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Settling assays testing the substrate preference of larvae of the cold-water coral *Desmophyllum pertusum* (syn. *Lophelia pertusa*)

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The cold-water coral *Desmophyllum pertusum* form complex habitats for associated fauna at landscape scale, however, these habitats have been degraded by human activities over the last decades. In all European OSPAR regions the status is poor and declining, with increasing realization that active restoration measures are needed to restore some of the lost complexity. The prerequisite for successful larval recruitment is still unknown for this species. The aim of this study was to find the optimal material composition of artificial reef (AR) units for larval recruitment to guide a large-scale restoration effort in the Skagerrak, Sweden. We tested nine different substrates, including different blends of concrete, metallurgic slag, and ceramic materials in a settling assay with settling-competent larvae. Substrates containing Ground Granulated Blast Furnace Slag (GGBFS) and silica oxide produced, together with samples of 3D printed concrete, significantly higher values both considering time spent on substrate as well as attachment rate compared to concrete made of standard Portland cement (PC). We propose that this is due to the higher content of magnesium in the GGBFS compared to PC. Incorporation of GGBFS in the concrete can potentially increase longevity of the ARs and will lower the carbon footprint. With increased larval interest in concrete with GGBFS this is a triple win and increases the potential for a successful restoration effort.

KEYWORDS

deep-sea corals, planulae, attachment rate, restoration, mineral composition

Introduction

The Cold-Water Coral (CWC) *Desmophyllum pertusum* (Linneaus, 1758) (syn. *Lophelia pertusa*) is an ecosystem engineer (Jones et al., 1994) that create complex habitats for a rich array of associated species (Buhl-Mortensen et al., 2010). It is a reef-building scleractinian coral producing a branching skeleton through clonal growth, with branching emanating at points where a new polyp has developed on the rim of the calice of its parental polyp. If left undisturbed the reefs can expand several meters high and several square kilometers wide, thus adding complexity at landscape scales. The three-dimensional structures capture organic matter and larvae from the passing currents, concentrating both nutrients and fauna within the reef matrix (Douarin et al., 2014; Bourque and Demopoulos, 2018), thus providing a rich feeding ground for fish and other mobile macro fauna. These corals are associated with constrictions and sills or elevations where current velocities are high. The *Desmophyllum pertusum* habitats are, however under stress. Within all OSPAR regions they are under decline due to mechanical damage caused by unsustainable human activities such as fishing with bottom-contacting gear, oil and gas extractions, dumping of waste or dredged materials, eutrophication, and increased sedimentation (Hall-Spencer and Stehfest, 2009). In the 2022 OSPAR (Oslo-Paris Convention) Status Assessment of *L. pertusa* reefs the overall status were poor in all OSPAR regions while fishing pressure had remained at the same level as previously (OSPAR, 2022). Seventy percent of the *L. pertusa* occurrences are outside of protected areas and many of them are in poor condition. On top of this, we have the added stresses of climate change, i.e., ocean warming, acidification, and oxygen depletion (Orr et al., 2005). Morato et al. (2020) estimated that there will be a reduction of suitable habitats for *D. pertusum* of at least 79 per cent by the year 2100 according to the RCP8.5 climate scenario. Once a reef habitat has been damaged and the three-dimensional complexity has been reduced or completely lost there is little evidence for spontaneous recovery (Watling and Norse, 1998; Gianni, 2004; Althaus et al., 2009). Even after being protected for 16 years, no visible recovery was observed at the Darwin Mounds marine protected area (Bett et al., 2025), indicating protection is not sufficient in some cases. The extensive ecosystem degradation in all biospheres has led to a call by the UN to "*prevent, halt and reverse the degradation of ecosystems worldwide*" under Resolution 73/284, proclaiming the years 2021–2030 the *United Nations Decade on Ecosystem Restoration*. This call includes deep-sea benthic ecosystems and CWC reef habitats. The issue of restoration of deep-sea habitats has already been addressed in the literature (e.g., Van Dover et al., 2014; Montseny et al., 2021; Aguzzi et al., 2024) and several restoration projects funded by the European Union's LIFE Nature and Biodiversity programme is ongoing, including LIFE *Lophelia* under which the present study was conducted.

To be able to successfully apply active restoration measures in the deep-sea there are knowledge gaps that needs to be bridged. While the prerequisite for coral larval settlement is quite well understood in tropical corals, the needs of deep-sea coral larvae are still largely unknown. *Desmophyllum pertusum* is the most well-studied CWC species, however, what chemical or textural cues that are needed to trigger settling behavior in its larvae remains elusive. In laboratory studies, the larvae have been found to wedge themselves in between coral calyx lamellae or other tight spaces and use cnidae for temporary anchoring, with cnidae discharge triggered by turbulence (Strömberg et al., 2019). Permanent settling and metamorphosis in the laboratory are, however, not yet observed for the species.

Observations of the brooded larvae of the deep-sea scleractinian *Goniocorella dumosa* in New Zealand showed that larvae settled on any available substrate, such as coral skeleton, silicone tubing and other plastic materials (Beaumont et al., 2024). Larvae of the brooding deep-sea octocoral genus *Driftia* have also been shown to be unselective and readily settle on several types of substrates provided in a laboratory setting (Sun et al., 2010). There was a preference for rougher and conditioned surfaces with bacterial film, however, the larvae also settled in clean Petri dishes. The larvae of brooding species are mostly demersal, crawling over the substrates in the vicinity of the parental colonies after planula release. If the parental colonies have thrived enough in the location to produce larvae, there is probably no need to evolve mechanisms for detection of chemical cues and finding specific substrates. In broadcast spawning species, such as *D. pertusum*—where fertilization and embryo development are external—there is probably a stronger drive for selection on larvae to develop environmental sensing capabilities. The larvae of *D. pertusum* drift with the currents for several weeks before settling (Strömberg and Larsson, 2017), and the adult corals thrive in high-current velocity areas. Larvae thus need to both overcome the challenge of attaching to substrates in high flow and sense the suitability of the substrate before settling.

In tropical corals several compounds from coralline algae and bacterial film have been isolated and found to trigger settling and metamorphosis in larvae (Gleason and Hofmann, 2011, and references therein). Both water soluble cues acting at a distance and non-water-soluble cues acting in close vicinity of the substrates have been identified. It is also known that there are different chemical cues for settlement and metamorphosis so that metamorphosis can be triggered before settling has taken place (Grasso et al., 2011). In addition to calcium carbonate rich substrates, other inorganic cues have also been shown to enhance settling rates in some species. Strontium and magnesium added to lime mortar settling plugs increased settling rates in some species (Levenstein et al., 2022; Yus et al., 2024). Sound has been shown to affect swimming direction, with coral larvae swimming towards sounds generated by reef associated organisms (Vermeij et al., 2010). Larvae have also been shown to react to surface microtopography and chosen to settle in micro cavities with a close fit to the larval width without presence of chemical cues (Whalan et al., 2015). Once a suitable substrate is located by the larvae, presence of kin can also affect the choice to settle or not settle. Many species of corals have been shown to form kin aggregations, also called *gregarious settling*, i.e., settling in close vicinity to each other (Lewis, 1974; Amar et al., 2008; Jiang et al., 2022). The primary polyps will fuse with neighboring young colonies, forming chimeras that enhance survival and growth. This occurs most frequently with closely related kins, but to some percentage also with less closely related kins.

Previously, *D. pertusum* colonies have been found attached to oil rigs and other manmade installations in the North Sea and Gulf of Mexico (Roberts, 2002; Larcom et al., 2014). The initial substrate has not been reported from the oil rigs, i.e., if corals have recruited directly to the metal surfaces, on aragonitic deposits from galvanic or electrolytic accretions (applied to protect the rig from corrosion), or if they have settled after primary recruitment of calcifying fauna such as barnacles, serpulid polychaetes, or bryozoans. Recent observations of *D. pertusum* recruitment on moorings and buoys revealed that they had settled on the calcified plates of barnacles (Strong et al., 2023) but were not present on boulders in the area as was noticed for *Madrepora oculata*. Young colonies of *D. pertusum* have also been observed on the parchment-like tubes of *Eunice norvegica*, a bristle worm strongly associated with *D. pertusum* reefs (pers. comment André Freiwald). In the present study area in Skagerrak at the west coast of Sweden, there are dense aggregations of drop stones and large boulders, or exposed rock, but these have never been observed to house young *D. pertusum* colonies. There have, however, been observations of young domeshaped colonies attached to dead coral framework at the Tisler reef in Ytre Hvaler in Norway (personal observation) indicating that larvae had recruited to the dead coral skeleton. From these sparse observations we hypothesize that these larvae need an elevated high-complexity substrate, and probably a substrate rich in calcium carbonate, or other biogenic materials. The CWCs of the NE Atlantic recolonized after the last glacial maximum (LGM) under environmental conditions very different from todays and probably recruited directly on glacial moraine (Frank et al., 2011). With increasing land runoff and sedimentation rates in this coastal area it is less likely that coral larvae can find suitable sediment free substrates.

In this study we have focused on testing settling preferences in *D. pertusum* larvae on different materials that could be used for the production of artificial reefs (ARs) for active restoration. There are six sites targeted for restoration within the Natura 2000 Kosterfjord-Väderöfjord area and Kosterhavet marine National Park in Skagerrak, Sweden. We tested four different concrete mixes including concrete made from regular Portland Cement (PC) as controls and three mixes with added Ground Granulated Blast Furnace Slag (GGBFS) and silica oxide. In addition, we tested two samples of 3D printed concrete with smooth and rough texture, pieces of metallurgic slag from the metal industries and two different ceramic materials. These materials have been used for AR production in restoration efforts in tropical reefs with some success (e.g., Spieler et al., 2001; Mohammed et al., 2012; Perkol-Finkel and Sella, 2014; Sella and Perkol-Finkel, 2015; Antink et al., 2018; Boström-Einarsson et al., 2020; Ly et al., 2021). GGBFS is known to increase the compressive strength of concrete (Osmanovic et al., 2018), and silica oxide protects submerged concrete structures, and their metal reinforcement bars from chloride intrusion (Baht and Tengli, 2019). GGBFS has been used in the Netherlands for marine concrete installations since the 1920s (Polder et al., 2014; Gulikers, 2016) vouching for the durability of the product. The carbon footprint of the final product is reduced when GGBFS is mixed with the cement (Song and Saraswathy, 2006). Thus, there are several potential benefits of using GGBFS and silica in concrete structures in the marine environment, both concerning the durability of the structures and the carbon footprint. The aim was to investigate how the different material compositions would affect larval recruitment. *D. pertusum* is a slow growing species, and ARs need to have a long lifespan to support the growth of newly established reefs.

Materials and methods

Collection and maintenance of parental colonies

Parental colonies were collected from the Tisler reef in Ytre Hvaler National Park, Norway, neighboring the Kosterhavet National Park in Sweden. Both are marine protected areas established in 2009. CITES permits for export/import of corals between the countries, and all permits necessary for working in Norwegian waters were in place. Tisler is a large and healthy reef with reproductively active corals. Sampled corals were brought to the Tjärnö Marine Laboratory (TML), situated adjacent to the Kosterhavet National Park. TML belongs to the Department of Marine Sciences at the University of Gothenburg. The corals were kept in tanks in a constant temperature room with flow-through of deep water from an intake at 45 m depth close to the laboratory. Water and air temperature in the room was set to 7 °C–8 °C, the *in situ* temperature at Tisler during the spawning season. Sub-samples of polyps from each sample were dissected to determine the sex. *Desmophyllum pertusum* is a gonochoric coral, with colonies being either male or female. If pockets of oocytes or spermatocytes are found in one polyp, this is generally valid for the entire sample. Pieces of each sample were then mounted on concrete bases by means of D-D Aquascape Aquarium epoxy putty for an upright position in the tanks, mixing males and females. The concrete bases were marked with sex and ID number of the individual samples. Collections were done in November-December of 2022 before the spawning season in January-February of 2023. In addition to the flow-through, the tanks were equipped with Nero 3 and 5 propellers (Aqua Illumination) to provide turbulence. The flow-through water was filtrated with a series of filters: 1) sand filter; 2) UV-filter Deltec UV-C (80 W) and TropTronic UVC (85 W); and 3) 50 µm plus 5 µm Ametek polypropylene cartridge filters mounted in sequence, all in mentioned order.

Adult corals were fed with Red Plankton (Ocean Nutrition) twice per week, and additional Fauna Marine LPS (Large Polyp Stony) once per week until spawning started. The feeding was paused for the duration of the spawning season and was not resumed until the spawning season was over. In the end of December-beginning of January the corals were checked hourly for signs of spawning so that gametes could be collected whenever spawning occurred.

Preparation of larvae

The larvae used for the experiments were all from a spawning event occurring on March 1st in 2023. Gametes were siphoned out of the aquarium with the parental colonies and collected in 2.5 L glass bowls for fertilization. At day five, as they developed into swimming blastulae, they were transferred to Erlenmeyer flasks kept upside down for maximum volume in the upper portion of the flasks. The blastulae and larvae are actively swimming upwards during the first weeks and gather in the top portion of the containers. Flasks were filled up completely with filtered sea water, care was taken that no air bubbles were present. Embryos were kept at 7 °C–8 °C and reached the planula stage at day 10. For reference, the embryo development in *L. pertusa* was described in further detail in Larsson et al., 2014.

Larvae begin to descend looking for a substrate at around 5 weeks. As larvae were reaching settling maturity 500 larvae were removed from a culture flask, put into a glass crystallization cup and fed with the fine fraction of homogenized and centrifuged *Calanus finmarchicus* copepods (Ocean Nutrition, Red Plankton). The copepods had been homogenized in a small amount of filtered sea water with a hand-held blender and then centrifuged in 3,000 rpm for 20 min to separate the fine fraction of protein from the larger particles of chitinous exoskeleton and excess lipids. This has successfully been used for larval feeding in previous years (Strömberg et al., 2019). The fine fractions were transferred to 1.5 mL plastic vials and put in the freezer until use.

TABLE 1 The nine substrates tested. PC = Portland Cement; GGBFS = Ground Granulated Blast Furnace Slag; SiO = Silica Oxide; M1-M3 = mix 1–3; 3D-1 = sample of 3D printed concrete with smooth surface; 3D-2 = sample of 3D printed concrete with rough surface.

Substrate	Explanation	Ratio (%)	Components
PC	controls	100	PC
M1	mix 1	50:50	PC:GGBFS
M2	mix 2	40:40:20	PC:GGBFS:SiO
M3	mix 3	30:40:30	PC:GGBFS:SiO
3D-1	smooth	ConcretePrint	
3D-2	rough	ConcretePrint	
MS	metallurgic slag	Höganäs AB	
CC	ceramic cylinder	Aquael BioCeraMAX 600	
CT	ceramic tile	Standard tile	

After being fed, the larvae were stained with Nile Red fluorescent stain with DMSO as the stain carrier according to protocols in Alemán-Nava et al. (2016); Doropoulos and Roff (2022). One milligram of Nile Red was dissolved in 2 mL DMSO as a stock solution, and a portion of the stock solution was mixed in 100 mL of filtered sea water to a concentration of c. 2.5 µg/mL. The 500 larvae were incubated in a glass crystallization cup for 2 hours in the Nile Red solution before experiments began. Feeding was done the day before the experiment, and staining was done in the morning before filming. The staining was primarily done to be able to trace the larvae in the wells without agitation of the fluorescence. We aimed to make repeat video documentation of the larvae in the wells, and finally document settled larvae with the help of blue light to agitate the stain to be able to spot the larvae. No permanent attachment with metamorphosis was achieved, however, and larvae were in increasingly poor condition or dying over the following days and could not be used for further experiments or documentation.

Preparation of substrates

Substrates for the settling experiments had been prepared in advance, with the different types of substrates cut to c. 10 × 10 mm pieces (using a Dremel tool), or pieces of substrate in that size range picked out. In total we tested nine different substrates, including the four mixes of concrete tested specifically to be able to choose a mix to use for the artificial reefs (AR) developed and produced within the LIFE *Lophelia* project. These mixes consisted of standard Portland Cement (CEM I 42.5 N SR3 MH/LA) as controls, and three different mixes with added Ground Granulated Blast Furnace Slag (GGBFS) and silica oxide (SiO) as described further in Strömberg and Larsson (2025) in this issue (Table 1). We chose the standard PC concrete as controls since this is one of the most common materials used for reef restoration by ARs, and the aim was to test whether the GGBFS and silica additions would have a positive or negative effect compared to plain PC. The panels from which the pieces were cut had been cast in January-February 2022, more than a year prior to this experiment, ensuring an extensive level of carbonation. In addition to these four, we also tested substrates cut from two samples of concrete delivered from ConcretePrint, the company contracted to produce the 3D printed concrete AR models for the project. These were samples of 3D printed concrete, one smooth sample (3D-1) and one sample with a rougher texture (3D-2). Both samples were of a customized mix used by the company based on CEM I with added silica slurry for workability and extrudability, retarder, fine silicious sand, and plastic fibers as reinforcement. The exact proportions are not known. In addition to these different types of concrete, we tested pieces of metallurgic slag (Petrit-E) delivered by Höganäs Sweden AB, and two different ceramic materials. We used one rough textured ceramic cylinder that is used as filtration media in aquaria (Aquael, BioCeraMAX 600), and standard mosaic ceramic tiles (for kitchens and bathrooms, brand unknown) with its textured back side as the substrate. The ceramics were added as a secondary control since it is a common material in settling trials.

Three replicates per substrate were prepared and mounted in standard 6-well plates with wells of 34 mm diameter. Substrates were randomly distributed over five plates. A gray coral epoxy putty (D-D Aquascape Aquarium epoxy) was used to mount the substrates to level them with the surrounding putty. The putty was weighed to 12 g per well to get the same volume in all wells. This was done to have a limited depth of motion available for the larvae to keep them in a narrow focal plane to make it easier to analyze the videos. Substrates covered 26.2% ± 4.9% (mean ± SD) of the surface area in the wells. All the plates with substrates were put on flow-through for 6 days with filtered sea water as described for the maintenance of adults and larvae. This was not to condition the substrates with bacterial film, but simply to neutralize the pH of the concrete and rinse off any contaminants.

Running the experiment

On 3 April 2023, the larvae were filmed to document their behavior around the different substrates in the 6-well plates. The larvae were 33 days old (4weeks + 5 days) at this time. 15 larvae were added in each well, one well at a time. The larvae were transferred with a glass pipette and filmed for 5 min with a Canon EOS 5D Mark II mounted on a stand with adjustable height. The camera's height over the wells was kept constant for every video. A Canon macro lens (EF 100 mm, f1:2.8) was used during filming. The larvae were left in the wells after filming and the aim was to repeat video documentations and check for settlement and metamorphosis after a few days, however, this could not be done due to poor condition of the larvae. During this first filming, however, larvae were in good condition.

Behavioral analysis

The produced videos were analyzed in the *Behavioral Observation Research Interactive Software* (BORIS version 7.13.8, Friard and Gamba, 2016). The larval behavior was divided into different categories, all belonging to the *state* type of behavior, meaning they have an appreciable duration (in contrast to *events* that are instantaneous, Altmann, 1974). An ethogram of eight observed behaviors was established: 1) *pelagic swimming*; 2) *foraging in the pelagic*; 3) *resting on the epoxy putty*; 4) *resting*

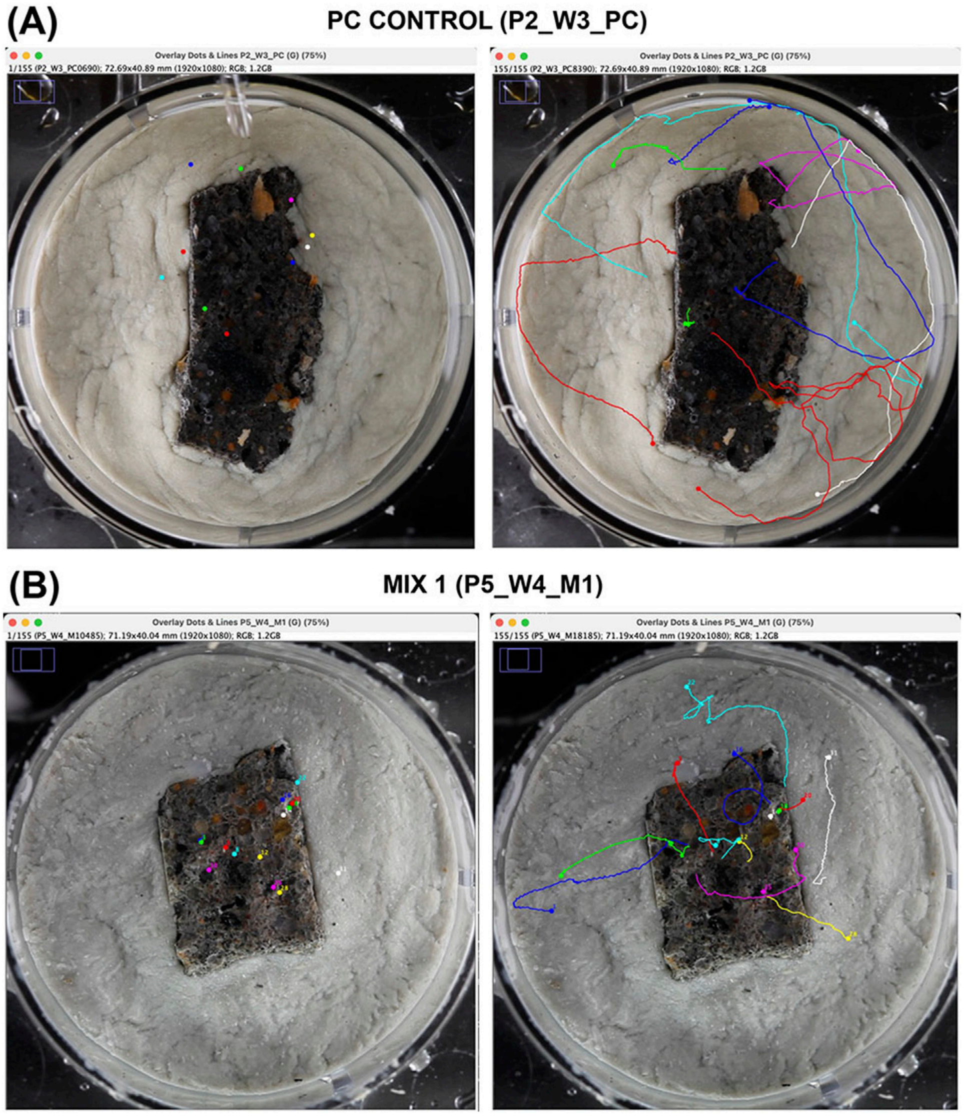

FIGURE 1
A schematic image that illustrate larval movement across the various substrates. Left images shows the starting position, right images the end position and tracks. The dots are at the end of the tracks. **(A)** Well 3 with plain Portland cement (PC) controls shows that larvae spend very little time on the substrates, only one larva is probing the substrate (the short green track). **(B)** Well 4 with substrate M1 shows how several larvae are moving slowly across the substrate, bottom-probing, and eventually attaching (the short tracks). Wells are 34 mm wide.

around the edge of the well; 5) *resting in the pelagic*; 6) *inside a crevice exploring;* 7) *bottom-probing on substrate;* and 8) *attachment to substrate*. The pelagic swimming was characterized by regular straight larval swimming, spinning around the oral-aboral axis at a distance from the bottom. Foraging is characterized by swimming in a spiral motion, slower than the regular pelagic swimming. There could be some residue from the food left in the water as larvae were pipetted from the feeding bowl to the staining and then into the 6-well plates. Resting on the putty or around the edge was characterized by temporary attachment to, or in contact with the surface, either still or rotating on the spot. Resting in the pelagic was characterized by larvae being still in the water, just rotating or moving water across the ectoderm by ciliary motion. This can also be a way of foraging. Bottom-probing is characterized by a slow meandering motion in close contact with the surface, and this was usually the way of motion they exhibited also inside the crevices. Attachment to substrates was characterized by being still in direct contact with the substrate. From the videos it was difficult to see if larvae were rotating on the spot or being still, and if they were attached or just resting. Sometimes they do attach and must be removed with some force, using a pipette. If not attached, they usually resume swimming after a while. The behaviors were considered positive when it was in contact with the substrate (6–8), and negative in contact with the putty. The pelagic swimming, resting, and foraging were considered negative behaviors since it meant no contact with either surface. The time spent on each of these eight behaviors was annotated in BORIS for all visible larvae.

Tracking and attachment rates

In addition to the analysis in BORIS we carried out manual tracking (Fiji/ImageJ, plugin ManualTracking, Schindelin et al., 2012) of all visible larvae to visualize the larval behavior and to be able to evaluate the condition of larvae. It was also used for counting the number of larvae that attached to the substrates during the video sequences. The videos were saved as individual frames (JPEG images) and all frames with a visible pipette tip was removed from the beginning of each image series. This was done to track the larval movements without the influence of the turbulence from the pipette. The number of frames analyzed in ImageJ/Fiji differed slightly among videos, resulting in a small variation of video lengths (5.166 ± 0.115 min, mean ± SD). The difference in video lengths between treatments was not significant when checked with a one-way ANOVA ($F_{8,18}$ = 1.903, p-value = 0.122). Two examples of the tracking of larval movements across the substrates are shown in Figure 1.

TABLE 2 Number of larvae in each replicate well across all substrates. In total 356 larvae of the 405 added were traced. 15 larvae were added to each well, some were lost in the handling or not visible in the videos. The lowest number of larvae per well was 11, making this a well-balanced analysis despite the loss of some larvae.

Substrate	Well_1	Well_2	Well_3	n
PC	11	12	15	38
M1	15	14	15	44
M2	13	14	14	41
M3	12	13	15	40
3D-1	15	12	14	41
3D-2	11	13	13	37
MS	15	12	13	40
CC	13	12	12	37
CT	11	14	13	38
sum	116	116	124	356

Statistical analyses

Most statistical analyses were done in RStudio (R Core Team, 2024: R version 4.4.1, and previous versions). R codes for the analyses and graphs are compiled in the Supplementary Material, with some complementary graphs of raw data (Supplementary Figures S1, S2).

To visualize the results from the BORIS analysis, we produced a stacked bar-plot using ggplot2, displaying the proportion of time spent on each behavior across all substrates.

The assumptions of normality and homogenous variances were checked by frequency distributions (Supplementary Figures S3), Q-Q plots (Supplementary Figures S4), and residual plots (Supplementary Figures S5). Homogeneity of variances was also checked with a Bartlett's test for equal variances.

To analyze the data, we used the sum of time spent on behaviors in contact with the substrates and planned to analyze it with a nested ANOVA, however, analyses of behavioral data commonly do not meet the assumptions of normality and homogenous variances, as were the case for these data. Instead, we used a Log Response Ratio (LRR) analysis (Hedges et al., 1999; Nakagawa and Cuthill, 2007) to compare the effect sizes among treatments with standard Portland cement used as the control (0 effect). Well was the replicate unit (n = 3) used to calculate the mean and standard deviation (SD) of all substrates, with the average time spent on the substrate per well as the numerical value. The natural logarithm (ln) of the Response Ratio, the LRR variance, and the 95% confidence interval (CI) was then calculated according to Equations 1 and 2 in Hedges et al. (1999). To further facilitate the interpretation of the results, the LRR effect sizes were translated into percentage change according to Equation 2 in Pustejovsky (2018).

In addition to analyzing time spent on substrates, we further isolated the number of larvae that attached to the substrates within the time frame of filming, and that stayed on the substrate the remainder of the video duration. Since attachment vs. no attachment is a binary outcome, and the numbers were below five in some treatments, the analysis was performed with a Fisher's Exact Test which is a more robust frequency test when numbers are low. A hierarchical design is not possible for this test so here we used pooled data from all three wells within treatments. The analysis was run with Monte Carlo simulations with seed set to 1234 (set.seed (1234)). This provides a simulated p-value, and setting the seed at the same number will give reproducible results. This was followed up by planned comparisons with Fisher's Exact tests, testing each substrate against PC as controls. Since the Fisher's exact test was ran with a Monte Carlo simulation, no correction was applied for the planned comparisons.

Results

Larval behavior across substrates

The following analyses are based on a total of 356 larvae of the 405 used for the experiment. Of the 15 larvae added to each well, between 11 and 15 were traceable in BORIS and included in the analysis (Table 2).

The results from the analysis in BORIS shows clear differences in the proportion of time spent on the eight defined behavioral patterns across the substrates (Figure 2). A large portion of time was spent in the pelagic, swimming. The pelagic swimming was recognized as such since the shadow of the larvae was offset, in contrast to when larvae were in contact with the substrate and the shadow was in direct association with the larvae. In wells with regular Portland Cement (PC) and the two ceramic materials (CC, CT), time spent swimming was >60%. Least time spent swimming (<40%) was observed in M1, M3, 3D-1, and 3D-2. The three behaviors that indicate interest in the substrate and deemed as positive were: 6) exploring a *crevice* within the substrate; 7) *bottom-probing* with larvae actively exploring the substrate in direct contact with it; and 8) *attachment* to the substrate. These behavioral patterns are shown in shades of green in Figure 2. On PC controls and ceramic materials, the larvae spent the least amount of time on the substrates, while larvae on M1, M3, and 3D-1 spent the highest proportion of time on substrates. Furthermore, on substrate 3D-1 larvae spent the longest

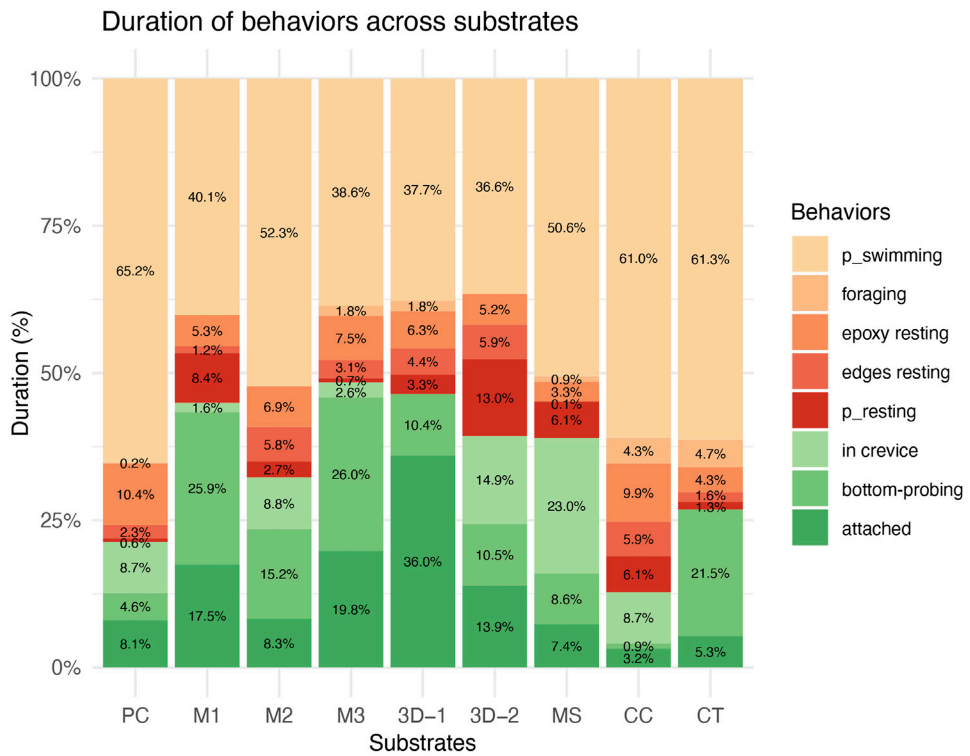

FIGURE 2
Duration of larval behaviors across the various substrates based on the annotations in BORIS (Behavioral Observation Research Interactive Software, version 7.13.8). All behaviors in contact with the substrates are shown in green, and the rest in shades of red. Percentages of time spent on each behavior is centered on each section of the bars. A few zero values were removed.

time attached to the substrates (36%), followed by M3 (19.8%) and M1 (17.5%). Time spent bottom-probing in close contact with the substrate was almost equal in M3 and M1 with c. 26%, followed by CT with 21.5%. On the metallurgic slag (MS), larvae spent the most time exploring crevices (23%), followed by the other rough textured substrate (3D-2) with 14.9%.

Checking the assumptions

The data did not meet the assumptions of normality and homogeneity of variances, as is common for behavioral data (Lantz, 2013). The same pattern was found for the entire dataset, across substrates, and across wells (Supplementary Material). The overall frequency distribution had a bimodal, or U-quadratic, distribution with high frequencies of zeros, and high frequencies of values approaching one in some treatments (Supplementary Figures S3). A Bartlett's test confirmed that the assumptions of equal variances was violated, and Log Response Ratios (LRR) was therefore chosen for the analysis (Bartlett's K-squared = 19.174, df = 8, p-value = 0.01396).

Time spent on substrate

The LRR analysis shows that time spent in contact with the substrates was significantly higher on two of the GGBFS enriched substrates (M1 and M3) and on the two 3D-printed substrates (3D-1 and 3D-2, Figure 3) compared to the PC controls. The other GGBFS enriched substrate (M2), the metallurgical slag (MS), and one of the ceramic materials (CT) all had positive effect sizes but with the 95% CI overlapping the control. The other ceramic material (CC) had a mean larval time spent on the substrate that was lower than the control. Compared to the control (PC), larvae in average spent 109, 46, and 136% more time exploring the surface for M1, M2, and M3 respectively; and 127, 85, and 81% more time for 3D-1, 3D-2, and MS respectively. When considering time spent on the ceramic materials, larvae on CT spent 27% more time compared to the control, while larvae on CC spent 38% less time than on the control.

Attachment rate across substrates

The number of attached larvae, i.e., larvae that attached during the filming period and remained attached to the same spot, completely still, until the end of the video, varied across treatments. The highest attachment rates were recorded for substrates M1, M3, and 3D-1 (Figure 4; Table 3). Note that for substrates with higher complexity (3D-2 and MS) where larvae spent a lot of time exploring pits and crevices, attachment might have been underestimated due to the fact that larvae were not always visible. The result of the Fisher's Exact test on number of attached larvae showed that there were significant differences in attachment rates across substrates (p-value = 0.000021).

To explore which pairwise comparisons that rendered significant results each substrate was tested against PC as

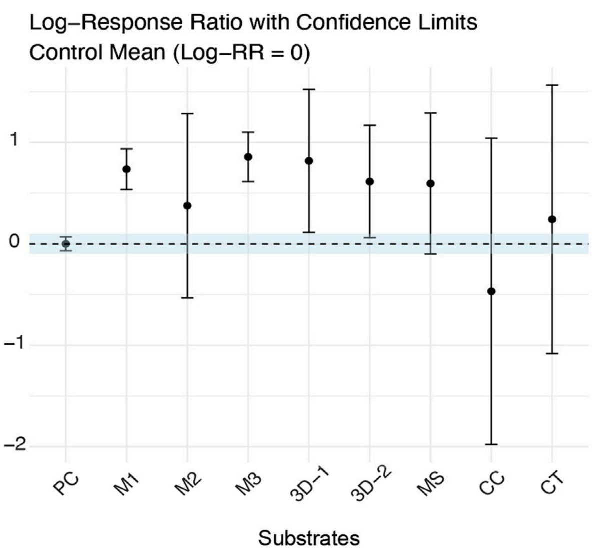

FIGURE 3
Log-response ratios averaged across wells showing Log_RR values with 95% confidence intervals (CI). Mix M1, M3, 3D-1, and 3D-2, had 95% CIs that was above the control mean (PC). Compared to the control, larvae in average spent 109, 46% and 136% more time exploring the surface for M1, M2 and M3, respectively; and 127, 85% and 81% more time for 3D-1, 3D-2 and MS respectively. Regarding the ceramic materials, larvae on CT spent 27% more time compared to the control while larvae on CC spent 38% less.

TABLE 3 Number of attached vs. not attached larvae across substrates, counts and percentages. Substrates M1, M3, and 3D-1 had the highest counts (highlighted in bold), with an attachment rate of 37.2%–44.7%.

	Not attached		Attached		
Substrate	Count	%	Count	%	n
PC	30	83.3	6	16.7	36
M1	27	62.8	16	37.2	43
M2	30	78.9	8	21.1	38
M3	23	62.2	14	37.8	37
3D-1	21	55.3	17	44.7	38
3D-2	26	76.5	8	23.5	34
MS	36	90.0	4	10.0	40
CC	36	94.7	2	5.3	38
CT	36	90.0	4	10.0	40
TOT SUM	265	74.4%	79	22.2%	N = 356

controls in a planned comparison with a Fisher's Exact test for the individual pairs. PC:M1 and PC:3D-1 was confirmed to be significant (p-values: 0.04842; 0.01213, respectively), and PC: M3 was close to significant (p-value = 0.06542). All with odds ratios around 3–4 (Figure 5; Table 4). M3 had a higher percentage of attached larvae (38%) than M1 (37%), but with lower numbers of larvae (n = 37) used in the tests compared to M1 (n = 43). MS, CC, and CT had less than five larvae attached. These low numbers are the reason Fisher's Exact test was applied.

Discussion

The main aim of this study was to find the most appropriate composition of the material used for the production of artificial reefs (ARs) to be deployed at the six restoration sites within the Natura 2000 Kosterfjord-Väderöfjord area and Kosterhavet marine national park in Skagerrak, Sweden. The target organism for the restoration effort is *Desmophyllum pertusum,* a large-scale ecosystem engineer providing complex habitat for associated fauna. A settling assay testing nine different substrates was performed in a laboratory setting with settling competent larvae of *D. pertusum*. Of the nine tested substrates, the larvae were significantly more interested in two of the three concrete substrates mixed with GGBFS (M1, M3), and both tested 3D printed concrete substrates (3D-1 and 3D-2), when looking at time spent on substrate compared to plain Portland cement (PC) that was used as the control. All tested substrates except one of the two ceramic materials performed in average better than the PC control. We included the ceramic materials since settling panels made of ceramics is commonly used in experiments with tropical coral larvae. Settling trials are often made with only a few different types of materials, or the same material with different surface texture or roughness. Testing if one blend of concrete is better than another is, however, not interesting unless you can show that the blend of concrete is also better than other common materials.

Some notes on larval behaviors

The Log Response Ratio (LRR) analysis included the three positive behaviors in direct contact with the substrates, i.e., bottom-probing, exploring a crevice, or time spent attached to the substrate. The results differed across the replicate wells with some of the substrates producing more consistent results among wells and others less so. In one of the MS wells with very low larval interest there was rust developing on the substrate and the avoidance was almost complete. A few larvae were only swiftly brushing against the edges of the slag. In the other wells with poor outcomes some of the larvae started swimming from a position above the putty rather than above the substrate. As the larvae were released from the pipette they sometimes got swept off the substrate although they were released above it. Most of them swam around the perimeter of the well rather than crossing over the substrate in the middle. There was, however, an example of two larvae that started off substrate and then swam directly towards the substrate and quickly attached to it on one of the pieces of metallurgic slag. It is thus likely that the larvae would have found the substrates if there had been an attractive enough chemical signal released from it. The distance from which they started swimming in relation to the substrate might also have influenced their capacity for detecting the signal. Within wells with substrates made of PC, the larvae mostly started swimming above the substrate, then immediately swam off and cruised the perimeters, indicating that the substrate did not have an appealing chemical signature.

When analyzing attachment rates in isolation, M1 and 3D-1 was significantly higher than the PC control, with attachment rates of 37.2 and 44.7 per cent, respectively, compared to 5.3–23.5 per cent

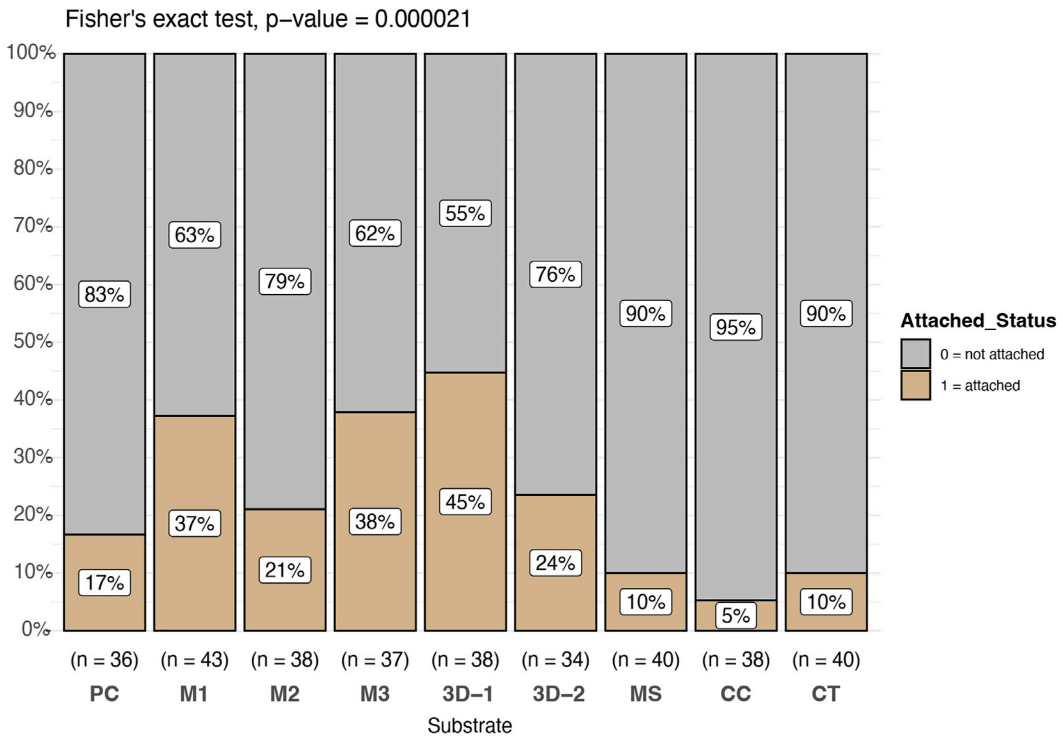

FIGURE 4
Stacked bar plot showing the results of the Fisher's Exact Test. Simulated p-values were run with Monte Carlo simulations ($1e^{+06}$ = 1 000 000 simulations, two-sided, set.seed (1234)). Percentages of larvae that attached vs. not attached across all substrates are shown in corresponding section of the bars.

for the other substrates. Larvae on PC substrates had an attachment rate of 16.7 per cent. M3 did not reach significant levels in this test but was close.

M1 contained a 50:50 mix of standard Portland Cement (PC) and Ground Granulated Blast Furnace Slag (GGBFS). M2 and M3 contained PC, GGBFS, and silica oxide (SiO_2) in different proportions. The higher concentration of silica oxide in M3 gave the substrate a lower pH (11.6) than the other mixes (12.5–12.7). There is no apparent explanation to why the M2 mix would be less appealing to the larvae than M1 and M3. In one of the wells with the M2 mix attachment rates was very high with eight attached larvae, shared only with M1 and 3D-1. In the other two wells there were, however, zero attached larvae, and even bottom-probing behavior was very low. In two of the wells time spent bottom-probing were on par with the results on substrates M1 and M3.

The overall low settling rate in this study could be due to that not all larvae had reached settling competency, and that it would have been beneficial to repeat the assays with larvae a week or two older. Maintaining water quality and larval health for extended periods is, however, a major challenge and we did not have enough healthy larvae left to run a second trial.

On the longevity of artificial reefs

Silica oxide and aluminum oxide are acidic, contributing to lowering the pH of the concrete. These are common oxides in volcanic materials used by the Romans for building hydraulic concrete structures in the marine environment, such as piers and harbors (Oleson et al., 2004; Jackson et al., 2018). Structures that remain today after being submerged for two millennia, although eroded. The extreme longevity of Roman concrete was one of the reasons why we chose to test the metallurgic slag and slag cement that has a similar mineral composition as the volcanic ash and rocks. The composition of minerals in volcanic materials produce a calcium-aluminum-silicate-hydrate (C-A-S-H) binder in contrast to the C-S-H binder of PC. The curing process is slower at the beginning but continues while the structure is submerged in seawater and have 'self-healing' properties, and the final compressive strength is higher than that of standard Portland cement (Jackson et al., 2018). The slag cement has similar properties as the volcanic materials and can increase the durability of the ARs. GGBFS has been used in cement mixes with PC for marine installations due to its properties with a replacement rate of 30–85 mass per cent (Osmanovic et al., 2018; Das et al., 2015; Bhat and Tengli, 2019). The inclusion of GGBFS—due to the higher silica content—protects the concrete from chloride intrusion and thereby protect any installed reinforcement bars from corrosion (Song and Saraswathy, 2006).

Regular PC contain a high amount of calcium oxide (CaO), but also silica oxide (SiO_2), and low amounts of aluminum oxide, magnesium oxide, and iron (ferric oxide, Zhang et al., 2011). The main hydraulic component is calcium silicate, producing a calcium silicate hydrate (C-S-H) acting as a binder. The mineral oxides in the cement will carbonate during curing and produce magnesium carbonate ($MgCO_3$), and calcium carbonate ($CaCO_3$) in the form

TABLE 4 The results of the planned comparisons with the Fisher's Exact as a *post hoc* test of attachment rates across substrates, with all substrates compared against PC as controls. Significant values were found for M1 and 3D-1, highlighted in bold numbers. PC:M3 was close to significant (underlined p-value). All with odds ratios around 3.

Comparison	95% CI		Sample estimates	p-value
	Lower	Upper	Odds ratio	
PC: M1	0.9195890	10.497742	2.9228370	0.04842
PC: M2	0.3541615	5.2561313	1.3281680	0.769
PC: M3	0.9086414	11.074067	2.9968680	0.06542
PC: 3D-1	1.2351590	14.465707	3.9698490	0.01213
PC: 3D-2	0.4040149	6.1097586	1.5289720	0.557
PC: MS	0.1059113	2.6149908	0.5598866	0.5032
PC: CC	0.0260521	1.7295141	0.2823960	0.1473
PC: CT	0.1059113	2.6149908	0.5598866	0.5032

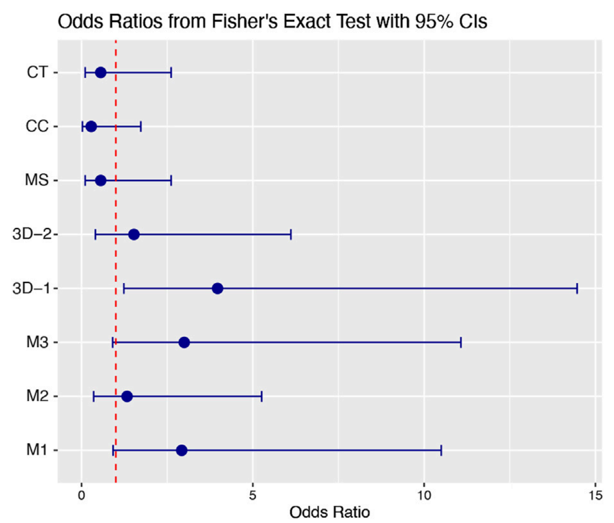

FIGURE 5
Odds ratios ± CI from the planned comparisons with Fisher's Exact test, with all substrates compared against standard Portland cement (PC) as controls. PC:M1 (p-value = 0.04842) and PC:3D-1 (p-value = 0.01213) were significantly higher than the other PC: substrate comparisons. PC:M3 was close to significant (p-value = 0.06542).

of calcite, but to some extent also aragonite, the same form of calcium carbonate that corals produce in their skeleton. The GGBFS contain higher levels of magnesium oxide, silica oxide, and aluminum oxide than PC (Saranya et al., 2019).

Comments on the 3D printed samples

The complete composition of the 3D printed product samples is unknown. This is a composition developed by ConcretePrint in collaboration with researchers at KTH, the technical university of Stockholm. It is based on CEM I (Portland cement), with additives such as silica slurry, retarder, fine grained silicious sand, and a plastic fiber as a reinforcement. The proportions of the components are not known to us. It has been developed to have specific properties for additive manufacturing (3D printing), i.e., to optimize extrudability and buildability, and for maximal compressive and flexural strength for the purpose of printing large objects, mainly houses. Initially, we incorporated the samples in the test to see if this concrete would have any adverse effects on the coral larvae, in case we would not be able to make an adapted blend for the project. Later, ConcretePrint was able to adapt the recipe for our needs and could incorporate 50 percent GGBFS (50:50 PC:GGBFS) and substitute the plastic fibers with basalt fiber, without affecting the workability of the wet cement. Strength development also looked good, although delayed, as expected with the incorporation of GGBFS. This final blend was, however, never tested on larvae since it was finished when larvae were no longer available. It would be interesting to test the original blend against the adapted blend to see if our adapted blend shows as good result as the original blend tested here. Simply substituting the plastic reinforcement fibers to basalt fiber or some other natural fiber would most likely be enough to make it safe for benthic fauna. Most additives in concrete are benign, except for air-entraining agents that often consist of plastic spheres, or a detergent that potentially could disrupt cell membranes of delicate marine fauna. Air-entraining agents were excluded from the adapted blend, although it might have been present in the original blend that we tested in this experiment.

Potential chemical cues for settling

Coral larvae are known to settle readily on substrates containing calcium carbonate, although settling can be enhanced with the addition of strontianite ($SrCO_3$) and magnesium in the form of magnesium carbonate and magnesium sulphate ($MgSO_4$) (Levenstein et al., 2022; Yus et al., 2024). The higher content of magnesium oxide in the GGBFS could thus be the cause for the increased interest and attachment in the mixed concretes. The GGBFS used in this experiment (Slag Bremen, Holcim AB, Bremen, Germany) contains 7 mass per cent magnesium in the form of magnesium oxide (MgO), almost three times more than in PC with 2.5 mass per cent. Strontium is present in seawater at a concentration of c. 7–8 mg/L (Angino et al., 1966) and will probably be incorporated into the concrete as it is submerged, while also readily available for the live corals from the ambient sea water. Both strontium and magnesium are present in coral skeleton with evidence of a vital effect of magnesium with bands of higher-than-expected concentrations within the skeleton (Meibom et al., 2008). A study by De Weerdt et al. (2016) showed that after 16 years of submersion in seawater concrete beams had a 200–300 µm deep magnesium enriched zone at the surface, making magnesium available for settling fauna. This layer was present in all three tested concrete blends, both with and without slag cement. In the study they used a low substitution of PC with slag of only 30 per cent. The magnesium was in the form of brucite ($Mg(OH)_2$) and magnesium silicate hydrate (M-S-H). If this is as attractive as magnesium carbonate and sulphate for coral larval settling needs to be tested.

Looking at potential natural substrates for *D. pertusum* larvae, calcifying benthic fauna could be of interest. There are some very common groups such as barnacles, serpulid polychaetes, and bryozoans that produce a solid substrate of calcite or aragonite ($CaCO_3$), with aragonite being a more easily soluble form of $CaCO_3$. Barnacles form low-magnesium-calcite with Mg/Ca ratios of less than 50 mmol/mol, and high Sr/Ca ratios (Ullmann et al., 2018). Serpulids produce tubes of a mix of calcite, magnesian calcite ($(Ca,Mg)CO_3$) and aragonite (5–10 Mol per cent $MgCO_3$) of different ratios (Bornhold and Milliman, 1973). The ratio of aragonite and magnesian calcite is temperature dependent, such that they both increase with increasing water temperatures while cold-water species are composed of primarily calcite. However, a generic control of mineral composition is also present, such that some species have a higher-than-expected ratio of aragonite and magnesian calcite irrespective of the environment. Bryozoans produce a low-to intermediate magnesian calcite, with arctic species being predominantly calcitic (Smith et al., 2006; Kuklinski and Taylor, 2009). Some bryozoans are too delicate to be plausible as substrates for *D. pertusum*, but many have a very robust morphology and are more likely. Several species of barnacles have been found at the major reef site at Väderö Islands, e.g., the large deep-sea barnacle *Chirona hameri*, and the smaller *Balanus balanus*. Several species of serpulid polychaetes have also been observed in abundance in the area. (Strömberg and Larsson, 2025, in this issue).

The observation of Strong et al. (2023) that *D. pertusum* had settled on the plates of barnacles but were not observed directly on the moorings and buoys—or on adjacent boulders—indicate that they might prefer settling on biogenic materials, thus displaying secondary recruitment on other biota rather than opportunistic recruitment on clean surfaces. Thus, it is probably beneficial for coral larval recruitment to have calcifying fauna already recruited on the ARs, and not necessarily a negative competition situation. It is possible that the addition of GGBFS into the concrete makes it more similar to these biogenic materials.

Conclusion

Larvae of *D. pertusum* show higher preference for substrates containing Ground Granulated Blast Furnace Slag (GGBFS) over standard Portland cement, possibly due to the higher content of magnesium or overall higher mineral complexity. The pH of the concrete could also play a role, however, the M1 mix with 50:50 PC: GGBFS and no added silica oxide was equally appealing to the larvae as the M3 mix with higher silica content and lower pH. We did not specifically test the pH in the wells, so it is not known if the lower pH of the M3 mix still had an effect at the time of the trial.

Larvae also showed higher interest in 3D printed concrete, compared to standard Portland cement. Since the full recipe of the 3D concrete is unavailable due to patent protection, we cannot speculate in the causes. It does contain silica slurry, but there is no known source of magnesium, other than that formed as a reaction at the concrete-water interface which would be present also on the plain PC substrates. The surface texture of the concrete could also have an effect. With more silica the concrete has a finer and denser texture.

Metallurgic slag seems to be appealing to the larvae when clean, however, when the iron content in the slag is high and rust develops on the surface, the larvae avoid the substrate. This needs to be corroborated by further testing.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The dataset is available via SND (Swedish National Data service), at https://doi.org/10.5878/3dtz-9b23.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

SS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. MR: Data curation, Software, Visualization, Writing – original draft. PV: Data curation, Software, Visualization, Writing – original draft. AL: Formal Analysis, Funding acquisition, Project administration, Validation, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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