Investigation of waterborne chemical cues for coral larval settlement

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Final Report

Prepared By:

Valerie J. Paul, Cassady L. Dougan, Jennifer M. Sneed

Smithsonian Marine Station

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Management Summary

One of the research priorities of the FY 2024-2025 Coral Protection and Restoration Program priorities is to better understand coral sexual reproduction and recruitment (or lack thereof) as it relates to ecosystem restoration in wild populations of priority species with a focus on the factor(s) causing reproductive and/or settlement failure at different life stages. Priority coral species for 2024-2025 include *Colpophyllia natans*, *Diploria labyrinthiformis*, *Montastraea cavernosa*, *Orbicella faveolata*, and *Pseudodiploria strigosa*. In this study, we demonstrate that waterborne chemical cues induce larval settlement and metamorphosis in seven different spawning coral species. Interestingly, the brooding coral *Porites astreoides* did not seem to respond to waterborne cues but settled in response to unfiltered seawater, suggesting a difference in larval response between brooding and spawning corals. Identification of waterborne compounds effective at inducing coral larval settlement will fill an important knowledge gap for coral restoration efforts. These studies help identify which organisms within the environment facilitate settlement; these organisms could then be incorporated into outplanting efforts. Once the chemical 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

This study aimed to address critical knowledge gaps in coral larval settlement processes. We explored the role of waterborne chemical cues in promoting settlement and metamorphosis, which are fundamental steps for coral sexual propagation. Over 35 bioassays were conducted with seven spawning and two brooding species of coral larvae and demonstrated that dissolved compounds in aquarium seawater can effectively induce metamorphosis and settlement for most spawning coral species. Seawater from a diverse coral reef ecosystem consistently induced settlement, suggesting the critical role of biodiversity in producing effective cues. Filtered aquarium 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, providing a pathway for identifying specific biochemical inducers. The isolation and characterization of these waterborne cues may lead to new methods that improve recruitment outcomes, even on degraded reefs with limited natural cues. Our research findings emphasize the importance of preserving species-rich habitats on coral reefs to maintain natural settlement dynamics. Our future directions involve further fractionation and analysis of active compounds to continue to isolate and characterize specific settlement inducers. Plans also include broader testing across coral species and aquarium coral reef environments to refine applications of these cues for restoration projects. This study highlights the importance of integrating chemical ecology into coral reef restoration, paving the way for more effective and sustainable conservation practices.

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Eluate & Fraction Identification Code Format

Source Seawater. Type of Resin. Month & Year Prepared (concentration)

* If IDs end in "B", the eluate was the second prepared of the same type within the same month. If IDs end in "NP", ethyl acetate was used to elute the compounds. *

1. INTRODUCTION

1.1. Background

Successful coral settlement and metamorphosis (hereafter referred to as settlement) are key to success in the sexual propagation of corals. The settlement process 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 the 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 (Harrington et al. 2004, 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 in Caribbean corals (Ritson-Williams et al. 2009, 2010, 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). Sometimes settlement can be high, but at other times very low, which leads to unpredictable outcomes. This settlement failure is likely a result of not understanding or being able to control inductive cues on settlement tiles and other substrates. Development of a consistent, low-cost natural 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).

We have recently focused attention on chemical cues for settlement and metamorphosis across multiple coral species such as *Colpophyllia natans, Acropora spp., Pseudodiploria spp., Orbicella faveolata* and others (Ritson Williams et al. 2016, Sneed et al. 2024). This has been greatly facilitated by access to coral larvae from ex situ breeding programs such as the Florida Aquarium Coral Conservation Program and the SeaWorld Orlando Florida Coral Rescue Center, which reliably produce coral larvae at multiple times of the year. We have been able to study the growth and survival of new recruits that settled in response to different chemical inducers. Last year we made a remarkable discovery that filtered seawater from our coral reef aquarium at the Smithsonian Marine Ecosystems Exhibit at the St. Lucie County Aquarium facilitated coral larval settlement without any other cues. This means that waterborne metabolites from an intact coral reef ecosystem can be highly effective as a settlement inducer with over 50% settlement, comparable to positive cues such as CCA. Sterile filtering the

seawater did not significantly diminish its effectiveness for most coral species, indicating the settlement was induced by dissolved compounds in the seawater.

We then used solid-phase extraction methods with C18 resin to extract the inductive compounds from the seawater. The seawater was passed over the C18 to retain the settlement cues on the C18 resin, which was then eluted with organic solvents. These extracts (also called eluates) also induced settlement, demonstrating that we had a method to obtain the inductive compounds from seawater. 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. 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 released by corals and other coral reef organisms such as crustose coralline algae may help explain the inability of degraded reefs to promote larval settlement and recruitment.

1.2. Project Goals

The specific aims of this study were to characterize which reef species contribute to the positive waterborne cues, to begin to isolate and characterize these cues and develop methods to utilize them for reef restoration.

We sought to address the following questions:

1. What combinations and densities of conspecific corals or multi-species assemblages are needed to produce waterborne cues sufficient to induce settlement and metamorphosis?
2. Do different species of coral larvae respond in similar or different ways to the waterborne cues?
3. What are the compounds in seawater that induce larval settlement and metamorphosis?
4. Are the same isolated chemical cues effective on larvae of different coral species?

2. METHODS

2.1. Task 2: Assessing waterborne cues with different species of coral larvae

Aquarium seawater for testing was collected from nine different tank locations across four facilities (Smithsonian Marine Ecosystems Exhibit (SMEE) at the St. Lucie County Aquarium in Fort Pierce, FL, The Florida Aquarium Coral Conservation Program (TFA) at Apollo Beach, FL, Nova Southeastern University (NSU) in Dania Beach, FL, and Smithsonian Marine Station (SMS) at Fort Pierce, FL) as described below (Table 1).

Table 1. Identification codes and descriptions of the 9 aquarium seawater sources. The presence of a capital "F" in front of the ID indicates sterile filtration. Both filtered and unfiltered seawater were tested.

Seawater ID	Facility	Volume (gal)	Collection Location	Coral Contents	Other Organisms
(F)DR	SMEE	150	Whole Tank	*Acropora cervicornis* *Acropora palmata* *Acropora prolifera* *Orbicella faveolata* *Porites astreoides* *Porites divaricata* *Porites porites* *Pseudodiploria strigosa* *Siderastrea siderea* *Orbicella franksi*	Juvenile Reef Fish Snails Crabs Brittle Stars Sea Stars Shrimp Urchins Anemones Crustose Coralline-Algae Sea Cucumbers Soft Corals
(F)Acer	SMEE	1800	Between branches of *Acropora cervicornis*		
(F)Apal	SMEE	1800	Above *A. palmata*		
(F)G2-1	TFA	1060	Sump	*C. natans* *Dichocoenia stokesii* *D. labyrinthiformis* *Meandrina meandrites* *Musa angulosa* *Porites porites* *Pseudodiploria strigosa*	Snails Crabs Urchins Peppermint Shrimp
(F)G2-2	TFA	985	Sump	*C. natans* *D. labyrinthiformis* *Meandrina meandrites* *Musa angulosa* *Mycetophyllia lamarckiana* *Pseudodiploria strigosa*	Snails Hermit Crabs Urchins Peppermint Shrimp
(F)PC3	TFA	333	Sump	*Acropora palmata*	Snails Crabs Urchins Peppermint Shrimp
(F)Pcli	NSU	500	Sump	*Pseudodiploria clivosa*	
(F)Mcav	NSU	500	Sump	*Montastraea cavernosa*	
FCCA	SMS	0.06	Whole Dish	n/a	*Hydrolithon boergesenii*

Coral species tested included *Colpophyllia natans* (*C. natans* or Cnat), *Pseudodiploria strigosa* (*P. strigosa* or Pstr), *Pseudodiploria clivosa* (*P. clivosa* or Pcli), *Orbicella*

faveolata (*O. faveolata* or Ofav), *Montastraea cavernosa* (*M. cavernosa* or Mcav), *Acropora palmata* (*A. palmata* or Apal), *Mycetophyllia ferox* (*M. ferox* or Mfer), *Diploria labyrinthiformis* (*D. labyrinthiformis* or Dlab), and *Porites astreoides* (*P. astreoides* or Past).

Prior to collecting seawater from the coral aquarium chosen for testing, collection jars were cleaned with reverse osmosis water, dried, and rinsed with seawater from their respective aquarium tank prior to filling. To obtain seawater from desired locations within a tank, a bulb baster was used to transfer seawater into the appropriate collection jar (Figure 1). A portion of all aquarium seawater collections were filter sterilized using 0.2 µm PES membrane filter units under vacuum to remove phytoplankton, bacteria and sediment (i.e. all particles > 0.2 µm).

Figure 1. Diagram of (F)Acer and (F)Apal aquarium seawater collections using a bulb baster.

Coral larval settlement bioassays were conducted in sterile, polystyrene 6-well plates with 10 mL of seawater and 10 larvae in each well (Figure 2). Treatments included unfiltered and filtered aquarium seawater along with filtered sterilized natural seawater (FSW) as a negative control. When obtainable, small pieces (~ 4 mm x 4 mm) of *Hydrolithon boergesenii*, a crustose coralline alga (CCA) known to induce settlement of multiple species of Caribbean coral larvae (Ritson-Williams et al. 2016), in FSW were used as positive controls. Treatments were randomized and replicated five times across the well plates. Microscopes were used to confirm the selection of swimming planula larvae with no visible deformities for all bioassays.

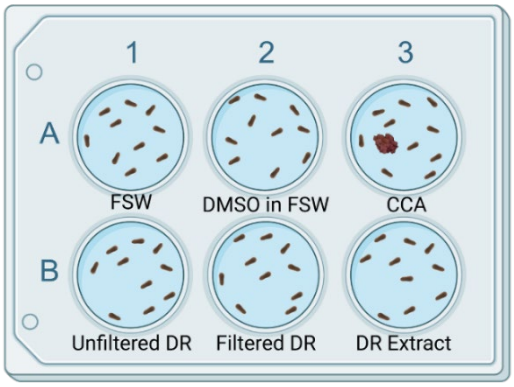

Figure 2. Diagram of an example 6-well plate from a larval settlement bioassay. The DR Extract is an example of an FDR C18 eluate in FSW. Five replicates were used for each treatment and control.

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

In preparation for waterborne compound extraction, a 1-10 L sample of selected aquarium seawater was collected and filtered following methods previously described for Task 2. All chromatography columns of C18 and/or HP20 resin were conditioned using methanol and rinsed with HPLC-grade water prior to use. The C18 columns used were prepacked 10 g Chromabond® columns, and the HP20 columns were glass columns packed with 10 g superclean HP20 (Supelco®). Batches of 1-10 L of filtered aquarium seawater were decanted onto the columns under vacuum (Figure 3). Columns were first rinsed with HPLC-grade water to reduce salt accumulation, and this rinse was not retained. Methanol and/or ethyl acetate were used to elute the retained compounds from the column, and these mixtures of organic compounds are referred to as eluates. To fractionate the crude eluates, a series of HPLC-grade water and methanol varying in concentration and polarity were used to separate the eluted compounds into fractions with a final ethyl acetate rinse of the column to remove any remaining nonpolar compounds.

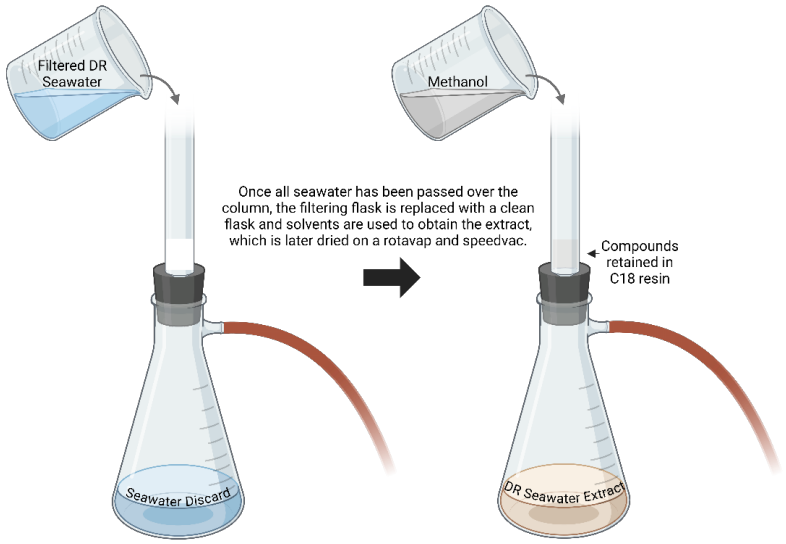

Figure 3. Diagram of FDR seawater being vacuum filtered over a C18 column, and the retained compounds being eluted with methanol under vacuum.

The eluates and fractions were transferred into pre-weighed scintillation vials and concentrated in vacuo at 35 °C (Thermo Savant SPD121P SpeedVac Concentrator) until solvents were removed. Residual water was frozen at -80 °C and removed via lyophilization at -45 °C and 0.15 mbar (Labconco FreeZone 6). Extracted compounds were weighed then redissolved in volumes of dimethyl sulfoxide (DMSO) corresponding to their natural concentrations in aquarium seawater. To ensure that we did not miss bioactivity and to account for possible losses during the chromatography steps, most eluates and fractions were tested at twice natural concentrations based on volume. To test the dissolved eluates and fractions in coral larval settlement bioassays, 10 µL of the samples dissolved in DMSO were added to wells containing 10 mL FSW. 10 µL DMSO was added to FSW as a negative control. We have begun preparing NMR spectra and will be obtaining high resolution LCMS spectra to characterize the most active waterborne compounds.

2.3. Data Analysis

For all coral larval settlement bioassays, the number of settled, metamorphosed but unattached, and swimming larvae were recorded in each well after 24 and 48 hours. The mean proportions of settled (metamorphosed and attached) larvae within each treatment were statistically analyzed using R. Data were tested for normality and homogeneity using the Shapiro-Wilk and Bartlett tests respectively. Since the data did not meet the normality assumptions, they were analyzed using the nonparametric Kruskal-Wallis and Dunn's post-hoc tests. Treatments were considered significantly different from appropriate controls when $p < 0.05$. All unfiltered and filtered aquarium seawater treatments were compared to FSW, while seawater eluates and fractions were compared to the negative DMSO control.

3. RESULTS

3.1. Task 2: Assessing waterborne cues with different species of coral larvae

Overall, 35 larval settlement bioassays testing aquarium seawater and corresponding eluates have been conducted across the 7 spawning and 2 brooding coral species. For the filtered seawater treatments, significant settlement was observed for FDR, FAcer, FApal, FPC3, and FCCA.

For *Colpophyllia natans*, significant aquarium seawater treatments included unfiltered (Kruskal Wallis test, $p = 0.003$) and filtered ($p = 0.01$) seawater collected from the deep refuge and near the *Acropora cervicornis* (*A. cervicornis* or Acer) within the coral reef system at SMEE (Figure 4). This result also stands true for *O. faveolata* (Figure 5) and *P. strigosa* (Figure 6).

Within the filtered seawater treatments, larvae settled in response to all tested treatments, with settlement significantly higher than FSW for filtered and unfiltered DR and Acer water with ~48% settled *C. natans* larvae observed for the FDR treatment (Kruskal Wallis test, $p = 0.0006$) (Figure 4). The mean proportions of settled *C. natans* in FDR and FAcer were similar to their equivalent unfiltered treatments. Approximately 20% of *C. natans* larvae settled in both filtered and unfiltered Apal seawater (Figure 4).

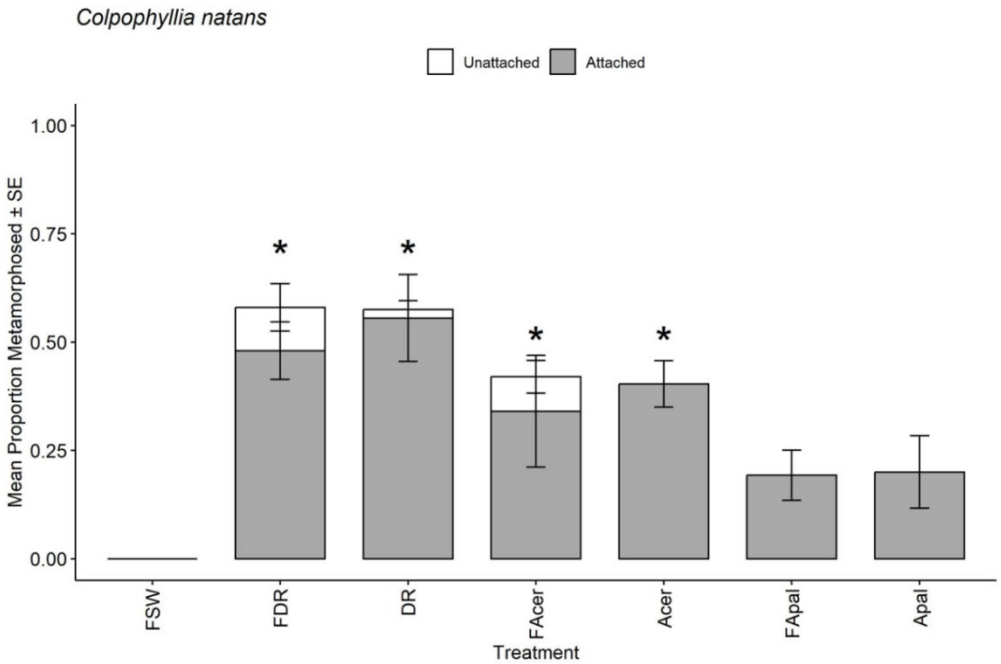

Figure 4. Mean proportions of attached (settled) and unattached metamorphosed *C. natans* larvae in filtered and unfiltered aquarium seawater from SMEE. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, FSW ($p < 0.05$).

O. faveolata larvae demonstrated higher mean settlement scores for all filtered aquarium seawater tested when compared to the unfiltered water (Figure 5), although both filtered and unfiltered seawater were significantly different from the FSW control. On average, the ratio of attached to unattached metamorphosed *O. faveolata* larvae was nearly 1:1 for the significant treatments (FDR, DR, Facer, Acer, and the CCA piece).

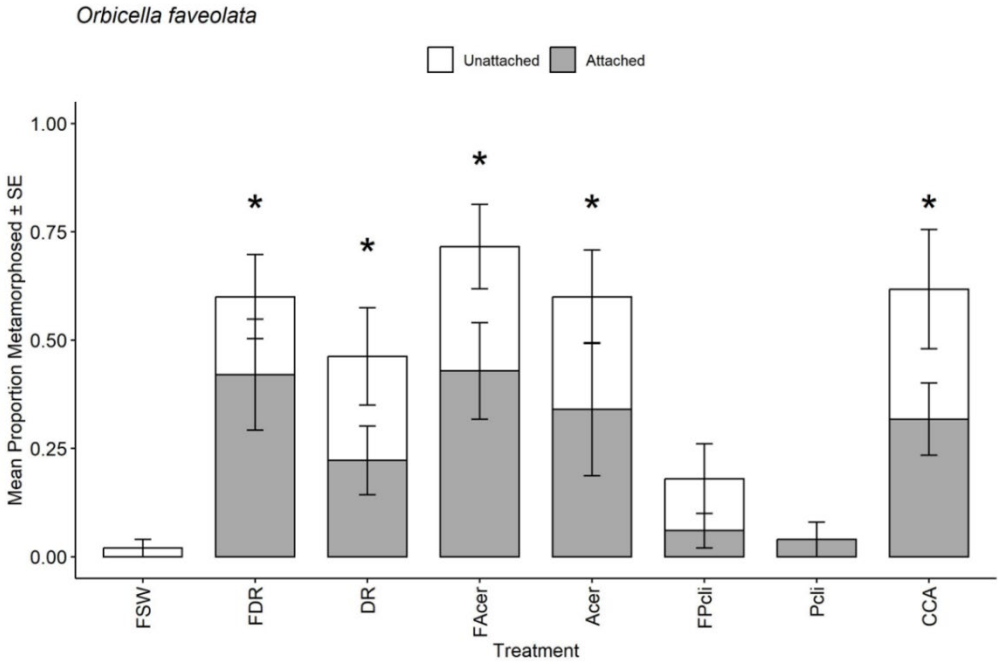

Figure 5. Mean proportions of attached and unattached metamorphosed *O. faveolata* larvae in filtered and unfiltered aquarium seawater from SMEE and NSU. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, FSW ($p < 0.05$). A piece of CCA is used as a positive control.

A significant proportion of *P. clivosa* larvae settled in FAcer (Kruskal Wallis test, p = 0.0006), and a slightly larger proportion metamorphosed but did not attach (Figure 6). The number of attached larvae was similar to the CCA positive control. Filtered deep refuge water had a small effect, but proportion attached was small and not significantly different from FSW.

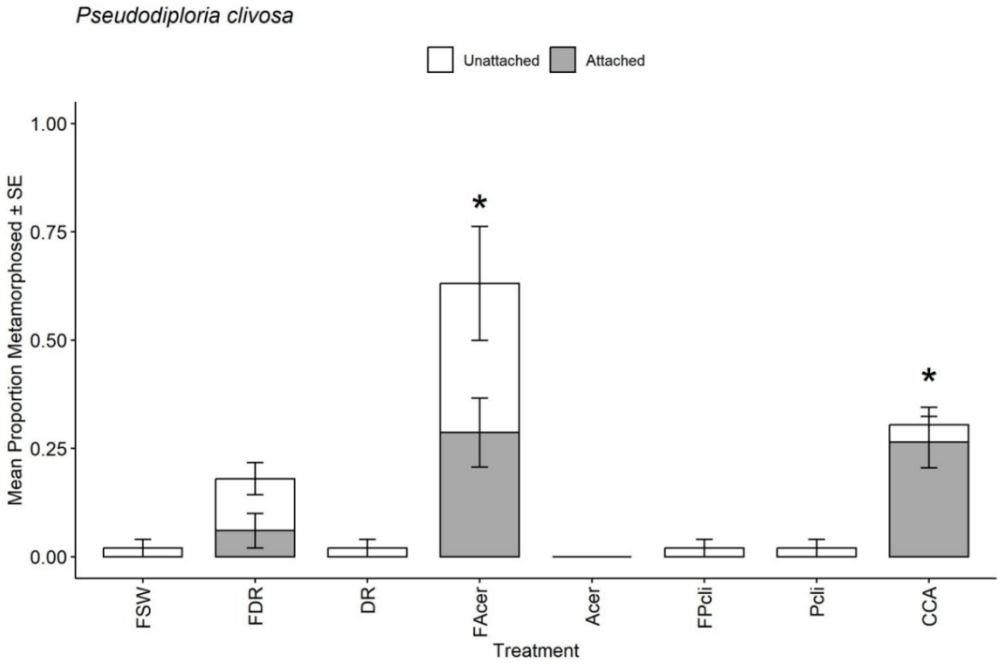

Figure 6. Mean proportions of attached and unattached metamorphosed *P. clivosa* larvae in filtered and unfiltered aquarium seawater from SMEE and NSU. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, FSW ($p < 0.05$). CCA is a positive control.

P. strigosa larvae seem to be less selective about the composition of waterborne cues. Larvae of *P. strigosa* settled in all thirteen aquarium seawaters tested, from which ten of those treatments exhibited statistical significance with greater than 68% settlement on average (Figure 7) and comparable to the CCA positive control. However, settlement was not observed in FSW, the negative control treatment, indicating some type of cue is required.

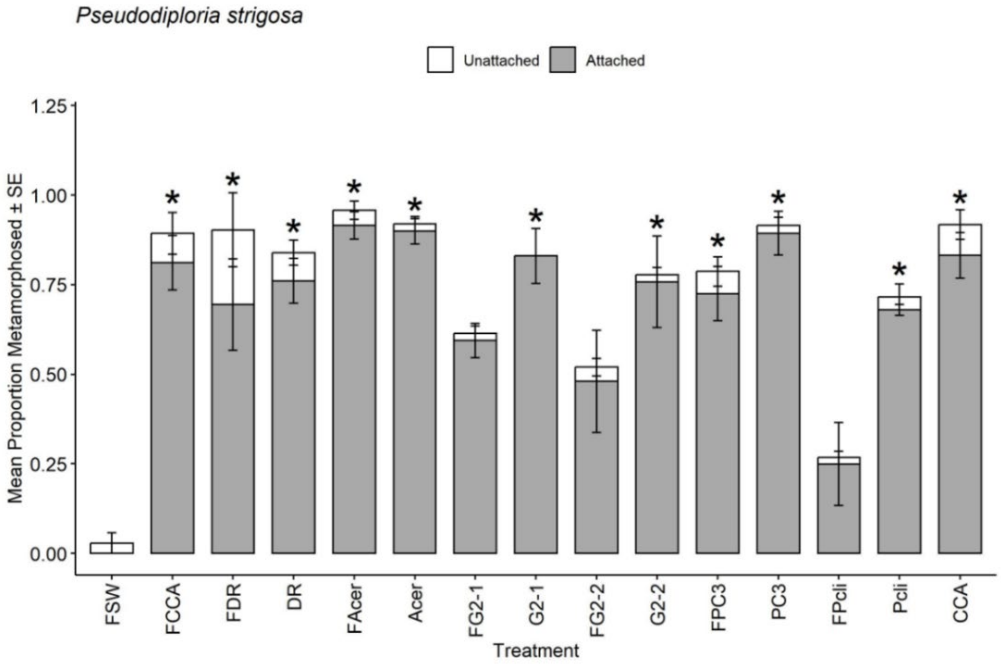

Figure 7. Mean proportions of attached and unattached metamorphosed *P. strigosa* larvae in filtered and unfiltered aquarium seawater from SMS, SMEE, TFA, and NSU. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, FSW ($p < 0.05$). CCA is a positive control.

P. astreoides only responded to unfiltered deep refuge seawater with nearly 72% settlement ($p = 0.006$) (Figure 8). Metamorphosis was not observed for the filtered equivalent deep refuge treatment, suggesting these larvae are responding to something in the aquarium water other than dissolved chemical cues.

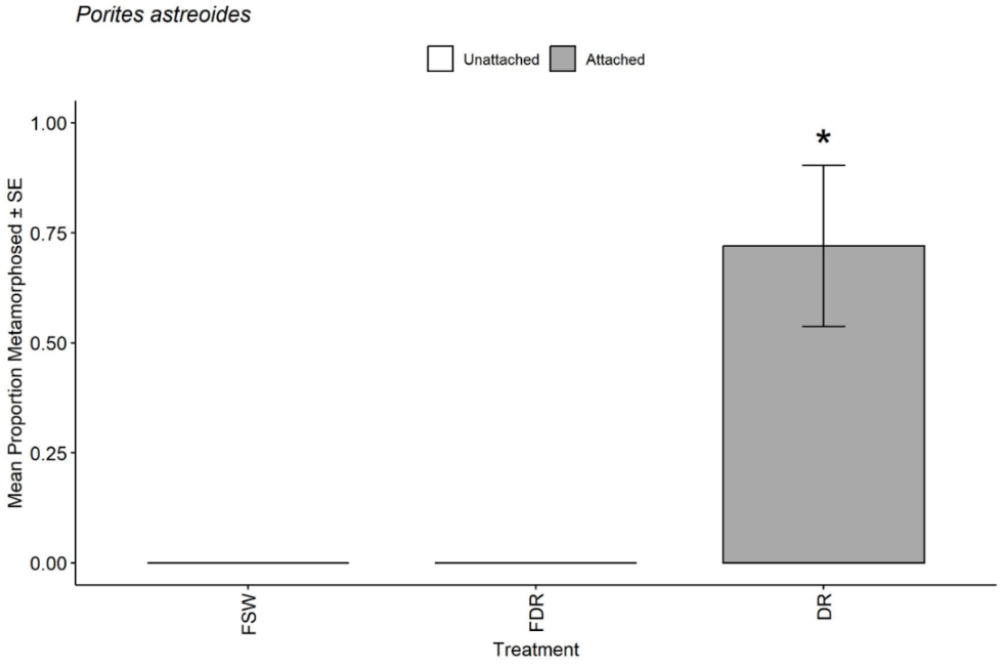

Figure 8. Mean proportions of attached and unattached metamorphosed *P. astreoides* larvae in filtered and unfiltered deep refuge seawater from SMEE. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, FSW ($p < 0.05$).

In contrast, *D. labyrinthiformis* responded to all aquarium seawater sources, but only the filtered treatments, suggesting that an inhibitory component to metamorphosis for this species was removed during filtration (Figure 9). Significant settlement was observed for FDR (Kruskal Wallis test, $p = 0.01$) and FApal ($p = 0.009$). Similar to the positive control, CCA, a negligible proportion of metamorphosed larvae did not attach within the significant aquarium seawater treatments.

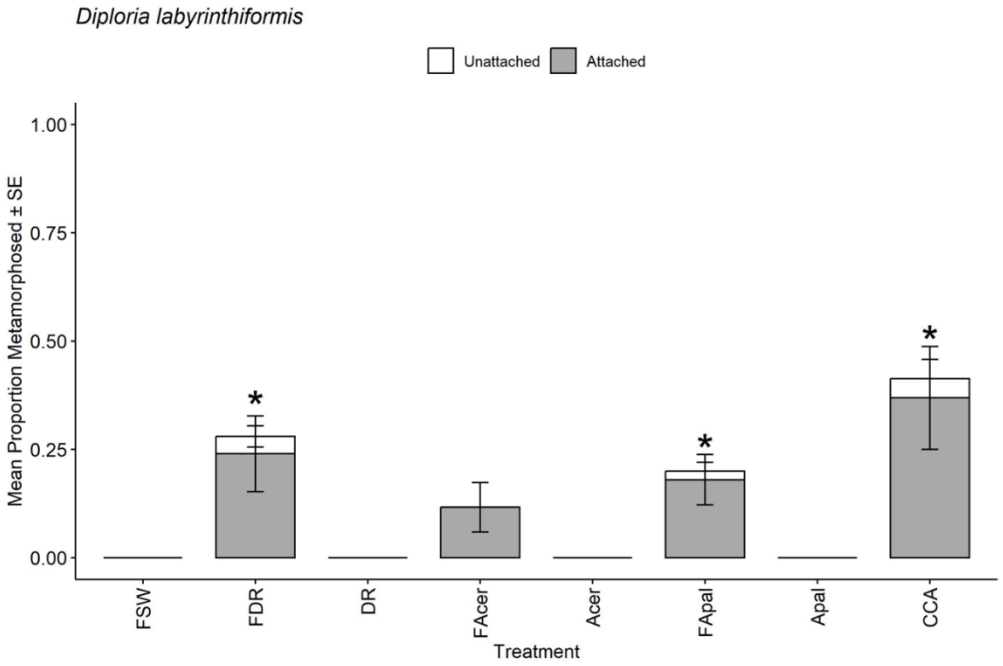

Figure 9. Mean proportions of attached and unattached metamorphosed *D. labyrinthiformis* larvae in filtered and unfiltered deep refuge seawater from SMEE. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, FSW ($p < 0.05$). CCA is a positive control.

The following table displays the results of all aquarium seawater bioassays to compare each treatment by species (Table 2).

Table 2. Average proportion of metamorphosed and attached larvae across all aquarium seawater samples tested by coral species. Values are indicated by a gradient ranging from light to dark orange, with darker shades representing higher values. For information on the contents of the tanks for each aquarium seawater source, see Table 1. All larvae settled on the CCA piece, *Hydrolithon boergesenii.*

Year	Species of Larvae	Aquarium Seawater																	
		FSW	CCA	DR	FDR	Acer	FAcer	Apal	FApal	G2-1	FG2-1	G2-2	FG2-2	PC3	FPC3	Pcli	FPcli	Mcav	FMcav	FCCA
2024	Cnat.Oct	0		0.56	0.48	0.40	0.35	0.20	0.19											
	Cnat.Sept	0.02	0.32	0.42	0.24	0.58	0.63													0.14
	Pstr.Oct	0.24		0.39	0.23	0.50	0.41	0.38	0.16											
	Pstr.Aug	0	0.83	0.76	0.69	0.90	0.92			0.83	0.59	0.76	0.48	0.89	0.72	0.68	0.25			0.81
	Pcli.Aug	0	0.26	0	0.06	0	0.29									0	0			0.76
	Ofav.Aug	0	0.32	0.22	0.42	0.34	0.43									0.04	0.06			
	Mcav.Aug	0	0.38	0	0	0	0											0	0	0.02
	Apal.July	0	0.60	0.17	0.12	0.24	0.02			0.02	0	0	0							0.60
2025	Past.April	0		0.72	0															
	Dlab.May	0	0.37	0	0.24	0	0.12	0	0.18											

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

To confirm the appropriate chromatography resin for retaining waterborne compounds, C18 and HP20 eluates of filtered aquarium seawater were prepared and tested in settlement bioassays.

On average, more than 50% of *C. natans* larvae significantly settled in the FCCA (Kruskal Wallis test, $p = 0.002$), FAcer ($p = 0.005$), and FApal ($p = 0.02$) C18 eluate treatments (Figure 10). The mean proportion of settled *C. natans* larvae was approximately 40% for both FDR eluate treatments, which was not statistically different from the DMSO control.

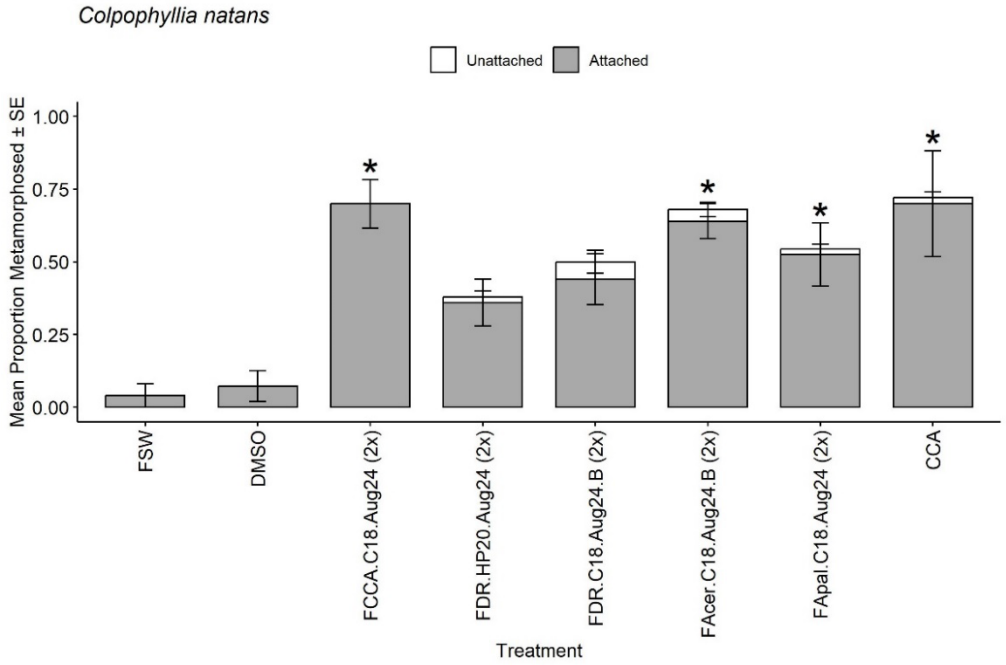

Figure 10. Mean proportions of attached and unattached metamorphosed *C. natans* larvae in FSW with SMEE and SMS seawater eluates. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, DMSO ($p < 0.05$). FSW and DMSO in FSW are negative controls; CCA is a positive control.

In a similar bioassay of *P. clivosa*, mean larval settlement for the FDR C18 eluate was significantly different from the negative control (Kruskal Wallis test, p = 0.02) while larvae did not respond to the comparable HP20 eluate (Figure 11). More than 50% of *P. clivosa* larvae metamorphosed after exposure to the FCCA C18 eluate with about half exhibiting significant settlement (attached) (p = 0.004).

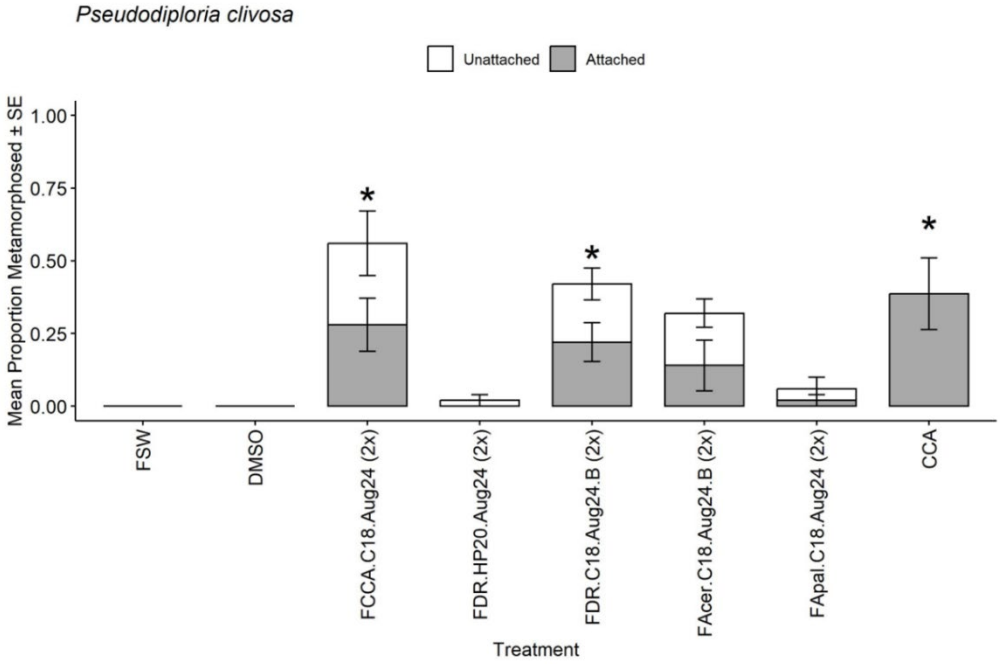

Figure 11. Mean proportions of attached and unattached metamorphosed *P. clivosa* larvae in FSW with SMEE and SMS seawater eluates. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, DMSO ($p < 0.05$). FSW and DMSO in FSWare negative controls; CCA is a positive control.

On average, the FDR eluates from each of the resins resulted in 100% metamorphosis for *P. strigosa* larvae, however, a greater proportion of the larvae introduced to the HP20 eluate remained unattached (Figure 12). The FDR C18 eluate resulted in significant *P. strigosa* settlement (Kruskal Wallis test, $p = 0.002$), while the FDR HP20 eluate did not. Significant treatments also included C18 eluates of FCCA ($p = 0.001$), FAcer ($p = 0.005$), and FApal ($p = 0.01$) and the CCA positive control.

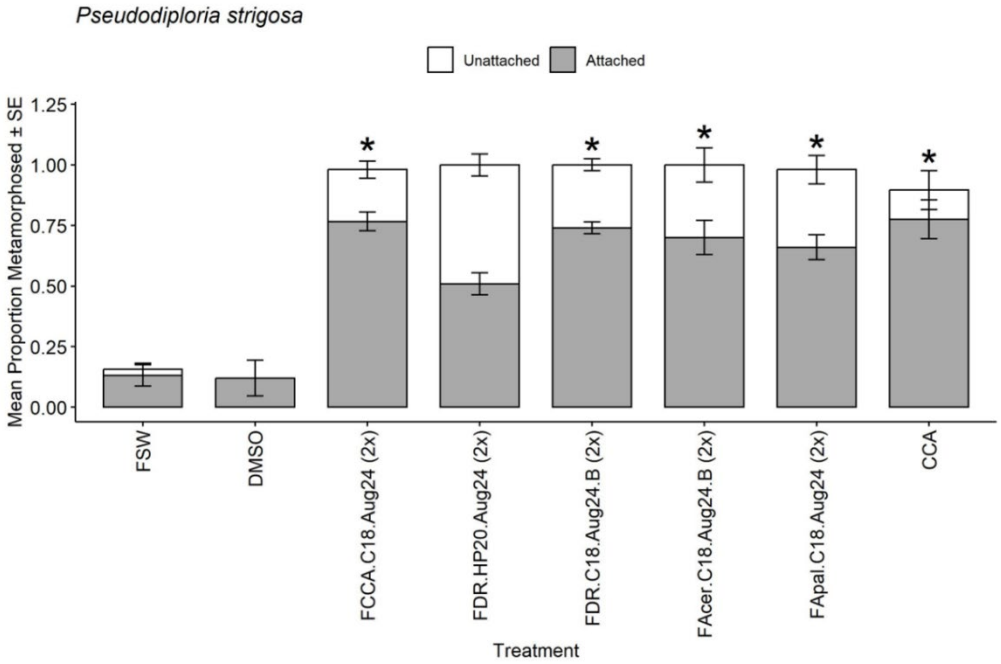

Figure 12. Mean proportions of attached and unattached metamorphosed *P. strigosa* larvae in FSW with SMEE and SMS seawater eluates. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, DMSO ($p < 0.05$). FSW and DMSO in FSW are negative controls; CCA is a positive control.

The following table displays the results of all aquarium seawater eluate bioassays to compare larval response to each treatment by species (Table 3). Overall, highest average settlement is observed within the FDR and FCCA eluate treatments. Variations in response across the different species to each eluate treatment are prominent.

Table 3. Average proportions of metamorphosed and attached larvae across all aquarium seawater eluates tested by species. Values are indicated by a gradient ranging from light to dark green, with darker shades representing higher values.

Year	Species of Larvae	Seawater Eluates (tested at 2x unless otherwise noted)									
		FSW	CCA	DMSO	FDR.C18.July24	FDR.C18.July24.NP	FDR.C18.July24.RE	FDR.C18.July24.RE.NP	FAcer.C18.July24	FAcer.C18.July24.NP	FCCA.C18.Aug24 (1x)
2024	Cnat.Oct	0		0							0.49
	Cnat.Aug	0.08	0.81	0.04	0.69		0.43		0.32		
	Pstr.Oct	0.06		0.08							0.84
	Pstr.Sept	0.10	0.68	0.08	0.72		0.64		0.71		
	Pstr.Aug	0.03	0.84	0	0.87		0.74		0.68		
	Pcli.Aug	0	0.43	0	0.45		0.47		0.44		
	Ofav.Aug	0	0.58	0	0.10		0.06		0.06		
	Mcav.Aug	0	0.48	0	0	0	0.02	0	0.06	0	
	Apal.July	0	0.76	0	0	0	0	0	0	0	
2025	Mfer.Jan	0		0							

Year	Species of Larvae	Seawater Eluates Continued (tested at 2x unless otherwise noted)							
		FCCA.C18.Aug24	FDR.C18.Aug24 (1x)	FDR.C18.Aug24	FDR.C18.Aug24 (4x)	FAcer.C18.Aug24	FDR.HP20.Aug24	FDR.C18.Aug24.B (1x)	FDR.C18.Aug24.B
2024	Cnat.Oct							0.21	0.26
	Cnat.Aug	0.70	0.62	0.49	0.29	0.61	0.36		0.44
	Pstr.Oct							0.81	0.58
	Pstr.Sept	0.77	0.20		0.67	0.32	0.51		0.74
	Pstr.Aug		0.83	0.56	0.43	0.35			
	Pcli.Aug	0.62	0	0	0.14	0.25	0		0.22
	Ofav.Aug				0.18	0.06			
	Mcav.Aug								
	Apal.July								
2025	Mfer.Jan							0	

Year	Species of Larvae	Seawater Eluates Continued (tested at 2x unless otherwise noted)					
		FAcer.C18.Aug24.B (1x)	FAcer.C18.Aug24.B	FApal.C18.Aug24 (1x)	FApal.C18.Aug24	FG2-1.C18.Jan25	FPC3.C18.Jan25
2024	Cnat.Oct	0.22		0			
	Cnat.Aug		0.64		0.53		
	Pstr.Oct	0.69		0.54			
	Pstr.Sept		0.70		0.66		
	Pstr.Aug						
	Pcli.Aug		0.14		0.02		
	Ofav.Aug						
	Mcav.Aug						
	Apal.July						
2025	Mfer.Jan	0		0		0	0

Once it had been confirmed that adding aquarium seawater C18 eluates to swimming coral larvae in FSW induces metamorphosis and settlement, fractionation techniques with C18 columns were used to separate the eluates into fractions containing smaller groups of compounds. In Figures 13 through 15, the FDR fractions tested were eluted according to the fractionation scheme with concentrations of different solvents listed below (Table 4).

Table 4. Solvent concentrations used to elute compounds retained from FDR on C18 column.

Fraction	Solvent
FDR.C18.Aug24.FR1	1:3 MeOH:H_2O (HPLC Grade)
FDR.C18.Aug24.FR2	1:1 MeOH:H_2O (HPLC Grade)
FDR.C18.Aug24.FR3	3:1 MeOH:H_2O (HPLC Grade)
FDR.C18.Aug24.FR4	100% MeOH
FDR.C18.Aug24.FR5	100% EtOAc

Fractions 1 (Kruskal Wallis test, $p = 0.009$) and 2 ($p = 0.03$) of the FDR C18 eluate induced significant larval settlement of greater than 55% for *C. natans* (Figure 13). Negligible settlement was observed for fractions 3 through 5, similar to the DMSO control.

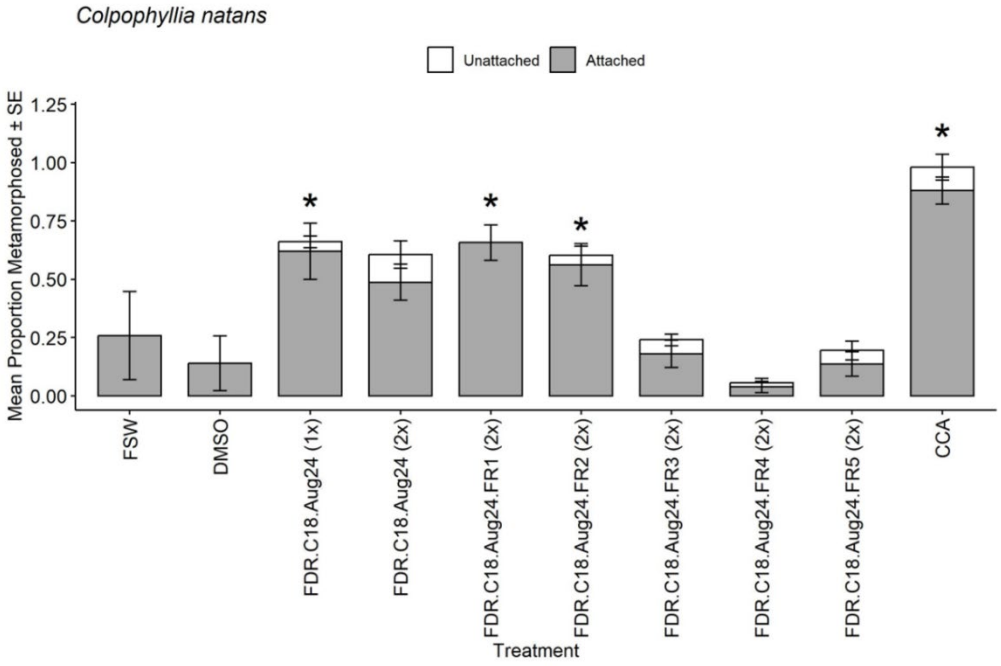

Figure 13. Mean proportions of attached and unattached metamorphosed *C. natans* larvae in FSW with deep refuge eluates and fractions. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, DMSO ($p < 0.05$). FSW and DMSO in FSW are negative controls; CCA is a positive control.

In a similar bioassay with *P. strigosa* larvae, the same three treatments induced significant settlement. On average, fractions 1 (Kruskal Wallis test, $p = 0.01$) and 2 ($p = 0.02$) of the FDR C18 eluate induced significant larval settlement of greater than 75% (Figure 14). Interestingly, the same FDR C18 eluate tested at 1x natural concentration induced a greater proportion of settlement than at 2x for both *C. natans* and *P. strigosa* larvae.

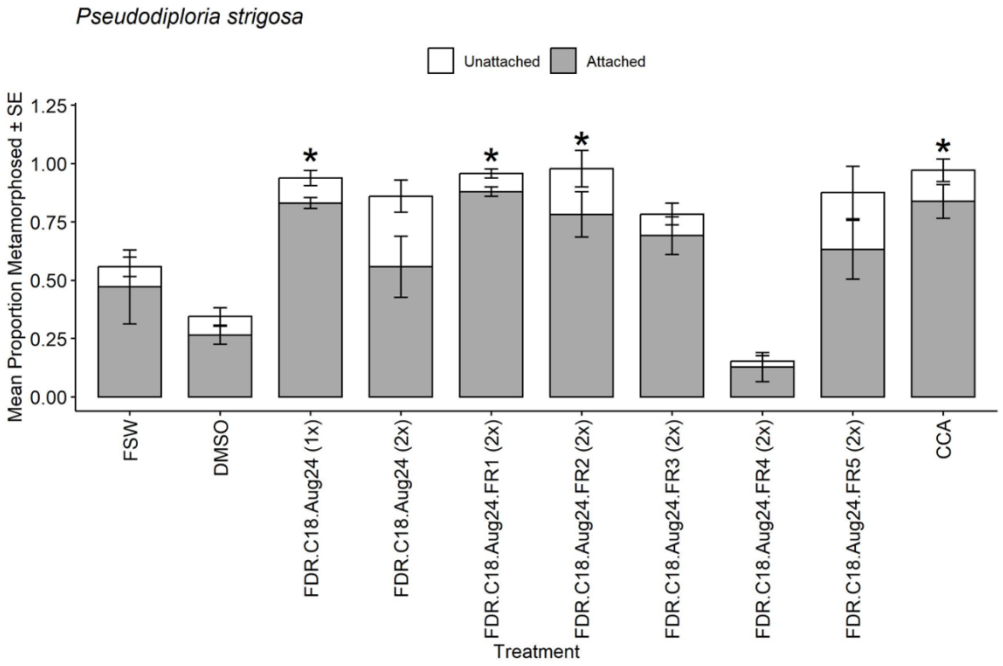

Figure 14. Mean proportions of attached and unattached metamorphosed *P. strigosa* larvae in FSW with deep refuge eluates and fractions. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, DMSO ($p < 0.05$). FSW and DMSO in FSW are negative controls; CCA is a positive control.

Most recently in 2025, *D. labyrinthiformis* larvae were exposed to an FDR eluate and fractions eluted according to the solvent concentrations listed in Table 4. This species seemed to be more selective about the composition of waterborne cues across the fractions (Figure 15). Settlement was only observed for fraction 2, which follows the trend of the early more polar fractions being the greatest settlement inducers. Significant settlement was only observed for the complete eluate which suggests that the fractions may need to be tested at a higher concentration or that some additive effects of different compounds were lost, or some material was lost or degraded during fractionation.

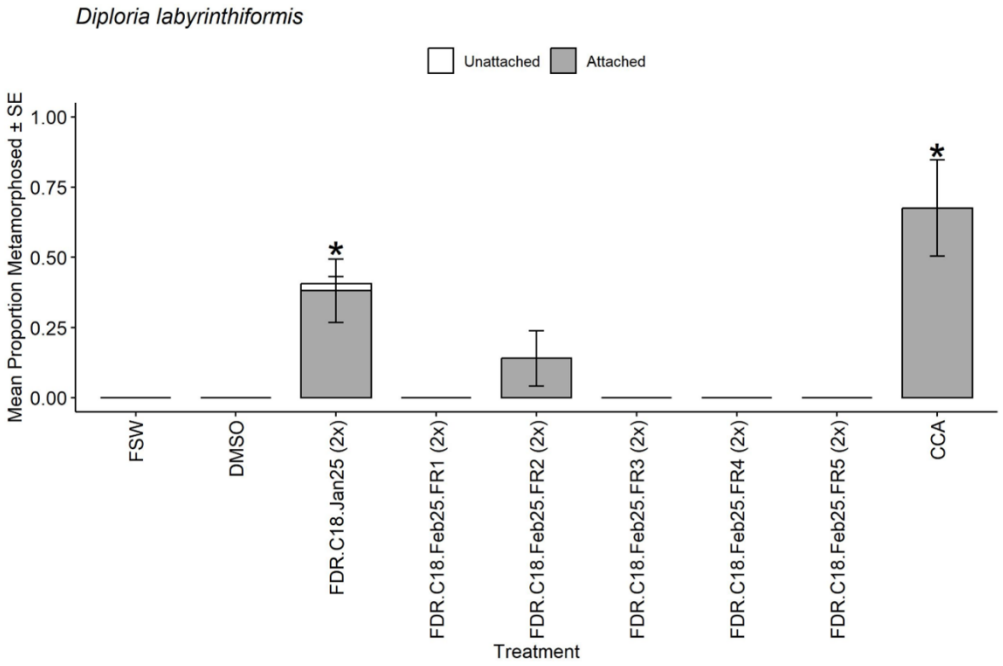

Figure 15. Mean proportions of attached and unattached metamorphosed *D. labyrinthiformis* larvae in FSW with deep refuge eluates and fractions. Asterisks indicate significant metamorphosis of attached larvae (shown in grey) compared to the negative control, DMSO ($p < 0.05$). FSW and DMSO in FSW are negative controls; CCA is a positive control.

4. DISCUSSION AND MANAGEMENT RECOMMENDATIONS

This research provides strong evidence that waterborne chemical compounds play an essential role in inducing larval settlement across multiple coral species. Coral larvae from diverse species of spawning corals, including *A. palmata*, *O. faveolata*, *P. clivosa*, *C. natans*, *P. strigosa*, *D. labyrinthiformis*, settled in polystyrene well plates with no other added cues, often at rates comparable to those exposed to small pieces of the preferred crustose coralline alga (CCA) *Hydrolithon boergesenii*, which was used as a positive control in most bioassays. The findings align with former studies in supporting the concept that unique environmental factors can drive metamorphosis and settlement in corals (Sneed et al. 2014, 2024, Ritson-Williams et al. 2016, Petersen et al. 2023, Randall et al. 2024). However, our results differ from most prior studies by showing that completely soluble compounds in seawater can serve as positive settlement cues. Seawater from the biodiverse reef ecosystem at SMEE consistently induced significant settlement in multiple species of coral larvae, highlighting the importance of species-rich environments in producing effective cues.

Our bioassays demonstrated that aquarium seawater, sterile filtered to 0.2 μM to remove bacteria, phytoplankton and other particles, retains its effectiveness in cuing settlement for most spawning species. This suggests that cues can be primarily attributed to dissolved compounds rather than particulate or microbial components, which are removed during filtration. Conversely, *P. astreoides*, a brooding coral species, seemed to depend on the removed components since settlement was only observed in unfiltered aquarium seawater. This result supports prior research on the role of bacterial signals in settlement induction and indicates a need for species-specific approaches in the application of waterborne cues for coral restoration (Sneed et al. 2014, 2024, Petersen et al. 2023). While it was clear that reproductive strategies influenced larval response to waterborne cues, differences in preference to specific cues were also observed among the spawning species.

The most settlement overall was exhibited by *P. strigosa* larvae, which seemed to be less particular when introduced to aquarium sourced waterborne cues. Nonetheless, negligible metamorphosis was observed for *P. strigosa* in the negative controls of most bioassays. When settlement was present in the negative controls, it was likely attributed to age of larvae (i.e. older larvae that were less discriminating). Therefore, the results suggest that some type of waterborne cue is required for settlement of *P. strigosa*. The reduced specificity by *P. strigosa* larvae suggests that this species is a generalist that could settle in many different habitats, which could be of utility for restoration purposes.

Other notable differences occurred among settlement behavior of different spawning coral species. Some species were more selective for different cues than others. In contrast to *P. strigosa*, which seemed to be more of a generalist in its settlement behavior, *P. clivosa* larvae only settled with waterborne cues of filtered Acer seawater and a small amount of settlement with filtered DR seawater. The unfiltered water did not induce settlement. A similar pattern was observed with *Diploria labyrinthiformis* larvae, which did not settle in the presence of unfiltered seawater. This suggests that

inhibitory substances were filtered out during the sterile filtration process, but we do not know if these were bacteria or phytoplankton or sediments. Differential filtration methods through different filter pore sizes might be a next step to help determine what the negative cues are. *Colpophyllia natans* larvae settled almost equally in response to filtered or unfiltered seawater and were not inhibited by anything in the unfiltered seawater.

Another difference in behavior among larvae of different spawning coral species was in the proportion of fully attached and metamorphosed settlers versus those that metamorphosed and remained unattached. *P. clivosa* and *Orbicella faveolata* had the highest proportions of unattached larvae. We do not know if this is a consequence of testing larvae in plastic (polystyrene) well plates where they might not be able to securely attach or whether this might occur in nature. We sometimes observed the metamorphosed but unattached spat attach in the dishes after a few days, but whether larvae that metamorphose in the water column can attach under natural field conditions with more water motion would be difficult to determine.

Using solid-phase extraction, waterborne compounds were successfully retained on C18 resin, eluted with methanol or various solvent mixtures, and tested in bioassays. We compared a similar method with HP-20 resin packed in glass columns and did not see settlement results as effective as with the C18 columns. Both *C. natans* and *P. strigosa* larvae settled in response to the HP-20 eluates, but at lower proportions than to the C18 eluates. *P. clivosa* did not settle at all in response to the HP-20 eluate but responded well to a C18 eluate. Given the less effective and inconsistent results with the HP-20 resin we stopped using this resin. The C18 columns were very effective and had the added advantage of being prepacked and ready to use, saving time and adding consistency to our methods of eluting the settlement inducing compounds from the seawater.

These compounds are thought to include a range of metabolites originating from reef-associated organisms, such as crustose coralline algae (CCA) and corals. The C18 seawater eluates prepared from FDR, FAcer, FApal, and FCCA were all effective at inducing larval settlement. Recent efforts in our laboratory have focused on using fractionation techniques to separate the retained compounds from FDR into smaller groups of compounds for testing in bioassays. Results show that more polar fractions (usually fraction 1 and/or fraction 2) contain the most effective inducers. Further work is needed to begin the identify these compounds.

Next steps include further fractionation of these active compounds, including high performance liquid chromatography (HPLC), which may lead to isolation of the compound or group of compounds responsible for cuing coral larval metamorphosis and settlement. We also plan to fractionate additional eluates for testing, such as FAcer, which has shown strong inductive properties across coral species. By expanding the range of aquarium seawater sources characterized we may identify common compounds that occur in different aquaria or a broader spectrum of bioactive compounds, potentially increasing the range of cues available for restoration applications.

We also found that filtered CCA (*Hydrolithon boergesenii*) seawater induced settlement in larvae of four coral species (Table 2) and that C18 eluates of CCA seawater effectively induced larval settlement for *C. natans*, *P. strigosa* and *P. clivosa* larvae with a weaker response in *Acropora palmata* larvae. 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 seemed to be less successful at obtaining active eluates. They had to test eluate concentrations at 10X natural concentrations to observe significant amounts of larval settlement.

Our study also points to the importance of preserving healthy reef ecosystems. While we know that favorable substrates are integral, we have also learned that waterborne biochemical cues released into seawater by coral reef-associated organisms are important to larval settlement. Based on results of this study, these include corals such as *Acropora cervicornis* and *A. palmata* as well as some species of CCA. We found that larvae of different coral species can respond differently to these cues and that diverse cues seem to be important. Therefore, the abundance of waterborne cues can decline with biodiversity loss across coral reef ecosystems and impair coral recruitment. Thus, protecting and restoring intact coral reef habitats should remain a priority to ensure the natural propagation of corals.

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