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**DOI**

[10.1021/acssuschemeng.1c08313](https://doi.org/10.1021/acssuschemeng.1c08313)

**Publication date**

2022

**Document Version**

Final published version

**Published in**

ACS Sustainable Chemistry and Engineering

**License**

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**Citation for published version (APA):**

Levenstein, M. A., Marhaver, K. L., Quinlan, Z. A., Tholen, H. M., Tichy, L., Yus, J., Lightcap, I., Wegley Kelly, L., Juarez, G., Vermeij, M. J. A., & Wagoner Johnson, A. J. (2022). Composite Substrates Reveal Inorganic Material Cues for Coral Larval Settlement. *ACS Sustainable Chemistry and Engineering*, 10(12), 3960-3971. <https://doi.org/10.1021/acssuschemeng.1c08313>

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# Composite Substrates Reveal Inorganic Material Cues for Coral Larval Settlement

Mark A. Levenstein,\* Kristen L. Marhaver,\* Zachary A. Quinlan, Haley M. Tholen, Lucas Tichy, Joaquín Yus, Ian Lightcap, Linda Wegley Kelly, Gabriel Juarez, Mark J. A. Vermeij, and Amy J. Wagoner Johnson\*



Cite This: *ACS Sustainable Chem. Eng.* 2022, 10, 3960–3971



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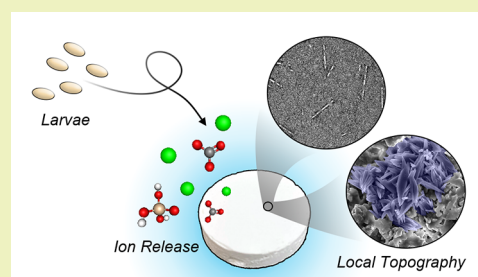


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Supporting Information

**ABSTRACT:** The widespread loss of stony reef-building coral populations has been compounded by the low settlement and survival of coral juveniles. To rebuild coral communities, restoration practitioners have developed workflows to settle vulnerable coral larvae in the laboratory and outplant settled juveniles back to natural and artificial reefs. These workflows often make use of natural biochemical cues, which are presented to swimming larvae to induce settlement. This paper establishes the potential for inorganic cues to complement these known biochemical effects. Settlement substrates were fabricated from calcium carbonate, a material present naturally on reefs, and modified with additives including sands, glasses, and alkaline earth carbonates. Experiments with larvae of two Caribbean coral species revealed additive-specific settlement preferences that were independent of bulk surface properties such as mean roughness and wettability. Instead, analyses of the substrates suggest that settling coral larvae can detect localized topographical features more than an order of magnitude smaller than their body width and can sense and positively respond to soluble inorganic minerals such as silica ( $\text{SiO}_2$ ) and strontianite ( $\text{SrCO}_3$ ). These findings open a new area of research in coral reef restoration, in which composite substrates can be designed with a combination of natural organic and inorganic additives to increase larval settlement and perhaps also improve post-settlement growth, mineralization, and defense.



**KEYWORDS:** coral reef restoration, crystallization, topography, chemical cues, larval settlement, coral propagation

## INTRODUCTION

Coral reefs are essential aquatic ecosystems that sustain biodiversity and provide tremendous value to the global economy through fishing, tourism, biochemical products, and the protection of shorelines and coastal infrastructure.<sup>1–4</sup> Unfortunately, these ecosystems have been in decline for decades due to factors including nearshore construction, natural disasters, sewage and fertilizer pollution, overfishing, disease outbreaks, and increasing sea surface temperatures.<sup>5–8</sup> One of the primary mechanisms by which reefs can recover from such large-scale disturbances is through the sexual propagation of foundational reef-building coral species.<sup>9,10</sup> The operational term for this process is “coral larval recruitment”. During the process of coral reproduction and recruitment, corals first release either swimming larvae or gametes that develop into larvae. These larvae then travel distances ranging from centimeters up to several kilometers in search of a suitable location to settle, that is, where they can attach to the reef, undergo metamorphosis, mineralize a skeleton, and eventually mature into an adult coral colony.<sup>11–13</sup> However, natural recruitment has an exceptionally low success rate, which has fallen to near zero due to human pressures.<sup>14,15</sup> Thus, many research and restoration efforts now

focus on better understanding this process and developing practical interventions.<sup>16,17</sup>

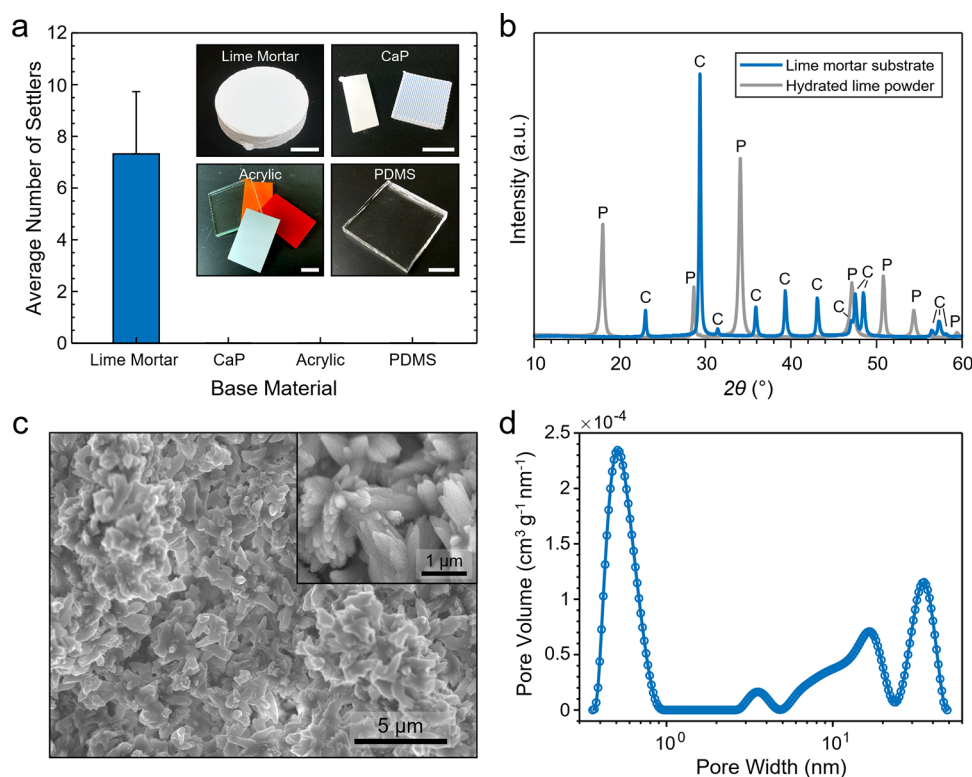
Larval navigation and settlement behavior are influenced by a variety of natural cues, the best known being the biochemical signals arising from mineralized crustose coralline algae (CCA) and CCA-associated bacteria.<sup>18–20</sup> Researchers and restoration practitioners routinely harness these cues to induce larval settlement in the laboratory. For instance, artificial substrates intended for settlement are often conditioned for months on a natural reef or in flow-through aquaria in order to develop algal and microbial films.<sup>21–24</sup> Alternatively, higher concentrations of biochemical cues are presented to larvae by placing fragments or powders of CCAs directly onto settlement substrates,<sup>25–27</sup> by introducing soluble extracts isolated from CCAs into settlement containers,<sup>25,28–30</sup> or by incorporating CCA extracts into solid resins.<sup>20,31,32</sup> Once larvae have settled

**Received:** December 11, 2021

**Revised:** February 14, 2022

**Published:** March 7, 2022





**Figure 1.** Production, selection, and characterization of lime mortar as a substrate material for coral larval settlement. (a) Settlement of *D. labyrinthiformis* larvae in choice experiments with substrates made from the indicated base materials. Error bars represent the standard error of the mean ( $n = 4$  replicate bins,  $n = 200$  larvae per bin). Material types were replicated within the bins to present a variety of colors/textures, but these features had no observable effect on larval settlement for the non-preferred base materials. Thus, data were pooled by material class (see Table S1 in the Supporting Information for a full list of substrates). The insets show an example photo of each of the substrate types (scale bars are 1 cm). (b) XRD patterns of raw kalkwasser powder and lime mortar substrates showing full conversion of portlandite (P) to calcite (C) crystals over the 7 days of accelerated carbonation. (c) Scanning electron micrographs of the top surface of a carbonated lime mortar substrate, revealing an interconnected network of scalenohedral calcite crystals of several microns in length. (d) Non-local density functional theory model of the pore size distribution of carbonated lime mortar substrates obtained from  $N_2$  gas sorption measurements.

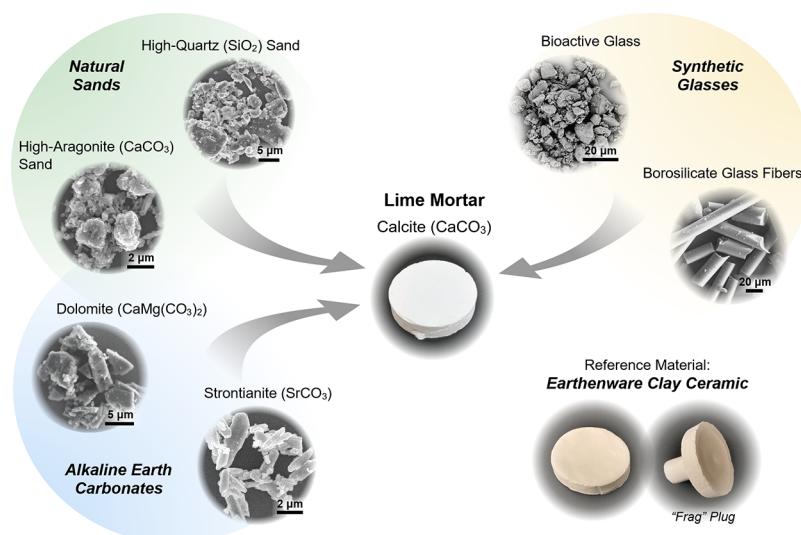
and matured into juvenile corals over several weeks to months in protected aquaria or nurseries, they are then outplanted back to degraded reefs to bolster restoration efforts.

Although not yet routinely applied by coral restoration practitioners, there are also many physical cues that can influence the behavior of marine larvae. These include factors such as the specific sounds,<sup>33</sup> light regimes,<sup>34</sup> and hydrodynamic conditions of reefs,<sup>35</sup> as well as the properties of natural or artificial substrate materials.<sup>36–38</sup> In particular, many studies have noted that coral larvae and other fouling organisms display settlement preferences based on substrate surface topography.<sup>39–41</sup> These observations have often been rationalized using Attachment Point Theory, which states that fouling organisms generally prefer substrates with surface features or roughness on a length scale that maximizes the settler-substrate contact area, that is, on a length scale close to the organism's body width.<sup>42,43</sup> Some studies have also reported settlement preferences based on substrate color<sup>22,44</sup> or wettability.<sup>39,45</sup> However, like studies of surface roughness, these studies have focused on extrinsic and/or bulk properties of the substrate rather than the intrinsic properties of the underlying material.

As reef-building organisms, corals are complex arrangements of both soft organic tissues and hard bio-inorganic skeletons. Thus, it is plausible that corals have evolved to seek out specific inorganic chemicals or materials during the larval stage that would indicate whether a location will be favorable for

mineralization and skeletal attachment and support. Indeed, a growing number of field studies have demonstrated that larval settlement rates of calcifying marine invertebrates vary on common structural materials (e.g., concrete, stainless steel).<sup>46,47</sup> Further work has assessed the ability of different materials to promote the growth of settlement-inducing algae and microbial communities.<sup>48,49</sup> However, to our knowledge, larval settlement cues related solely to inorganic material composition have not yet been investigated systematically.

Here, we present the development of composite substrates containing potential inorganic larval settlement cues and the discovery of species-specific settlement preferences in the absence of CCA or other biochemical settlement inducers. We produced lime mortar ( $CaCO_3$ ) substrates with a range of additives—including sands, glasses, and carbonates of alkaline earth elements that are essential to coral mineralization—to determine whether larvae exhibit preferences to settle on particular materials. In randomized settlement choice experiments with larvae of the Caribbean elkhorn coral *Acropora palmata* and grooved brain coral *Diploria labyrinthiformis*, larvae distinguished between substrates containing different inorganic additives and different concentrations of a glass fiber additive. Analysis of substrate properties using 3D laser scanning confocal microscopy and direct contact angle measurements revealed no correlation between settlement preference and mean surface roughness or wettability. Instead, our results support the existence of more localized surface



**Figure 2.** Illustration of the additives selected for incorporation into lime mortar substrates to investigate coral larval settlement preferences. The additives can be grouped into three categories: natural sands, synthetic glasses, and alkaline earth carbonate minerals. The plain lime mortar substrates and substrates containing additives were also compared to substrates made from a standard earthenware clay ceramic material. This type of ceramic is commonly used in coral research and propagation to make fragmentation or “frag” plugs (right image). Here, ceramic disks were used without the addition of a stem (left image).

recognition capabilities in coral larvae. Also, we identify a possible mechanism driving the larval settlement behavior in our experiments, specifically the release of ions from soluble strontianite ( $\text{SrCO}_3$ ) and silica ( $\text{SiO}_2$ ) additives that were incorporated into substrates.

## EXPERIMENTAL SECTION

**Study Sites and Species.** Larval settlement experiments were conducted on the Southern Caribbean island of Curaçao, and coral gametes were collected from two sites on the leeward coast of the island: *A. palmata* from Sea Aquarium ( $12^\circ 4' 59''\text{N}$ ,  $68^\circ 53' 43''\text{W}$ ) and *D. labyrinthiformis* from the Water Factory (also known as Koredor;  $12^\circ 6' 34''\text{N}$ ,  $68^\circ 57' 23''\text{W}$ ; Figure S1). Prior to settlement experiments, gametes were reared into larvae based on previously established methods,<sup>23,27,50</sup> which are summarized in the Supporting Information. These species were selected for study because they are important reef-building taxa distributed widely across the Caribbean, Florida, and Gulf of Mexico and whose early life stages have been well-characterized.<sup>24,27</sup> A relatively stress-resistant coral, *D. labyrinthiformis*, spawns multiple times per year and settles quickly, allowing iterative testing of materials. Conversely, *A. palmata* is an ecologically distinct, shallow-water species that no longer recruits in large numbers. It was listed as critically endangered on the IUCN Red List in 2008. Advances in the settlement and propagation of this species would therefore be beneficial for the conservation of the species overall.

**Substrate Preparation.** Lime mortar substrates were fabricated from an unaged lime putty prepared by mixing aquarium-grade  $\text{Ca}(\text{OH})_2$  (Kalkwasser powder, ESV Aquarium Products) and fresh tap water into a paste by hand (1:1 mass ratio). The mortar was molded into cylindrical disks and kept in a high  $\text{CO}_2$  environment for 7 days to harden the substrates by carbonation. Substrate additives were ground with a mortar and pestle and mixed with the  $\text{Ca}(\text{OH})_2$  powder before adding water. The concentration of all additives was fixed at 10 wt % of the dry mixture unless otherwise noted. The reference unpigmented earthenware ceramic substrates were obtained from Boston Aqua Farms. A more detailed description of the lime mortar fabrication process and the fabrication of additional substrate types are provided in the Supporting Information along with details on substrate characterization methods.

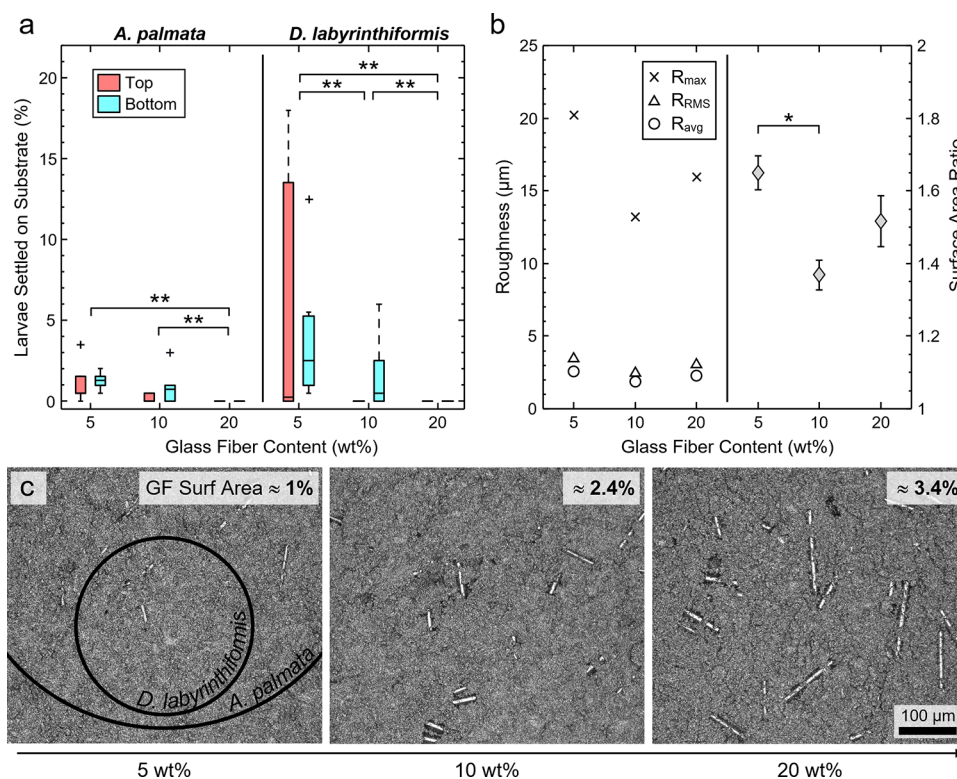
**Larval Settlement Experiments.** Test substrates with different compositions were placed together in replicate 1 L polystyrene bins,

each containing 800 mL of filtered seawater (FSW), to evaluate larval settlement choices. FSW was prepared using a succession of stacked sediment filters with pore sizes of 50, 20, 5, and  $0.5 \mu\text{m}$  ( $\text{H}_2\text{O}$  Distributors, Marietta, GA). Settlement experiments were initiated by pipetting 200 larvae ( $\pm 5\%$ ) into each bin and ended after 7 days by recording settlement locations on the top and bottom of each substrate. Data were evaluated by one-way ANOVA with post hoc Tukey testing to determine significance differences; more detailed statistical methods are provided in the Supporting Information.

## RESULTS AND DISCUSSION

**Coral Larvae Exhibit a Preference for Lime Mortar Substrates.** In search of a more effective larval settlement substrate, we first conducted a preliminary trial of substrates with different topographies, additives, and base materials (Figure 1a). Substrates were made from lime mortar ( $\text{CaCO}_3$ ), calcium phosphate (CaP), poly(methyl methacrylate) (acrylic), and polydimethylsiloxane (PDMS) and subsequently modified through surface molding and the inclusion of additives such as sands and glasses (Figure S2 and Table S1; additives discussed below). *D. labyrinthiformis* larvae were presented with these substrates in a randomized settlement choice experiment. Strikingly, regardless of the additive composition or surface topography, *D. labyrinthiformis* larvae only settled on substrates made with lime mortar as the base material (Figure 1a). Therefore, lime mortar was selected as the base material for further characterization, optimization, and larval experiments.

**$\text{CO}_2$  Accelerates Lime Mortar Carbonation.** Lime mortar is typically prepared by mixing hydrated lime powder ( $\text{Ca}(\text{OH})_2$ , portlandite) with water and allowing the paste to carbonate in air. While this process is more sustainable than the use of Portland cement—since atmospheric  $\text{CO}_2$  is sequestered while the mortar carbonates<sup>51</sup>—mortar carbonation is slow (i.e., months to years). Further, incomplete carbonation leaves unreacted  $\text{Ca}(\text{OH})_2$  within substrates, which can produce high pH conditions that could be harmful to corals. Therefore, the carbonation of lime mortar substrates was accelerated in a high  $\text{CO}_2$  environment. Powder X-ray

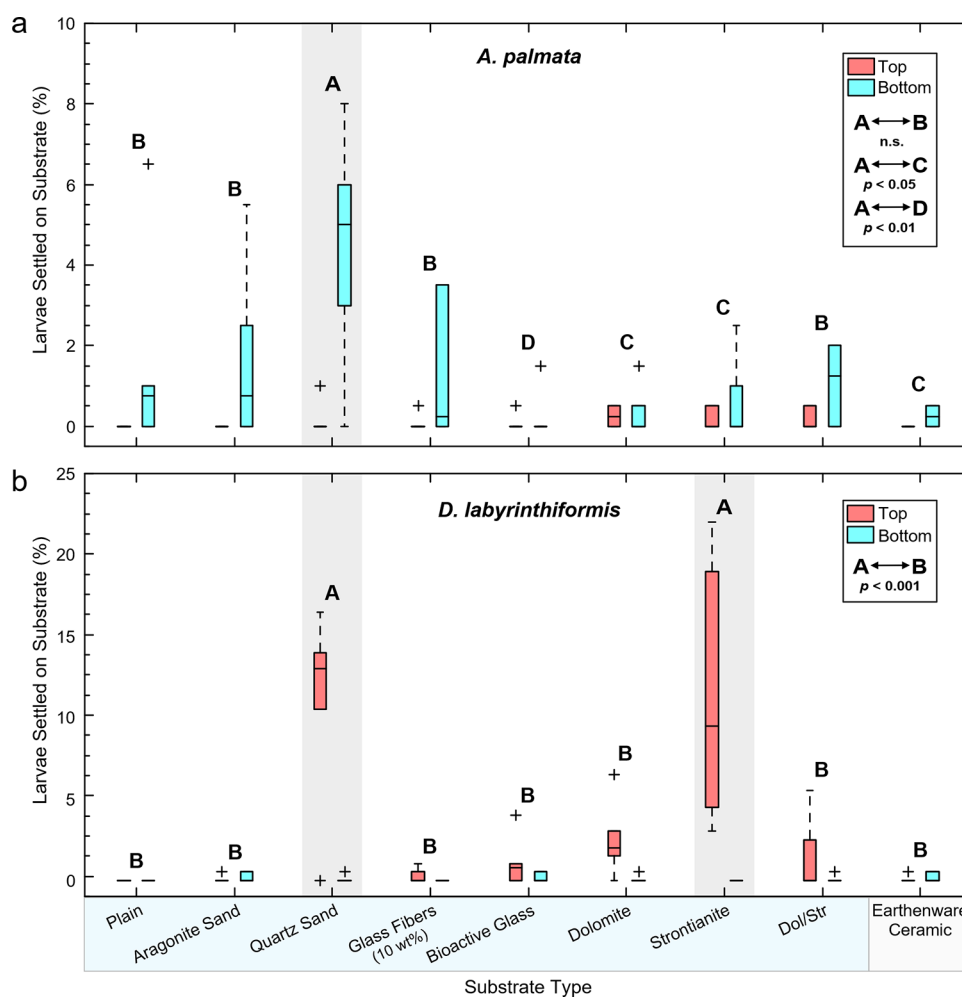


**Figure 3.** Coral larvae exhibit a substrate preference in response to the glass fiber content of lime mortar. (a) Results from settlement choice experiments with lime mortar substrates containing 5, 10, or 20% glass fibers by dry weight. The results are presented as the percent of the initial cohort of larvae added to each bin ( $n = 200$ ) that settled on the top or bottom of a substrate ( $n = 3$  replicates per experiment for *A. palmata* and  $n = 4$  for *D. labyrinthiformis*; choice of 6 substrates per replicate). In box-and-whisker plots, the black lines display the median value, boxes encompass the inner quartile range (IQR, between upper and lower quartiles), whiskers denote data within  $1.5 \times$  IQR, and the crosses represent individual data points outside this range. Significant differences in the proportion of total settlement (i.e., settlement on the top and bottom of the substrate) between substrate types are denoted by asterisks (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; post hoc Tukey HSD). (b) Quantification of surface roughness and surface area of substrates containing glass fibers by 3D laser scanning profilometry. Roughness results are reported as the arithmetic mean roughness ( $R_{avg}$ ), root mean square (RMS) roughness ( $R_{RMS}$ ), and maximum peak height ( $R_{max}$ ). Surface area is presented as the surface area ratio, that is, the total surface area divided by the planar measurement area ( $20\times$  field-of-view  $\approx 0.367$  mm<sup>2</sup>). Error bars denote the standard error of the mean. (c) Representative laser scanning confocal micrographs of lime mortar substrates revealing the differences in the percent of substrate surface area occupied by glass fibers (GF Surf Area) based on concentration. For comparison of substrate characteristics to coral size, the large and small arcs overlaid on the 5 wt % image represent the average diameter of *A. palmata* and *D. labyrinthiformis* larvae, respectively. The scale bar is 100  $\mu$ m and applies to all three panels.

diffraction (PXRD) confirmed that  $>99\%$  conversion of  $\text{Ca}(\text{OH})_2$  into  $\text{CaCO}_3$  could be achieved within 7 days using this method (Figures 1b and S3). The carbonated mortars had a composition of  $99.4 \pm 0.26$  wt % calcite,  $0.26 \pm 0.28$  wt % portlandite, and  $0.29 \pm 0.04$  wt % quartz (mean  $\pm$  standard deviation,  $n = 3$ ). Microscopic analyses of the carbonated substrates revealed a dense network of interconnected scalenohedral calcite crystals of  $\sim 1\text{--}3$   $\mu$ m in size, punctuated by similarly sized gaps and pores (Figure 1c). In addition to these macropores ( $>50$  nm), complementary gas sorption measurements showed that the substrates also had a range of micro- ( $<2$  nm) and mesopores (2–50 nm; Figure 1d), which were especially concentrated around 0.5 and 35 nm, respectively. Analysis of gas sorption data also provided a Brunauer–Emmett–Teller (BET) specific surface area of 8.29 m<sup>2</sup> g<sup>-1</sup>.

**Additives Modify Lime Mortar Properties.** To investigate the settlement preferences of coral larvae in response to diverse inorganic cues, we modified the base lime mortar material with two additives from each of three material groups: (1) natural sands, (2) synthetic glasses, and (3) alkaline earth carbonates (Figure 2). Natural sands are traditionally used as

the “aggregate” that adds strength to mortars and cements.<sup>52</sup> From this category, we selected a standard, quartz-rich sand and a high-aragonite sand. The selection of two different types of sand enabled the comparison of larval preference for an abundant non-Ca-based mineral (here silica/quartz) and the mineral found in abundance on reefs in both the skeletons of living corals and the reef structure that they leave behind (i.e., aragonite). From glasses, we selected borosilicate glass fibers and a bioactive glass powder. Glass fibers are a standard insoluble material added to increase the strength of composite resins, and bioactive glasses are partially soluble silicates that release  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions and aid in bone growth<sup>53</sup> and  $\text{CaCO}_3$  mineralization.<sup>54</sup> Finally, we also selected two alkaline earth carbonates as additives: strontianite ( $\text{SrCO}_3$ ) and dolomite ( $\text{CaMg}(\text{CO}_3)_2$ ). Strontium and magnesium are known to be essential for maintaining coral health in aquarium systems due to their role in coral mineralization.<sup>13,55,56</sup> We hypothesized that the presence of these elements in substrates could influence larval settlement and might even aid in the initial calcification and survival of settled polyps. The compositions of the additives were characterized by PXRD and energy-dispersive X-ray spectroscopy (EDX; Figures S4–

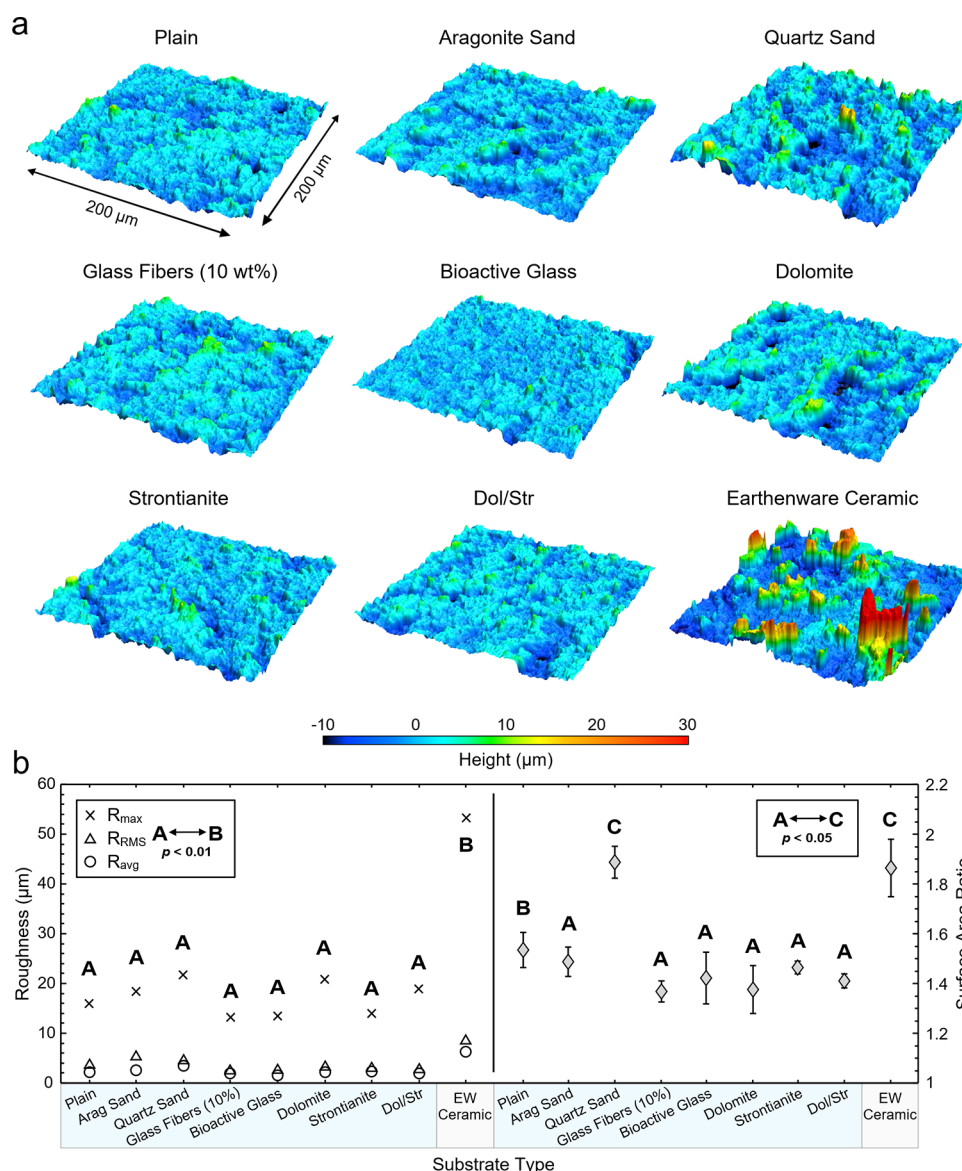


**Figure 4.** Results from settlement choice experiments using coral larvae of two species and lime mortar substrates with a variety of additives. (a) *A. palmata* and (b) *D. labyrinthiformis* larvae were presented with lime mortar-based substrates containing the indicated additives ( $n = 6$  replicates per experiment, choice of 9 substrates per replicate). One substrate type also contained a mixture of dolomite and strontianite additives (Dol/Str). A substrate made from lime mortar without an additive (Plain) and a common earthenware ceramic substrate were used as internal references. Results are presented as the percent of larvae that settled on the top or bottom of a substrate ( $n = 200$  larvae per bin). Data are presented as box-and-whisker plots: black lines denote the median value, boxes encompass the inner quartile range (IQR, between upper and lower quartiles), whiskers denote data within  $1.5 \times$  IQR, and the crosses denote individual data points outside this range. Significant differences in the proportion of total settlement (i.e., settlement to either the top or the bottom) between substrate types are denoted by letters above each substrate type (self-defined groups A, B, C, and D based on post hoc Tukey HSD tests). There were no significant differences between groups B, C, and D (n.s.).

S6). To evaluate the settlement enhancing ability of these additives, lime mortar composites were also compared to standard earthenware (EW) clay ceramics often used for coral restoration and fragmentation applications.<sup>17</sup> The composition of the raw clay and the concentration of key elements in the final EW ceramic are reported in [Supplementary Tables S2 and S3](#), respectively.

Before presenting the substrates to coral larvae, we studied the effect of the additives on both mortar carbonation and the final structure of the composite substrates after carbonation was complete. None of the additives strongly inhibited carbonation ( $>98\%$  conversion for all additives). However, most additives did affect the final polymorphism of the mortar. In addition to calcite, the presence of aragonite was detected in substrates containing quartz sand (1 wt % aragonite), glass fibers (1.5 wt %), bioactive glass (0.9 wt %), strontianite (22.7 wt %), and in substrates containing the combination of dolomite and strontianite (6.4 wt %; [Figure S3](#)). Additionally, substrates containing aragonite sand had  $\sim 2.5$  wt % more

aragonite than expected based on the amount of sand added: the final substrates had 7.7 wt % aragonite in total, whereas only 5.1 wt % was added initially (when considering the purity of the aragonite sand and the substrate mass gain due to carbonation). Except for the aragonite sand additive, PXRD of the raw additive powders confirmed that the aragonite was produced solely as a result of carbonation and not due to aragonitic impurities in the powders ([Figure S4](#)). The particularly large amount of aragonite formed in the presence of strontianite is attributed to the documented stabilization of aragonite by  $\text{Sr}^{2+}$  ions.<sup>57</sup> The only additive that did not appear to result in some aragonite formation during carbonation was dolomite. The surfaces of fully carbonated substrates were additionally analyzed by Raman spectroscopy to confirm that the phases identified by PXRD were also present at the substrate surface ([Figure S7](#)). Despite these differences in both the added and resultant compositions of the mortars, the final pore size distribution and specific surface area of substrates



**Figure 5.** Topographical characterization of lime mortar and earthenware (EW) ceramic substrates used in larval settlement choice experiments. (a) Representative 3D laser confocal surface maps of settlement substrates obtained using a 50 $\times$  objective for visualization of finer details. (b) Quantitative analysis of 3D surface maps obtained using a 20 $\times$  objective for a larger field-of-view. Roughness is reported as the arithmetic mean roughness ( $R_{avg}$ ), root mean square (RMS) roughness ( $R_{RMS}$ ), and maximum peak height ( $R_{max}$ ). There were no significant differences in mean roughness between lime mortar-based substrates, but the EW ceramic substrate was significantly rougher than all lime mortar substrates ( $p < 0.01$ , self-defined groups A and B; post hoc Tukey HSD). Surface area is presented as the surface area ratio, that is, the total surface area divided by the planar measurement area (20 $\times$  field-of-view  $\approx 0.367$  mm<sup>2</sup>). Error bars denote the standard error of the mean. Significant differences in the surface area ratio between substrate types are denoted by letters above/below each substrate type (self-defined groups A, B, and C; post hoc Tukey HSD). There were no significant differences between groups A and B or B and C.

containing different additives were similar (Figure S8 and Table S4).

**Coral Larvae Settle onto Substrates with Lower Glass Fiber Content.** When *A. palmata* and *D. labyrinthiformis* larvae were presented with a choice between lime mortar substrates containing three different concentrations of glass fibers (5, 10, and 20 wt %), both species displayed a settlement preference for substrates with lower glass fiber content (Figure 3a), and no larvae of either species settled on the substrates containing 20 wt % glass fibers. Settlement of *A. palmata* on 5 and 10 wt % substrates was higher than on 20 wt % substrates ( $p = 0.002$  and  $p = 0.012$ , respectively). Settlement of *D. labyrinthiformis* on 5 wt % substrates was higher than on both

10 wt % ( $p = 4.83 \times 10^{-5}$ ) and 20 wt % ( $p = 1.11 \times 10^{-6}$ ) substrates, and settlement on 10 wt % substrates was higher than on 20 wt % substrates ( $p = 0.003$ ).

In the absence of any chemical differences between the three substrate types in either the base material or additives, we might expect that differences in surface topography due to differences in the glass fiber content would be the main factor influencing larval settlement behavior. For marine larvae in general, rougher surfaces with a greater number of points for larval attachment are recognized as promoting settlement.<sup>40</sup> However, all three substrates tested here were smooth, and the inclusion of the glass fibers (16  $\mu$ m average diameter, 32.5  $\mu$ m average exposed fiber length on the substrate surface; Figure

S9) did not modify roughness on the length scales typically considered by Attachment Point Theory, that is, close to the larval size. For reference, the average size of *A. palmata* larvae is  $\approx 700 \mu\text{m}$  and that of *D. labyrinthiformis* is  $\approx 300 \mu\text{m}$ .<sup>27,58</sup> This qualitative description was confirmed by 3D surface analysis of the substrates, which revealed no large differences in mean roughness as a function of glass fiber content (Figure 3b). This analysis also provided the relative surface area available for larval contact on each substrate type, presented as the surface area ratio, or the factor by which the surface area is increased from a perfectly flat plane (Figure 3b). While there was a significant difference in the surface area ratio of substrates containing 5 and 10 wt % glass fibers ( $p = 0.024$ ), we found no significant correlations between larval settlement and mean roughness or surface area ratio for either species (Figure S10). Therefore, the observed preference for substrates with lower glass fiber content suggests that larvae may be able to sense individual glass fibers or groupings of fibers, neither of which strongly influenced these average surface metrics.

As would be expected, we observed that the number of exposed glass fibers at the substrate surface increased with the glass fiber concentration (Figure 3c). Although the absolute change in the surface area coverage of glass fibers was small, it would not have been possible for the larvae of either species to settle on the 20 wt % glass fiber substrates without contacting multiple fibers (Figure 3c). If the fiber ends felt sharp to the larvae, or if fiber smoothness, curvature, or another property in some way inhibited larval attachment, then it is conceivable that the larvae might have chosen to avoid the fibers. Indeed, analysis of the substrate surfaces did reveal a strong negative correlation between *A. palmata* settlement and the surface area occupied by exposed glass fibers ( $r = -0.999$ ,  $p = 0.032$ ; Figure S11). There was also a negative correlation between the exposed glass fiber surface area and *D. labyrinthiformis* settlement, although this was not statistically significant ( $r = -0.96$ ,  $p = 0.182$ ).

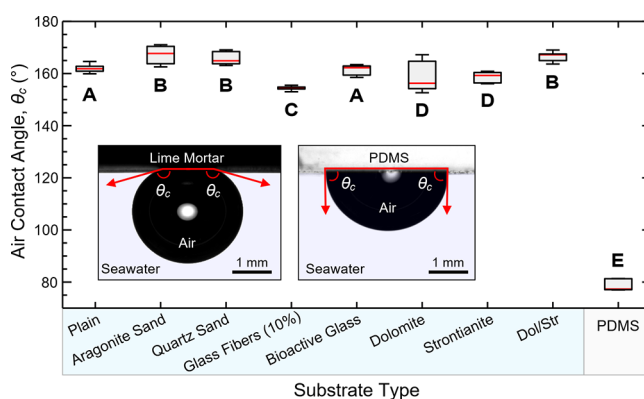
Our results then indicate that larvae are sensitive to substrate heterogeneities with characteristic length scales more than an order of magnitude smaller than their size—here, dispersed  $16 \mu\text{m}$  diameter glass fibers. As most previous, controlled laboratory studies of Attachment Point Theory have utilized homogeneous substrate materials such as micropatterned PDMS,<sup>39,40</sup> these results warrant further investigation and a possible extension of Attachment Point Theory to consider heterogeneous substrates and composite materials. Our results also suggest that future materials studies should test a range of additive concentrations as these can have a large effect on larval settlement preference.

**Coral Larvae Settle onto Substrates Containing Quartz Sand or Strontianite.** Next, we examined settlement preferences in relation to material composition by presenting larvae with a choice between substrates containing different inorganic additives (Figure 2). *A. palmata* larvae exhibited a slight preference for substrates containing quartz sand ( $p < 0.05$  compared to bioactive glass, dolomite, strontianite, and earthenware ceramic; Figure 4a), and *D. labyrinthiformis* larvae exhibited a strong preference for substrates containing quartz sand or strontianite ( $p < 0.001$  compared to all other treatments; Figure 4b). Notably, the earthenware ceramic substrates—commonly used in larval settlement and coral propagation as “frag plugs” and included here as a reference material—had some of the lowest settlement rates for both species. We also observed that *A. palmata* larvae preferred to

settle on the bottoms of substrates, while *D. labyrinthiformis* larvae preferred the substrate tops (Figure 4).

Like the glass fiber experiment, subsequent surface analysis of the substrates did not reveal major topographical differences that could explain the observed settlement preferences (Figure 5a). There were no significant differences in the mean roughness of any of the lime mortar-based substrates, which were all smoother than the earthenware ceramic substrates ( $p < 0.01$ ; Figure 5b). Additionally, quartz sand and earthenware ceramic substrates had a greater surface area ratio than all substrates except for plain lime mortar ( $p < 0.05$ ; Figure 5b), yet as in the glass fiber experiment, these differences were not correlated with larval settlement (Figure S12).

Further, we performed inverted contact angle analysis to determine if changes in surface wettability caused by the additives could explain the observed differences in substrate preference (Figure 6, insets). According to Cassie's law, for a



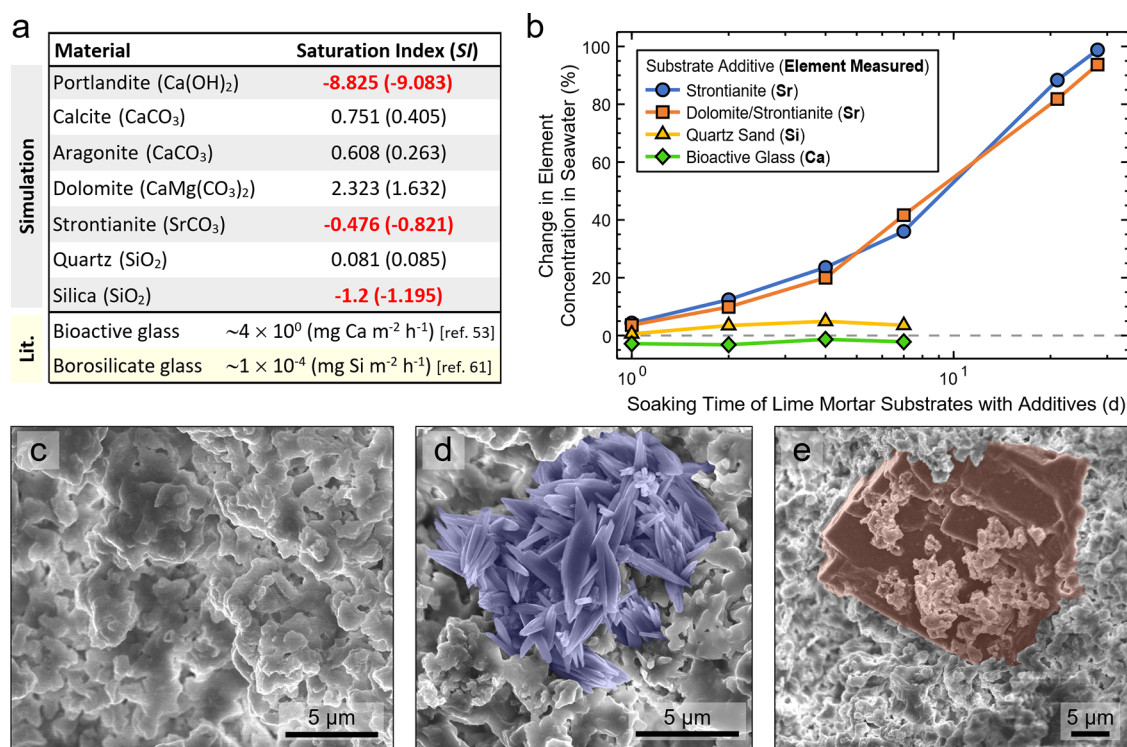
**Figure 6.** Measurement of the wettability of lime mortar substrates by air contact angle. Higher air contact angles ( $\theta_c$ ) correspond to greater hydrophilicity (i.e., wettability). Lime mortar substrates were compared to a known hydrophobic material, PDMS, to confirm that inverted measurements (air-in-seawater) can capture changes in wettability. In the box-and-whisker plots, the red lines display the median value, boxes encompass the inner quartile range (IQR, between upper and lower quartiles), and whiskers represent data within  $1.5 \times \text{IQR}$ . Significant differences in wettability between substrate types are denoted by letters below each box plot (self-defined groups A, B, C, D, and E are statistically distinct from each other;  $p < 0.001$ ; post hoc Tukey HSD). The insets display representative micrographs that illustrate the inverted experimental setup and the difference in contact angle between lime mortar substrates and PDMS.

chemically heterogeneous surface with two components, the effective contact angle of a droplet ( $\theta_c$ ) is related to the inherent contact angles of both components and the fraction of the surface that they each occupy.<sup>59</sup> For the lime mortar substrates containing additives, this relationship can be described by the equation:

$$\cos \theta_c = \alpha_1 \cos \theta_1 + \alpha_a \cos \theta_a \quad (1)$$

where  $\alpha_1$  and  $\alpha_a$  are the surface area fraction and  $\theta_1$  and  $\theta_a$  are the inherent contact angle of the lime mortar base and additive materials, respectively. Assuming the small differences in surface roughness (Figure 5b) did not contribute significantly to the measured contact angle, these measurements provide direct insight into the contribution of the inherent additive wettability (i.e.,  $\theta_a$ ) to the overall wettability of the substrate. Indeed, lime mortar-based substrates displayed significantly





**Figure 7.** Release of soluble additives from lime mortar substrates. (a) Mineral solubility table from seawater chemical equilibrium simulations using input data from Piscaderabaai, Curaçao. The saturation indices of undersaturated minerals are red. The values in parentheses were simulated using standard seawater conditions. Kinetic release rates of Ca and Si from bioactive glass and borosilicate glass fibers, respectively, from literature are included in units of mass released per material surface area per time. (b) Release of the indicated elements during substrate soaking experiments obtained by ICP-OES. The results are reported as the percent change in the concentration of the indicated element during larval settlement experiments assuming uniform mixing. Error bars of the uncertainty in concentration of the element measured by ICP-OES are smaller than the data markers. (c–e) SEM micrographs of lime mortar substrates. (c) Lime mortar with quartz sand, demonstrating the general morphology of the mortar network. (d) Lime mortar with strontianite, illustrating needle-like aragonitic deposits (false-colored blue). (e) Lime mortar with dolomite/strontianite, illustrating large deposits of rhombohedral dolomite crystals (false-colored red).

distinct wettability values depending on the additive ( $p < 0.001$ ; Figure 6). Compared to the known hydrophobic material PDMS, all lime mortar substrates were hydrophilic, presenting average air contact angles between  $150^\circ$  and  $170^\circ$  (with the theoretical maximum being  $180^\circ$ ). However, we found no correlation between these differences in wettability and the larval settlement preferences observed for either coral species (Figure S13).

**Soluble Additives Are Released in Seawater.** After excluding differences in average surface topography and wettability as factors that could be responsible for the observed differences in larval substrate preference, we turned our attention to the possibility that larvae were chemically attracted to the specific additives in the composites themselves: *A. palmata* to quartz sand and *D. labyrinthiformis* to quartz sand and strontianite. A chemical equilibrium model was developed to simulate the solubility of the substrate additives in seawater and to identify the undersaturated additives that would produce a net release of ions into the water column that could be sensed by larvae. While most of the mineral additives were supersaturated in seawater, and therefore resulted in a net uptake of ions by the material, amorphous silica (SiO<sub>2</sub>) and strontianite were both undersaturated (Figure 7a). Although crystalline quartz (SiO<sub>2</sub>) was supersaturated, the highly basic lime mortar carbonation process ( $\text{pH} \approx 12$ ) solubilized some of the quartz sand,<sup>60</sup> as confirmed by inductively coupled plasma optical emission spectrometry (ICP-OES). Therefore, both preferred substrate types contained soluble compounds

that could have diffused into the surrounding seawater during the larval settlement experiments. Because the thermodynamic solubility products ( $K_{\text{sp}}$ ) for bioactive and borosilicate glass required for solubility modeling are not available, we estimated their potential Ca and Si release, respectively, from the literature.<sup>53,61</sup> In high salt solutions, bioactive glass is approximately 4 orders of magnitude more soluble than borosilicate glass (Figure 7a).

Lime mortar substrates containing soluble additives (quartz sand, strontianite, dolomite/strontianite, and bioactive glass) were placed in artificial seawater to experimentally determine the release profiles of key ions over time (Figure 7b). The bioactive glass substrates showed a net uptake of Ca<sup>2+</sup> ions from the artificial seawater, likely due to competition between the release of Ca<sup>2+</sup> from bioactive glass particles and the sequestration of Ca<sup>2+</sup> by the overgrowth of supersaturated calcite crystals in the mortar. The quartz sand substrates released approximately 0.11% of their Si content (0.27 mg) over the first 4 days of soaking, likely as SiO(OH)<sub>3</sub><sup>-</sup> ions. Assuming similar release characteristics during larval settlement experiments, this would have resulted in a 5% increase in Si concentration in the settlement bins. No additional net Si release was observed, but based on ICP-OES measurements of solubilized quartz sand, we estimate up to 60% of soluble silica remained after 4 days. The strontianite substrates released approximately 2.4% of their Sr content (6 mg) over the first 7 days of soaking, increasing the concentration of Sr<sup>2+</sup> in the settlement bins by 36% (Figure 7b). Continued monitoring of

the substrates over 28 days of soaking showed that the  $\text{Sr}^{2+}$  release followed a bounded exponential profile that resulted in a doubling of the initial  $\text{Sr}^{2+}$  concentration. Surprisingly, despite containing half the amount of strontianite (see Supporting Information), the substrates containing a mixture of dolomite and strontianite had remarkably similar  $\text{Sr}^{2+}$  release characteristics compared to the substrates containing strontianite only. This indicates that the release of  $\text{Sr}^{2+}$  ions was diffusion-limited under these solution conditions. Yet, interestingly, the *D. labyrinthiformis* larvae did discriminate between the strontianite and the dolomite/strontianite (Dol/Str) substrates during settlement, showing a significant preference for the substrates containing strontianite only (Figure 4b).

**Coral Larval Attraction Is Mediated by Ion Release and Surface Recognition.** Scanning electron microscopy of substrates containing quartz sand, strontianite, and dolomite/strontianite revealed a similar general surface morphology (Figure 7c). However, important local topographical differences were observed between strontianite and dolomite/strontianite substrates. Clusters of needle-like aragonite crystals were distributed across the surface of the strontianite substrates (Figure 7d). Conversely, no aragonitic needle clusters were observed on the surfaces of dolomite/strontianite substrates, which contained 3.5 $\times$  less aragonite in total (by PXRD; Figure S3). We also observed large,  $\sim 20$ – $50$   $\mu\text{m}$  rhombohedral dolomite crystals on the surfaces of dolomite/strontianite substrates (Figure 7e). These results suggest that the differences in local surface features may have been responsible for the preference of *D. labyrinthiformis* for strontianite substrates over dolomite/strontianite substrates, considering their similar  $\text{Sr}^{2+}$  release rates.

Although it is unclear whether the larvae were perhaps attracted to the needle-like aragonitic deposits in the strontianite substrates or repelled by the large dolomite crystals in the dolomite/strontianite substrates, this high degree of selectivity in response to substrate composition and heterogeneity is consistent with that observed in the glass fiber experiment (Figure 3). Therefore, given the preference of *D. labyrinthiformis* for substrates containing strontianite but not strontianite/dolomite, we propose that a two-step mechanism of attraction may be at work: (1) larvae sense  $\text{Sr}^{2+}$  ions in the water column and are drawn to their source; (2) subsequently, larvae probe local surface features (aragonite needles or dolomite rhombohedrals) and choose their preferred settlement location based on micro-scale topography and/or material composition. It is also possible that the release of ions creates a local pH gradient around substrates, which should be investigated as substrate materials are further refined and tested in the future. Local surface features may have also played a role in the preference of *A. palmata* and *D. labyrinthiformis* larvae for substrates containing quartz sand. However, given the selection of substrates we used, we could not resolve whether the preference for quartz sand resulted from the features of the substrate surface itself or from the release of silicates into the water column. Nevertheless, these experiments demonstrate the remarkable degree of specificity with which coral larvae choose a settlement substrate and the importance of inorganic substrate composition and micro-scale topography for larval settlement in general.

## CONCLUSIONS

Most research into the settlement of marine invertebrate larvae to date has focused on the effect of biochemical cues and larva-sized topographical features on larval substrate preferences. Here, we show that the inorganic composition and micro-scale topography of substrate materials can also strongly influence the settlement of larvae of two Caribbean coral species. The settlement of *A. palmata* and *D. labyrinthiformis* larvae on lime mortar substrates with lower glass fiber content demonstrates that larvae are sensitive to micrometer-scale substrate heterogeneities even when these do not modify average surface parameters such as mean roughness and surface area. Notably, these surface features created by exposed glass fibers were also more than an order of magnitude smaller than the topographical features expected to modify larval settlement preference according to Attachment Point Theory. We therefore suggest that Attachment Point Theory could be extended to consider composite substrates and that new quantitative metrics are needed to better compare localized surface features. Additional experiments with several inorganic additives reinforced our finding that coral larvae are remarkably sensitive to such micro-scale surface features while also revealing that larvae display species-specific attraction to particular materials: both *A. palmata* and *D. labyrinthiformis* responded to a quartz-rich sand, while only *D. labyrinthiformis* responded to strontianite ( $\text{SrCO}_3$ ). We propose that these attractions are mediated by the release of ions into the water column, which are then sensed by swimming larvae as they navigate toward substrates for further investigation of their settlement suitability.

Our findings help to highlight the hidden and under-recognized layers of interaction that exist in the already complex ecological process of coral larval recruitment. Fortunately, this complexity also creates opportunity: materials scientists and engineers can develop new tools that can be harnessed by restoration practitioners to rebuild coral communities and other threatened aquatic ecosystems. This work goes some way toward this goal by demonstrating a substrate system made from a material that is already present naturally on reefs and that can be modified with settlement-inducing minerals. We hope our results will stimulate new research into understanding inorganic and materials-based cues for larval settlement and thus bolster efforts in sustainable materials engineering for coral reef restoration.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssuschemeng.1c08313>.

Extended experimental methods, description of preliminary substrate selection experiments, maps and photos of study sites and species, PXRD, Raman, SEM, EDX, and gas sorption characterization of substrates, composition of selected raw materials, settlement correlation plots, and seawater modeling input parameters (PDF)

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## Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors acknowledge the National Science Foundation (NSF) for funding through the Convergence RAISE program (Award # IOS-1848671) and the Government of Curaçao Ministry of Health, Environment, and Nature (GMN) for research and collecting permits provided to CARMABI. Z.A.Q. was supported by the NSF Graduate Research Fellowship Program (Award # DGE-1842470). Coral spawning research at CARMABI in 2019 was also supported by the Paul G. Allen Family Foundation (to K.L.M.). Substrate characterization was carried out in part in the Materials Research Laboratory

(MRL) Central Research Facilities, University of Illinois. The authors thank MRL scientists Dr. Julio Soares, Dr. Kathy Walsh, and Dr. Offir Cohen for their assistance and advice and Dr. Valérie Chamberland and Kelly Latijnhouwers of SECORE International for the collection of *A. palmata* gametes and for their general support and ever helpful discussions. The authors also thank Dr. Alice Webb for providing the local seawater data used in the solubility model. Finally, the authors would like to thank others at CARMABI who assisted them during the 2019 spawning season, including Matthew-James Bennett, Evan Culbertson, Tonia Doblado Speck, Daisy Flores, Nina Le Trocquer, Megan Ramirez, Zach Ransom, Sophie Schönherr, and the staff of The Diveshop Curaçao.

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