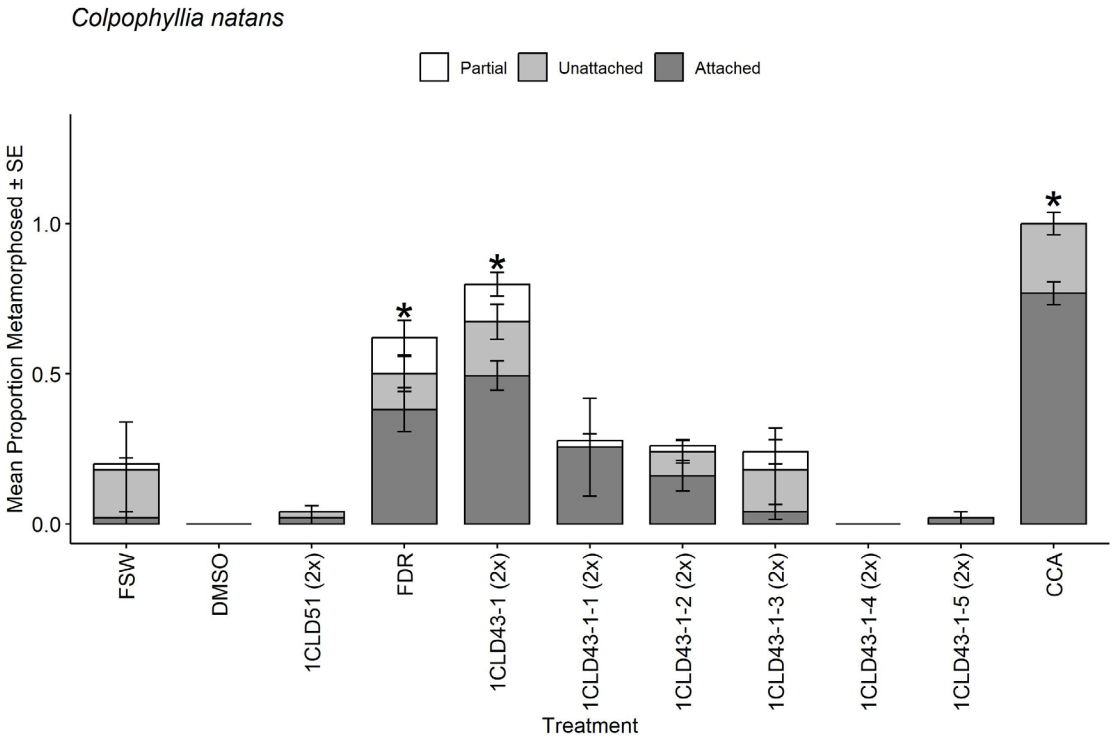

Figure 19. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to subfractions derived from parent fraction 1CLD43-1. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

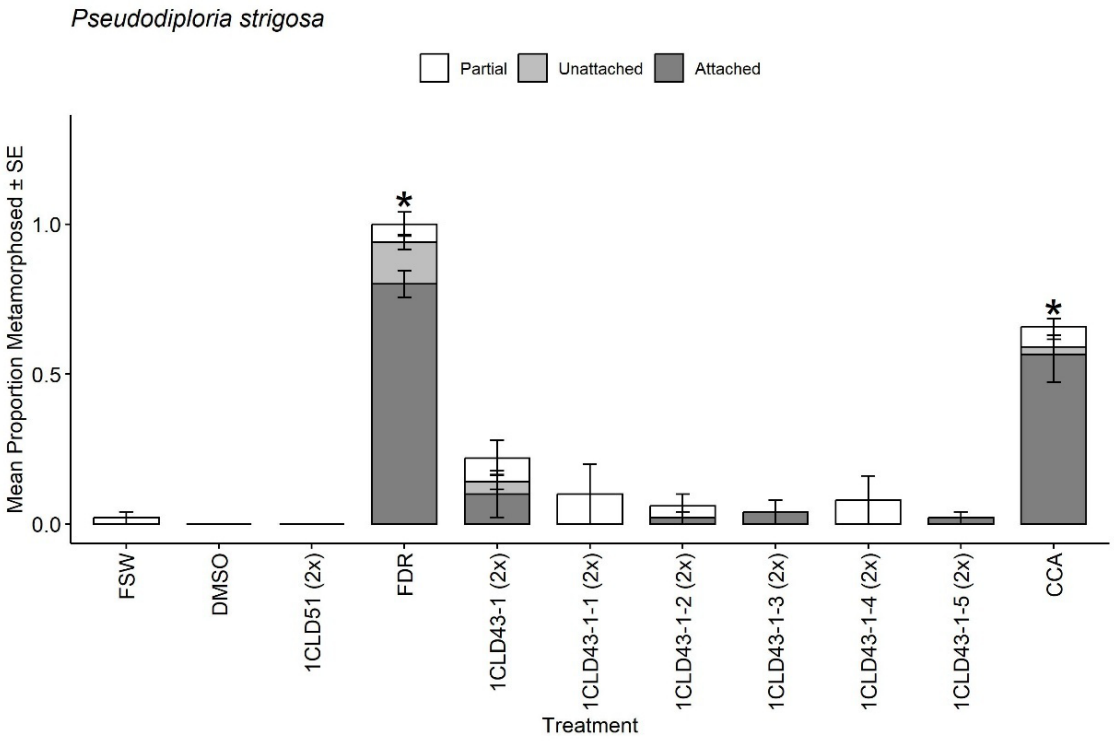

Figure 20. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae exposed to subfractions derived from parent fraction 1CLD43-1. Asterisks

indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

Active parent fraction 2 from the 1CLD43 FDR fractionation series was also further separated into five subfractions and evaluated in settlement bioassays (Table 4).

Table 4. Solvent system for 1CLD43-2 subfractions.

Fraction ID	Solvent
1CLD43-2-1	80% H_2O - 20% MeOH
1CLD43-2-2	60% H_2O - 40% MeOH
1CLD43-2-3	40% H_2O - 60% MeOH
1CLD43-2-4	20% H_2O - 80% MeOH
1CLD43-2-5	100% MeOH

The parent fraction, 1CLD43-2, continued to induce significant settlement in both *C. natans* and *P. strigosa* larvae (Figures 9 and 10). Following subfractionation, settlement responses were reduced relative to the parent fraction. None of the resulting subfractions induced settlement comparable to the parent fraction. However, low levels of settlement and metamorphosis were observed in subfraction 1CLD43-2-2 in both species. Based on these results, subfraction 1CLD43-2-2 was selected for further separation by HPLC.

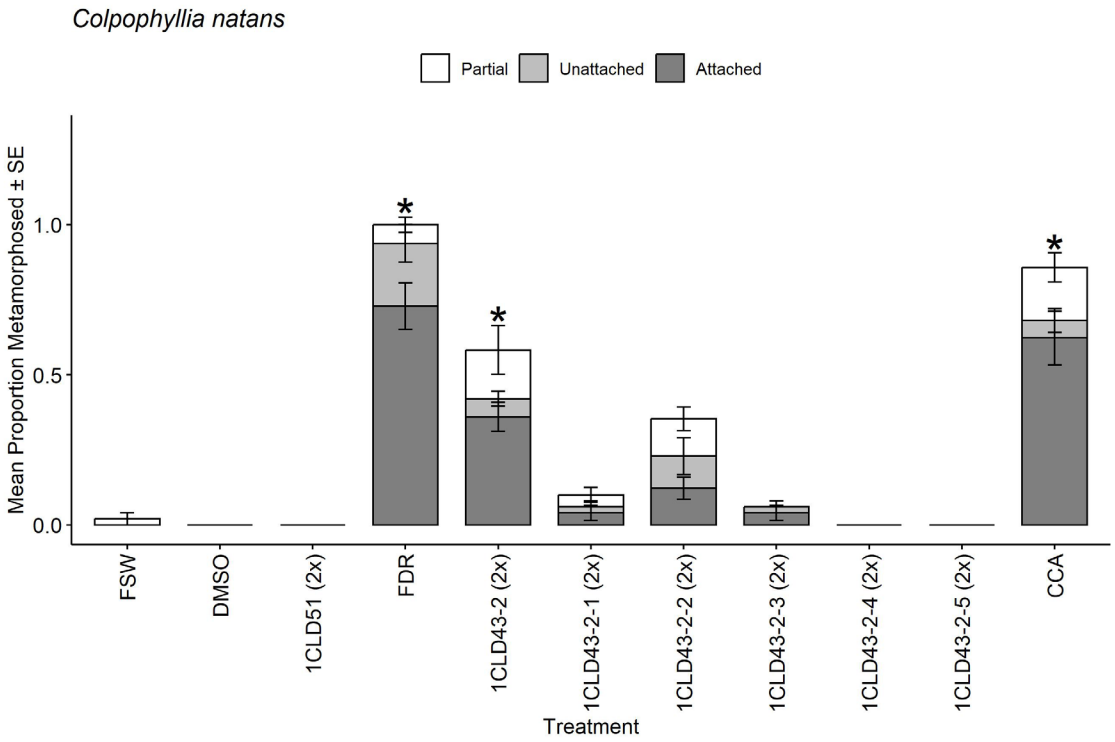

Figure 21. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to subfractions derived from parent fraction 1CLD43-2. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

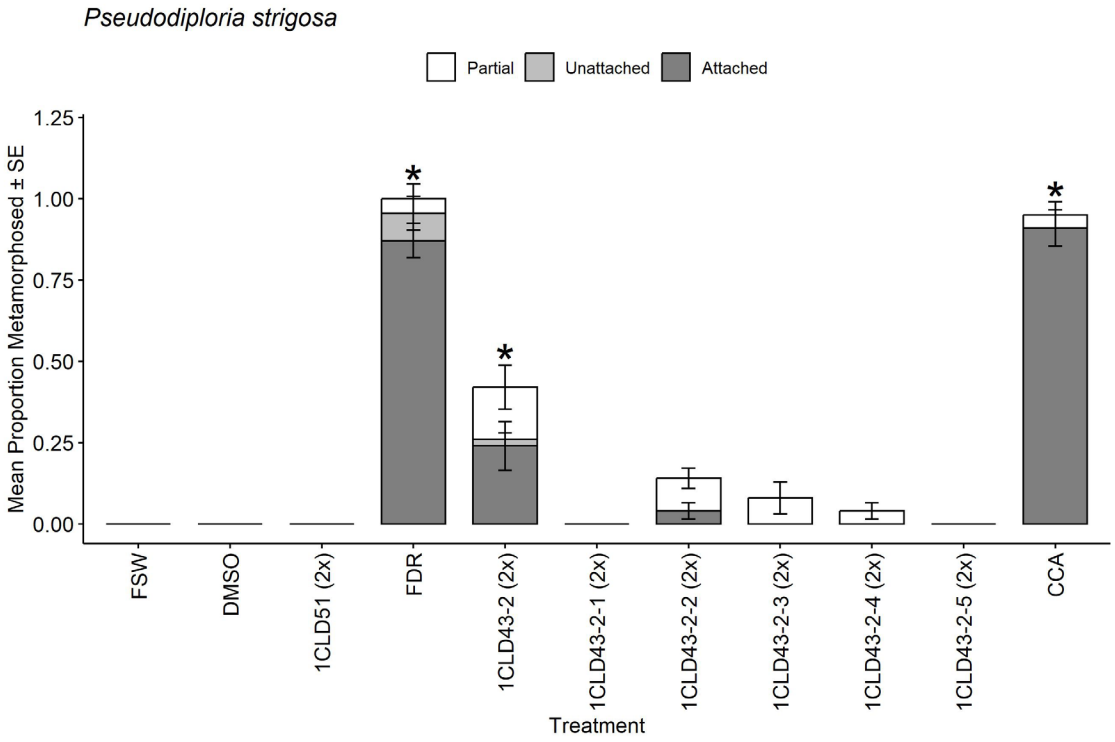

Figure 22. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae exposed to subfractions derived from parent fraction 1CLD43-2. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

3.2.5. HPLC Fractionation of Active FDR Subfractions

To further characterize active samples, subfraction 1CLD43-1-1 was subjected to HPLC separation and the resulting fractions were evaluated in settlement bioassays. For *C. natans*, HPLC fraction 1CLD43-1-1-1 induced a mean settlement proportion of 0.52 ± 0.13, which was significantly greater than the negative control ($p = 0.01$; Figure 23). The remaining HPLC fractions induced little or no settlement. In contrast, none of the HPLC fractions derived from subfraction 1CLD43-1-1 induced significant settlement in *P. strigosa* larvae (Figure 24) although some activity was observed in several fractions.

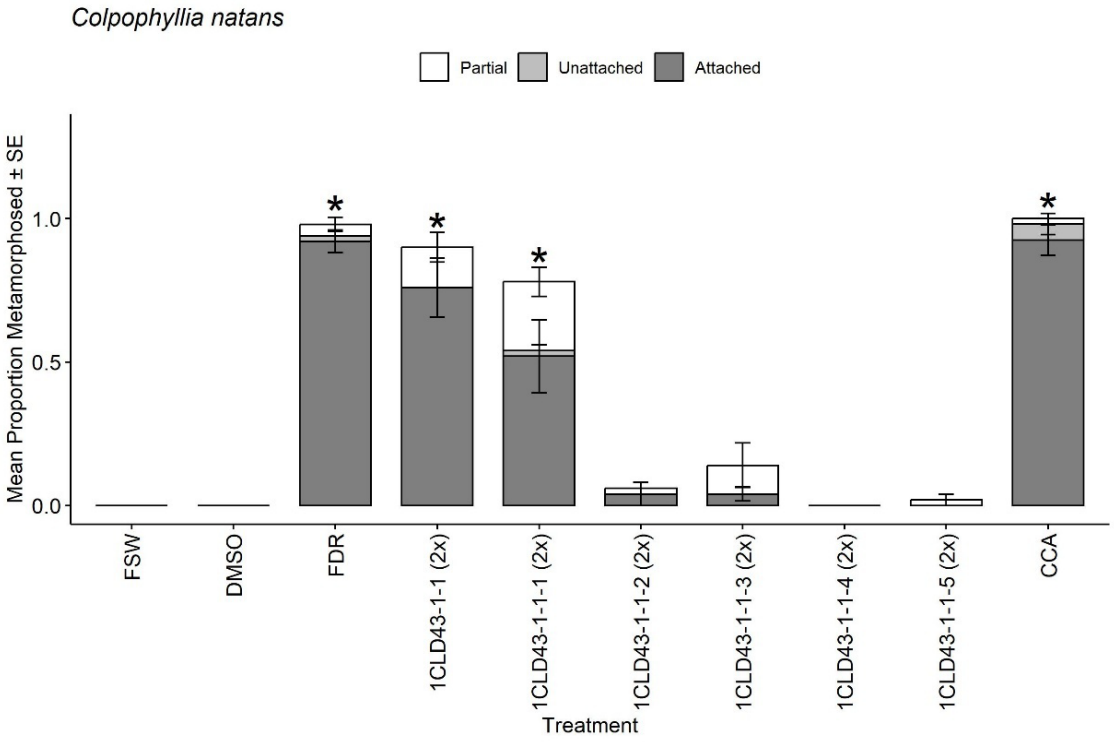

Figure 23. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to HPLC fractions derived from subfraction 1CLD43-1-1. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

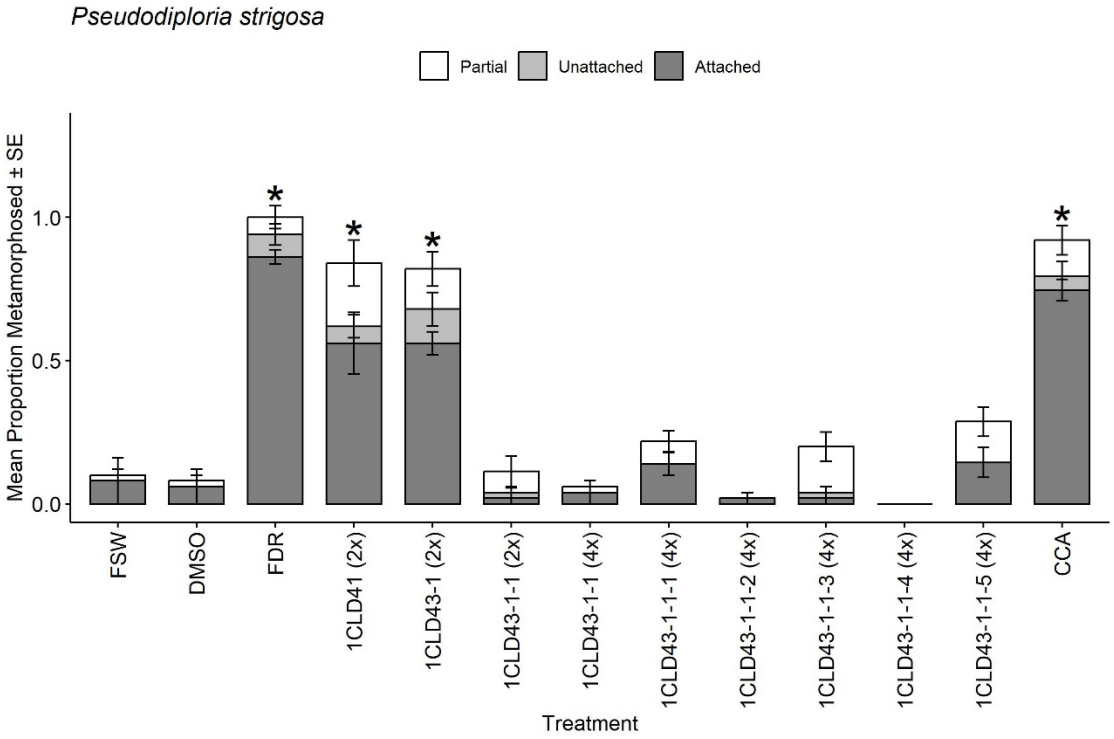

Figure 24. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae exposed to HPLC fractions derived from subfraction 1CLD43-1-1.

Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

Subfraction 1CLD43-1-2 was also subjected to HPLC separation and the resulting fractions were evaluated in settlement bioassays. Although the parent subfraction induced settlement, the resulting HPLC fractions produced comparatively low settlement responses in both *C. natans* and *P. strigosa* larvae. Similar response patterns were observed when fractions were tested at both 2x and 4x natural concentrations.

HPLC fractionation of subfraction 1CLD43-2-2 revealed species-specific settlement responses. For *C. natans*, settlement responses to the resulting HPLC fractions were generally low, and none induced significant settlement (Figure 25). In contrast, several HPLC fractions induced significant settlement in *P. strigosa*, including 1CLD43-2-2-3 (0.44 ± 0.04, $p = 0.001$), 1CLD43-2-2-4 (0.28 ± 0.02, $p = 0.01$), and 1CLD43-2-2-5 (0.38 ± 0.02, $p = 0.002$) (Figure 26). Further characterization of the active fractions is planned by liquid chromatography-high resolution mass spectrometry (HRLCMS).

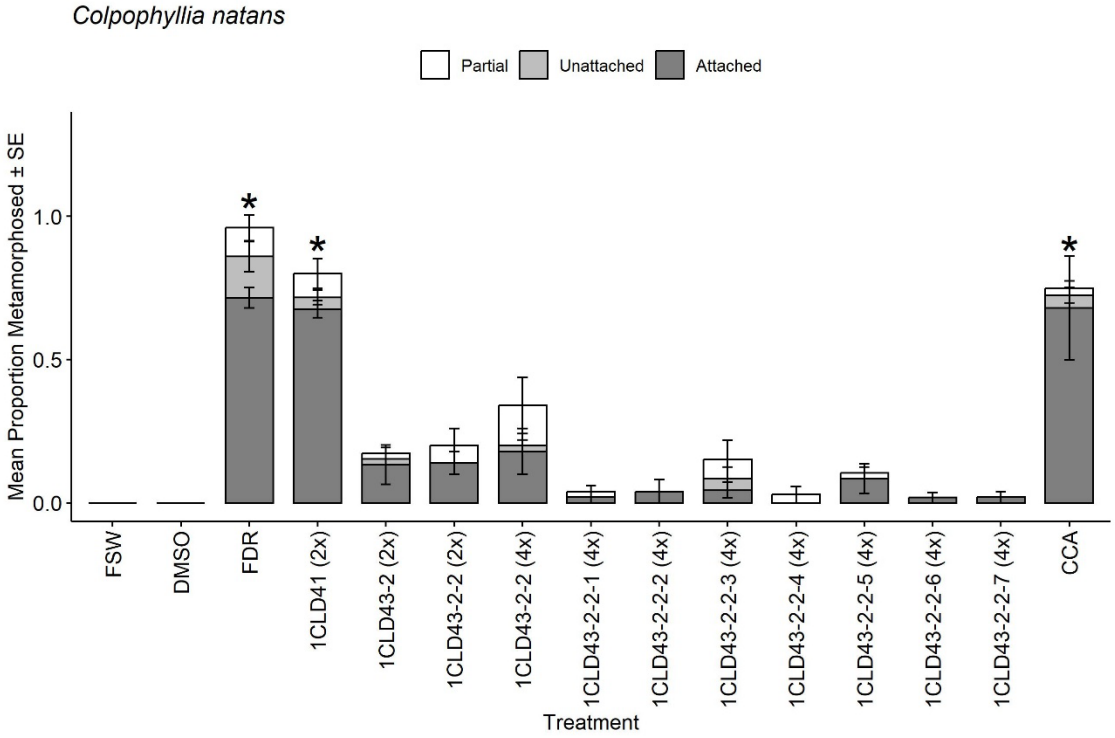

Figure 25. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to HPLC fractions derived from subfraction 1CLD43-2-2. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

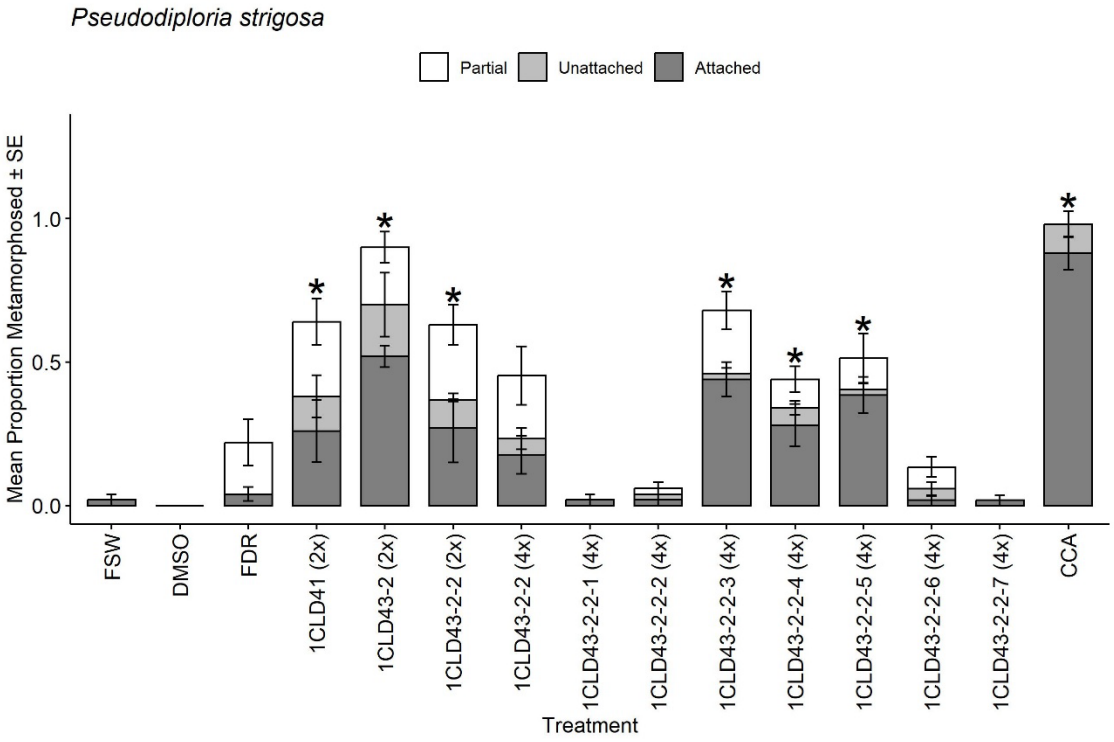

Figure 26. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. strigosa* larvae exposed to HPLC fractions derived from subfraction 1CLD43-2-2. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

3.2.6. Fractionation of FAcer Eluates

Because FAcer eluates induced significant settlement responses in *C. natans* larvae, a parallel characterization effort was conducted using FAcer fractions (Table 5).

Table 5. Solvent system for 1CLD44 fractionation.

Fraction ID	Solvent
1CLD44-1	80% H_2O - 20% MeOH
1CLD44-2	60% H_2O - 40% MeOH
1CLD44-3	40% H_2O - 60% MeOH
1CLD44-4	20% H_2O - 80% MeOH
1CLD44-5	100% MeOH

Fractionation of the FAcer eluate identified fractions 1CLD44-1 and 1CLD44-2 as the most active fractions. Fraction 1CLD44-1 induced a mean settlement proportion of 0.59 ± 0.06, which was significantly greater than the negative control ($p = 0.01$) and comparable to FAcer eluate 1CLD52, while fraction 1CLD44-2 induced a mean settlement proportion of 0.35 ± 0.13 ($p = 0.046$) (Figure 27). Based on these results, fractions 1CLD44-1 and 1CLD44-2 were selected for further separation and characterization.

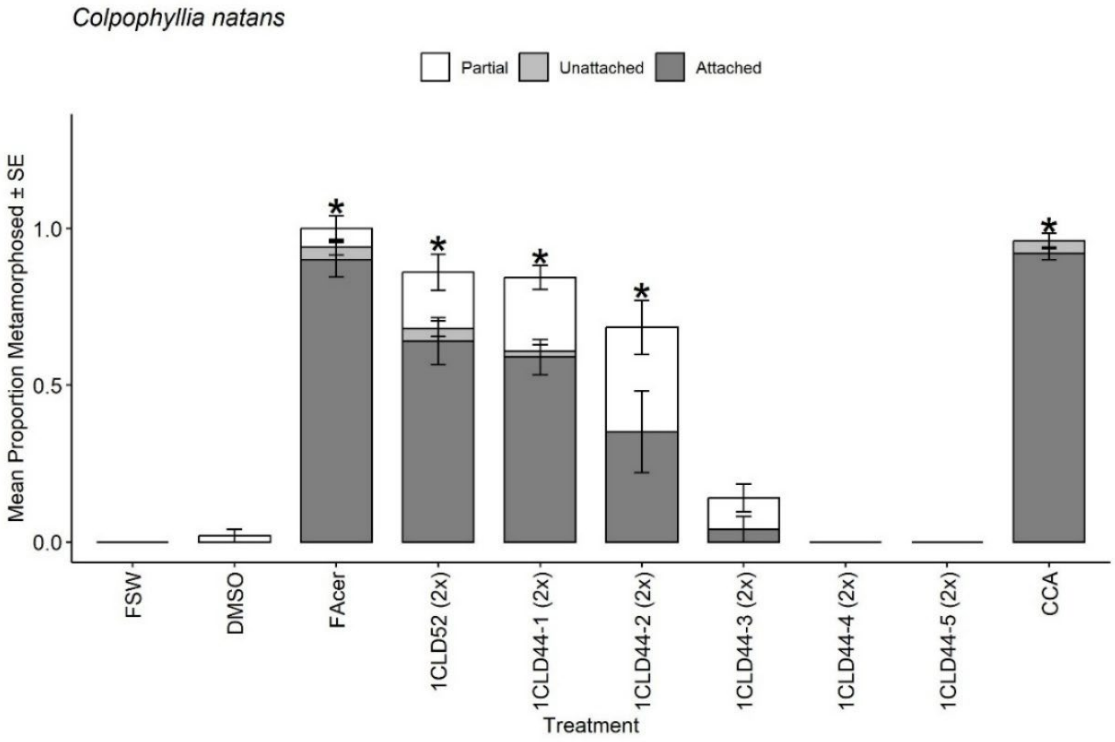

Figure 27. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to FAcer fractions. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

Fractions 1CLD44-1 and 1CLD44-2 (1CLD44-1+2) were combined and further separated into five subfractions (Table 6).

Table 6. Solvent system for 1CLD44-1+2 subfractions.

Fraction ID	Solvent
1CLD44-1+2-1	90% H_2O - 10% MeOH
1CLD44-1+2-2	80% H_2O - 20% MeOH
1CLD44-1+2-3	50% H_2O - 50% MeOH
1CLD44-1+2-4	100% MeOH

The combined fraction 1CLD44-1+2 was more active than either one alone. For *C. natans*, subfraction 1CLD44-1+2-1 induced a mean settlement proportion of 0.30 ± 0.10, which was significantly greater than the negative control ($p = 0.03$), whereas settlement responses in the remaining subfractions were lower (Figure 28). Based on these results, subfraction 1CLD44-1+2-1 was selected for continued characterization. Further characterization of the active fractions is planned by liquid chromatography-high resolution mass spectrometry (HRLCMS).

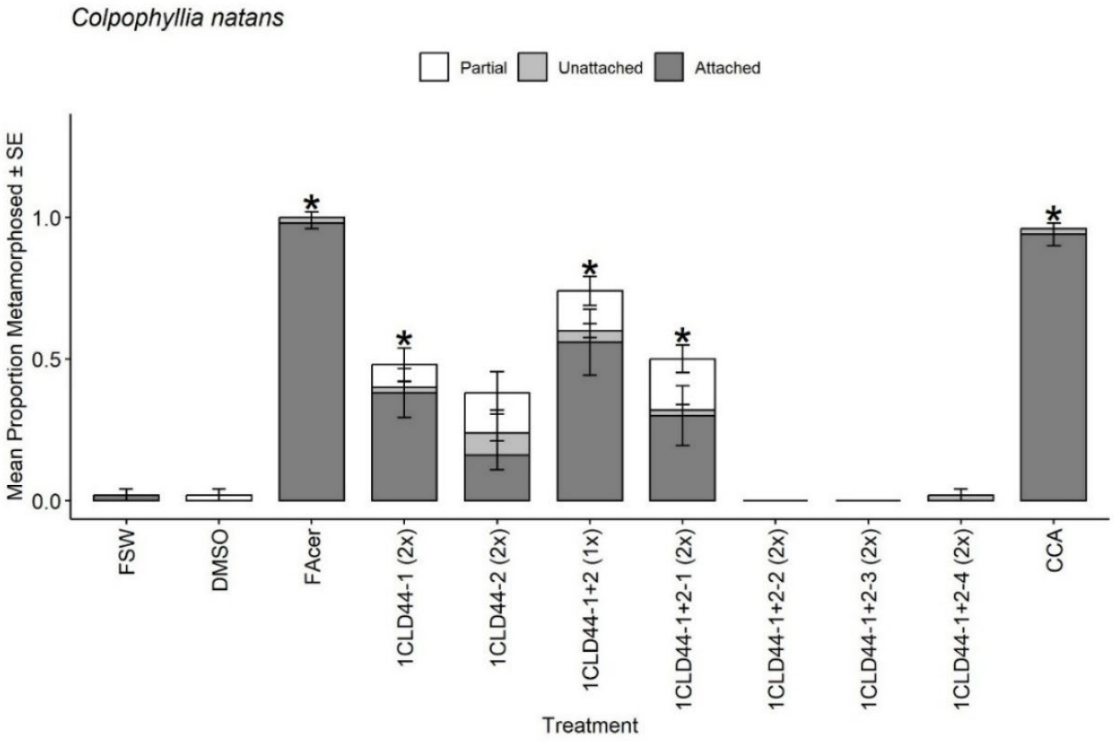

Figure 28. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *C. natans* larvae exposed to FAcer subfractions. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

3.2.7. Responses of Additional Coral Species

Settlement responses of additional coral species were evaluated using selected seawater samples. *Diploria labyrinthiformis* larvae exhibited settlement responses to both FCCA and FDR seawater treatments, with significant settlement observed in FDR (Figure 29). Settlement responses to FCCA were also elevated relative to the negative control, although differences were not statistically significant. In separate assays, settlement responses to seawater eluates were less consistent and require further investigation.

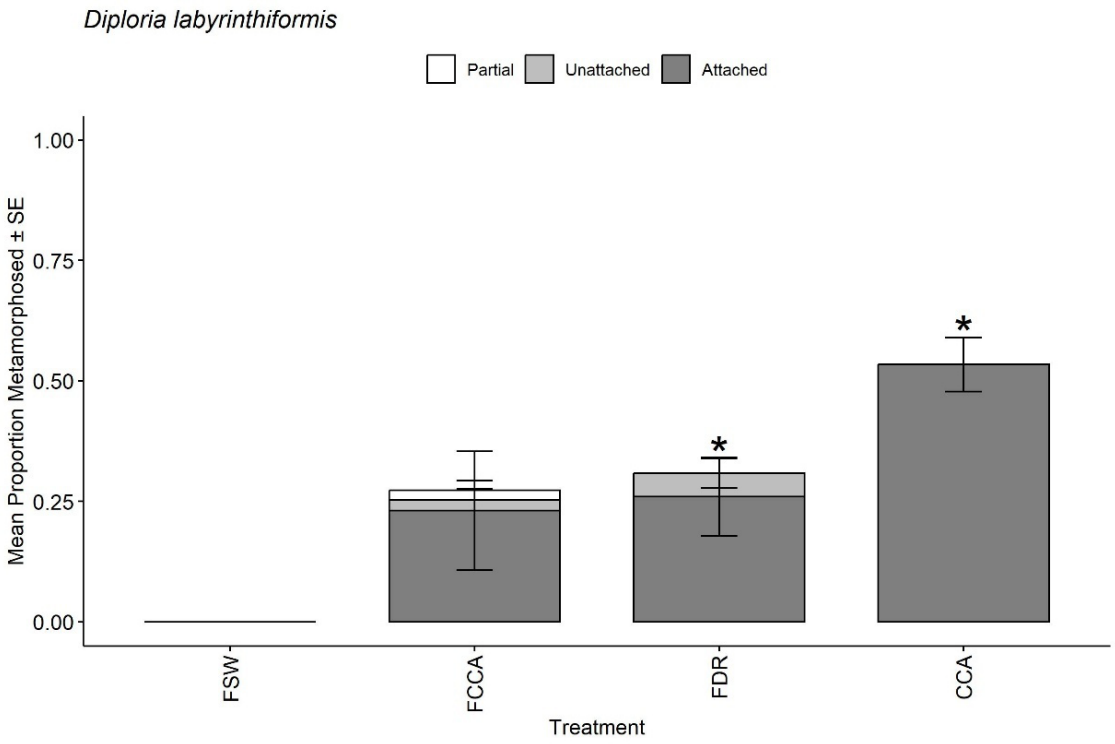

Figure 29. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *D. labyrinthiformis* larvae exposed to FCCA and FDR. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the DMSO negative control ($p < 0.05$).

Acropora cervicornis larvae responded to several unfiltered aquarium seawater treatments but exhibited little or no response to the corresponding filtered seawater samples (Figure 30). Additional testing was conducted using larger filter pore sizes to evaluate whether settlement responses were associated with particulate components removed during filtration. No consistent trend was observed.

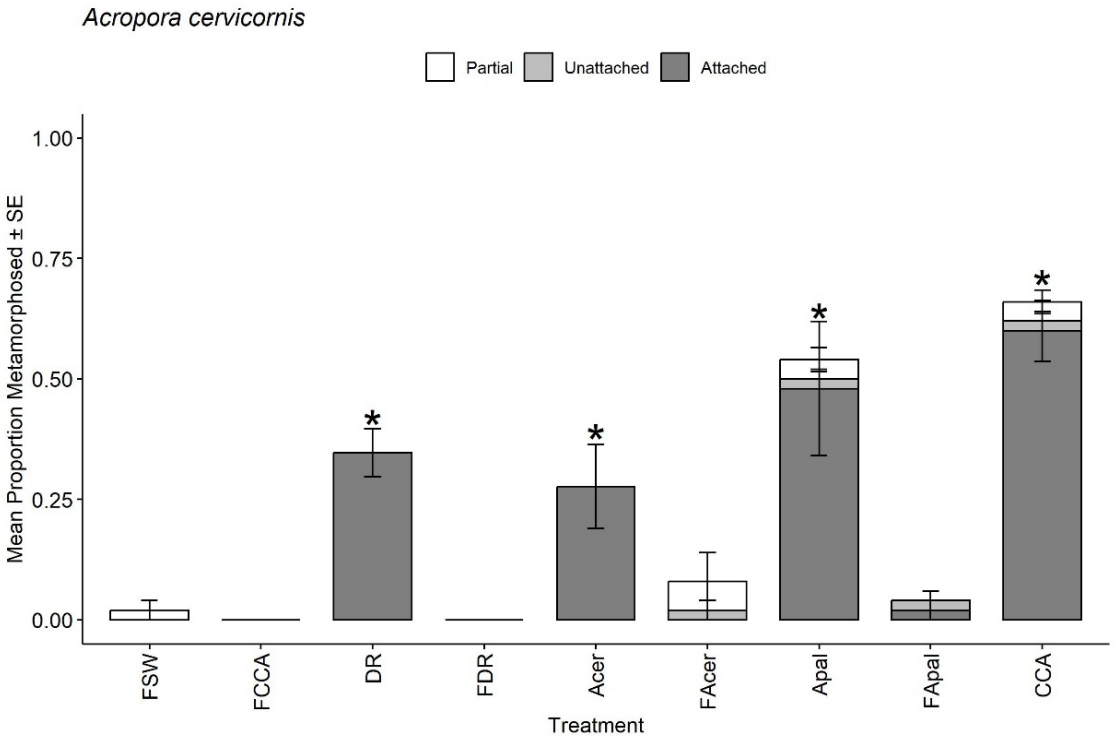

Figure 30. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *A. cervicornis* larvae in FCCA and aquarium seawater collected from multiple locations within the coral reef exhibit system at SMEE. Asterisks indicate significant metamorphosis of attached larvae (settled; dark grey) compared to the FSW negative control ($p < 0.05$).

Porites astreoides larvae exhibited little to no response to either filtered or unfiltered deep refuge seawater during this reporting period (Figure 31).

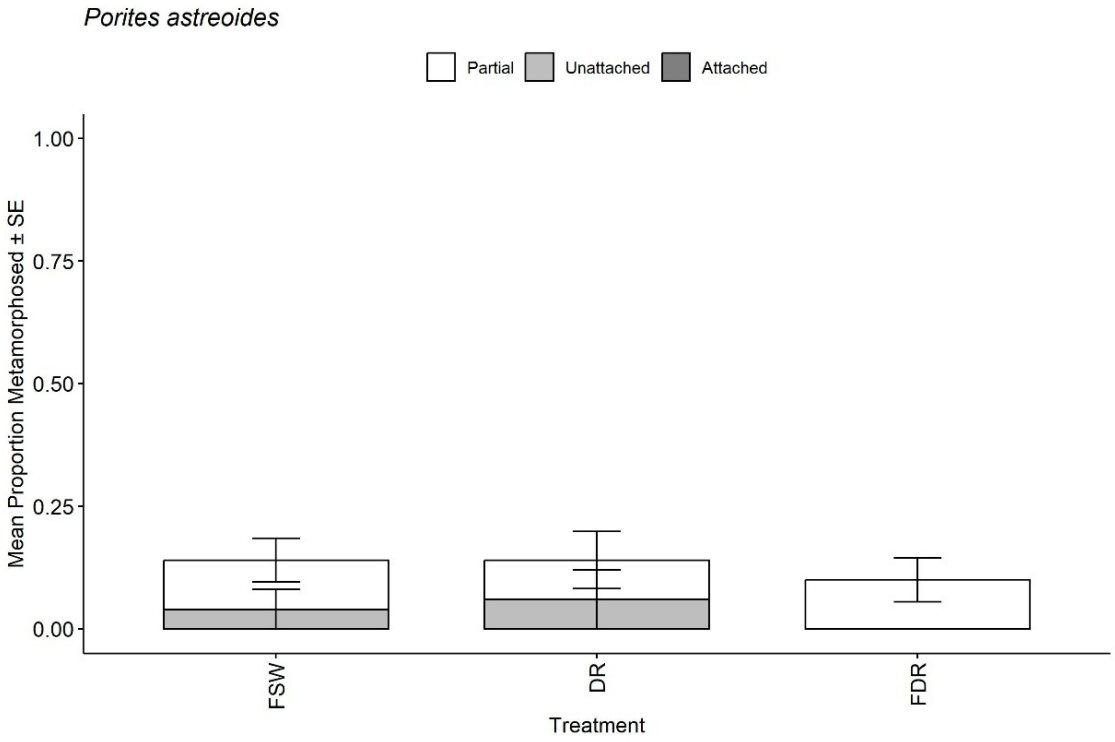

Figure 31. Mean proportions of attached (dark grey), unattached (light grey), and partially (white) metamorphosed *P. astreoides* larvae in DR and FDR.

4. DISCUSSION AND MANAGEMENT RECOMMENDATIONS

Successful coral restoration depends not only on larval settlement but also on post-settlement survival. This study aimed to identify chemical cues that increase coral settlement and assess their applicability in reef restoration.

Task 2 examined the effects of settlement substrate conditioning coupled with chemical settlement cues on the microbiomes of larvae, new recruits, and juveniles of *C. natans* and *P. strigosa*. Effects on recruitment (settlement and survival to one month) and recruit survival at six months were also monitored. The results above indicate that both substrate conditioning and chemical cues influenced recruitment and survival through six months. While settlement cues affected one-month recruitment patterns, it appears that tile conditioning had a stronger influence on post-settlement survival. For *C. natans*, there was a higher percent recruitment at the one-month time point on conditioned versus unconditioned tiles when looking at larvae that were settled with either DMSO or FSW. However, those that were settled on unconditioned tiles were 30% more likely to survive six months than those settled on conditioned tiles when settled with DMSO or FSW. The same pattern was seen in *P. strigosa* where larvae were generally more likely to settle on conditioned tiles than unconditioned tiles but at six months, no matter the chemical cue used during settlement, recruits were more likely to have survived on unconditioned tiles than conditioned. This pattern could be a result of individuals on conditioned tiles experiencing more competition than those on unconditioned tiles. Newly settled coral recruits often need to compete for space on a conditioned tile with surrounding encrusting

algae and with other corals that settled close by (Vermeij, M. J. A. & Sandin, S. A, 2008). This competition may lead to mortality at early life stages that is not seen when larvae are able to settle on a clean, unconditioned tile.

Exposing larvae to TBP before settlement allows restoration practitioners to use unconditioned tiles instead of conditioned ones as larvae will reliably settle without the presence of other settlement cues from microbial biofilms or crustose coralline algae (CCA) (Sneed et al., 2014). In addition to having increased percent survival, using unconditioned tiles will also decrease the labor time and cost that goes into conditioning substrates and cleaning tiles after post settlement. TBP effects on survival could not be assessed for *C. natans* due to low recruitment numbers, however TBP treated *P. strigosa* had final recruit numbers on unconditioned tiles comparable to untreated larvae on conditioned tiles, which represents the current standard operating procedure. Settling corals with TBP will reliably produce settlement levels on unconditioned tiles that will allow for successful restoration, while avoiding the costs and variability associated with substrate conditioning. It is important, however, to optimize the concentration of TBP when it is being used in systems like TFA where there are likely additional waterborne settlement cues in the settlement systems. Most of the *C. natans* larvae that were exposed to TBP settled on the settlement bins themselves instead of the settlement tiles. Based on this experiment and other observations made when using TBP, we hypothesize that the presence of additional waterborne cues can lead to high levels of unspecific recruitment. This is something currently being investigated at SMSFP.

Given the effectiveness of TBP, it is important to understand the potential impacts it has on recruitment and juvenile microbiomes. Understanding this will allow restoration practitioners to make more informed decisions on how to best apply this method to the coral restoration pipeline. While the microbiome data are still being processed, it is not expected that the use of TBP will have an impact. This is because the larvae were only exposed to the chemical for one hour and were subsequently rinsed before being added to the settlement bins. The use of TBP has potential to substantially improve propagation success, efficiency, and overall production output. It is important to optimize its usage and continue studying any post settlement effects to use this tool for reef restoration at its highest potential.

While TBP serves as a promising candidate for the chemical induction of settlement, we continue to explore the presence of other natural settlement cues as well. This will provide additional information on environmental conditions on reefs that may facilitate settlement in situ and additional methods for inducing larval settlement for restoration. Task 3 focused on identifying waterborne chemical cues from reef environments and determining their effects on the settlement of different coral species. This research provides strong evidence that waterborne chemical cues play an important role in larval settlement across multiple coral species. Settlement responses were observed in several spawning species of coral larvae exposed to aquarium seawater collected from the Smithsonian Marine Ecosystems Exhibit (SMEE), seawater associated with the crustose coralline alga *Hydrolithon boergesenii* (FCCA), and eluates prepared from these seawater sources. The findings align with previous studies demonstrating that environmental cues

influence metamorphosis and settlement (Sneed et al. 2014, 2024; Ritson-Williams et al. 2016; Petersen et al. 2023; Randall et al. 2024). However, our results differ from prior studies by demonstrating that dissolved compounds retained from aquarium seawater can serve as positive settlement cues in the absence of added substrates. Aquarium seawater from a biodiverse reef exhibit repeatedly induced settlement in multiple coral species, highlighting the importance of species-rich environments as sources of effective settlement cues.

FCCA and several types of filter-sterilized aquarium seawater from SMEE retained settlement-inducing activity for multiple species, suggesting that dissolved compounds alone were sufficient to induce settlement. This finding indicates that microbes, phytoplankton, and other particulate components removed during filtration are not the sole contributors to settlement-inducing activity. Different metamorphosis and attachment responses were observed across species. *Colpophyllia natans, Pseudodiploria strigosa,* and *Diploria labyrinthiformis* responded to filtered aquarium and FCCA seawater treatments, whereas *Acropora cervicornis* larvae responded primarily to unfiltered seawater. Additional experiments using larger pore-size filtration were conducted to determine whether settlement responses could be attributed to particulate components removed during sterile filtration. No consistent relationship between filter pore size and settlement was observed. Similarly, *Porites astreoides* exhibited little response to either filtered or unfiltered deep refuge seawater. Together, these observations suggest that coral species differ in their sensitivity and specificity to waterborne settlement cues and may rely on different combinations of environmental signals during settlement.

Eluates prepared from active seawater samples retained settlement-inducing activity, demonstrating that dissolved cues could be recovered using C18 extraction methods and evaluated in bioassays. FDR eluates repeatedly induced settlement in both *C. natans* and *P. strigosa*, and fractionation of two independently prepared FDR eluates from 3.6 L and 10 L of seawater revealed remarkably similar activity patterns. In both preparations, significant settlement responses were concentrated within fractions 1 through 3, and fraction 2 induced significant settlement in both species in each fractionation series. The recurrence of activity within the same fraction range across independently prepared FDR eluates demonstrates that the recovery of settlement-inducing activity within these fractions could be replicated despite differences in starting seawater volume and minor variations in solvent gradients. Similarly, FAcer eluates retained activity following extraction, and parent fractions 1CLD44-1 and 1CLD44-2 induced significant settlement responses in *C. natans*. Further fractionation narrowed activity to subfraction 1CLD44-1+2-1. Together, these findings demonstrate that settlement-inducing activity could be repeatedly recovered from multiple seawater sources and tracked through successive rounds of fractionation.

Additional separation steps revealed increasingly distinct response patterns among coral species. Although *C. natans* and *P. strigosa* initially responded to many of the same FDR parent fractions, their responses diverged following additional fractionation. HPLC fractionation of subfraction 1CLD43-1-1 identified a single fraction, 1CLD43-1-1-1, that

induced significant settlement in *C. natans*, whereas none of the corresponding HPLC fractions induced significant settlement in *P. strigosa*. In contrast, HPLC fractions derived from subfraction 1CLD43-2-2 induced significant settlement in *P. strigosa* but not *C. natans*, with activity concentrated in fractions 1CLD43-2-2-3, 1CLD43-2-2-4, and 1CLD43-2-2-5. These findings suggest that multiple compounds may contribute to settlement-inducing activity and that coral species differ in their responsiveness to individual components within complex mixtures. Continued characterization of both active and inactive fractions may provide important insight into the diversity and specificity of coral settlement cues.

FCCA remained an important source of settlement-inducing activity throughout this study. Filtered seawater associated with *Hydrolithon boergesenii* induced settlement responses in multiple coral species, and eluates prepared from FCCA also retained activity. Similar results have been previously reported with CCA seawater (Quinlan et al. 2023). The Quinlan et al. study also used solid-phase extraction with small-scale C18 and ~~PPL~~ columns but required eluate concentrations of approximately 10× natural concentrations to observe significant amounts of larval settlement. The repeated recovery of activity from FCCA eluates further supports the hypothesis that CCA release dissolved compounds capable of influencing coral larval settlement.

One of the primary challenges associated with the characterization of coral settlement cues is that the active compounds occur at low concentrations in seawater. Although fractionation successfully narrowed activity to a subset of subfractions, only limited quantities of active material were recovered. Despite this challenge, settlement-inducing activity was retained throughout successive fractionation steps, supporting continued efforts toward compound identification.

Future work will focus on continued chemical characterization of settlement-inducing cues through additional HPLC fractionation. Resulting fractions will be analyzed by liquid chromatography-high resolution mass spectrometry (HRLCMS) in collaboration with the Neha Garg laboratory. Comparisons among active and inactive eluates, fractions, and subfractions may help identify compounds associated with settlement induction and prioritize candidates for further investigation.

These findings have several implications for coral restoration and conservation. Because larvae of different coral species responded differently to the same seawater sources and fractions, restoration strategies should account for species-specific settlement requirements rather than relying on a single cue or approach. The repeated settlement responses observed in seawater from biodiverse reef communities suggest that maintaining ecological diversity helps preserve the diversity of settlement cues available to recruiting larvae. Understanding which reef-associated organisms contribute to settlement-inducing activity may help identify species and communities that support coral recruitment and guide restoration and conservation efforts. Improved understanding of settlement-inducing cues could enhance the effectiveness of restoration approaches that rely on sexually propagated corals by facilitating more reliable larval settlement and recruitment. Together, these findings expand our understanding of the environmental

signals involved in coral recruitment and highlight the value of conserving and restoring diverse reef ecosystems

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