UNIVERSITY OF CALIFORNIA SAN DIEGO

# Development of coral micropropagates for coral restoration

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

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The thesis of Emily Jade Walton is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

# DEDICATION

I want to dedicate this thesis to those in my life who have made it possible. I wouldn't be able to be here without the support of my family, my friends, and my lab mates. I extend my heartfelt gratitude to my mother, Priscilla Stegall, for her unwavering support and belief that I can do anything I set my mind to. Thank you for taking me to the Dallas World Aquarium all those years ago and igniting this interest in all things marine. I want to thank my brother, Trey Walton, for being a constant friend throughout my life.

I am deeply grateful to Martin Tresguerres for opening the doors to his lab to me and providing me with support throughout my time as an undergrad to now with this master's thesis. A special appreciation goes to Daniel Wangpraseurt for his invaluable mentorship and guidance throughout this project. Both of these individuals have had me think critically about my own work and the importance of scientific discovery. My sincere thanks go to both the Tresguerres Lab and Coral Reef Engineering group for their assistance and encouragement which have been instrumental in shaping this research.

Finally, I'd like to thank Dr. Smith and Dr. Wegley Kelly for being a part of my thesis committee.

# EPIGRAPH

“The sea, once it casts its spell, holds one in its net of wonder forever”
Jacques Yves Cousteau

“I was taught that the way of progress was neither swift nor easy”
Marie Curie

"For myself, I like a universe that includes much that is unknown and, at the same time, much that is knowable. A universe in which everything is known would be static and dull, as boring as the heaven of some weak-minded theologians. A universe that is unknowable is no fit place for a thinking being. The ideal universe for us is one very much like the universe we inhabit. And I would guess that this is not really much of a coincidence."
Carl Sagan

"An understanding of the natural world is a source of not only great curiosity, but great fulfilment."
David Attenborough

“That’s really cool!”
My mom

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# LIST OF ABBREVIATIONS

LED Light-Emitting Diode

FSW Filtered Sea Water

OCT Optical Coherence Tomography

PAM Pulse-Amplitude Modulated

$F_t$ Transient Fluorescence

$F_V/F_M$ Variable Fluorescence over Maximum Fluorescence

$CaCO_3$ Calcium Carbonate

PVC Polyvinyl Chloride

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ABSTRACT OF THE THESIS

Development of coral micropropagates for coral restoration

by

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Master of Science in Marine Biology

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Coral reefs, vital ecosystems supporting marine biodiversity, face unprecedented threats from anthropogenic climate change. The resulting reef degradation necessitates urgent restoration research. While conventional methods have focused on fragmentation, the potential of coral micropropagates for restoration remains unexplored. Polyp bailout, where polyps detach in response to acute stress, offers a promising source of micropropagates. This study pursues two objectives: 1) developing a hypersalinity-induced bailout protocol and 2) assessing micropropagate attachment to common restoration substrates. Specifically, this study tests the

viability of micropropagates following hypersalinity stress using chlorophyll *a* fluorimetry and morphological characterization. Following successful micropropagate development, attachment and growth are observed over 14 days on $CaCO_3$, PVC, and cement. A salinity gradient of approximately 1 ppt/hr induced bailout for *Stylophora pistillata*, producing micropropagates characterized by healthy morphology and average $F_V/F_M$ values of 0.45 +/- 0.04. Micropropagates attachment was highest on $CaCO_3$ (73.81% +/- 10.75%) surpassing PVC (47.62% +/- 11.66%) and cement (4.76% +/- 7.38%). Post-settlement, micropropagates displayed varying growth rates, with PVC (0.057 +/- 0.008 $mm^2$/day) exhibiting the highest growth rate, followed by $CaCO_3$ (0.037 +/- 0.002 $mm^2$/day), and cement (-0.0068 +/- 0.008 $mm^2$/day). These results showthat viable *S. pistillata* micropropagates can be produced via hypersalinity stress in a reproducible manner and that artificial substrates made from CaCo3 produce successful attachment and healhty lateral tissue growth. Together, these findings suggest that polyp bailout has potential as a technique for generating micropropagates for coral restoration.

# 1. INTRODUCTION

## 1.1. Coral reefs and a changing climate

Corals serve as a key organism that form the foundation of coral reef ecosystems. In particular, reef-building corals serve as the primary architects of coral reef ecosystems (Henkel et al., 2010). Reef-building corals, belonging predominantly to the phylum Cnidaria and class Anthozoa, secrete $CaCO_3$ skeletons (Henkel et al., 2010). These skeletal structures, collectively known as coral reefs, harbor a diversity of life forms spanning from microorganisms to large marine vertebrates (Hughes et al., 2017). The structural complexity of coral reefs provides vital shelter, breeding grounds, and protective habitats for numerous marine species (Hughes et al., 2017). The crevices and interstices of coral colonies offer safety for juvenile fishes, shelter against predation, and the intricate niches required for successful ecosystem interplay (Shulman et al., 1983; Shulman et al., 1985; Allgeier et al., 2017).

Beyond their role as habitat providers, reef-building corals contribute to the intricate balance of marine ecosystems through their participation in nutrient cycling, carbon sequestration, and shoreline protection (Eliff et al., 2017; Froelich et al., 1983; Ceh et al., 2013; Zarate-Barrera & Maldonado, 2015). Their symbiotic relationship with photosynthetic dinoflagellates (zooxanthellae) enables efficient nutrient recycling through the assimilation of metabolic waste products (Pawlik et al., 2016; Ceh et al., 2013). Moreover, their photosynthetic activity results in the fixation of atmospheric carbon dioxide (Zarate-Barrera & Maldonado, 2015; Goiran et al., 1996).

However, the ecological importance of reef-building corals is intertwined with their vulnerability to anthropogenic stressors such as climate change, overfishing, and pollution (Hoegh-Guldberg., et al. 2007; Hooidink et al., 2016; Pandolfi et al., 2003). The ongoing decline

of coral reefs highlights the urgency of comprehensive conservation and restoration efforts. The conservation of these keystone species resonates well beyond the marine realm, as the vitality of coral reefs is inextricably linked to the health of our planet's ecosystems and the well-being of future generations (Hughes et al., 2018). Due to anthropogenic climate change coral reefs are undergoing rapid changes in terms of overall health and structure (Dubinsky et al., 2011). This decline has led to an increase in research focused on better understanding how corals react to anthropogenic change. Two primary techniques involved within coral restoration are coral gardening and the development of artificial reef structures (Burt et al., 2009; Lima et al., 2019; Rinkevich et al., 2021). Coral gardening is the process of cultivating coral fragments in nurseries under monitored and controlled conditions (Rinkevich et al., 2021). Once fragments are matured, they are outplanted onto degraded reefs (Forrester et al., 2012). Artificial reefs are man-made structures that mimic natural reef habitats (Burt et al., 2009; Lima et al., 2019).

**1.2. Polyp bailout**

The life cycle of scleractinian coral colonies starts with the settlement of planula larvae which develop into a primary polyp that then divides to form a colony through the process of budding (Hughes 1983). This continuous polyp division results in a coral colony composed of thousands of individual polyps. Coral polyps are connected to one another by a thin connective tissue known as the coenosarc (Hall & Hughes, 1996). Under acute stress, this colonial unit can break down, eventually resulting in the detachment of individual polyps from the coral colony (Sammarco et al., 1982). This process, known as polyp bailout, serves as an escape response to extreme stressors such as hypersalinity, high (and low) temperatures, and acidification (Kvitt et al., 2015; Shapiro et al., 2016). The morphology of the coral polyp undergoes well-documented changes (Shapiro, 2016). First, the tentacles of the polyp retract which is followed by the

thinning of the coenosarc tissue (Shapiro, 2016). Lastly, individual polyps retract into their corallites before detaching completely from the coral skeleton (Shapiro, 2016). In undergoing this process, polyps retain their symbionts and are able to reattach themselves to a surface to grow into a new colony (Shapiro, 2016; Chuang & Mitarai, 2020).

The ability for polyps to settle after bailout has created interest in utilizing this stress response as a method of micropropagation. Compared to traditional methods of fragmentation, polyp bailout can produce dozens of micropropagates from a singular coral fragment. This large output of polyps has promise in being utilized as a method of coral micropropagation. Previous research that has studied polyp micropropagation has focused upon short-term observations centered on creating microscale coral polyp model systems (Shapiro et al., 2016, Pang et al., 2020). The coral-on-a-chip system was used to monitor individual polyp growth and characterize the reattachment of polyps on a glass surface following bailout (Shapiro et al., 2016). Within this study, resettled polyps were fixed and examined for physiological parameters such as skeletogenesis, and responses to bacterial infection, and bleaching (Shapiro et al., 2016). While these pioneering studies have established basic protocols for polyp bailout no work has been done documenting the long-term settlement behavior of bailed polyps on commonly used restoration substrates. These previous studies were not directed at using polyp bailout as a method of restoration but rather studying the physiological parameters of the micropropagate itself. Long-term observations, longer than 15 days, of bailed micropropagates have also yet to be well documented.

**1.3. Thesis aims**

This thesis aims to first, develop a technique for inducing hypersalinity polyp bailout within *S. pistillata*, and second, document long-term health parameters within settled polyps

upon commonly used reef restoration substrates. To evaluate the viability of bailed polyp settlement, three substrates commonly used in coral fragmentation and restoration were chosen. Coral fragmentation and restoration endeavors require careful consideration of the substrates utilized to facilitate viable attachment, growth, and survival of corals. Among substrates commonly used, this work utilized PVC, $CaCO_3$, and Portland cement (Adey et al., 1975; Boström-Einarsson et al.,2020; Levenstein et al., 2022). $CaCO_3$ mirrors the composition of coral skeletons and has been used in coral gardening and fragmentation of corals (Levenstein et al., 2022). PVC structures and pipes have been extensively used in coral nurseries and the formation of artificial reef frameworks (Adey et al., 1975). Cement serves as an inexpensive and scalable substrate that has become increasingly popular in the development of artificial reefs (Boström-Einarsson et al., 2020; Burt et al., 2009).

I developed a method for artificially inducing polyp bailout within the stony coral, *Stylophora pistillata* by exposing a coral nubbin to a hypersaline environment. To explore the viability of polyp bail-out for micropropagation and coral restoration, I will compare the settlement and recovery of hypersalinity bailout on different substrates. Through artificially inducing polyp bailout I hope to observe fine-scale interactions between the polyp and its environment such as growth and photosynthetic efficiency over its recovery period. A better understanding of how corals interact with their environment on a fine scale will provide insight into how a changing climate will affect reefs, thereby guiding future mitigation and restoration work.

## 2. METHODS

### 2.1. Corals

Colonies of *S. pistillata* were maintained in flow-through aquaria at Scripps Institution of Oceanography. Water temperature was kept constant at 25 ℃ with an aquarium heater (EnjoyRoyal, USA) and irradiance was provided at 100 μmol photons $m^{-2}$ $s^{-1}$ downwelling irradiance at a 10hr/14hr light-dark cycle that included moonlight simulation (4hrs during dark cycle) (Orbit Marine LED Current Loop).

### 2.2. Induction of polyp bailout

We developed an approach to create coral micropropagates via hypersalinity-induced coral polyp bailout (Shapiro et al., 2016). Polyp bailout was evaluated for a range of hypersalinity regimes and experimental protocols. To create different salinity gradients over time, we evaluated the use of a peristaltic pump, manual adjustments in water salinity, as well as natural evaporation of water for different water volumes. Preliminary experiments with manual increases in salinity with the addition of high salinity water over a set time period and the use of a peristaltic pump proved to be difficult to replicate. Detailed experiments were thus exclusively performed via the creation of salinity gradients as induced by natural evaporation of water. Six different volumes (25 mL, 50 mL, 75 mL, 100 mL, 150 mL, 200 mL) of filtered natural seawater (0.35 um) (FSW) at 35 PPT were evaporated in a small glass container (8 cm diameter) to create different rates of salinity increase over time. Evaporation experiments were performed in a 25 ℃ room with the container placed on a magnetic stirrer at 40 rpm to create gentle water movement. Incident irradiance was provided by an LED panel that delivered a downwelling irradiance at 100 μmol photons $m^{-2}$ $s^{-1}$. An air pump was connected to a small pipette to ensure that the oxygen content of the ambient water was saturated. Salinity was measured hourly using a refractometer

(Agriculture Solutions, USA). To test the effect of different salinity gradients on inducing viable coral micropropagates, we used fragments of *S. pistillata* that were about 2 cm in length. For each salinity gradient, we exposed 3 fragments to hypersalinity stress. Each fragment was continuously monitored for polyp detachment via visual observation. *S. pistillata* required gentle agitation for the polyps to be released from the skeleton. This was done by utilizing a plastic pipette to gently agitate the fragment with water. Bailed polyps were then transferred to a petri dish filled with 35 ppt seawater to reacclimate. After a 15-minute period of acclimation, bailed polyps were evaluated for healthiness using stereomicroscopy and variable chlorophyll *a* fluorimetry.

**2.3. Viability of coral micropropagates**

The health and viability of bailed polyps was determined by morphology and variable chlorophyll *a* imaging. Polyps were either graded as healthy or degraded. This was done by a visual assessment that characterized health from polyp morphology utilizing a stereoscope (Olympus SZ61, Japan). Bailed polyps that were deemed healthy had intact tentacles, a clear mouth, and stomach. Polyps that were characterized as degraded were lacking one or more of these characteristics. Viable and healthy polyps also often demonstrated a spinning behavior which has been marked in other studies as well (Shapiro, 2016).

After polyps were identified for viability through morphology, PAM fluorimetry was then utilized to assess several photosynthetic parameters to further assess polyp viability and healthiness. We used an imaging pulse amplitude-modulated chlorophyll *a* fluorometer (Imaging PAM, mini version; WALZ GmbH, Effeltrich, Germany). The Imaging-PAM employs a blue light (460 nm) via an LED (light emitting diode) panel. We measured the maximum quantum yield of photosystem II ($F_V/F_m$) of bailed polyps following a dark acclimation period of 20

minutes. Subsequently, $F_V/F_M$ was measured by exposing the polyps to a saturation pulse (8200 µmol $m^{-2}$ $s^{-1}$). We also measured relative electron transport rates (rETR) to evaluate the light-use efficiency of the coral micropropagates (Ralph et al. 2008). For this, polyps were dark-adapted again for 20 minutes. rETR curves were performed spanning an irradiance regime from 0 - 783 µmol $m^{-2}$ $s^{-1}$, 12 light steps were conducted; 0, 1, 23, 43, 81, 145, 222, 269, 321, 403, 492, 783 µmol $m^{-2}$ $s^{-1}$ and at each light step samples were incubated for 30 s.

**2.4. Artificial substrates for micropropagate attachment**

To test the attachment and growth of coral micropropagates we selected a range of commonly used settlement substrates including $CaCO_3$, PVC, and Portland cement. $CaCO_3$ has been used in traditional fragmentation methods for hermatypic corals (Levenstein et al., 2022). Many restoration programs across the globe utilize cement as an inexpensive and scalable material for reef restoration. As of 2020, cement has been used as an attachment substrate within about 10 % of restoration studies (Boström-Einarsson et al., 2020). PVC has also been widely used in coral attachment studies (Mallela et al., 2017). This artificial substrate has been seen to support the recruitment of coral and CCA similar to that of natural reef substrate (Adey et al., 1975). PVC and cement substrates were cut to (10 cm x 5 cm) in size. $CaCO_3$ tiles were left at manufactured dimensions (3.175 cm x 3.175 cm). All substrates were fabricated with crevices (1mm deep and 2 mm wide) to facilitate micropropagate attachment. This was done through the use of a handsaw.

**2.5. Attachment of coral micropropagates**

To facilitate the attachment of micropropagates to different substrates, attachment experiments were performed under laminar flow. 18 Laminar flow chambers (12 cm x 6 cm x 3 cm) were custom-made using a CAD software (OnShape) (Figure 1). 18 Flow chambers and 36

nozzles were 3-D printed (PRUSA MK4 3D Printer) with black PLA filament. Flow chambers were fitted with laminar flow dividers (6 cm x 3 cm) and were laser cut from acrylic paneling. The constructed laminar flow chambers were then placed over holding tanks filled with 10 liters of FSW (35 ppt) to create a flowthrough system. To maintain constant flow, silicone tubing is placed on the inlet of the chamber, and water pumps from the holding tank pump water to the chamber. Water flow velocity within the laminar flow chambers was set to 1 cm/s as assessed visually via particle tracking. The water temperature was set to 25 ℃ via a submersible aquarium heater (EnjoyRoyal, USA).

Attachment experiments were performed with 7 replicate micropropagates in 9 tanks for each of the three substrates (PVC, $CaCO_3$, cement). Bailed micropropagates were induced to settle upon $CaCO_3$, PVC, and Portland cement substrates. To induce attachment and settlement, bailed polyps are placed in crevices (2 mm diameter x 1mm depth) developed on the substrates. Micropropagates were exposed to an incident irradiance of 100 μmol photons $m^{-2}$ $s^{-1}$ using white and blue LEDs (Orbit Marine LED Current Loop) that were set to a 10hr/14hr light-dark cycle. Attachment and micropropagate health were evaluated over a period of 21 days. To maintain salinity at 35 PPT, FSW changes were done daily to compensate for any evaporative losses. During these experiments, water temperature and salinity were monitored daily.

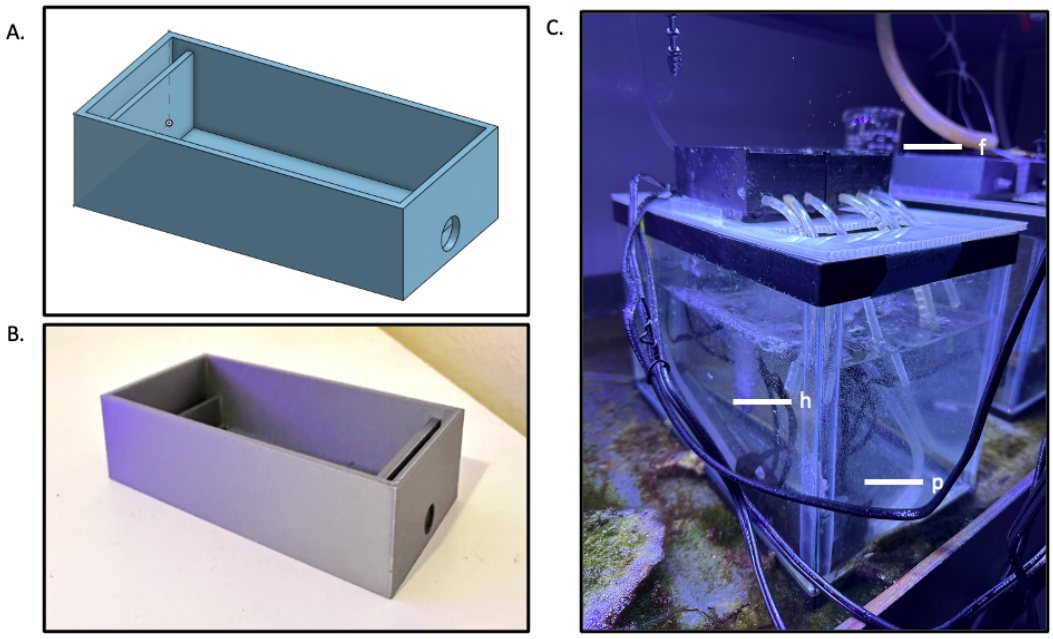


Figure 1. CAD model of laminar flow chamber (A). 3D printed laminar flow chamber in PLA (B). Recirculating tank system. Flow chambers marked as f. Water pumps marked as p. Heater marked as h (C).

### 2.6. Micropropagate growth rate.

To determine the growth rate of attached micropropagates, high-resolution images were taken over a 14-day period every 3 days using a stereoscope (Olympus SZ61, Japan). To not disturb the attachment period, photographic images were taken directly in the experimental set-up from above. Lateral tissue growth was approximated as the planar surface area visually covered by coral tissue. ImageJ (version 1.53, USA) was utilized to measure the planar surface area of attached polyps (Figure 2). This was done by calibrating the ImageJ software to convert pixels to a known measurement. Polyps were then outlined to measure the surface area in $mm^2$. The growth rate of polyps was analyzed by the percentage change in the surface area of the polyps over a 14-day growth period.

Figure 2. Example of planar surface area measurement of attached micropropagate on $CaCO_3$ using ImageJ. Outline of the coral tissue (yellow line) used to measure surface area. Scale bar is 1mm.

**2.7. Optical coherence tomography imaging and substrate characterization**

Optical Coherence Tomography (OCT) was used to non-invasively characterize the attachment of coral micropropagates as well as the surface structure of the artificial substrates. OCT imaging was performed as described previously (Wangpraseurt et al., 2017, 2019). These images were then used to calculate surface roughness. OCT was also used to take images of attached polyps to visualize the interface of attachment between substrates and coral polyps.

**2.8. Statistical analysis.**

All statistical analyses were performed through R Studio. Statistical significance in $F_V/F_m$ between coral fragments and micropropagates was performed through a 1-way ANOVA test. Micropropgate substrate settlement variance (polyp state: substrate) was analyzed using a 2-way ANOVA.

3. RESULTS

### 3.1. Hypersalinity Bailout method

Rates of salinity increase for 25 mL, 50 mL, 75 mL, 100 mL, 150 mL, 200 mL were as follows 3.5 ppt/hr, 2.5 ppt/hr, 1.8 ppt/hr, 1.2 ppt/hr, 0.9 ppt/hr, 0.5 ppt/hr. Smaller volumes of water resulted in faster rates of evaporation which led to a quicker rate of salinity increase. Faster rates of salinity increase resulted in bailed polyps that were not viable and did notretain their morphology. Bailout was induced by the coral fragment being exposed to a gradual increase of salinity through evaporation. A salinity increase of 0.9 ppt/hr produced the highest frequency of morphologically viable micropropagates.

*S. pistillata* polyp morphology responded to increases in salinity in a reproducible manner. Polyp retraction was observed at salinities from 44-46 ppt, followed by the separation and thinning of the coenosarc at 48-51 ppt, which is then followed by the total separation of polyps from the tissue of the coral skeleton at 52-56 ppt. This process resulted in the total bailout of the polyps from the coral skeleton at around 24-26 hours. Polyps bailed from the fragment as patches or as singular units (Figure 3).

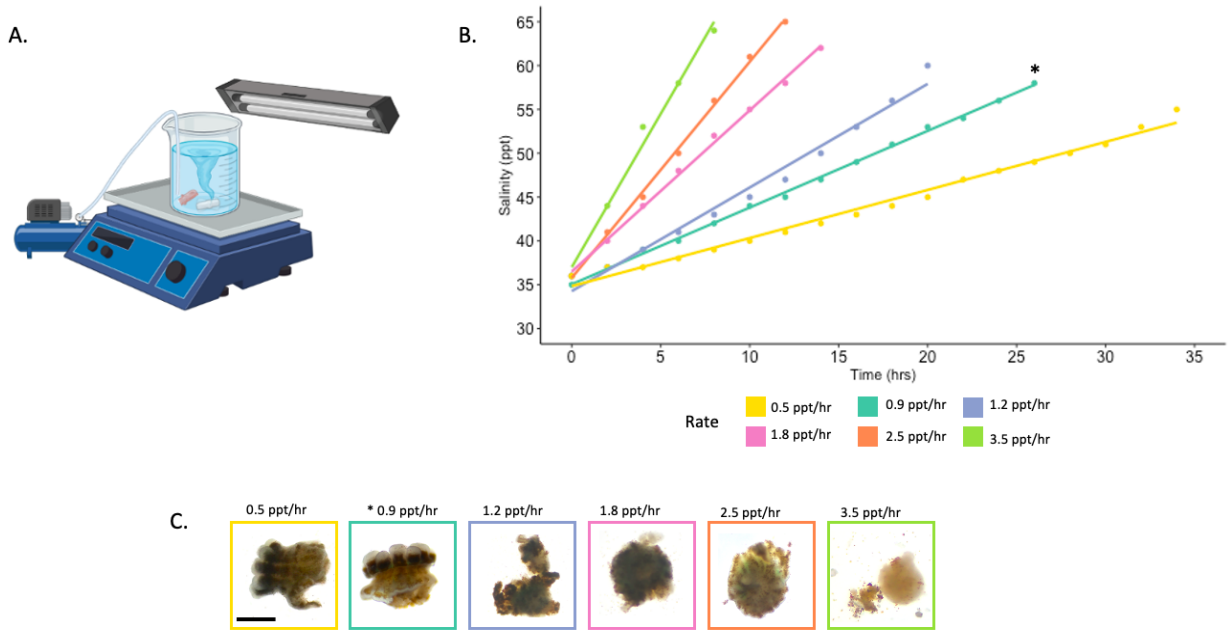


Figure 3. Set-up for evaporation-based hypersalinity polyp bailout (A). Evaporation-based salinity rates of different volumes; asterisk stands for finalized volume utilized for salinity bailout (B). Morphologies of polyps after bailout based upon different volumes and coinciding salinity rates (C).

20 polyps from coral fragments were used for bailout and 20 bailed polyps were used as areas of interest for $F_T$ and $F_v/F_M$ measurements. For both $F_v/F_M$ and $F_T$measurements, there was no significant differnece between coral fragments and micropropagates following bailout (ANOVA, $p<0.05$ p-value = 0.476). The average Ft for *S. pistillata* fragments was 0.19 +/- 0.03 which was similar to the Ft for bailed polyps (0.18 +/- 0.02). $F_v/F_M$ valuesfor coral fragments before bailout were on average 0.47 +/- 0.08 and the $F_v/F_M$ average for bailed polyps was 0.45 +/- 0.04 (Figure 4).

Measurements were also conducted to determine the $F_v/F_M$ healthiness cutoff for polyps. Polyps that did notretain all three aforementioned morphological characteristics were measured to determine an $F_v/F_M$ value for unhealthiness. Polyps that fell below the determined healthiness average of polyps regardless of morphology would not be chosen for latter settlement experiments. The average unhealthy $F_v/F_M$ measurement for polyps is 0.31 +/- 0.09.

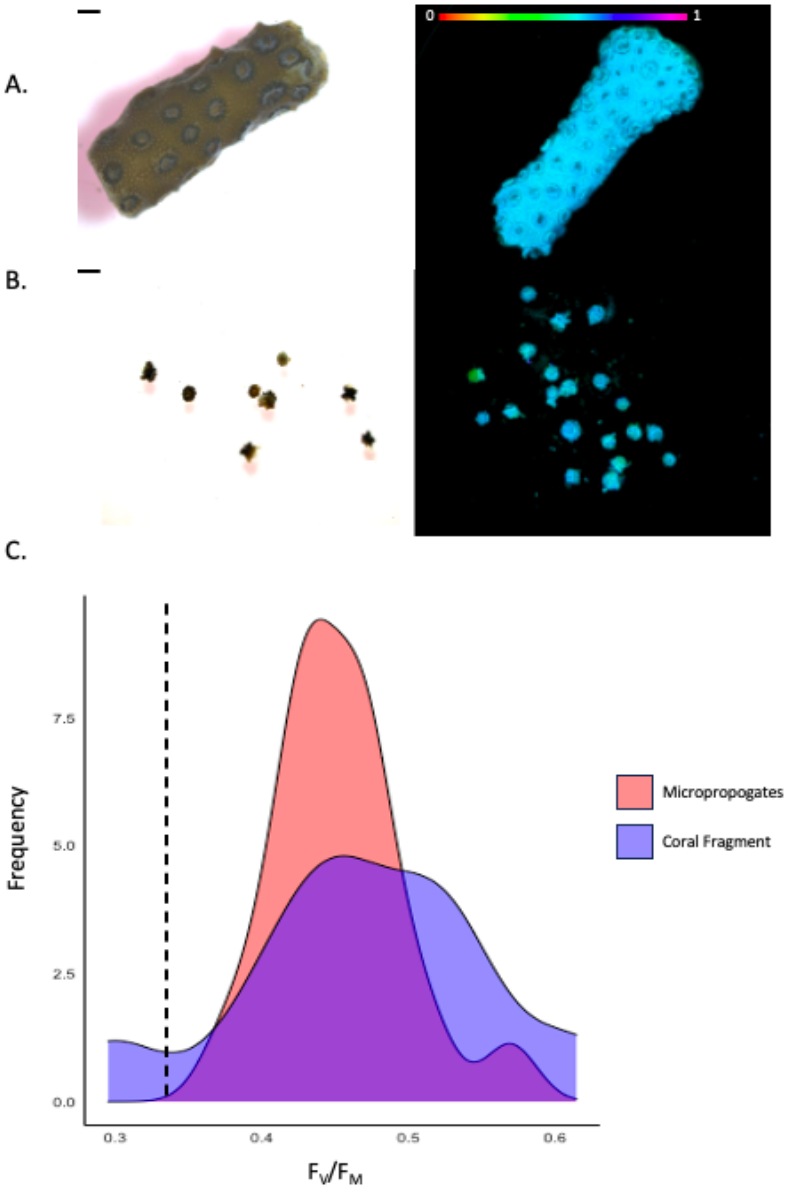


Figure 4. Photophysiological viability of coral micropropagates. (A) Microscopic image of coral fragment before hypersalinity bailout (left), scale bar = 1 mm and $F_v/F_M$ image of coral fragment(right) (A). Microscopic image of micropropagates (left) and $F_v/F_M$ image of micropropagates (right) Scale bar = 1mm (B). Maximum quantum yield ($F_v/F_M$) histogram of $F_v/F_M$ measurements of coral polyps before and after hypersalinity bailout(C). The dashed line is the cutoff for polyps chosen for settlement experiments(C).

Lastly, measurements for ETR were conducted to record the effective quantumyield of PSII of micropropagates over the settlement period. 5 micropropagates from each of the substrates were measured for ETR over a 14-day period at day 7, day 14, and day 21 (Figure 5).

For $CaCO_3$ and PVC, micropropagates displayed a recovery in their ETRs over the 14 days, while cement had an observed degradation in micropropagate ETR.

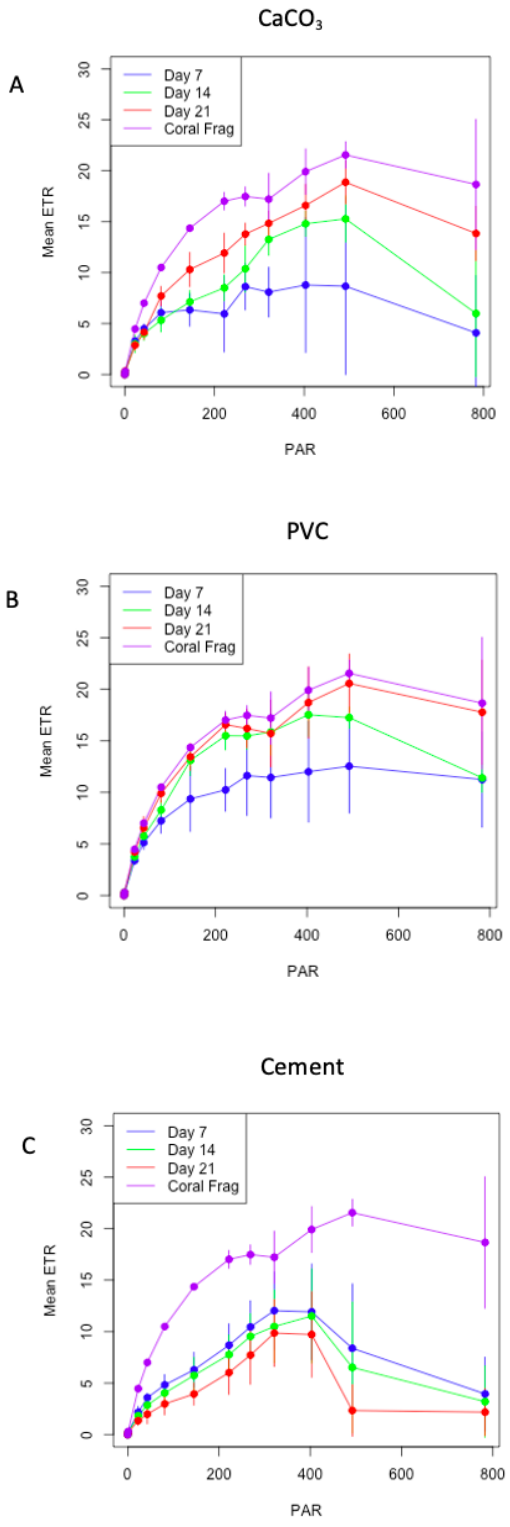


Figure 5. ETR curves of settled micropropagates and healthy coral fragments. ETR curves for day 7, day 14, and day 21 in comparison to healthy coral fragments for micropropagates settled on $CaCO_3$ (A), PVC (B), and cement (C).

### 3.2. Attachment of bailed polyps to engineered substrates

Initial attachment of the polyps onto substrates was observed within 7 days after polyp bailout. Settlement and attachment of bailed polyps varied between substrates. Three characteristics of attachment were recorded (Figure 6). These are attached & healthy, detached & healthy, and attached & degraded. $CaCO_3$ had the highest percentage of attachment of healthy coral polyps at 73.81 % +/- 10.75%, this was followed by PVC with 47.62 % +/- 11.66 %, and Portland cement with 4.76 % +- 7.38 %. PVC has the highest percentage of detached polyps at 45.24 % +/- 10.75 %, this was followed by Portland cement with 23.81 +/- 17.30 %, and $CaCO_3$ with 11.90 % +/- 14.05 %. Portland cement had the highest percentage of attached & degraded polyps at 71.43 % +/- 12.77 %, followed by $CaCO_3$ with 14.23 % +/- 9.04 %, and PVC with 7.14 % +/- 11.95 %. Two-way ANOVA revealed statistical significance between polyp state (p-value = 0.00146) (attached, degraded, detached) and substrate: polyp state (p-value <0.001).

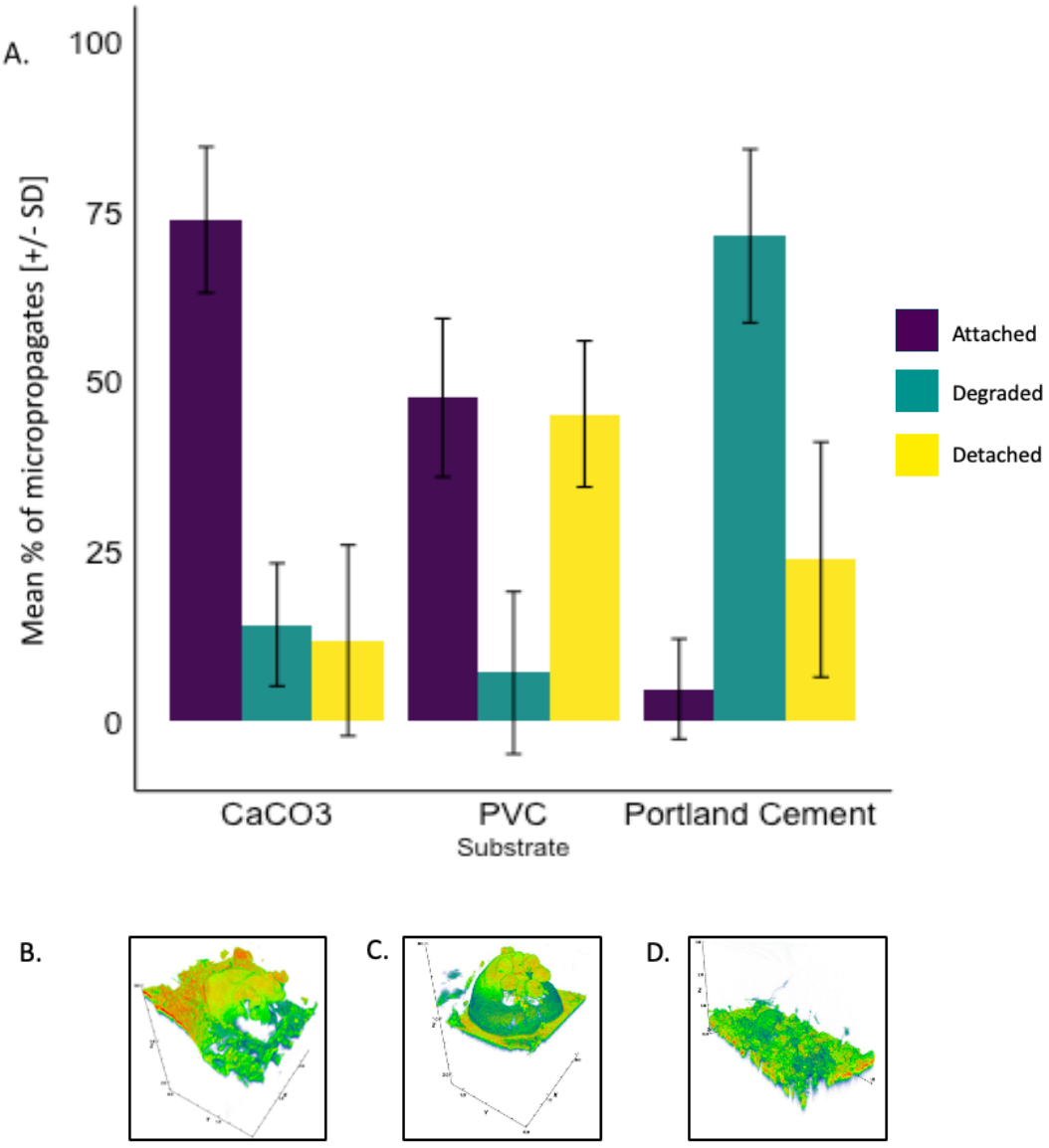


Figure 6. Attachment of micropropagates to substrates. Bar graph of the percentage of polyps for different polyp states (A). OCT image of attached polyp on $CaCO_3$ (B). OCT image of attached polyp on PVC (C). OCT image of degraded polyp on Portland cement (D).

### 3.3. Healthiness and growth rates of attached polyps

$CaCO_3$ had the highest observed $F_v/F_M$ value throughout the measured time points. At day 7 $CaCO_3$ had a measured $F_v/F_M$ value of 0.49 +/- 0.06, at day 14, 0.46 +/- 0.09, and at day 21, 0.48 +/- 0.08. PVC followed with a measured $F_v/F_M$ value of 0.36 +/- 0.04 at day 7, at day 14 0.38 +/- 0.03, and at day 21 0.35 +/- 0.05. Cement had the lowest observed $F_v/F_M$ values, with a

measured day 7 $F_v/F_M$ value of 0.28 +/- 0.04, at day 14 0.22 +/- 0.06, and at day 21 0.21 +/- 0.03.

Growth rate of polyps on $CaCO_3$ was 0.037 +/- 0.002 $mm^2$ over the 14 days. Growth rate of polyps on PVC was 0.057 +/- 0.008 $mm^2$ and growth rate of polyps on Portland cement was -0.0068 +/- 0.008 $mm^2$ (Figure 7).

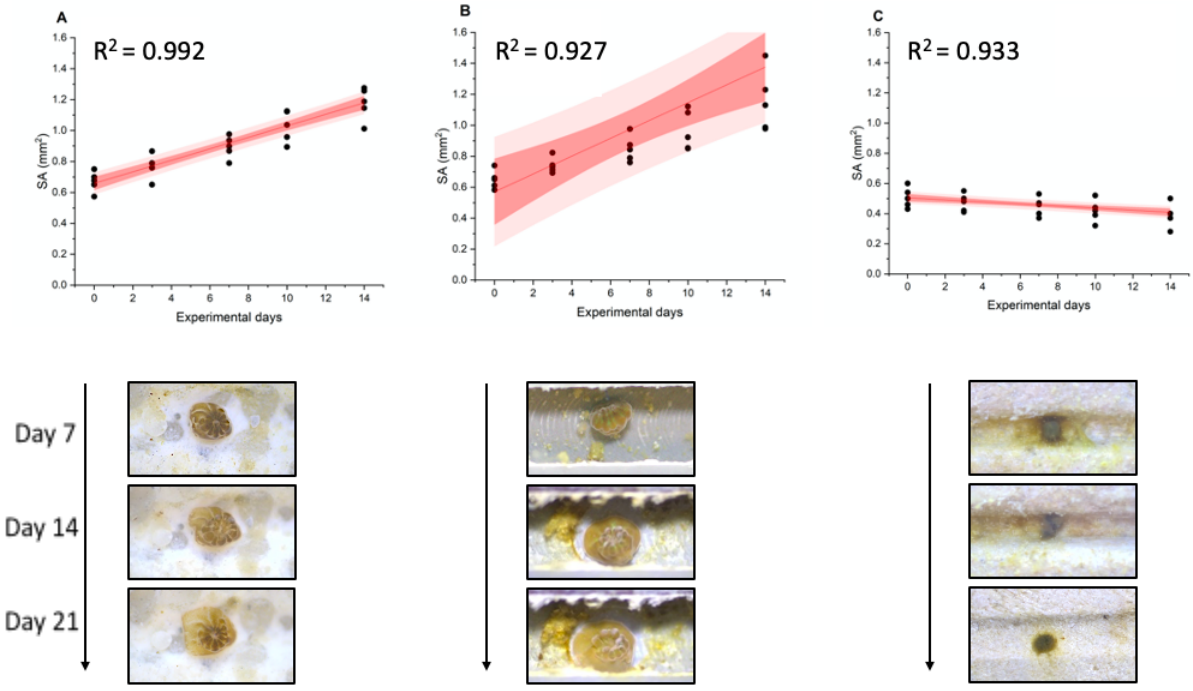


Figure 7. Change in planar surface area (SA) covered by coral tissue over 14 days post-attachment and example images at days 7, 14, and 21. for $CaCO_3$ (A), PVC (B) and portland cement (C).

### 3.4. Substrate characterization

OCT was utilized as a method to characterize substrate surface roughness (Figure 8). From a visual assessment, clear differences in surface morphology were observed. $CaCO_3$ and cement had high surface roughness while PVC was the smoothest substrate in comparison.

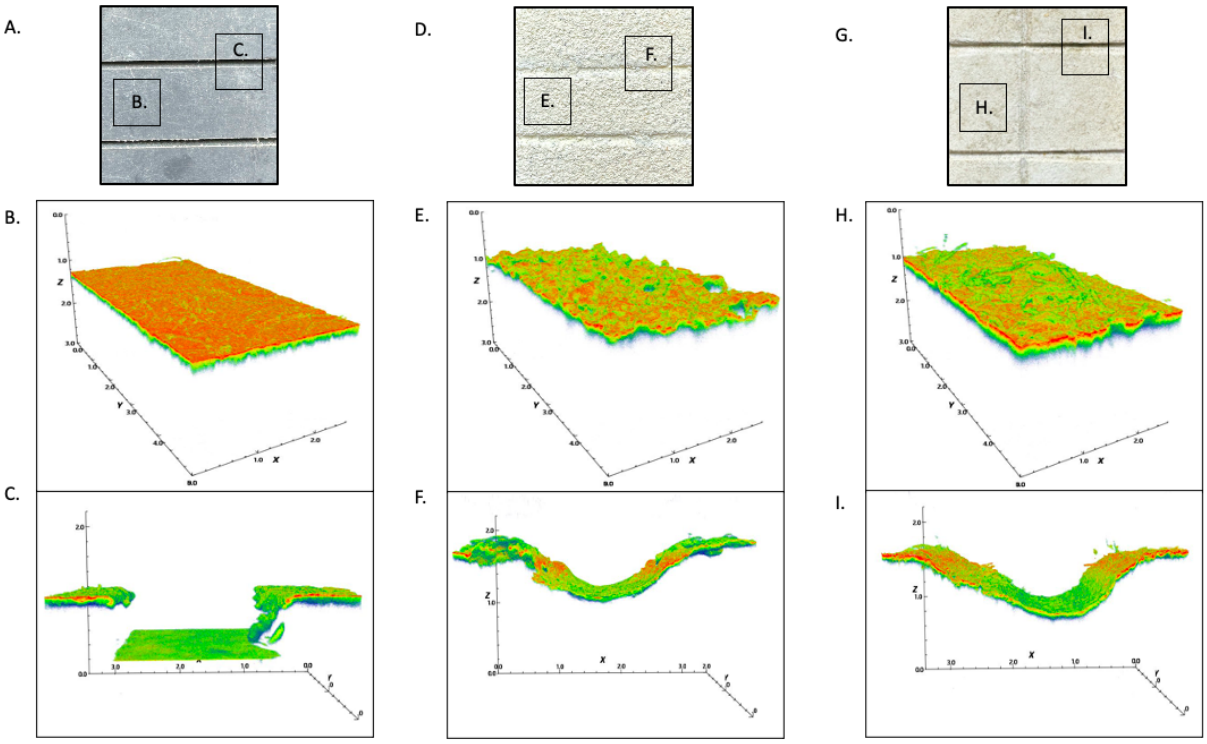


Figure 8. OCT images of substrates. PVC substrate marked with flat surface and crevice of material (A). OCT image of flat surface of PVC substrate (B). OCT image of crevice of PVC substrate (C). $CaCO_3$ substrate marked with flat surface and crevice of material (D). OCT image of flat surface of $CaCO_3$ substrate (E). OCT image of crevice of $CaCO_3$ substrate (F). Portland cement substrate marked with flat surface and crevice of material (G). OCT image of flat surface of Portland cement substrate (H). OCT image of crevice of Portland cement substrate (I).

# 4. DISCUSSION

In this study, we have successfully devised a rapid and easy technique for generating viable coral micropropagates of *S. pistillata* through hypersalinity-induced polyp bailout. Furthermore, we have established a method for the effective attachment of these micropropagates to various settlement substrates. Our work involved the development of a recirculating system designed for incubating polyp attachment and settlement, allowing us to closely monitor the growth and health of coral polyps over a 21-day period.

## 4.1. Hypersalinity Polyp Bailout

Polyp bailout, as an escape response to adverse environmental stressors, has been well-documented in coral biology (Sammarco, 1982). Previous research has identified several stressors, including hypersalinity, acidification, calcium deprivation, and thermal stress, as inducers of polyp bailout (Kruzic et al., 2007; Kvitt et al., 2015; Shapiro et al., 2016; Pang et al., 2020). Within laboratory settings, the induction of polyp bailout has often employed hypersalinity due to its well-established methodology (Chuang et al., 2021; Shapiro et al., 2016; Sammarco, 1982).

Our study builds upon the work of Shapiro et al. (2016), which demonstrated that two other coral taxa from the family Pocilloporidae (*Pocillopora damicornis* and *Seriatopora hystrix*) responded to hypersalinity stress with viable and reproducible bailout. However, a defined and replicable method for *S. pistillata* had not been well-established. In our research, we have successfully devised a specialized protocol for inducing polyp bailout in *S. pistillata*. Consistent with Shapiro et al. (2016), we observed polyp bailout occurring at salinity levels 17-21 ppt above ambient seawater conditions. It is noteworthy that, in contrast to previous observations in *P. damicornis*, the induction of bailout in *S. pistillata* required agitation and water circulation. The

similarities in polyp bailout responses across *P. damicornis*, *S. hystrix*, and *S. pistillata* suggest that this may be a common reaction to hypersalinity stress within the Pocilloporidae family.

By establishing a reliable hypersalinity bailout protocol, we have ensured the preservation of polyp tissue morphology and holobiont composition (Shapiro et al., 2016). Crucially, this allows the bailed polyps to successfully settle following the bailout event. Of particular significance within the coral holobiont are dinoflagellate algae. Our observations, conducted through chlorophyll *a* fluorescence fluorometry measurements, confirm that bailed polyps of *S. pistillata* retained their zooxanthellae over the 21-day experimental period. These findings not only advance our understanding of hypersalinity-induced polyp bailout but also provide valuable insights into the potential applicability of this technique in coral propagation and restoration efforts. Further research is warranted to explore the broader ecological implications and refine the methodology for practical implementation in coral restoration initiatives.

### 4.2. Attachment

Settlement of polyps was observed to varying degrees between substrates. This work serves as the first documentation of settlement upon materials besides glass (Shapiro et al.,2016; Pang et al., 2020). These substrates were chosen due to their use and prevalence within reef restoration work and traditional fragmentation. Polyps were observed on day 7 after bailout to assess the rate of settlement between the different substrates. The differences in substrate settlement suggest material preferences for *S. pistillata* micropropagates.

A comprehensive material characterization of the selected substrates will facilitate a better understanding of the observed differences in attachment behaviors. Initial observations suggest that the elevated levels of healthy and firmly attached micropropagates evident for the

$CaCO_3$ substrate may be attributed to its distinct chemical and structural properties. Notably, this substrate exhibits high surface roughness and shares a compositional similarity with natural coral skeletons. Such parallels in surface topography and chemical composition may account for the pronounced attachment success rates observed.

Conversely, the pronounced rates of micropropagate degradation witnessed within the Portland cement substrate appear to correlate with the leaching behavior inherent to cementitious materials. It is a well-documented phenomenon that cement, when exposed to environments of near-neutral solutions such as seawater, is susceptible to leaching (Chen et al., 2023). Due to the higher alkalinity within the concrete composition, hydroxyl and other associated alkalis can leach into the solution leading to a decrease in the local pH, potentially compromising the viability and adherence of micropropagates.

The high rate of detachment observed within PVC may correlate to its smooth surface roughness. As both $CaCO_3$ and cement had higher rates of attachment whether healthy or degraded respectively, they also had higher observed surface roughness in comparison to PVC. The lack of surface roughness on PVC may hinder the ability of coral micropropagates to settle and securely attach.

### 4.3. Future Research

The results of this study highlight the potential of the polyp bailout methodology to significantly enhance the production of viable polyp micropropagates. The ability to generate dozens of individual polyps from a single small coral fragment underscores the efficiency of this approach in coral propagation. However, as with any technique, there are important trade-offs to consider within the context of coral restoration efforts.

One of the primary trade-offs revolves around the relationship between micropropagate output and size. Polyp bailout, while producing a higher quantity of micropropagates compared to individual coral fragments, introduces the issue of smaller-sized micropropagates. This reduction in size may have ecological implications, particularly concerning their susceptibility to environmental stressors. This may align with observations from field studies, including those by Lirman et al. (2000), which have consistently demonstrated a connection between the size of coral fragments and critical ecological parameters. Smaller coral propagules exhibit the potential for increased yield, which is an advantageous trait for coral propagation efforts. However, this advantage comes at the cost of heightened vulnerability to various environmental stressors. Mortality rates and growth dynamics of smaller coral propagules are adversely affected by factors such as sedimentation, grazing, predation, and competition. These stressors can impede the survival and development of smaller micropropagates, potentially offsetting the initial gains in quantity achieved through polyp bailout. Therefore, while this methodology offers promise in terms of output, it necessitates careful consideration of its application in specific restoration contexts.

Our research also shows promise in the attachment and settlement phase of bailed polyps as well. With the use of settlement substrates commonly used within reef restoration this work shows preliminary preferences within settlement for *S. Pistillata*. This work serves as a longer term observational study, 21 days, of bailed polyp health and growth in comparison to past research (Shapiro et al., 2016; Pang et al., 2020). This has provided insight on the recovery and growth of polyp tissue and photosynthetic efficiency. The ecological implications of deploying polyp bailout as a coral propagation method warrant further investigation. Future research should aim to assess the performance of micropropagates produced through this technique under

different environmental conditions and how micropropagates compare to other fragmentation techniques within in situ environments. This will contribute to a more comprehensive understanding of the advantages and limitations of polyp bailout in the broader landscape of coral restoration strategies.

In conclusion, polyp bailout offers the potential for large-scale production of viable micropropagates, representing a valuable addition to coral propagation efforts. Nevertheless, the trade-offs related to micropropagate size and susceptibility to environmental stressors emphasize the need for a nuanced approach to its deployment. A holistic understanding of the ecological dynamics involved will better the use of this technique to maximize its benefits in coral restoration.

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