

Composite substrates for coral larval settlement and reef restoration based on natural hydraulic lime and inorganic strontium and magnesium compounds

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ABSTRACT

Coral reefs face unprecedented threats from climate change and human activities, making reef restoration increasingly important for the preservation of marine biodiversity and the sustainability of coastal communities. One promising restoration method relies on coral breeding and larval settlement, but this approach requires further innovation to achieve high rates of settlement and survival. In this study, we built on our previous work engineering lime mortar-based coral settlement substrates by investigating three different compositions of a natural hydraulic lime (NHL) base material as well as composite NHL substrates containing alkaline earth metals. These materials were tested with larvae of three reef-building Caribbean coral species: *Orbicella faveolata* (Mountainous star coral), *Diploria labyrinthiformis* (Grooved brain coral), and *Colpophyllia natans* (Boulder brain coral). We found that the base material composition, including its silicate and calcium carbonate (CaCO_3) content, as well as the addition of the inorganic additives strontium carbonate (SrCO_3), magnesium carbonate (MgCO_3), and magnesium sulfate (MgSO_4), all influenced coral larval settlement rates. Overall, NHL formulations with lower concentrations of silicate and higher concentrations of calcium, strontium, and magnesium carbonates significantly increased coral settlement. Further, when dissolved ions of magnesium and strontium were added to seawater, both had a significant effect on larval motility, with magnesium promoting settlement and metamorphosis in *C. natans* larvae, supporting the observation that these additives are also bioactive when incorporated into substrates. Our results demonstrate the potential benefits of incorporating specific inorganic ion additives such as Mg^{2+} and Sr^{2+} into substrates to facilitate early coral life history processes including settlement and metamorphosis. Further, our results highlight the importance of optimizing multiple aspects of coral substrate design, including material composition, to promote settlement and survival in coral propagation and reef restoration.

1. Introduction

Coral reefs are one of the most diverse ecosystems on the planet, providing habitat for a wide range of marine species and supporting the livelihoods of millions of people worldwide (De Groot et al., 2012;

Costanza et al., 2014; El-Naggar, 2020). These ecosystems are highly sensitive to climate change, pollution, overfishing, and coastal development, resulting in the ongoing loss of coral reefs globally (Hughes, 1994; Hughes et al., 2003; Huang, 2012; Hoegh-Guldberg et al., 2017). In response to this decline, a concerted effort has emerged among

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marine conservation organizations and scientists to develop more effective and scalable tools for restoring these ecosystems. One reef restoration strategy involves the fabrication and deployment of artificial structures (Lee et al., 2018) created using techniques such as molding or slip-casting. On an ecosystem-scale, the final goal of these artificial reef structures is to develop into self-sustaining natural reefs by offering suitable surfaces for coral larval recruitment and facilitating their growth into mature colonies, thus accelerating the permanent establishment of a robust reef ecosystem. On a smaller scale, artificial settlement substrates are being developed to support the recruitment, growth, and survival of juvenile corals, yet this remains a major challenge in applying coral larval propagation as a restoration technique.

Innovative approaches to support coral recruitment and promote settlement involve using biological cues such as bacterial isolates (Tebben et al., 2011), bacterial biofilms (Negri et al., 2001), and crustose coralline algae (CCA) (Gómez-Lemos et al., 2018). More recently, inorganic cues such as Ca^{2+} ions have been tested (Yang et al., 2022). Calcium is a known component of coral skeleton, but is presumed to initiate settlement in part due to the role of Ca^{2+} -binding proteins in various cell signaling pathways related, but not limited, to invertebrate settlement and metamorphosis (Carafoli et al., 2001; Yang et al., 2022). Additional alkaline ions, namely elemental strontium and magnesium, are found in coral skeletons beginning at very early life stages and it is hypothesized that these elements play an important role in coral skeletal calcification (Sun et al., 2015; Mass et al., 2017; Akiva et al., 2018; Neder et al., 2019). In fact, the Sr:Ca ratio in the skeleton of corals and coral fossils is widely used to estimate the ancient composition and temperature of seawater (Hughes et al., 2019; Sayani et al., 2022; Walter et al., 2023). Our previous work demonstrated increased coral settlement in response to strontium carbonate when incorporated into a lime mortar base (SrCO_3 ; (Levenstein et al., 2022). This work also demonstrated a settlement response in the presence of silica (SiO_2), but not in the presence of dolomite ($\text{CaMg}(\text{CO}_3)_2$, an insoluble form of magnesium). Here, we continue to investigate the role of inorganic elements and ions in novel coral settlement substrate compositions. We hypothesized that both the presence of silica and the addition of elemental and ionic strontium and magnesium would act as a settlement cue, increasing coral larval settlement. In general, materials capable of incorporating and facilitating the release of settlement cues are of high interest in coral propagation and reef restoration (Banaszak et al., 2023).

Currently, materials such as Portland cement are widely used in artificial reef construction and coral propagation (Chamberland et al., 2017; Paxton et al., 2020; Banaszak et al., 2023). However, these materials are often highly alkaline and common compositions can leach harmful chemicals into the water, causing detrimental effects on coral growth and survival (Hillier et al., 1999; Ali et al., 2011). Alternative materials gaining interest in restoration include non-hydraulic lime and natural hydraulic lime (NHL) mortars (Diaz-Basteris et al., 2022; Levenstein et al., 2022). Our recent research demonstrated that non-hydraulic lime mortars can be useful in coral reef restoration by providing a stable, calcium carbonate-based substrate for coral larvae to attach and grow upon (Levenstein et al., 2022). Calcium carbonate is the primary component of the coral skeleton, thus carbonated NHL mortars mimic certain aspects of the natural calcium carbonate composition of coral reefs. Incorporating calcium carbonate is hypothesized to promote coral attachment and growth in restoration materials (Spieler et al., 2001). Moreover, these mortars have several advantages over conventional materials, such as ceramic or Portland cement, that are currently used in coral reef restoration. They have a higher microporosity than fired ceramics (Silva et al., 2008) and polymers (Mayhoub et al., 2022), allowing for better water perfusion and nutrient exchange, which are essential for coral growth (Yanovski and Abelson, 2019). Before setting, the mortar paste is malleable, allowing it to conform to irregular surfaces and giving it the potential to mimic more natural reef structures and features. NHL mortar is also non-toxic and biocompatible, thus, it does not leach harmful chemicals into the water and is not considered to

pose any risks to marine life. Additionally, the preparation of lime mortar substrates allows for the incorporation of inorganic and organic additives that may further enhance early coral settlement and growth. To further build upon previous research, here we expand our investigation from non-hydraulic lime mortars to natural hydraulic lime (NHL) mortars.

Importantly, NHL mortars can be tailored to meet species-specific needs. The physical composition allows for the incorporation of a variety of features such as structure, texture, and micro-niches, all of which stand to influence the composition and abundance of colonizers, and therefore the success of an artificial reef (AR) (Baynes and Szmant, 1989; Glasby, 2000; Burt et al., 2009; Hill et al., 2021; Lymperaki et al., 2022). Producing substrates with a variety of these features provides a test bed to determine optimal parameters for coral settlement and growth. For this purpose, it is essential to understand the chemistry occurring during the hydration and carbonation processes when NHL mortars are employed, since the composition of the material is another important factor in the success of AR (Hylkema et al., 2020; Monchanin et al., 2021; Ramm et al., 2021; Reis et al., 2021). Here, we examined the effects of NHL composition and several inorganic additives on the swimming behavior and settlement of coral larvae. Two experiments investigated the impact of ion additives and base material formulation on the early life history of corals: (1) a behavioral experiment examined the impacts of dissolved Mg^{2+} and Sr^{2+} ions on the survival, swimming behavior, settlement, and metamorphosis of boulder brain coral, *Colpophyllia natans* larvae; and (2) a settlement study examined the effects of NHL-based substrates with and without ionic additives on the larval settlement rates of the mountainous star coral, *Orbicella faveolata* (Ellis and Solander, 1786), grooved brain coral, *Diploria labyrinthiformis* (Linnaeus, 1758), and boulder brain coral, *C. natans* (Houttuyn, 1772) larvae (Veron et al., 2024).

2. Materials and methods

2.1. Preparation of coral settlement substrates using natural hydraulic lime (NHL) base material

Three different commercial formulations of natural hydraulic lime mortar (NHL, Lime Works, USA) were used with increasing concentrations of calcium silicate (C2S); NHL2, NHL3.5, and NHL5 contained 17, 35, and 43 wt% C2S, respectively (values provided by the supplier, Lime Works, USA). NHL powder was mechanically homogenized with deionized water to yield pastes with a water:lime ratio of 0.7 (w:w). The NHL3.5 mortar was then used as a base material to produce various settlement substrate compositions containing a range of inorganic additives, including strontium carbonate (SrCO_3), magnesium carbonate (MgCO_3), and magnesium sulfate (MgSO_4 , all additives sourced from Sigma-Aldrich). For these NHL substrates with inorganic additives (Fig. 1a), NHL3.5 was dry-mixed with the selected inorganic additives using a shaker (Tornado II Paint Shaker 51000, Blair, Michigan, USA) prior to blending and homogenizing the NHL paste. NHL3.5 was used for these composite materials (instead of NHL2 and NHL5) due to its intermediate silicate content and following preliminary, small-scale trials demonstrating its ability to induce coral settlement without any biological or inorganic chemical additives.

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

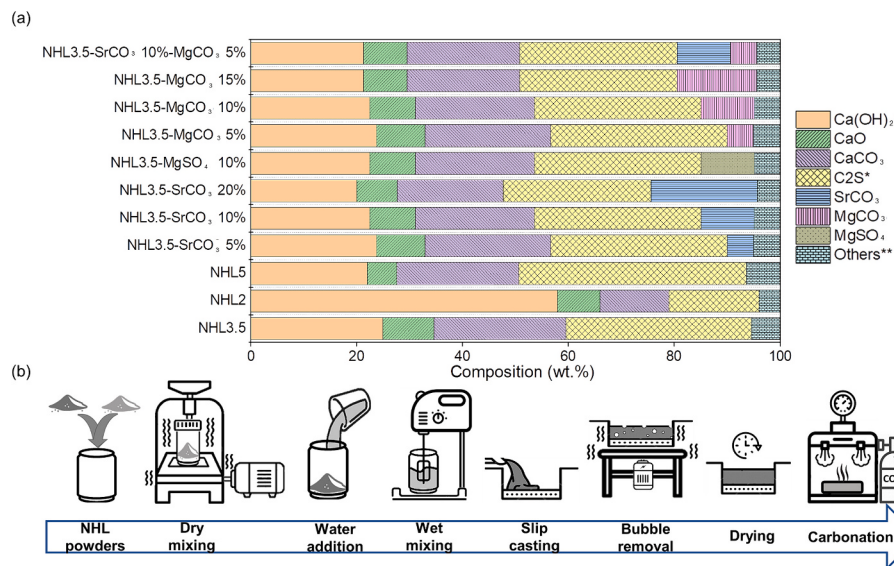


Fig. 1. a) Coral settlement substrate names and compositions. Substrate composition is shown as wt% of each component (both base material components and additives) in the mixture of dry NHL powder with additives, prior to the addition of water. *Dicalcium Silicate. ***“Others” includes the trace components tricalcium aluminate, dicalcium alumina silicate, brownmillerite, and calcium sulfate. b) Schematic of the protocol used for manufacturing NHL-based coral settlement substrates.

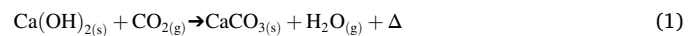
below.

NHL mortars contain a mixture of calcium hydroxide, calcium carbonate, and calcium silicates. These mortars are set by hydration, which refers to the effect of water binder ratio on the early hydration of NHL. After hydration, the carbonation of NHL leads to the transformation of the remaining calcium hydroxide into calcium carbonate (Ball et al., 2011; Figueiredo et al., 2016). This carbonation process is partly driven by the gaseous diffusion of CO₂ within the NHL microstructure. It is, therefore, a multi-decadal process under ambient conditions depending on the properties of the sample (Auroy et al., 2018). Thus, an alternative approach to accelerate carbonation is utilized to speed up this process. Using a modified pressurized CO₂ chamber, up to 95% carbonation can be achieved in 6 h, with the added advantage that the equilibrium pH of the final material decreases from 12.4 to 8.4, ensuring that there is no calcium hydroxide left that could alter the pH of the experimental system or ecosystem upon deployment. This expedited carbonation method is essential for rapid processing. In a previous study (Levenstein et al., 2022), a glovebox was used instead of a chamber, yielding comparable carbonation levels. Notably, the absence of a pressurized chamber extended the carbonation duration to 7 days, contrasting with the 16-h timeframe when utilizing the chamber.

2.2. Characterization of substrate composition, carbonation, hardness and durability

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

The carbonation rate of the substrates in the CO₂ chamber depended not only on the time of CO₂ exposure but also on the position inside the chamber (Fig. 3). To determine the influence of the location on carbonation, and to ensure that all substrates achieved full carbonation during production, the carbonation chamber was fully loaded with NHL2 substrates (26 per rack) and substrates were sub-sampled through time. The chamber was divided into 4 vertical racks and 4 quarter divisions to determine if there were gradients during the carbonation reaction (eq. 1).



The presence of vertical gradients in CO₂ concentration would result in variations in the carbonation extent of substrates in each rack. To examine potential carbonation gradients in the radial direction, each rack was subdivided into quarters. To determine the time required in the chamber until full carbonation of calcium hydroxide in the substrates, one substrate was randomly selected from each rack and position in the carbonation chamber after 3, 16, and 23 h. Substrates were ground into a fine powder prior to determining the presence of calcium hydroxide by FTIR (Frontier FT-IR, PerkinElmer, USA).

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

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

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

To further investigate the long-term stability and durability of NHL substrates when placed underwater, we out-planted substrates of 4 compositions (NHL3.5, NHL3.5-MgCO₃ 10%; NHL3.5-SrCO₃ 10%; and NHL3.5-SrCO₃ 10%-MgCO₃ 5%) at a common location on the coral reef in front of CARMABI Research Station, Curaçao (12°07'19.4"N 68°58'09.7"W). Individual substrates for each mixture were imaged monthly for three months (underwater) and then again at 20 months (in lab) and visually inspected. At the 20-month endpoint, the substrate composition was analyzed by XRD (D8 Advance X-ray diffractometer, Bruker, USA) to measure any changes in chemical composition that had occurred. Substrates were ground into a fine powder prior to determining the crystallography of the substrates.

2.3. Collection and fertilization of coral gametes

Coral gamete bundles were collected during a succession of natural spawning events in August, September, and October 2021. Gametes were collected on the coral reefs off the Southern Caribbean island

nation of Curaçao at the Zakitó dive site (also known as Water Factory; 12°0629.7"N 68°5712.6"W). Following methods described in previous works, egg-sperm bundles were collected from spawning coral colonies using weighted nylon mesh nets attached to plastic funnels and 50 mL conical polypropylene tubes (BD Falcon), thus concentrating the buoyant gamete bundles into a small volume for collection and transport (Hagedorn et al., 2021; Levenstein et al., 2022). *Orbicella faveolata* gametes were collected from parent coral colonies on the night of September 26th; *C. natans* were collected on August 30th and September 27th, and *D. labyrinthiformis* were collected on October 3rd. Once ~5 mL of egg-sperm bundles had accumulated in each conical tube, the tube was removed from the funnel and capped. Gametes were returned to the lab directly after the spawning event, cross-fertilized by mixing in 1 L polycarbonate fat separators (Norpro), and incubated for at least 1 h. After fertilization, embryos were rinsed with 0.5 µm filtered seawater (FSW; serial spun polypropylene sediment filters with pore size 50, 20, 5, and 0.5 µm; H2O Distributors). Fertilized gametes were then distributed into polystyrene clamshell food containers (Dart Container Corporation) containing 1 L of FSW at the approximate density of 1 larva/mL (~1,000 larvae per bin). Water changes were performed daily to reduce microbial load and maintain a stable pH in the rearing environment throughout development (Vermeij, 2006; Chamberland et al., 2017; Hagedorn et al., 2021).

All experiments were run in a temperature-controlled laboratory at CARMABI with experimental water temperatures kept consistent with that of the local reefs (27.5 ± 1.5 °C). All unsettled larvae were released into the sea at the end of each experiment. Settled coral recruits were either out-planted onto a restoration structure, moved to aquaria for long-term monitoring, or preserved for microscopic analysis.

2.4. Larval survival and swimming behavior experiment using dissolved magnesium and strontium ions

One advantage of using NHL as a coral settlement substrate is the ability to incorporate diffusible additives that can be slowly released into the surrounding seawater, supporting initial settlement or longer-term survival and growth. To test for survival and swimming behavior effects of ion additives, we conducted a laboratory experiment to mimic the environment that coral larvae would experience when in proximity to NHL substrates that release magnesium and strontium ions. To mimic these conditions, MgSO_4 and SrCO_3 stock solutions were prepared based on the observed 3-day dissolution rates of Sr^{2+} from the NHL composite substrate (Fig. S1). The effect of dissolved inorganic additives on larval survival, swimming behavior, and settlement was then monitored to understand how the proposed additives affect coral larvae during dispersal and settlement. Each experimental block consisted of one six-well plate, using the four outside wells, with one well per treatment in each plate. Wells within a block were assigned a treatment using a random number generator. In total, there were four treatments: each inorganic additive individually (Mg^{2+} or Sr^{2+}), the combined inorganic additives (Mg^{2+} and Sr^{2+}), and a control (FSW). For Sr^{2+} treatments, a stock solution with SrCO_3 was prepared by dissolving 0.336 g of SrCO_3 (Sigma-Aldrich, purity $\geq 98\%$) into 200 mL of FSW and stirring on low heat (40–50 °C) for 24 h. 2 mL of the stock solution was then diluted in 498 mL FSW to obtain a final ion concentration of 12 ppm of Sr^{2+} (150% with respect to ambient seawater). For Mg^{2+} treatments, 1.6 g of MgSO_4 anhydrous (Sigma Aldrich, $\geq 99.5\%$) was dissolved directly into 500 mL of FSW to obtain a final concentration of 1950 ppm of Mg^{2+} (150% with respect to ambient seawater).

Two days after fertilization, 800 swimming planula larvae were pooled from rearing bins using a 70 µm nylon cell strainer (Fisher Scientific) and rinsed in FSW. Larvae were then evenly allocated to 20 replicate wells per treatment, 10 larvae per well. Larval behavior was scored at 09:00 h daily for six consecutive days. Behaviors and survival were scored using four distinct categories: actively motile or swimming, motionless, settled and metamorphosed, or dead. Dead larvae lose their

rounded bilateral symmetry and appear frayed or become completely absent (Marhaver et al., 2013) and are thus distinguishable from motionless larvae. Settled and metamorphosed coral larvae have successfully adhered to the well and developed a polyp mouth. The proportion of motile larvae was calculated using the total number of larvae observed alive in each well. A linear mixed-effects model was fit to compare the effect of factors Mg^{2+} and Sr^{2+} on the proportion of both motile and alive larvae in a given well using days 1–6 as the repeated measures response variable. Well location and plate number were included in the linear regression as random factors. Due to non-normality, a non-parametric permuted ANOVA (# permutations = 10,000) was performed to compare the effects of ion additives on the proportion of larvae that underwent successful settlement and metamorphosis on the final day of the experiment. Originally, a two-way permuted ANOVA (# permutations = 10,000) was run with Mg^{2+} and Sr^{2+} as factors. Due to no significant interaction, the model was simplified to two one-way permuted ANOVAs. An additional permuted ANOVA was performed to compare the effects of Mg^{2+} and Sr^{2+} on survival. Well number was included in all models as a random factor.

2.5. Larval settlement experiment using NHL base materials and inorganic additives

In the field of larval propagation, settlement is often promoted by adding known positive settlement cues such as CCA (Morse et al., 1988; Heyward and Negri, 1999a, 1999b; Tebben et al., 2015). Here, all settlement studies were conducted without such cues to best elucidate larval response to NHL-based substrates and the aforementioned inorganic additives of interest. Prior to beginning the settlement experiments, substrates in the lab were conditioned in FSW for 3 days. Substrates were turned over every 12 h to release any air bubbles that had escaped from the substrate pores and accumulated on the substrate surfaces. Substrates were then each placed in a 200 mL polystyrene cup with 150 mL of FSW to evaluate the effects of NHL composition, inorganic additive type, and additive concentration on larval settlement. 100 (± 5) larvae were pipetted into each cup, for a total of eight replicate cups per substrate type. All larvae were healthy, motile, and between 2 and 3 days old at the start of the experiments. Larval settlement on the top and bottom of each substrate was recorded following 13 days of exposure.

The total number of settlers on each substrate was used for statistical analysis. Due to non-normality, settlement data were analyzed via two separate one-way permuted ANOVAs (# permutations = 10,000) to compare the effect of NHL composition and inorganic additive type and weight % (Fig. 1) on larval settlement for each of the three coral species. A series of Wilcoxon rank-sum pairwise comparisons were performed to distinguish differences between NHL and inorganic additives compositions separately.

3. Results

3.1. Characterization of substrate composition of NHL base materials and composite materials

The kinetics of accelerated carbonation of NHL mortars was examined by combining XRD and FTIR spectroscopy. The extent of carbonation was determined by FTIR. A peak at ~ 3650 cm^{-1} indicates the presence of calcium hydroxide, signifying incomplete carbonation (Fig. 2a). The XRD data for NHL3.5 before and after accelerated carbonation confirmed that the pressurized CO_2 chamber achieved full carbonation of the material (Fig. 2c). The dry NHL powder contained 25% calcite (CaCO_3), demonstrated by the diffraction peak at 29.6° . The characteristic peaks of portlandite (Ca(OH)_2) occurred at 18° and 34.2° . After carbonation, those peaks disappeared, while the intensity of the calcite peaks increased.

Further, the presence of the SrCO_3 additive was confirmed by peaks

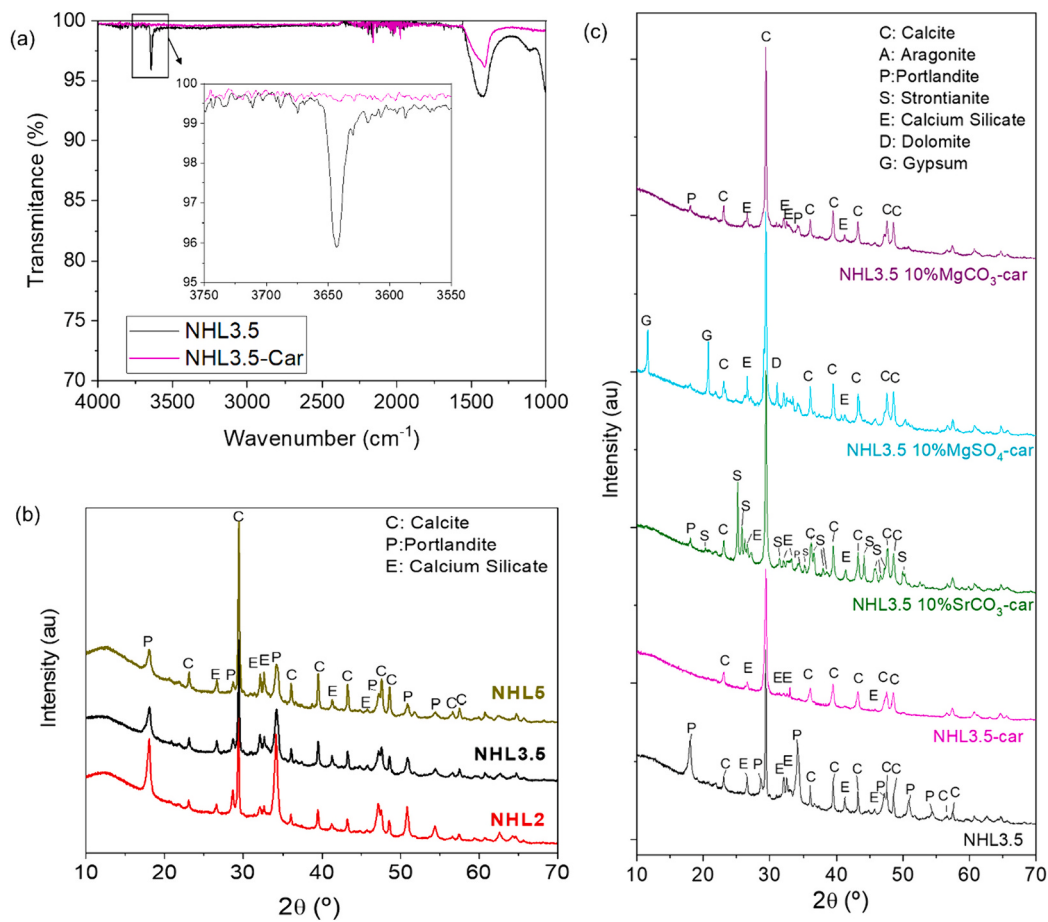


Fig. 2. (a) Infrared spectra of NHL3.5 substrates before and after carbonation obtained via Fourier transform infrared spectroscopy (FTIR). A peak at 3650 cm⁻¹ reveals the presence of Ca(OH)₂, indicating incomplete carbonation. (b) X-ray diffractogram comparing the different commercial NHL powders used in this study. The NHL powders were subject to analysis before carbonation. (c) X-ray diffractogram of unmodified NHL3.5 substrates before and after carbonation, and of the post-carbonated (“-car”) NHL3.5 composite substrates containing 10 wt% MgCO₃, 10 wt% SrCO₃, and 10 wt% MgSO₄.

at 25.3° and 26.0°, indicating its stability after the fabrication process (Fig. 2c). In contrast, the addition of MgSO₄ provoked a reaction and formation of both gypsum, illustrated by peaks at 11.7° and 20.8° in cyan, and of dolomite, illustrated by a peak at 31°. It is also noteworthy that the addition of amorphous MgCO₃ did not shift any peaks; although this material is invisible in XRD, we confirmed that it did not modify either the intensities nor the positions of peaks of other crystalline phases that would occur if the carbonate reacted with the other materials present. This result suggests a high stability of the MgCO₃ during the production process.

The composition of three different NHL-based substrates (NHL2, NHL3.5, and NHL5) was qualitatively determined from the ratio of the silicate peak at 27.3° and the carbonate peak at 29.4° (Fig. 2b). As expected, NHL2 had the highest portlandite content (peak at 18.1°) and lowest silicate content (peaks at 26.2°, 32.1°, 32.7°, and 41.1°). NHL5 had the highest silicate content and the lowest amount of calcium hydroxide. NHL3.5 had intermediate levels of silicate and calcium hydroxide. This material was chosen as the base substrate material for testing the inorganic additives (SrCO₃, MgCO₃, and CaSO₄) at different concentrations. All compositions are listed in Fig. 1a.

3.2. Evaluation of the degree of carbonation of NHL substrates within pressurized CO₂ chamber

To ensure that all substrates were fully carbonated in the production process, we investigated the degree of carbonation within the pressure chamber and determined whether substrate location affected the rate of

carbonation results, specifically whether carbonation was faster for substrates located on the lower shelves (Fig. 3a and b). Overall, carbonation was faster for substrates located on the lower shelves (Fig. 3c). No radial gradient was observed. FTIR spectra are provided in the supplementary information (Fig. S2).

3.3. Assessment of material durability, measured by indentation techniques and underwater tests

The hardness of substrates intended for use in coral reef restoration is an important material property. We use hardness as a simple proxy to indicate handleability and stability in the marine environment. Substrate stability is important for settlement whereas an unstable surface will hinder settlement and stable attachment, reducing the success of restoration efforts (Ceccarelli et al., 2020). The hardness of the substrate can also affect its ability to withstand environmental stresses such as wave action and storm surge (Lymperaki et al., 2022). A soft material may be more vulnerable to damage or displacement during high-energy events, which could undermine the stability of newly established corals.

Vickers micro-indentation tests were used to measure the hardness of all NHL base materials and composites (Fig. 4). The sample composition with the highest Vickers hardness was NHL5 followed by NHL3.5, NHL3.5-MgSO₄ 10%, NHL3.5-SrCO₃ 10%, NHL2, NHL3.5-SrCO₃ 10%-MgCO₃ 5%, NHL3.5-MgCO₃ 10%. The incorporation of inorganic additives decreased the substrate's hardness. MgSO₄ and SrCO₃ additives reduced the substrate hardness while MgCO₃ additives further decreased hardness even below that of the NHL2 without additives.

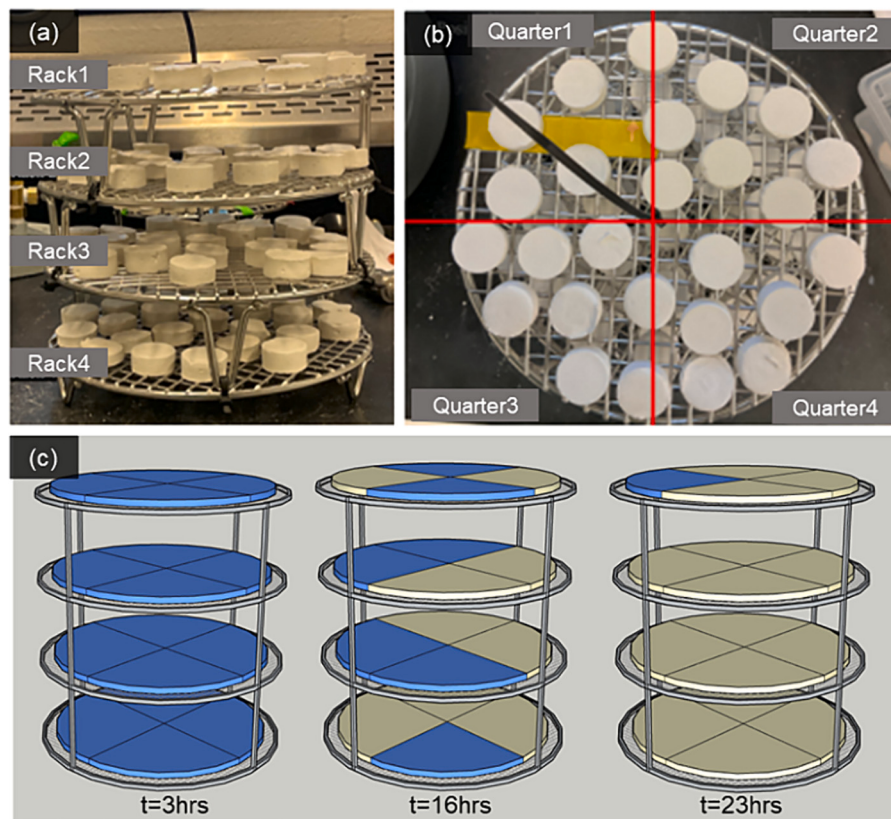


Fig. 3. Carbonation chamber shelves and inner structure. Photos of the (a) vertical division into 4 racks and (b) sectorial division in 4 quarters. (c) Schematic showing the degree of carbonation after 3 h, 16 h, and 23 h. Light yellow: full carbonation, blue: incomplete carbonation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

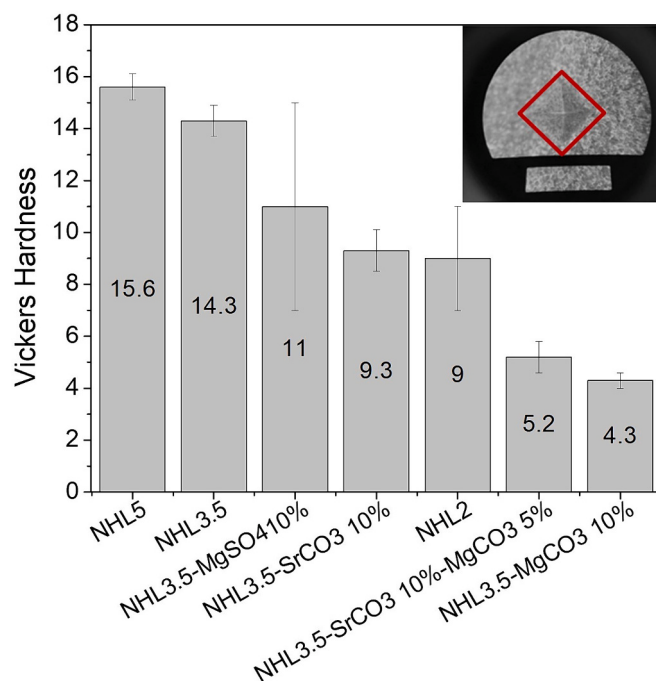


Fig. 4. Substrate hardness of NHL base materials and composites. Average Vickers Hardness for the seven substrate compositions of interest (% referred to as wt% of the additive mixed with dry NHL3.5). Inset: image of one indentation created during hardness testing. $n = 3$ replicate measurements per substrate type. Error bars represent SD.

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

The substrate composition was further investigated by XRD after 20 months on the reef, confirming that out-planting over this period did not induce significant compositional changes. X-ray diffractograms of NHL3.5, NHL3.5-MgCO₃ 10%, and NHL3.5-SrCO₃ 10%-MgCO₃ 5% are presented in Fig. S3 of the supplementary information.

3.4. Coral larval behavior in response to dissolved strontium and magnesium

To understand the potential effects of inorganic ion dissolution from composite substrates, we investigated the survival and behavior of coral larvae during the planktonic stage when exposed to ions in solution. Survival, swimming behavior, and settlement rates were measured in response to inorganic additives at 150% ambient seawater concentrations and in the absence of a settlement substrate. Survivorship remained high throughout the study, with the mean survival remaining

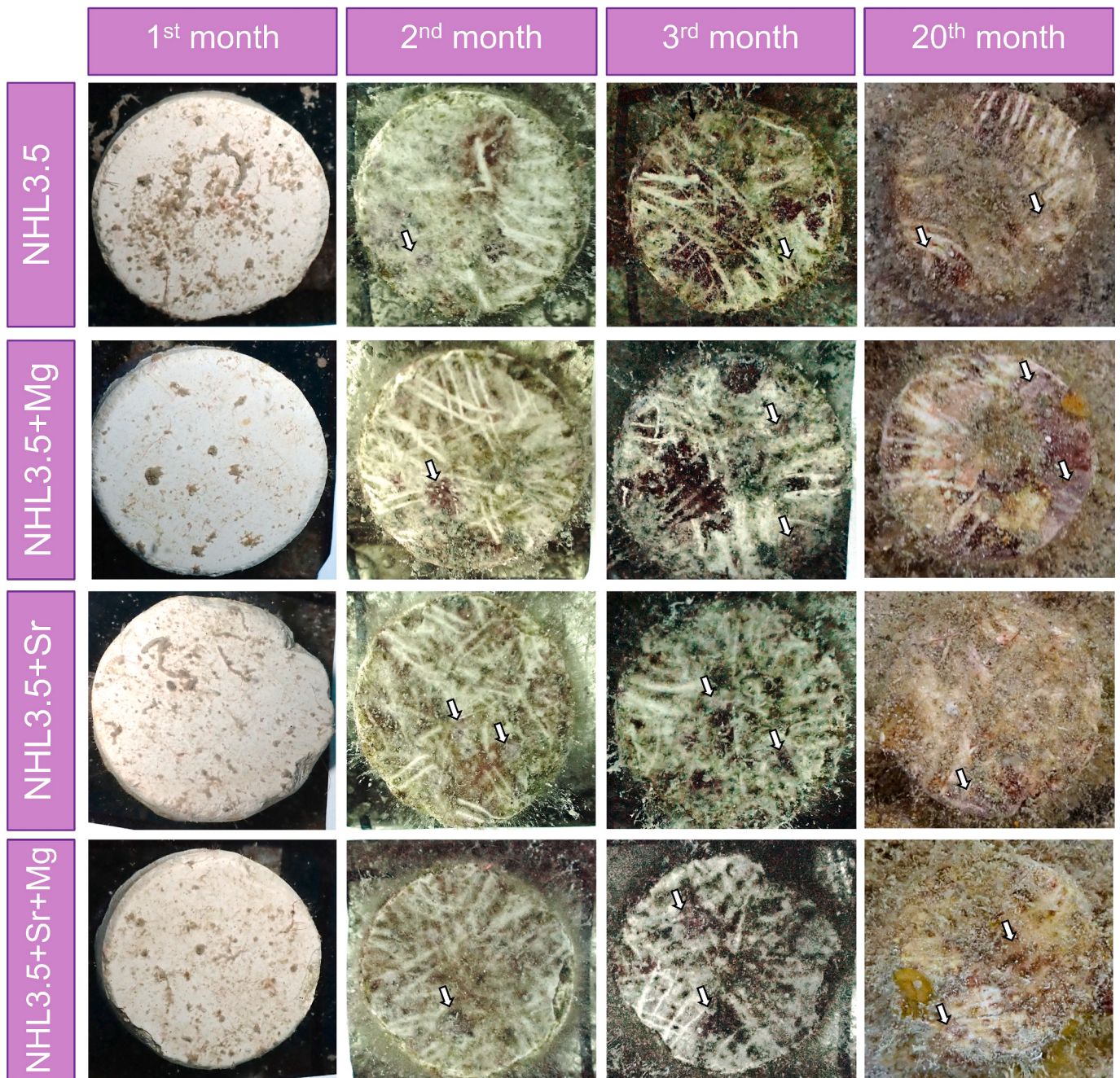


Fig. 5. Growth and grazing of marine fouling organisms on NHL-based substrates deployed underwater for 20 months. Images show the progression of fouling by turfing macroalgae and the grazing (scraping) activity by marine organisms over time. Substrates were outplanted on the reef in front of CARMABI Research Station in Curaçao at 12 m depth. Black and white arrows mark areas where crustose coralline algae (CCA) was observed growing on the substrates. All substrates are approximately 2.54 cm in diameter.

above 97% for all treatments (Fig. S5). Mg^{2+} and Sr^{2+} had no significant effect on larval survival throughout the duration of the experiment ($p > 0.05$, Fig. S5). Time had a significant effect on survivorship ($p < 0.01$, Fig. S5), with an overall trend of decreased survivorship over time.

Dissolved Mg^{2+} elicited the strongest effect on coral larval motility ($p < 0.01$, Fig. 6a). This was most evident in the first three days of the experiment. On day 1, Mg^{2+} treatments had a mean larval motility of 94% and 87% respectively. In comparison, to a mean larval motility in the FSW control of 62%. This treatment effect persisted through day 3, at which time mean larval motility in Mg^{2+} treatments decreased to 59% and 52% respectively, in comparison to 40% in the FSW control. This effect diminished by day 6, at which time Mg^{2+} treatments had a mean

larval motility of 23% and 21% respectively, in comparison to 32% in the FSW control. Dissolved Sr^{2+} also caused a significant increase in larval motility ($p < 0.01$, Fig. 6a). Independent of Mg^{2+} , the effect of Sr^{2+} became apparent on day 2, with a mean larval motility of 71%. Larval motility remained higher in this treatment than in the control through day 5, at which time the Sr^{2+} treatment had a mean motility of 46%, in comparison to 30% in the FSW control. Similar to the dissolved Mg^{2+} , this effect of Sr^{2+} diminished by day 6, at which point the mean larval motility in this treatment decreased to 39%.

In tandem with increases in initial motility, Mg^{2+} treatments displayed significantly higher larval settlement and metamorphosis, with 5.5% and 8% mean settlement in Mg^{2+} and $Mg^{2+}Sr^{2+}$ treatments,

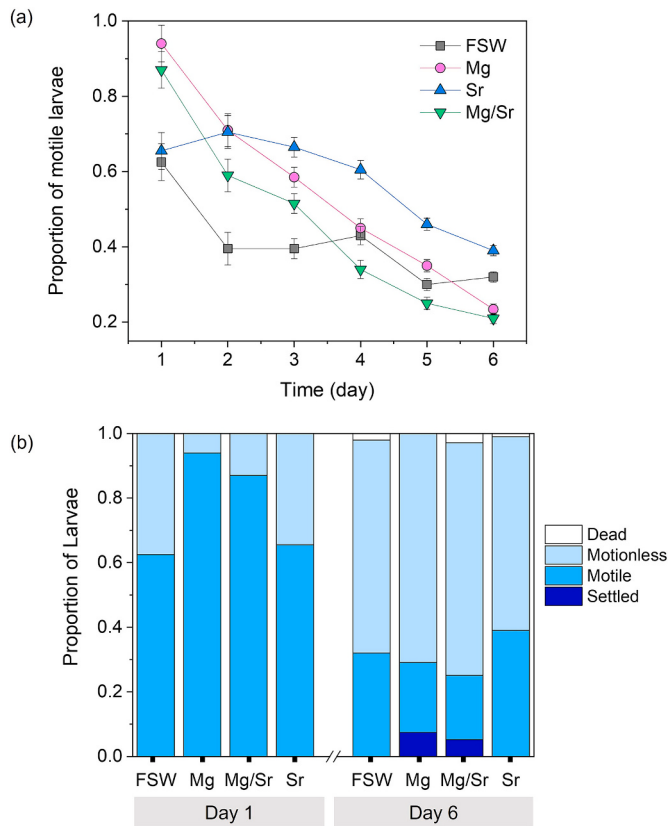


Fig. 6. Swimming behavior and settlement of *C. natans* coral larvae when exposed to solutions of two inorganic ion additives used in composite settlement substrates. Larvae were exposed to elevated concentrations (150% with respect to ambient seawater) of Mg^{2+} (dots), Sr^{2+} (triangles), both Mg^{2+} and Sr^{2+} (inverted triangles), or a filtered seawater (FSW, squares) control absent of inorganic additives (black squares). Behavioral data were collected at 09:00 h daily for six consecutive days ($n = 20$ replicates/treatment). (a) Values reported are the treatment means for the mean proportion of motile larvae in each replicate well. Error bars represent ± 1 standard deviation from the mean. (b) Stacked bar plot comparison of *C. natans* survival and swimming behavior by treatment: dead, motionless, motile, and settled. Data are shown for day 1 and day 6 of the experiment.

respectively (permuted one-way ANOVA, $p = 0.04$, Fig. 6b). Notably, no settlement was observed in the FSW control or Sr^{2+} treatment ($p > 0.05$, Fig. 6b).

3.5. Larval settlement onto NHL base materials and composite substrates

To examine the potential utility of NHL substrates and inorganic additives in coral propagation and reef restoration, we measured coral larval settlement preferences by scoring the number of coral larvae settled on each substrate in each replicate experimental cup. Box-and-whisker plots show settlement counts for *D. labyrinthiformis*, *O. faveolata*, and *C. natans*, respectively (Fig. 7). In general, larvae preferred the bottoms of the substrates, likely due to the protected, enclosed habitats created between the substrate and the cup. For statistical analysis, the sum of settlers on each substrate was considered whether on the top, bottom, or sides of the substrate. It's important to highlight that our study focuses on total areas no larger than 10 cm^2 per substrate. To put this into perspective, 0.2 settlers/ cm^2 equates to the presence of ~ 45 settlers on one face of a 15 cm by 15 cm tile. Both *D. labyrinthiformis* and *O. faveolata* larvae settled in higher proportions on NHL2 substrates compared to NHL3.5 and NHL5 ($p < 0.05$, Fig. 7a). While *C. natans* also settled on NHL2 to a slightly higher degree, there was no significant settlement preference ($p > 0.05$, Fig. 7a). NHL2

contains a smaller amount of silicates and more portlandite ($\text{Ca}(\text{OH})_2$) that transforms into calcite after carbonation. Thus the higher CaCO_3 content in NHL2, rather than the lower amount of silicates per se, may have attracted more larvae for settlement.

When comparing NHL3.5 substrates prepared with inorganic additives, *D. labyrinthiformis* larvae exhibited a statistically significant preference for substrates that contained $>10 \text{ wt\%}$ of both MgCO_3 and SrCO_3 compared to substrates that contained MgCO_3 $5 \text{ wt\%}$ and SrCO_3 $5 \text{ wt\%}$ ($p < 0.05$, Fig. 7b). *Orbicella faveolata* larvae did not settle on substrates with MgCO_3 $5 \text{ wt\%}$, settling in greater numbers on SrCO_3 $5 \text{ wt\%}$ and SrCO_3 $10 \text{ wt\%}$ ($p < 0.05$, Fig. 7c). No significant settlement preference was observed between other substrates for this species. Similar to *D. labyrinthiformis*, *C. natans* larvae were significantly more attracted to the combination of SrCO_3 and MgCO_3 but also settled to a lesser degree on substrates with $10 \text{ wt\%}$ of MgCO_3 ($p < 0.5$, Fig. 7d). However, for this species, no other significant differences were found among the different substrates besides the preference for NHL3.5 with $10 \text{ wt\%}$ of MgCO_3 .

4. Discussion

4.1. Material characterization and underwater durability of NHL-based coral settlement substrates

The results of this study highlight the influence of substrate composition and inorganic additives on coral larval behavior and settlement. We developed new coral settlement substrate materials employing a pressurized CO_2 chamber to accelerate the carbonation of NHL-based substrates. This carbonation starts in the lower racks and continues in the upper racks with time (Fig. 3). Due to the higher density of the gaseous CO_2 compared to the water vapor, the released H_2O (g) rises, creating a concentration gradient of CO_2 and potentially slowing the carbonation of the substrates located on the upper racks. The carbonation process transformed portlandite ($\text{Ca}(\text{OH})_2$) into calcite (CaCO_3), changing substrate composition and increasing hardness. The strength of NHL mortars is mainly associated with the hydration of the calcium silicate C2S. The calcium silicate hydrate (C-S-H) contributes to the self-setting property and spontaneously increases the strength of the material (Liu et al., 2019). Based on the C2S content of NHL (Fig. 1a), the expected results from the hardness tests agree with the observation of increasing strength with increasing C2S (i.e., NHL5 $>$ NHL3.5 $>$ NHL2). The incorporation of inorganic additives decreased the substrate hardness. This is explained by the chemical bonding and crystal structures of the two minerals, i.e., MgCO_3 has a less dense and more open crystal structure. The weaker ionic bonding between magnesium cations (Mg^{2+}) and carbonate anions (CO_3^{2-}) results in a less rigid structure, leading to lower strength compared to SrCO_3 . Furthermore, from simply handling the substrates, it was evident that the addition of MgCO_3 greatly reduced the strength of the mortar. However, this represents a potential trade-off in this material given that this additive may support coral skeleton growth; Mg^{2+} influences shape, morphology, and polymorphism of bioproduced CaCO_3 , all of which influence in vivo skeletal growth in coral (Zaquin et al., 2022). Overall, based on our results, we recommend incorporating between 5 and $10 \text{ wt\%}$ MgCO_3 into the substrates. With $>10 \text{ wt\%}$ MgCO_3 , substrates become fragile. This fragility was not observed in substrates with SrCO_3 . Notably, SrCO_3 incorporation of $>10 \text{ wt\%}$ demonstrates no significant enhancement in Sr^{2+} release over short periods of time (Fig. S1). However, it will be released for longer. Therefore, incorporating more of this carbonate is of potential significance, particularly if it promotes skeletal growth or positively modulates other local environmental factors pertinent to the sustained health of coral ecosystems over extended durations.

Substrates immersed in the ocean for 20 months showed no significant changes in material composition, confirming their general stability and durability on a relevant timescale to coral propagation and out-planting (Fig. S3). Substrates were subject to moderate fouling by turfing macroalgae and they also exhibited clear signs of grazing by

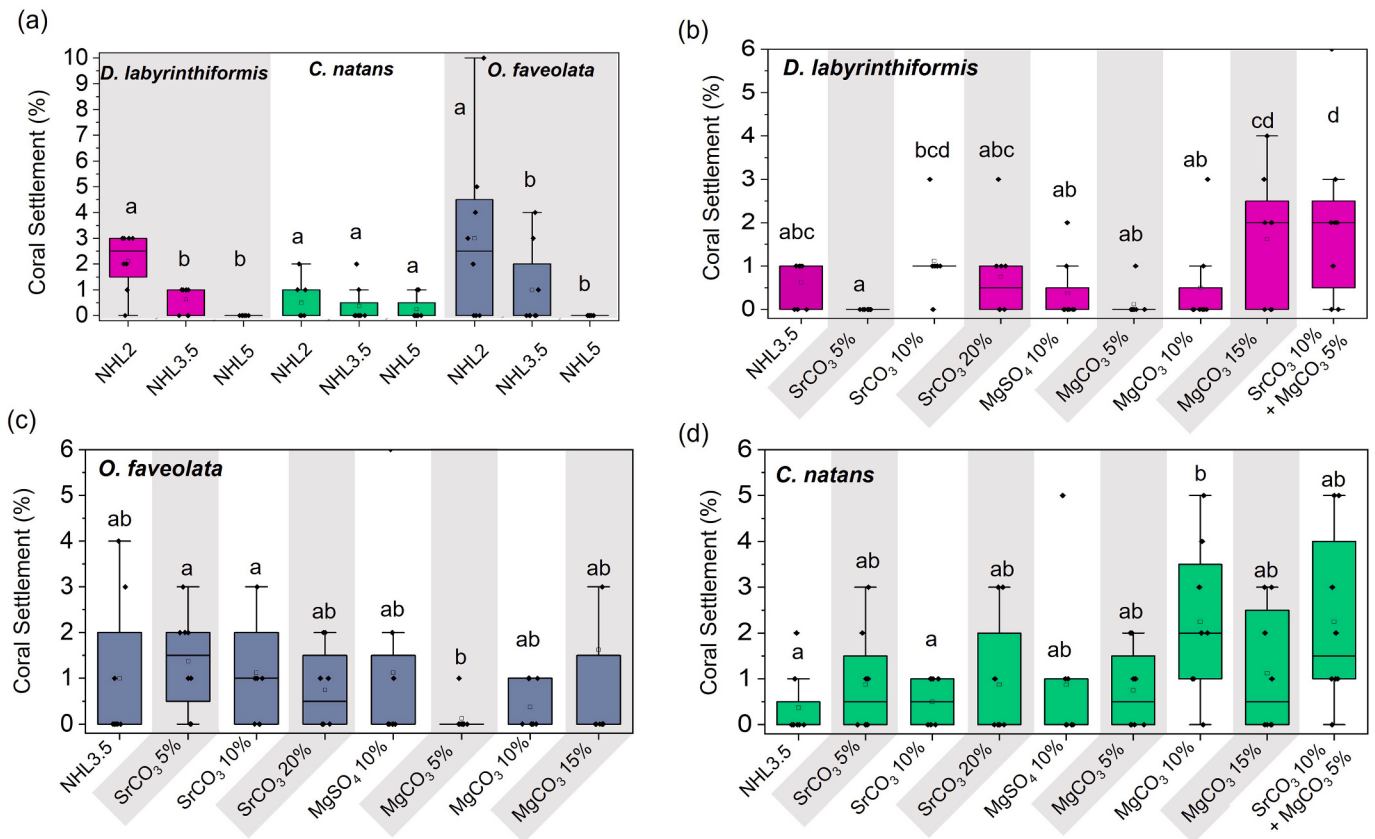


Fig. 7. Settlement rates of *D. labyrinthiformis*, *O. faveolata*, and *C. natans* coral larvae onto NHL substrates and composites. Box-and-whisker plots show the percentage of larvae that settled onto each substrate ($\sim 10 \text{ cm}^2$) type by day 13 of the experiment. (a) Settlement onto substrates composed of NHL2, NHL3.5, and NHL5 base materials only, with no additives. (b-d) Settlement of (b) *D. labyrinthiformis*, (c) *O. faveolata*, and (d) *C. natans* larvae onto NHL3.5 substrates with inorganic additives (referred to as wt% of the additive when mixed with dry NHL3.5). $n = 8$ replicates per experiment. Letters indicate statistically significant differences between substrate types within each species ($p < 0.05$).

marine organisms (Fig. 5), the latter of which creates newly available surfaces and grooves that can facilitate coral settlement. This is attributable to grazing organisms such as sea urchins and parrotfish feeding on the proliferating turf algae. Parrotfish grazing can facilitate coral recruitment by removing macroalgae, leaving clean spaces for coral larval settlement (Mumby, 2009). While the proliferation of turfing algae is a potential deterrent to coral settlement and survival on these substrates, the relatively high density of grazing marks is an important indicator that these substrates can be effectively grazed back to bare substrate, creating repeated opportunities for coral settlement and growth. This grazing behavior does introduce a potential risk of inadvertent harm to corals (Miller, 2014). Simultaneously, it holds promise for fostering coral growth by diminishing benthic competition (Neil et al., 2024). Recognizing this delicate balance underscores the need for ecologically-informed material development in reef restoration, prioritizing options that can be grazed to a bare substrate without harming juvenile corals, in contrast to the prevailing use of excessively sharp and hard materials that are difficult or impossible for most herbivores to scrape clean.

The presence of CCA growing on our substrates shows their potential as viable options for promoting coral recruitment. The presence of various CCA species is well established as a coral settlement cue (Morse et al., 1988; Heyward and Negri, 1999a, 1999b; Abdul Wahab et al., 2023); and CCA is almost always present within healthy reefs (Tebben et al., 2015; Gómez-Lemos et al., 2018; Yang et al., 2022). The fact that our NHL-based substrates fostered CCA growth and facilitated successful grazing by herbivorous animals demonstrates their potential viability as a material for promoting coral recruitment in restoration programs. Nevertheless, it is also important to consider the potential challenges

associated with algal fouling and longer-term erosion when using NHL-based substrates for coral reef restoration. Further investigations are needed to gain a more comprehensive understanding of the dynamics of fouling and colonization of these novel substrate compositions and to optimize substrate compositions that further promote the growth of CCA over turfing algae, a key element of their overall effectiveness and utility in coral reef restoration.

4.2. Exposure to inorganic ions in solution stimulates larval swimming behavior and settlement

Most coral larvae are lecithotrophic, meaning they have fixed energetic reserves on which they rely during planktonic dispersal and settlement. Thus, pre-settlement and settlement swimming and searching behaviors and the ultimate choice of settlement location represent particularly crucial aspects of their life history. While coral larvae of some species have been found to swim for up to hundreds of days (Graham et al., 2008), swimming is energetically expensive (Bennett and Marshall, 2005) and any perturbation to larval baseline metabolism can have long-lasting, if not lethal consequences (Vermeij, 2006; Kwok and Ang, 2013; Hartmann et al., 2015). Therefore, potential chemical effects on larval swimming, attraction to the substrate, settlement success, and survival are important to consider when engineering restoration materials.

Our results show that in solution, Mg^{2+} and Sr^{2+} stimulated significant increases in larval motility throughout day three and day four of the study, respectively (Fig. 6a). Ecologically, larval swimming signifies the ability to actively search for a final place of settlement (Vermeij et al., 2010; Marhaver et al., 2013). Thus we infer that Mg^{2+} and Sr^{2+}

ions promote the search for suitable settlement habitats. This is supported by the increased settlement we observed on substrates containing MgCO_3 and SrCO_3 (Fig. 7a), as well as the settlement and metamorphosis seen on day six in the treatments containing dissolved Mg^{2+} (Fig. 6b). An initial increase in motility may be interpreted as a stress response (Vermeij et al., 2006), however toxicological studies predominantly find sublethal or deleterious effects to be demonstrated by a reduction in larval swimming (Wallace and Estephan, 2004; Faimali et al., 2006), with few studies reporting hyperactivity (Roast et al., 2000), and coral larvae exhibit decreased motility without settlement in response to a multitude of stressors (Faimali et al., 2006; Kwok and Ang, 2013; Hartmann et al., 2015; Antonio-Martínez et al., 2020). On the final day of the study, over 50% of larvae were motionless, but this did not significantly differ across treatments and is thus not likely an effect of the inorganic ion additives (Fig. 6b) but rather an artifact of poor water quality or energetic depletion.

Importantly, no significant differences in survival were found between treatments over the six-day exposure period. This finding indicates that the proposed additives, Mg^{2+} and Sr^{2+} , at 150% relative to ambient ocean concentrations are not immediately perilous to *C. natans* larvae (Fig. S5). Lethality may be of concern at higher concentrations, which has been found with the settlement inducer Ca^{2+} (Yang et al., 2022) as well as with environmental pollutants (Kwok and Ang, 2013); this threshold remains to be determined for Mg^{2+} and Sr^{2+} . Therefore, we conclude that Mg^{2+} and Sr^{2+} at 150% ambient concentrations display no deleterious effect on larval survival, swimming, or settlement, with both ions increasing larval swimming in the initial 4 days of exposure, and Mg^{2+} promoting metamorphosis and settlement. Future studies will examine the biochemical pathways underlying treatment differences to fully elucidate the behavioral response to Mg^{2+} and Sr^{2+} ions observed in this study.

4.3. Coral larval settlement choices are influenced by inorganic substrate composition

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

For the three coral species studied here, settlement preferences also varied based on the type and number of inorganic additives incorporated into the NHL3.5 base material. *Diploria labyrinthiformis* larvae preferred substrates with 10 and 15 wt% MgCO_3 and 10 or 20 wt% SrCO_3 . *Orbicella faveolata* larvae avoided substrates with only 5 wt% of MgCO_3 . *Colpophyllia natans* larvae were attracted to substrates with high concentrations (>5 wt%) of either SrCO_3 or MgCO_3 (Fig. 7). In general, for *D. labyrinthiformis* and *C. natans*, the incorporation of both carbonates together leads to a higher number of recruits compared to the plain NHL3.5. In the case of *O. faveolata*, the evaluation of this combination was precluded by the insufficient availability of samples for testing. Given these findings, future studies will investigate substrates comprised of NHL2 (e.g., substrates with a higher content of CaCO_3) with added

SrCO_3 and MgCO_3 . Overall, we found that coral settlement preference depends on the compositions of the NHL base material as well as the inorganic additives incorporated into the substrates. Importantly, we have shown that we can influence coral larval settlement and thus facilitate the coral recruitment processes using inorganic materials alone.

5. Conclusions

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

Furthermore, coral larval settlement was enhanced via the addition of inorganic additives into the NHL-based substrates. In particular, the combination of strontium and magnesium carbonates enhanced settlement. The long-term stability and durability of NHL substrates were confirmed through observations of substrates out-planted underwater for 20 months. Turf algal fouling was observed on all substrates; however, this was partially offset by light surface erosion attributed to grazing animals. Although accidental grazing behavior may pose a risk to corals, its potential to foster growth by reducing benthic competition underscores the need for materials to allow effective grazing without harm to juvenile corals. Achieving this balance is essential for advancing ecologically-sensitive reef restoration strategies. In addition, the growth of CCA, a known biological cue that promotes coral settlement, was also observed on the NHL substrates. Overall, our findings contribute to a better understanding of the interactions between substrate composition, inorganic additives, and coral larval swimming and settlement behaviors, highlighting the potential of NHL-based substrates in future coral reef restoration applications.

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CRediT authorship contribution statement

J. Yus: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **E.N. Nixon:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **J. Li:** Investigation. **J. Noriega Gimenez:** Investigation, Formal analysis. **M.J. Bennett:** Writing – review & editing, Investigation. **D. Flores:** Investigation. **K.L. Marhaver:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **L. Wegley Kelly:** Writing – review & editing, Supervision, Funding acquisition. **R.M. Espinosa-Marzal:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **A.J. Wagoner Johnson:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoleng.2024.107236>.

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