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Porous ceramics with tailored pore size and morphology as substrates for coral larval settlement

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ABSTRACT

The growing demand for stony corals as ornamental aquarium animals requires defined aquacultural breeding strategies. For the sexual propagation of corals, material substrates are needed, that attract larvae and support their settlement and development. In this study, five types of highly porous ceramic materials were developed following the example of coral skeleton. The applicability of these settlement substrates was tested using larvae of the stony coral *Pocillopora damicornis*. Partial sintering of pressed clay pellets, freeze casting of clay and alumina-mullite based slurries and direct foaming of high alkane phase emulsified suspensions (HAPES) using alumina were employed. By the addition of mm-sized spherical polystyrene beads as sacrificial templates during freeze casting (alumina-mullite), superficial pores in the size of the larvae were created. The inorganic substrates featured open porosities between 35% (pressed clay) and 83% (foamed alumina), pore sizes ranging from nm to mm-scale and pore morphologies dominated by interparticle porosity (pressed), lamellar pores (freeze casting) and cellular pore types (direct foaming). The ceramic substrates were incubated in artificial sea water for 3 months to induce necessary biofilm formation and algae growth. Afterwards, individual substrates were exposed to 5 coral larvae, and their settlement behavior was monitored over 14 days. At the end of this period, all ceramic materials were successfully accepted as settlement substrates, with a mean settlement rate of 46.2%, and no significant differences between the substrate types. On samples with large surface superficial pores, a significantly reduced survival of settled larvae (79%) compared to the other porous materials (93–98%) was determined, suggesting a non-ideal surface topography. While alumina foam samples (HAPES) exhibit the most promising results in terms of settlement and survival of larvae, clay-based substrates provide a more economic solution for the sexual propagation of corals in aquaculture.

1. Introduction

All around the globe, the productivity and biodiversity of coral reefs are endangered not only due to climate change, but also by excessive and selective harvesting of marine organisms [1–5]. The preservation of these ecosystems is of utmost importance, since they provide food for both animals and humans, they are a habitat to sessile and freely moving organisms and they serve as coastal protection [6,7]. Live corals, especially stony, reef building corals (Scleractinia), are of huge interest for the marine ornamental trade [8–12]. Per year, ornamental fish, corals and other invertebrates with a value of 200–330 million USD are traded globally [13]. For corals alone, prizes range from USD 5 to USD 499.99 per piece [5].

To serve the needs of this multi-million dollar industry and comply with international trade regulations, nowadays, an increasing amount of traded corals is produced in aquaculture [4,5,14]. During the sexual propagation of stony corals, planktonic larvae settle and develop into sessile polyps [15]. The settlement on a suitable material substrate is one bottleneck in this process [15], and it is dependent on appropriate settlement cues. If those are not provided, the corals will remain in their

Abbreviations: ANOVA, Analysis of variance; APD, Anionic polyelectrolyte dispersant; BET, Brunauer-Emmett-Teller (analysis); CCA, Calcareous coralline algae; HAPES, High alkane phase emulsified suspension; HSD, Honest significant difference; SD, Standard deviation; SE, Standard error; SEM, Scanning electron microscopy; SLS, Sodium lauryl sulfate; SSA, Specific surface area; PAA, Polyacrylic acid; PSD, Pore size distribution; PVC, Polyvinyl chloride; RH, Relative humidity; RT, Room temperature

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larval states and eventually die [16,17]. Thus, the development of appropriate substrates for larval settlement is a key parameter for sexual propagation of corals.

On the one hand, environmental conditions such as water temperature [18], salinity [19], water movement [20] and quality [7], chemical attractants [21,22] and light conditions [15,23–26] influence the larval behavior during settlement. On the other hand, relevant substrate related settlement cues are material type [24,25,27], its orientation [28–32], its preconditioning in water [24,25,33] and its surface topography [27].

In terms of surface structure and morphology, in general, rough rather than smooth surfaces seem to act as suitable settlement substrates [32,34]. Certain types of corals have shown to settle preferentially in small pits and grooves [25,27,35–37]. These types of structures can protect the larvae from predators and serve as refugia [35,38]. Norström et al. [39] observed, that coral larvae - when confronted with pieces of coral skeleton – most likely settle on skeleton with a morphology similar to their own. This pattern can be either explained by the structure and shape of the substrate [40] as well as by chemical settlement cues [22]. The same reasoning applies to an increased larval settlement rate on cement tiles containing 10% of coral rubble [41,42].

Concerning the substrate material, successful coral larval settlement has been observed on polymers [43–46], glass surfaces [31,47], fragments from coral skeleton [39,48,49] and calcareous coralline algae (CCA) [49] as well as different kinds of ceramic materials [7,15,25–27,35,50–55], with comparatively high rates of settlement on ceramic tiles [31,56]. Ceramic materials also have the advantage of not causing toxic effects in a marine environment due to their chemical inertness, long-term durability during coral larval settlement and growth [57–59] and facile tailoring of their pore structure. For the production of defined porous ceramic structures, various processing routes are available, such as methods involving partial sintering, sacrificial templating and direct foaming [58,60]. The resulting materials can reach total porosities in a range from 20% to 97%, pore sizes from nm to mm range and different pore shapes, while still maintaining sufficient mechanical strength for 'bioceramic' applications [58,59].

After settlement of larvae, the environmental conditions have to be continuously monitored during the nursery phase to enable high coral quality and growth [7,15]. In this stage, the primary polyps have to be fed, to reach a size which ensures their resilience against sedimentation, algal competition and predation and transfers preferential settlement into the growth of healthy corals [7].

In this study, larvae from the stony coral *Pocillopora damicornis* were used for settlement experiments. This species represents a biological model in scientific research [61]. Furthermore corals from the family of Pocilloporidae are also highly demanded by the marine ornamental trade, because they are colorful, easy to maintain and relatively inexpensive, but also characterized by a high growth rate, their resilience towards fluctuations in water quality and light intensity and a low vulnerability to common diseases [15]. *Pocillopora damicornis* is a brooder species, i.e. fertilization takes place inside the adult organism, and based on the lunar cycle, settlement competent larvae with a diameter between 0.5 and 2 mm are released to the environment every month [38,41,62–64]. They are typically able to settle within hours [7]. Besides small amounts of organic components, the skeleton of adult corals consists to 99.9% of aragonite [65]. It shows a mean total porosity of 54% [66]. During the analysis of coral skeletons of different coral species, bimodal pore size distributions with small pore sizes between 2 and 20 µm and larger pore sizes in a range from 125 to 500 µm have been observed [67–69].

The objective of this work is the fabrication of suitable porous ceramic substrates for coral larval settlement in aquaculture with strong focus on the pore size and pore morphology. Based on the properties of coral skeleton, highly porous materials are fabricated to control the settlement of larvae during sexual proliferation of corals. The porous structure is tailored using different kinds of processing routes. Partial sintering of pressed pellets, direct foaming of high alkane phase emulsified suspensions (HAPES), freeze casting and conventional templating methods are employed to vary the pore size from nm to the mm range. The pore shapes are controlled by the fabrication process to achieve interparticle, cellular and lamellar pore structures, respectively. Additionally mm-sized polymeric spheres are used to generate spherical template pores as larval refugia on the surface of one type of substrate. Both the applicability of high purity ceramic powders, and commercially available red clay granulates are evaluated. After sintering, the settlement substrates are analyzed concerning their pore size range, mean pore size, open porosity and specific surface area. Prior to the conduction of settlement experiments the ceramic substrates are preconditioned in artificial sea water for 3 months. Finally, larval settlement and survival of settlers on different types of porous ceramic substrates are investigated in detail over a period of 2 weeks.

2. Materials and methods

2.1. Materials

For the fabrication of settlement substrates, ceramic powders and reagents were purchased and used without further purification. Granulated clay powder (SZ/Rot-SK, granule diameter = 150–300 µm, specific surface area [SSA] = 23.6 m^2/g, Sibelco, Germany), α-alumina (CT 3000 SG, d_{50} = 500 nm, SSA = 7.5 m^2/g, Almatis, PA, USA) and two types of mullite powder (Alodur WFM, particle diameter = 45 µm or 80 µm, Treibacher Schleifmittel, Germany) were used as ceramic particles. Silica sol (Begosol K, 45 vol% SiO_2, particle size: 8 nm) was obtained from BEGO (Germany). In the freeze casting approach with template addition, polyacrylic acid (PAA) (Syntran 8220, 40 wt%, molecular weight 4000 g/mol, Interpolymer, Germany) was used as dispersant, distilled water was chosen as diluent and expanded polystyrene spheres (Styroporkügelchen COMFORT, particle size: 2–5 mm, Kissenwelt, Germany) served as sacrificial templates. For direct foaming, an anionic polyelectrolyte dispersant (Dolapix CE-64, Zschimmer & Schwarz, Germany) was added to stabilize alumina particles, the anionic surfactant sodium lauryl sulfate (SLS) (Lutensit AS 2230, BTC, Germany) was used to prepare stable emulsions of decane (≥ 99%, product no. D901, Sigma-Aldrich, Germany) in double deionized water with a resistance ≥ 18 MΩ.

All experiments in artificial sea water were conducted in the aquaria of the Marine Experimental Ecology Lab at the Leibniz Centre for Tropical Marine Research. The aquaria system consisted of two tanks in the same recirculation system with a total water volume of 1500 L. Only the upper tank was used for larvae cultivation and the settlement experiment. The water reprocessing treatment unit contained a protein skimmer, calcium reactor, phosphate absorber and a moving bed filter. An ambient light regime (12 h light and 12 h darkness) was established and the water temperature was held constant at 25 °C, showing a salinity of 35 ppt. Water parameters (pH, carbonate hardness, nitrate, nitrite, ammonia, and phosphate) were controlled once a week with test kits from Salifert (KN-Aquaristik GmbH, Germany). The tanks were aerated with Turbelle® Pumps (Tunze Aquarientechnik GmbH, Germany) and cleaned as necessary, usually once a week. Ten percent of the artificial seawater (osmosis water combined with Red Sea Salt) was exchanged every week. All corals were fed 3 times a week with freshly hatched larvae from brine shrimp (*Artemia salina* nauplii).

For the cultivation of coral larvae, coral colonies of the species *Pocillopora damicornis* were used. The colonies originated from De Jong Marinelife B.V. in the Netherlands and the corals' diameter ranged from 12 to 20 cm. The corals had been acclimatized in the experimental facilities for at least 4 years.

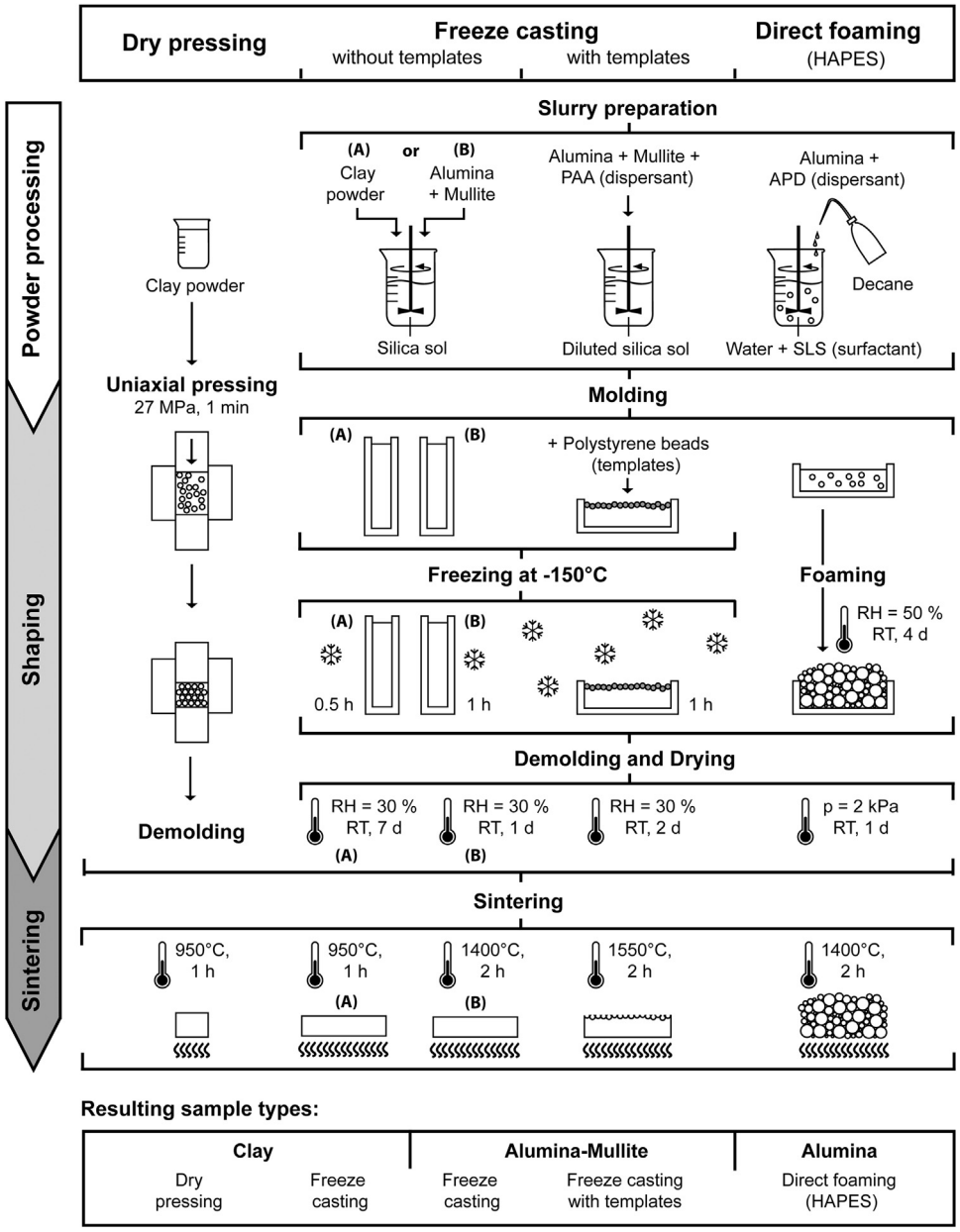

Fig. 1. Processing routes for the fabrication of porous substrates for coral larval settlement. From left to right: Uniaxial dry pressing, freeze casting without and with sacrificial templates (polystyrene beads) and direct foaming using high alkane phase emulsified suspensions (HAPES). PAA = polyacrylic acid, SLS = sodium lauryl sulfate (anionic surfactant), APD = anionic polyelectrolyte dispersant, RT = room temperature, RH = relative humidity.

2.2. Preparation of settlement substrates

For the evaluation of the pore structure's influence on coral larval settlement, five different settlement substrates were developed. Both the substrate material as well as the porous properties were varied, while the macroscopic geometry of the cylindrical samples was kept constant at a diameter of 12 mm and a sample height of 6 mm. The processing routes are summarized in Fig. 1.

2.2.1. Dry pressing

Ceramic green bodies were fabricated using one-sided uniaxial pressing in a matrix of 12.2 mm diameter at 3 kN (25.66 MPa) for 30 s. Per pellet, 1.4 g of granulated clay powder were used. The samples were partially sintered at 950 °C in a pottery kiln (CB 60A, Kittel Ofenbau, Germany) for 1 h with a heating and cooling rate of 4 K/min and 8.33 K/min, respectively.

2.2.2. Freeze casting without templates

For the preparation of freeze casted clay samples, 315 g of granulated clay powder as filler and 240 g of silica sol were mixed in a 500 mL polyethylene bottle and homogenized for 16 h using a ball mill (UR400, Germatec, Germany) and 40 g of Al_2O_3 milling balls (Germatec, Germany). The ceramic slurry was then filled into a mold consisting of a U-shaped polyvinyl chloride (PVC) plate in the size of 120 mm (adjustable to account for slurry amount) × 70 mm × 10 mm (height × width × depth), which was sealed by two aluminum plates. The mold was placed in a freezer (Sanyo MDF1155, Japan) at − 150 °C for 30 min. The frozen plates were removed from the mold and dried at room temperature (RT) and relative humidity (RH) of 30 ± 5% for 7 d. The resulting green bodies were sintered in the same manner as the dry pressed clay substrates (Section 2.2.1).

Freeze casted samples made of alumina/mullite were fabricated in a similar fashion. The ceramic powders – 50 g of alumina and 100 g of mullite powder (particle diameter = 80 µm) – were added to 50 g of

silica sol. The slurry was homogenized, processed and dried analogous to the clay slurry (see above) with an increased freezing time of 1 h and a reduced drying period of 1 d. The samples were finally sintered at 1400 °C for 2 h with heating and cooling rates of 2 K/min and 5 K/min, respectively.

2.2.3. Freeze casting with templates

Alumina/mullite substrates with both freeze casting and sacrificial template derived pores were prepared based on a processing route by Carlesso et al. [70]. In short, 150 g of silica sol were diluted with 30 g of distilled water and mixed with 150 g of alumina powder, 300 g of mullite powder (particle diameter = 45 µm) and 3.1 g of polyacrylic acid as dispersant. Homogenization of the ceramic dispersion was carried out as mentioned in Section 2.2.2. The slurry was filled into a PVC mold in the size of 10 mm × 70 mm × 120 mm (height × width × length). The dispersion's surface was covered with polystyrene spheres as sacrificial templates to produce large pores on top of the substrates. The mold was placed inside the freezer at − 150 °C for 3 h. After removal and demolding of the casted plates, they were dried at RT and RH of 30 ± 5% for 2 d. The plates were sintered using a 3-step sintering program: (1) Heating of the green bodies with a rate of 0.5 K/min up to a temperature of 200 °C and a dwelling time of 2 h, (2) further increase of the temperature up to 500 °C with the same heating rate and holding time, (3) a final temperature increase up to the maximum temperature of 1550 °C at a heating rate of 2 K/min and dwelling time of another 2 h and a subsequent cooling with a rate of 5 K/min.

2.2.4. Direct foaming (HAPES)

For the preparation of settlement substrates via direct foaming, the high alkane phase emulsified suspension (HAPES) technique according to Barg et al. [71] with slight modifications by Kroll et al. [72] was used. In the first step, a homogeneous aqueous alumina slurry with a particle content of 42.5 vol% and 0.74 wt% of anionic polyelectrolyte dispersant was prepared using a laboratory mixer (Dispermat LC, VMA Getzmann GmbH, Germany) at a stirring velocity of 2500 rpm for 20 min. Prior to emulsification at a stirring speed of 1100 rpm and reduced environmental pressure of 10 kPa (100 mbar), the anionic surfactant and decane were added to reach concentrations of 0.33 vol% and 70 vol%, respectively. The final emulsion was subjected to ambient pressure and poured into molds made of aluminum foil with a size of 20 mm × 120 mm × 120 mm (height × width × length). The samples were dried for 4 d at RT and RH of 50%. After demolding, an additional 1 d drying step at RT under reduction of pressure from 10 kPa (100 mbar) to 2 kPa (20 mbar) was performed. The dried plates were finally sintered at 1400 °C for 2 h with a heating and cooling rate of 2 K/min and 3 K/min, respectively.

2.2.5. Finishing and preconditioning

Cylindrical samples with a diameter of 12 mm were prepared out of the wetted freeze casted and directly foamed plates using a stationary drilling machine (SBE 710, Metabowerke GmbH, Germany) with a diamond hollow bit. The height of the samples was adjusted to 6 mm by grinding.

Prior to material characterization, all fabricated samples were cleaned in distilled water using an ultrasonic (US) bath and dried for 24 h at 70 °C. For the settlement experiments, the ceramics were fixed on light egg crate ceiling grille plastics and preconditioned for 3 months in the aquarium tank.

2.3. Characterization of settlement substrates

The microscopic surface structure of the fabricated settlement substrates, their porous properties and their specific surface area (SSA) were investigated by scanning electron microscopy (SEM), mercury intrusion porosimetry and nitrogen adsorption with Brunauer-Emmett-Teller (BET) analysis, respectively.

A CamScan scanning electron microscope (SEM, UK) was used for the identification and visualization of macropores and potential surface defects. The pore size distributions (PSD), open porosities P_{op} and pore size modes $\overline{d}_M$ are determined with the combination of two Hg porosimeters (Mercury Porosimeter Pascal 140 and 440, Porotec GmbH, Germany). In unimodal pore size distributions, the determined pore size range describes a region around the mean value, which comprises 90 vol% of the overall pore volume. In the case of bimodal pore size distributions, the evaluation of the pores sizes was limited by the measurement technique to pore sizes above 4 nm and below 120 µm and thus the whole span of measurement values is given as pore size range. A BELSORP-mini (BEL Japan, Inc., Japan) volumetric gas adsorption apparatus was used for N_2 adsorption experiments with subsequent BET analysis for the determination of the SSA.

2.4. Cultivation and collection of coral larvae

For the conduction of settlement experiments, brooded larvae of *Pocillopora damicornis* were collected each month after full moon from July 2016 until October 2016. One to three nights after full moon, spawning occurred and larvae were pipetted (Pipette Graduated 3 mL Sterile Pastette®) from the water surface into plastic containers inside the tank, in which the corals were kept overnight. These containers had windows covered with fine mesh to control water exchange/movement and coral larvae accumulation. Furthermore, airstones were used for oxygen production and water movement.

2.5. Larval settlement experiment

After the collection of larvae, the preconditioned ceramic substrates were placed one per well inside microwell plates (CELLSTAR® 12 Well Cell Culture Multiwell Plates) (Fig. 4.A.). The microwells were filled with 0.2 µm filtered tank sea water (AcroPak™ Capsules with Supor® Membrane, Sterile, Pall Laboratory). Five randomly pipetted larvae were added to each microwell. The microwell plates were kept opposite to the culture tank in a shelf under diffuse light from the surrounding light regimes above the aquaria system. Fifty percent of the water was exchanged with freshly filtered sea water each day.

The larvae were sighted and monitored after 1, 3 and 5 h after larvae addition, daily from day 1 to day 7 and 2 weeks after the beginning of the experiment using written records and photographic images (Canon Powershot G7). Larvae were categorized into 'dead', 'not settled', 'settled (other)' and 'settled (substrate)' (Fig. 4.B.). 'Dead' larvae were observed as missing, since they dissolved or got eaten by small predators such as juvenile brittle stars. 'Not settled' larvae included swimming and attached larvae, which had not started to develop their typical disc-shaped settler body. 'Settled (other)' larvae settled onto the microwell bottom and wall or under the water surface. 'Settled (substrate)' denotes larvae, that settled onto the ceramic substrates, either on top or on the side. The development of exemplary larvae settled on ceramic substrates was monitored qualitatively over a period of 7 months using photographic images. After 3 weeks, these substrates were transferred to the aquarium tank.

2.6. Statistical analysis

Material analysis was carried out in triplicates (n = 3). To describe the distribution of open porosity (P_{Op}), pore size modes ($\overline{d}_M$) and specific surface area (SSA) the arithmetic means ($\overline{x}$) and the standard deviations (SD) were calculated as follows:

$$\overline{x} = \frac{1}{n}\sum_{i=1}^{n} x_i \tag{1}$$

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{x})^2}{n-1}} \tag{2}$$

with n being the population size, i.e. the amount of experiments and x_i the observed values of the data set.

Larvae settlement experiments were performed using 20 individual cylindrical ceramic samples per substrate type (n = 20) and 5 coral larvae per experiment. For all biological experiments, the arithmetic mean and the standard error (*SE*) were calculated to evaluate the data. *SE* is defined as $SD/\sqrt{n}$.

Both results from material's characterization and settlement experiments were reviewed concerning their statistical significance using the analytics software STATISTICA 10 (StatSoft, Inc., OK, USA). An analysis of variance (ANOVA) with subsequent Tukey honest significant difference (HSD) test was carried out at a significance level $p < 5\%$. In general, Tukey HSD analysis for equally sized populations n was used. For a differentiated evaluation of the larvae's settlement location on the ceramic substrates (top/side, Fig. 6), the settlement of at least one coral larvae per substrate was the precondition for analysis. Since the coral larvae did not settle on the ceramic substrate in all 20 experiments, the population sizes varied: n = 20 for dry pressed clay substrates, n = 18 for both freeze casted alumina-mullite and foamed alumina samples, n = 16 in the case of freeze casted clay and n = 15 for alumina-mullite substrates fabricated by freeze casting with the addition of templates. Thus, this data set was analyzed using Tukey HSD analysis for unequal population sizes.

3. Results and discussion

3.1. Properties of fabricated settlement substrates

Using a variety of different ceramic powders and processing routes, five different types of larval settlement substrates with distinct material properties were fabricated. The macroscopic and microscopic surface structure of fabricated settlement substrates are displayed in Fig. 2, while the porous properties and specific surface areas (*SSA*) are summarized in Table 1. Representative pores size distribution of all materials are given in Fig. 3.

Prior to preconditioning of the ceramic substrates in artificial sea water, the ceramic substrates are characterized by different porous properties and specific surface areas (*SSA*), which are directly related to the applied fabrication process (Figs. 2, 3, Table 1). Considering all fabricated and tested ceramic substrates, achieved pore sizes ranged from 4 nm to 5 mm with open porosities between 35–83% and specific surfaces areas of 0.27–25.13 m^2/g.

During partial sintering of ceramic samples at 950 °C full densification of the samples during heat treatment is avoided [58,73]. The samples were sintered at a relatively low temperature, leading to the formation of sintering necks between the initial particles and interparticle pores [58,73]. Partially sintered, dry pressed samples exhibited a unimodal pore size distribution (Fig. 3A-1) with a pore size range from 16 nm to 1.91 µm and a mode of 115 ± 10 nm. With partial sintering, the pore size can be easily controlled at size usually 2–5 times smaller than the size of the initial powder [58,73]. From this results, a breaking of µm sized granules during dry pressing to fragments in a size range of 200–600 nm and the formation of interparticle pores during partial sintering can be inferred [73]. SEM images (Fig. 3) show a roughened sample surface, which is not reflected by the pore size distribution and can thus be attributed to surface defects related to the pressing dies used for the fabrication process. The total porosity of partially sintered ceramics is usually below 50% [58,73]. Characteristically, the pressed substrates included the lowest open porosity of 35 ± 4%, the smallest mean pore size and a consequently high *SSA* of 25.31 ± 0.34 m^2/g. In Comparison, alumina and alumina-mullite samples featured mainly µm-sized pores, which resulted in very small *SSA* values ranging between 0.27 and 0.45 m^2/g.

During freezing of a ceramic suspension, the used solvent transforms into periodically arranged crystals, which serve as sacrificial templates, while the initial particles are compacted in between the solidified solvent. The pore morphology is affected by solvent type and additives. The employed freeze casting processes were all water-based. During freezing water creates lamellar ice crystals, thus, the pore geometry of all samples was characterized by a lamellar structure (Fig. 2). The largest lamellar pores with a width of 40.7 ± 8.1 µm were observed in the samples produced with spherical template particles. Generally, the pore size is dependent on the direction of freezing, freezing time and freezing rate, where long freezing times and a slow freezing rates result in larger pores. Both the longer freezing time of 2 h and the reduced freezing speed due to insulation from the polystyrene spheres explain the observed relatively large pore size. The freeze-casted clay samples were subjected to the shortest freezing time of 0.5 h and under otherwise identical freezing conditions than the substrates made of alumina and mullite. Nevertheless, they exhibit a significantly higher lamellar pore width of 21.9 ± 3.1 µm compared to 8.9 ± 0.4 µm. This discrepancy could be related to the swelling of clay minerals upon the suspension in water and the low applied sintering temperature of 950 °C. Similar to dry pressed clay samples, this sintering temperature led only to partial sintering. Due to freeze casting, the porosity is dependent on the solid loading and ranges between 20% and 90%. Freeze casted clay samples and samples produced by a combination of freeze casting and template addition exhibited a similar open porosity of 52 ± 6% and 47 ± 3%, respectively. This is in agreement with a similar solid loading of the respective ceramic slurries of approximately 70 vol%. The fabrication of freeze-casted alumina-mullite samples with a higher solid loading of around 75 vol% resulted consequently in a lowered open porosity of 39 ± 1%. In contrast to the freeze casted alumina-mullite samples and due to the lower solid loading, the pores size distributions of the other freeze casted samples showed a considerable contribution from matrix derived pores in addition to freeze casting pores to the overall open porosity (Fig. 3). Concerning freeze casted clay samples, these matrix pores have a mean size of 0.008 nm ± 0.002 nm, in alumina-mullite samples made by freeze casting and template addition the mean size is 2.211 ± 0.057 µm. This indicates a relatively small size of suspended clay particles, which allows for a denser particle packing in combination with silica nanoparticles, compared to alumina and mullite particles with particle sizes of 500 nm and 45 µm, respectively. Besides the open porosity, the small matrix derived pores in freeze casted clay samples contribute significantly to its *SSA* to reach a value of 7.78 ± 0.03 m^2/g. Spherical polystyrene templates were used as sacrificial templates and decomposed during sintering, leaving pores in the shape of the initial placeholder, i.e. macroscopic pits with a diameter of 2–5 mm. The size of these pits was larger than the produced larvae and similar to refugia structures, that had been successfully used in other studies to increase coral larval survival [35,38].

The direct foaming technique is based on the incorporation of a gaseous phase into a suspension of ceramic particles, the stabilization of the cellular bubble structure and the subsequent drying and sintering of the ceramic foams. High alkane phase emulsified suspensions (HAPES) is a facile direct foaming technique based on the emulsification of alkanes in water and the transition of alkane droplets to the gaseous phase. The porosity is controlled by the amount of gas, that is introduced to the system which is usually > 80% [58]. Consequently, settlement substrates produced by HAPES exhibited the highest open porosity of 83 ± 1%. Barg et al. showed, that the stirring speed during emulsification can be used to control the pore size within a range of 3–200 µm [71,74]. This is in agreement with a very narrow pore size distribution (Fig. 3C-1) with a mean pore size of 7.3 ± 0.3 µm. Characteristically, foamed samples exhibited a cellular pore structure. Kroll et al. proofed the applicability of these kind of porous ceramics materials in a marine environment as spawning plates for fish breeding [72], which makes HAPES foams particularly promising as substrates for coral larval settlement.

In terms of porosity, ‘Clay - Freeze casting’ and ‘Alumina-Mullite-Freeze casting with templates’ samples were most similar to the

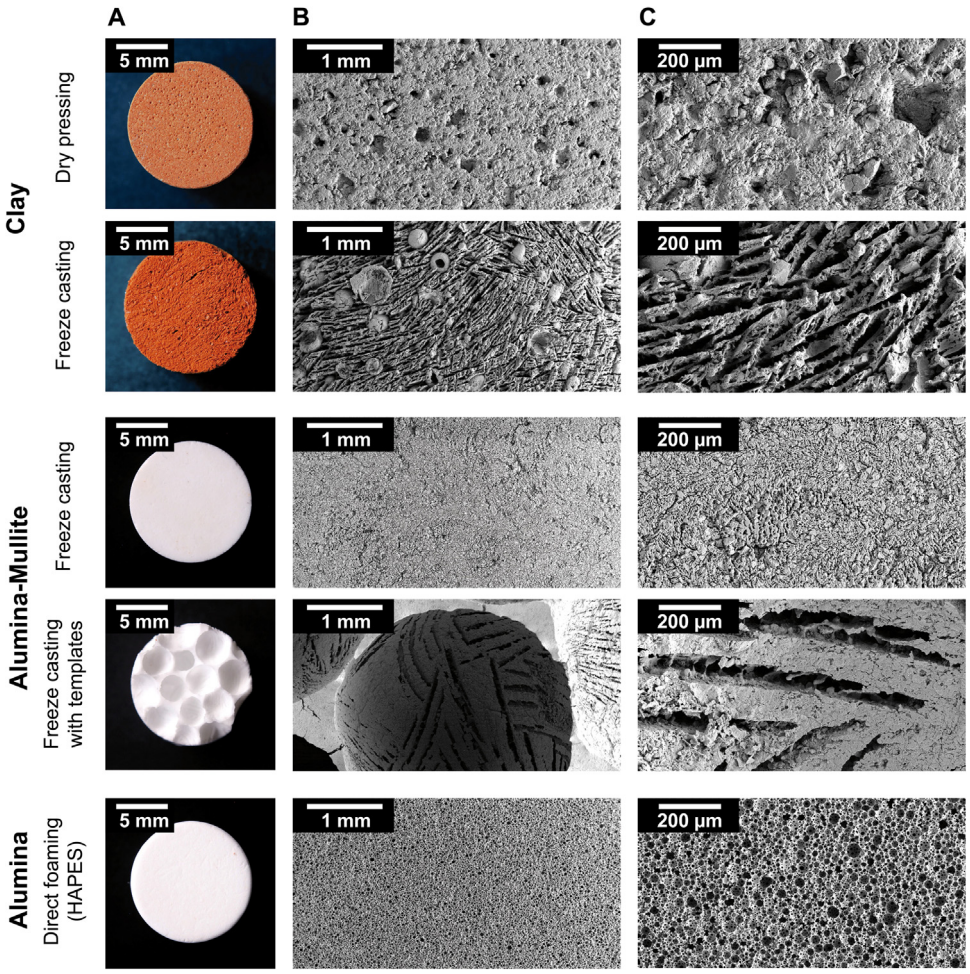

Fig. 2. Macroscopic and microscopic surface structure of substrates for coral larval settlement distinguished by material type and processing route. (A) Photographic images of whole substrate plates, (B) overview and (C) detailed SEM images of the substrates surface and pore structure.

structure of coral skeleton of *Pocillopora damicornis* [66]. The sizes of small pores present in various types of coral skeletons range from 2 to 20 µm [67–69]. Pores in 'Clay – Dry pressing' samples were characterized by pores smaller than 2 µm, while the freeze casting pores in alumina-mullite samples (with templates pores) were larger than 20 µm. Considering not only the width, but also the length of the freeze casting derived pores (Fig. 2), the size of pores in freeze casted clay samples also exceeds 20 µm. In terms of pore size and porosity, freeze casted alumina-mullite and HAPES samples most closely resemble the structure of coral skeleton.

3.2. Effects of substrate pre-conditioning

To increase the rates of coral larval settlement on different kinds of substrate materials, it is essential to precondition the samples by incubation in water. This treatment will result in biofilm formation and eventually in algae growth [24]. After preconditioning of the substrates for 3 months, their surfaces were overgrown with a thin biofilm which

Table 1
Porous properties and specific surface area of substrates for coral larval settlement differentiated by material type, processing route and sintering temperature $T_{Sint.}$. Mean pore sizes $\bar{d}_M$ (mode), pore size distributions (*PSD*) and open porosities P_{op} were measured by Hg porosimetry. Large superficial pores (> 120 µm) originating from polystyrene templates are not determined by this method (Freeze Casting + Templates). The specific surface area (*SSA*) was obtained by nitrogen adsorption experiments (BET analysis).

Sample specifications			Characterization methods				
Material type	Processing route	$T_{Sint.}$ (°C)	Hg porosimetry				N_2 adsorption
			PSD (µm)	P_{op} (%)		$\bar{d}_M$ (µm)	*SSA* (m^2/g)
Clay	Dry pressing	950	0.019–1.910	35 ± 4	(1)	0.115 ± 0.010	25.31 ± 0.34
	Freeze casting	950	0.004–121.783	52 ± 6	(1)	21.965 ± 3.106	7.78 ± 0.03
					(2)	0.008 ± 0.002	
Alumina-Mullite	Freeze casting	1400	0.032–31.502	39 ± 1	(1)	8.909 ± 0.398	0.27 ± 0.04
	Freeze casting + Templates	1550	0.004–109.670	47 ± 3	(1)	40.677 ± 8.135	0.30 ± 0.02
					(2)	2.211 ± 0.057	
Alumina	Direct foaming (HAPES)	1400	3.487–14.449	83 ± 1	(1)	7.347 ± 0.335	0.45 ± 0.02

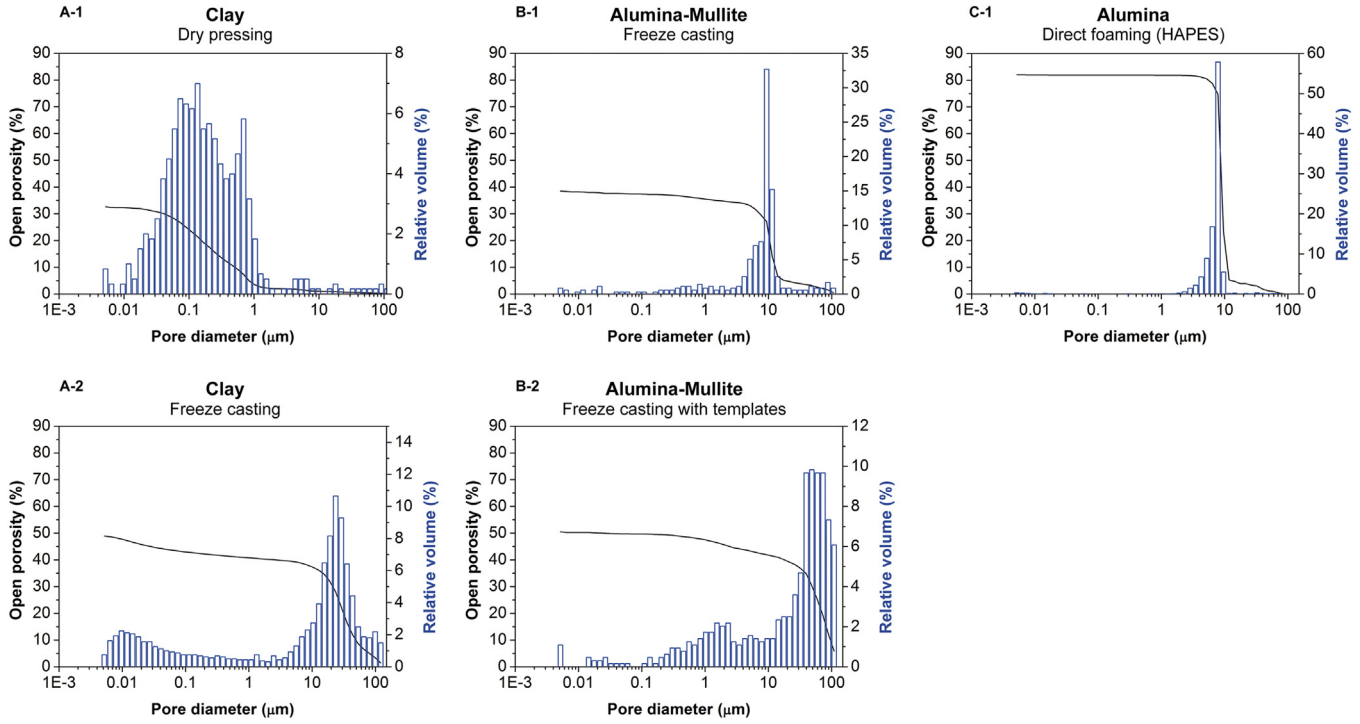

Fig. 3. Exemplary pore size distributions of substrate materials determined by Hg intrusion porosimetry: Dry pressed (A-1) and freeze casted (A-2) clay samples, freeze casted alumina-mullite samples without (B-1) and with template addition (B-2), as well as foamed alumina samples using the 'HAPES route' (C-1). The cumulated open porosity (%, black lines) and relative pore volume (%, blue columns) are plotted against the pore diameter (µm).

contained different marine algae, e.g. red algae in the order of Corallinales. Both crustose coralline algae and their associated bacteria could enhance coral larval settlement and metamorphosis during the settlement experiments. It can be noted, that complex multifactor interactions between settlement cues from biofilm and algae are possible [75].

Personal observations showed a relatively high abundance of different algae orders as well as hiding spots for predators (e.g. juvenile brittle stars) in the large superficial template pores of the freeze-casted alumina-mullite substrates. Increased algae growth could have been caused by a poor accessible of these structures to grazing organisms and the accumulation of sediments inside the pits [7,76].

During the removal and transfer of freeze casted clay substrates from the aquarium tank to the microwell plates, a poor mechanical stability of the substrates and the subsequent production of small fragments was observed. The growth of algae inside these highly porous

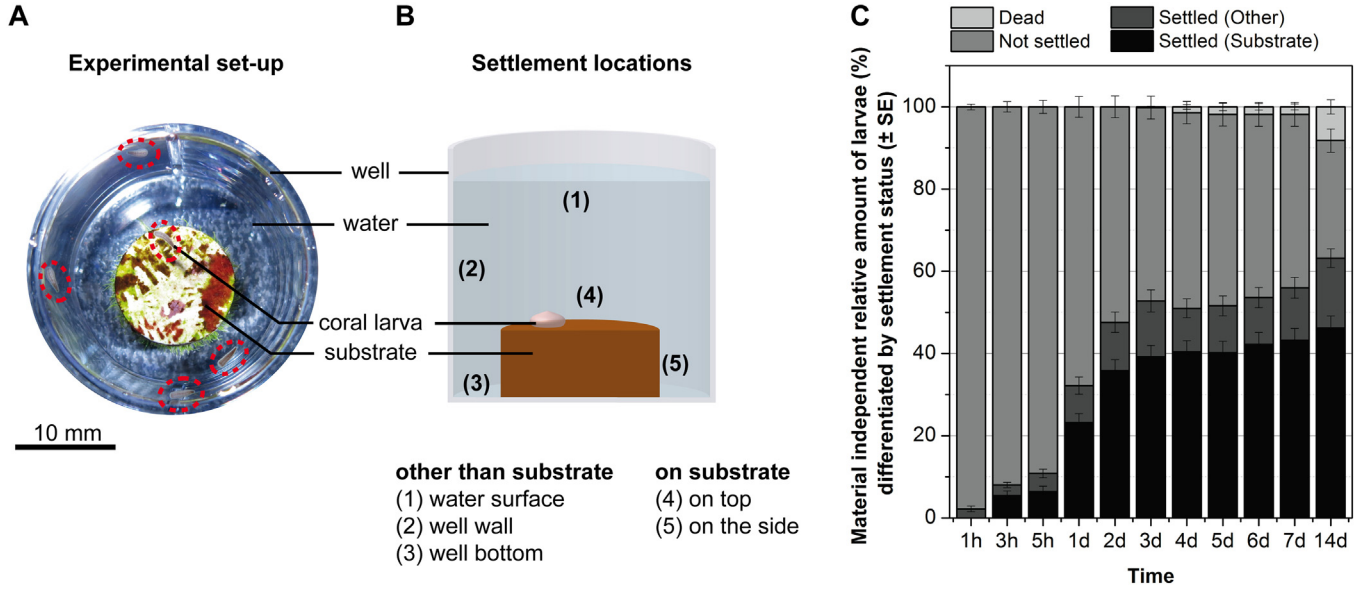

Fig. 4. (A) Exemplary top view photograph of the experimental set-up used for settlement experiments. The image was taken directly after addition of 5 coral larvae (indicated by red, dashed line). (B) Cross-sectional sketch of the experimental set-up indicating all possible larval settlement locations. (C) Material independent tendency in settlement behavior of coral larvae over time. The relative amounts of larvae differentiated by settlement status are given in percent. The status of larvae is subdivided into 'dead', 'not settled' (swimming, attached), 'settled on a location other than the substrate' (microwell walls and bottom, water surface) and 'settled on substrate'.

structures might have contributed to their weak mechanical strength.

3.3. Substrate dependent coral larval settlement and development

Fig. 4A and B show the experimental set-up and five possible settlement locations, respectively. The overall, material independent settlement behavior over a time period of 2 weeks is displayed in Fig. 4C. In general, a clear tendency is shown by the monitoring data over time: 'Not settled' larvae (swimming and attached) decreased over time while the categories 'dead', 'settled (other)' and 'settled (substrate)' increased.

After 14 days of coral larvae exposure to the settlement substrates, a highly significant total amount of 46.2% ($p \leq 0.001$) were settled on the ceramic substrates ('settled (substrate)'). Only 8.2% of the larvae were found dead, 28.6% did not settle and 17.0% settled on either the surface of the microwell or the water surface ('settled (other)'). These findings are consistent with results from previous studies, which observed an opportunistic settlement of larvae on both polymeric [43–46] and ceramic [7,15,25–27,35,50–55] substrates, but indicated a preferential settlement on ceramic materials [31,56]. This study indicates, that preconditioned, highly porous ceramic substrates were well accepted as settlement substrates for coral larvae, especially in consideration with the species *Pocillopora damicornis*. The mortality and any unspecific settlement ('settled (other)') can be explained by the consumption of energy resources stored inside the coral larvae [61]. To avoid depletion of energy and, eventually, death, the larvae can develop into a coral settler, which is able to actively feed on organic and inorganic nutrients with its tentacles [16,17].

The relative amount of settled larvae differentiated by substrate type is displayed in Fig. 5. It shows, that the first larvae settled already between the first and third hour after addition to the microwell plates. Most coral larvae settled between 5 h and 2 days. After 2 days, the settlement rates only slightly increased over time. These findings are in agreement with the larvae's ability to settle within hours after spawning and by observations indicating a decrease in settlement of *Pocillopora damicornis* after nine days [7,74]. It can be inferred, that the first 2 days after spawning are most crucial in influencing the larval settlement behavior of this species.

After 14 days, 36–53% of the coral larvae had settled on a ceramic substrate (Fig. 5), with a mean value of 46.2% (Fig. 4.C). Due to large standard deviations, no significant differences in larvae settlement between the five ceramic sample types were found, but freeze-casted clay samples and alumina-mullite samples prepared by freeze casting and template addition ('Alumina-Mullite-Freeze casting + templates') tended to attract less larvae than other porous samples: Only 36% of larvae settled on 'Alumina-Mullite-Freeze casting + templates' substrates. The increased algae growth on this type of samples might have induced direct and/or indirect effects from shading, smothering and fouling onto the coral larvae and settlers and thereby prevented settlement. In the context of biofouling mitigation, the dependence of larval settlement on the size of such surface structures has been investigated. The settlement of larvae from moss animals (Bryozoa) was most efficiently prevented by structures slightly smaller than the width of the organisms [77]. Larvae from barnacles were most likely to settle on structure smaller 1–5 µm or larger 64–256 µm [78]. This allows them for sufficient contact to the substrate with their feet (20–30 µm) and provides enough space for their whole body (500 µm) [78]. Analogous to these findings, the large freeze casting pores in these substrates might have hindered settlement due to difficulties in initial attachment [78]. Additionally, the large superficial pores created by spherical templates did not act notably as refugia during settlement. Sharp edges between individual pores and relatively steep pore walls, i.e. the pore geometry, might have interfered with the accessibility of the pores to both grazing organisms during preconditioning as well as the larvae themselves.

The amount of settled larvae on 'Clay - Freeze casting' samples was 42% and thus slightly diverged from the mean value. The formation of ceramic fragments or sediments during the fracture of these substrates might have lowered the amount of coral settlers. Freshly exposed fracture planes would be lacking sufficient preconditioning [79,80] and small ceramic pieces would not offer enough space for settlement. Furthermore, small fragments could cause sedimentation, which can be harmful to larval settlement [7], and cause collisions with the larvae during water exchange practices.

In Fig. 6, the settlement locations of coral larvae on ceramic substrates are differentiated into settlement on 'Side' and on the 'TopSide'. All substrates showed a trend towards settlement on the side of the ceramic substrates. Considering, that the lateral area of the cylindrical samples is twice the size of the top area, an increased amount of settled larvae on the side is expected (66.6%), if no additional factors are taking effect. Such factors could include light conditions [15,26,81], water movement [20], surface coverage with biofilm [30,80,82] and algae [7,21,22,79,83–86], and predation pressure [28–32]. 'Alumina – Direct foaming (HAPES)' substrates showed significantly more side-settlers ($p \leq 0,05$) in comparison to 'Clay – Dry pressing' substrates ($p \leq 0,01$), 'Clay - Freeze casting substrates' ($p \leq 0,05$) and 'Alumina-Mullite-Freeze casting + templates' substrates ($p \leq 0,05$). On these substrates, 95% of the larvae settled on the side, which is a value significantly higher than what could be expected based on the available lateral surface area of the cylinder (66.6%). Since the microwells represent a highly controlled environment, light conditions and water movement were very similar for all samples and should not have caused difference between the substrates. Due to the substrates' properties in terms of porous structure, surface area and ceramic material, local variations in biofilm formation and algae growth could have developed

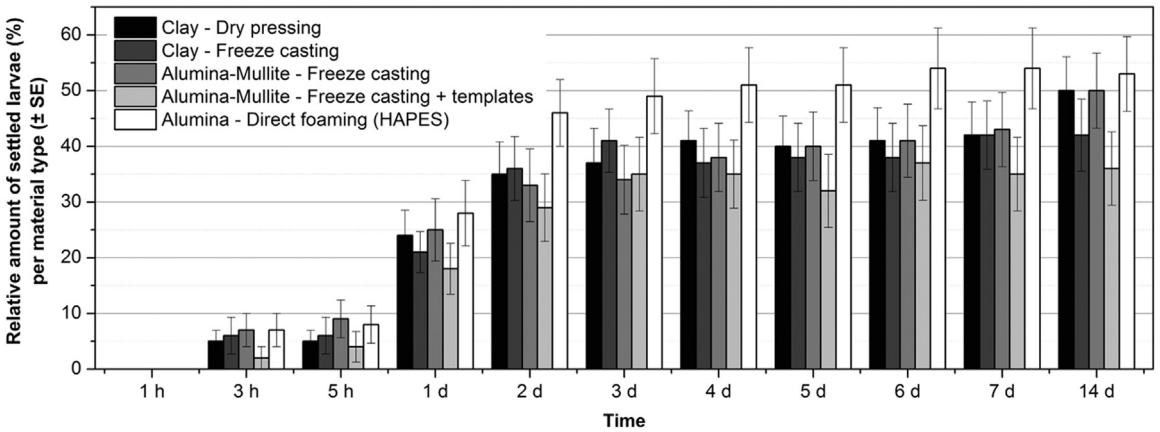

Fig. 5. Relative amount of settled coral larvae in % per type of porous ceramic material with standard error (SE) versus time after addition of larvae to the settlement substrates.

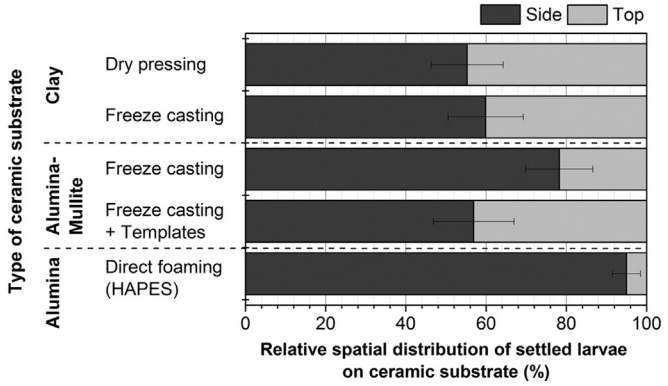

Fig. 6. Relative spatial distribution of settled coral larvae on ceramic substrates in % versus type of substrate material. The settlement positions are differentiated into settlement on 'Side' and on the 'Top' of the ceramic sample.

during sample preconditioning. This might have caused a preferential lateral settlement on ceramic foam samples. In nature, vertical surfaces ('Side') are often more sheltered areas and some species are known to preferentially attach to these structures [31,32]. Due to the unique porous properties of the alumina samples, which resemble the structure of natural coral skeleton quite closely, an attachment and settlement of larvae to these structures might be easiest. Thus, vertical settlement against the force of gravitation could be dependent on the substrate morphology, and in this case might have been most successful on directly foamed alumina samples.

The time-dependent relative amounts of surviving larvae after their settlement on ceramic substrates is shown in Fig. 7. After 3 days, mortality was first observed on 'Clay – Freeze casting' samples. After 4 days, a loss of larvae was determined for all samples except for foamed alumina samples. Mean survival of larvae after 14 days was 91.8%. At this point, larvae on 'Alumina – Direct foaming (HAPES)' samples showed the highest survival of 98% which differed significantly from the 'Alumina-Mullite – Freeze casting + templates' samples ($p \leq 0{,}01$) with 79% survival. Freeze casted clay ($p \leq 0{,}05$) and alumina-mullite samples ($p \leq 0{,}05$) also differed significantly from the 'Alumina-Mullite-Freeze casting + templates' substrates. 'Clay – Dry pressing' substrates just showed a trend ($p = 0{,}06$) towards a higher mean survival of larvae (93%) compared to the 'Alumina-Mullite-Freeze casting + templates' samples (79%). Factors, such as predation by juvenile brittle stars and an increased growth of algae (personal observation), could have played a role in relation to the lowest survival on 'Alumina-Mullite-Freeze casting + templates' samples. Additionally, it can be noted, that although all samples were characterized by the same overall height, there was a difference in lateral surface area, when comparing 'Alumina-Mullite-Freeze casting + templates' to the other substrate types. Due to the large superficial pores in a size range of 2–5 mm the vertical space available for settlement was locally reduced, which might also have affected mortality.

The exemplary development of 3 coral settlers on a dry pressed clay tile over a period of 7 month is illustrated in Fig. 8. It shows the continuing growth and calcification of the healthy organisms, both in the nursery phase in the microwell, as well as after transfer to the aquarium tank, 3 weeks after settlement. The small size of the substrate tiles allows for a flexible positioning within the tank. This case study suggests the successful development of corals settlers on the fabricated substrates beyond a period of 14 days and therefore indicates the applicability of the fabricated ceramics as larval settlement substrates for the aquaculture of corals.

4. Conclusions

This study shows, that different processing techniques can be successfully employed to produce tailored porous ceramic substrates suitable for the settlement of larvae during sexual propagation of corals. Using partial sintering of pressed pellets, freeze casting, direct foaming and sacrificial templating, materials with high porosities, relatively small specific surface areas, a broad variety of pore sizes and unique pore geometries were fabricated. Larvae of the coral species *Pocillopora damicornis* served as model organisms for settlement experiments. This type of stony coral is both attractive as marine ornamental and has been used in several scientific studies as physiological model.

Over a period of 14 days, the successful settlement of coral larvae on all 5 types of substrates was investigated. No significant differences between the different material types were found. The observed mean settlement rate after 2 weeks was 46.2% and confirms a successful coral larval settlement to highly porous ceramic materials. The highest settlement rate occurred between 5 h and 2 days after the beginning of the experiment, which appeared to be the most crucial time span during the settlement of *Pocillopora damicornis.* On basis of the structure of coral skeleton, ceramic substrates with open porosities between 35% and 83%, pore sizes between 4 nm and 5 mm and specific surface areas between 0.27 and 25.31 m^2/g were achieved. Depending on the processing route, the pore morphology was dominated by interparticle pores, lamellar or spherical geometries. Partial sintering of pressed pellets resulted in samples of smallest open porosity (35%) and main pore size of 115 nm. In freeze casted samples, the open porosity varied with solid loading of the used ceramic suspension between 39% and 52% and a characteristic lamellar pore structure. The width of the lamellae ranged between 8.9 and 40.7 μm. With the addition of spherical template pores during the freeze casting process, large spherical pores with diameters of 2–5 mm were created on the substrates surface, which were sufficient in size to be occupied by single coral larvae during the settlement process. Cellular pore structures with a mean

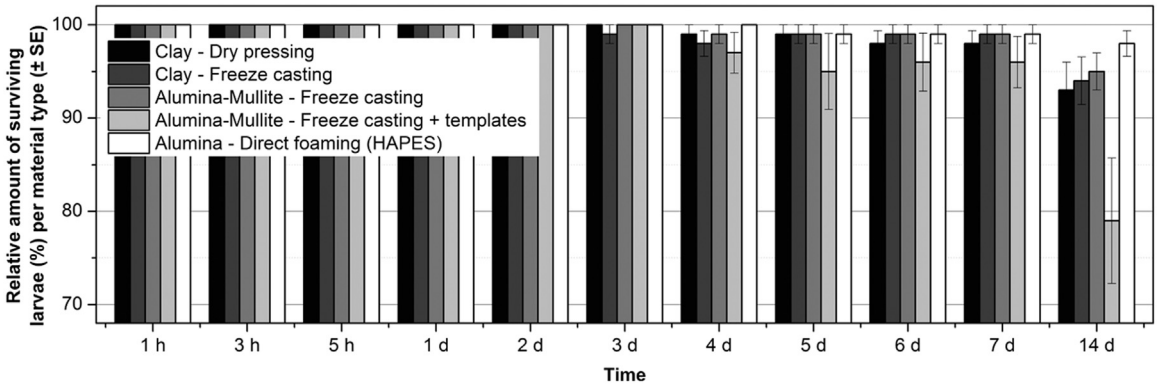

Fig. 7. Relative amount of settled and surviving coral larvae in % per type of porous ceramic material with standard error (SE) versus time after addition of larvae to the settlement substrates.

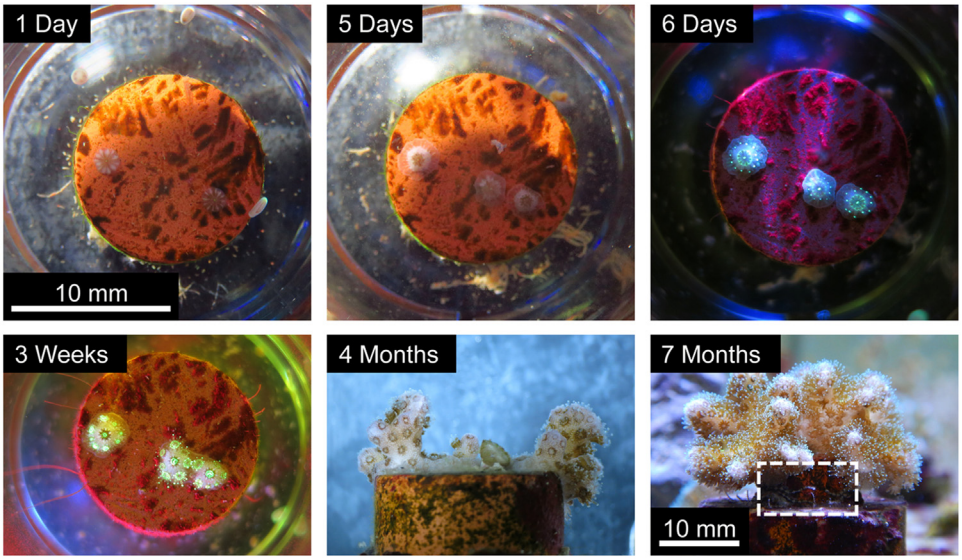

Fig. 8. Development of settled *Pocillopora damicornis* larvae on a dry pressed clay substrate over a period of 7 months. Up to 3-weeks-old corals were growing inside a microwell plate, afterwards they were placed in an aquarium. Images after 6 days and 3 weeks are taken with the help of UV radiation. Images from day 1 to month 4 are subjected to the same scaling. The dashed line (7 months) indicates the position of the inorganic settlement substrate.

diameter of 7.3 µm and 83% of open porosity were prepared by direct foaming using high alkane phase emulsified suspensions. The porous structure of both freeze casted alumina-mullite substrates and alumina foams most closely resembled the microstructure of coral skeleton. On ceramic foams, a preferential larval settlement on the side (95%) of the cylindrical samples rather than the top was shown, which significantly exceeded the rate on the other 4 substrates. This finding indicates, that the coral larvae prefer to settle on the more sheltered side of the substrate, if the substrate is such, that a stable attachment and subsequent settlement can be reached.

After 14 days, freeze casted alumina-mullite samples with large superficial pores exhibit the significantly lowest survival rate of settlers (79%) compared to otherwise similar values (93–98%). During the preconditioning of the ceramic samples in artificial sea water, biofilm formation and algae growth on their surfaces was observed. Large superficial pores featured a particularly high abundance of various algae, which can be explained by poor accessibility to grazing organisms and the accumulation of biomatter inside the pits. They additionally served as hiding spaces for small predators such as brittle stars. Both of these factors could explain the increased mortality of coral larvae on this type of samples, although similar structures have been reported to serve as refugia to coral larvae an enhance survival [35,38]. To promote larval settlement in sexual propagation of corals, specific considerations with respect to not only pit size, but also pit geometry seem to be necessary.

Based on this successful feasibility study on the sexual propagation of coral larvae from the stony coral *Pocillopora damicornis*, porous ceramic materials can be identified as suitable settlement substrates for aquacultural applications. A bioinspired fabrication of materials with close morphological resemblance to coral skeleton seems to have a beneficial effect on larval settlement. First studies on the long-term development of settlers suggest a successful transfer of settled larvae from the nursery environment to the aquarium tank and the development into healthy calcified corals. While ceramic foams exhibited the best results in terms of settlement and survival, pressed substrates made of clay may offer a low cost alternative which could find widespread use in the aquaculture of marine ornamentals.

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