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Which concrete substrate suits you? *Ostrea edulis* larval preferences and implications for shellfish restoration in Europe

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Abstract :

The European flat oyster, Ostrea edulis, is an important ecosystem engineer that has been progressively disappearing from European coasts over the last century mainly due to overexploitation, habitat degradation and disease. It is now the subject of many conservation and restoration programs throughout Europe, including the Flat Oyster REcoVERy (FOREVER) project in France.

Protecting and managing the remaining populations has become a nature conservation priority because this species is able to build biogenic reefs, very specific habitats that provide many ecosystem functions and services. The availability of suitable hard substrates for larval fixation is a critical factor during this reef-building process. Although natural substrates are in short supply, production and deployment is an easy step to help oyster reef restoration.

The present study was carried out to improve artificial reef design and the composition of the concrete used to build them, focusing on the impact of concrete formulation and surface texture on larval settlement in the field. Nine bio-sourced concrete formulations and ten surface textures were evaluated. The number of settled larvae counted on each concrete substrate reflected their preferences, and results demonstrated that microscale surface texture has a greater impact on recruitment than concrete formulation, with larvae preferring to settle in depressions on a rough rock-like texture and avoiding flat, horizontal and exposed areas.

Physical and mechanical properties of the different formulations were also evaluated. Although they had almost no impact on recruitment, these results could be helpful for artificial reef-building (in terms of 3D design, durability, manufacture and deployment) and guide choices of materials that should be used and the proportion and granulometry of incorporated shells to obtain the best surface texture.

Highlights

► *Ostrea edulis* larval preferences for different substrates were evaluated in situ. ► Various bio-sourced concrete mix formulations and surface textures were tested. ► Incorporation of seashell by-products influence mechanical properties of concrete. ► Substrate texture has a greater impact on larval settlement than its formulation. ► Substrate micro-topography has direct implications for flat oyster restoration.

Keywords : *Ostrea edulis*, Larval recruitment, Artificial reef, Concrete, Seashell by-products, Restoration

1. Introduction

The flat oyster *Ostrea edulis* is a European native species that once covered vast areas in the North Sea, on the Atlantic coast and in other European coastal waters including the Mediterranean region (zu Ermgassen et al., 2020; Pogoda et al., 2019). All these populations have been heavily fished by dredging over the last three centuries. More recently, the development of parasitic diseases (due to the emergence of *Marteilia* and *Bonamia*) combined with the proliferation of various predators dangerous to the species (especially sea stars, sea bream and oyster drills) and many human-induced stressors (pollutants, terrestrial outputs, coastal development) have caused a dramatic decrease in the last remaining flat oyster populations (Duchêne et al., 2015; Thurstan et al., 2013). Today, this species has disappeared from many locations in Europe and is registered on the OSPAR (Oslo-Paris Convention for the Protection of the Marine environment of the North-East Atlantic) list of threatened and/or declining species (Pogoda et al., 2019). In France, the flat oyster is confined to only a few localised environments, notably in Brittany and Normandy (Duchêne et al., 2015). However, these residual populations continue to be subjected to a range of threats that limit them still further, to the point that if no conservation and/or restoration actions are taken soon, the species and its associated habitats could disappear completely from French coasts.

On an ecological level, oysters are 'engineer species'. Like corals in tropical waters, they build calcareous biogenic habitats (from clusters and aggregates to massive reefs) that are favourable for many other organisms and thus increase the biodiversity of the surrounding environment (other invertebrates, algae, fishes, etc.; e.g., Beck et al., 2011). These reefs also provide many other ecosystem services, such as promoting sedimentation, reducing turbidity and eutrophication and helping to prevent coastal erosion by acting as breakwaters (Borsje et al., 2011; Meyer et al., 1997;

Salvador de Paiva et al., 2018). Finally, the flat oyster contributes to the local economy through aquaculture, fishing and recreational activities. For all these reasons, many projects are underway in Europe to preserve this species (Pogoda et al., 2019).

The European flat oyster is characterised by a biological cycle with a pelagic larval stage. The reproductive period that includes a brooding phase starting generally in June and ending in September (Bayne, 2017; Martin et al., 1995). Larvae can be observed throughout the summer, but are generally more abundant in July on the French Atlantic coast. After release by the brooding mothers, they develop in the water column for 2 weeks. Then, at the end of their pelagic life, they swim to the bottom searching for a substrate on which to settle. Oysters are able to settle on different types of hard substrate such as rocks, gravel or muddy sand with cultch (Shelmerdine and Leslie, 2009), but still have a preference for conspecific shells, especially those of living congeners already present on the seabed. Indeed, motile spat have been observed to settle preferentially on the growth rim of these shells (Kennedy and Roberts, 1999; Korringa, 1946). This behaviour offers some fitness advantages as it favours the formation of aggregates that, after a long period without major disturbances, enlarge to become new biogenic reefs, known as ‘oyster beds’. The proximity of individuals and cumulative recruitment conferred by the reef are important for reproductive success, individual growth and survival, thus ensuring the self-sustainability of the reef over time (Guy et al., 2018; Schulte et al., 2009).

Thus, the disappearance of flat oyster shells or other hard substrates from the bottom due to dredging activities combined with the effects of other stressors (environmental degradation, especially chemical contaminants and soil leaching causing increased sedimentation in estuaries, diseases, predation, etc.) leads to a progressive disappearance of the habitat favourable to the oyster (Beck et al., 2011;

Pogoda et al., 2019). It is now understood that one of the limiting factors for *O. edulis* stock recovery on which we can easily act, is the availability of suitable hard substrate material for oyster larval settlement (Smyth et al., 2018).

Since 2018, the Flat Oyster REcoVERy project (FOREVER) has been promoting the reestablishment of native oysters in Brittany (France). This multi-partner project is led by CRC (*Comité Régional de la Conchyliculture*) Bretagne Sud and involves IFREMER (*Institut Français de Recherche pour l'Exploitation de la Mer*), ESITC (*Ecole Supérieure d'Ingénieurs des Travaux de la Construction*) Caen, *Cochet Environnement* and *CRC Bretagne Nord*. The project consists of (1) inventorying and evaluating the status of the main wild flat oyster populations across Brittany, (2) making detailed analysis of the two largest oyster beds in the bays of Brest and Quiberon to improve understanding of flat oyster ecology and recruitment variability and to suggest possible ways of improving recruitment, and (3) proposing practical measures for the management of wild beds in partnership with members of the shellfish industry and marine managers. In this last action, the development of artificial substrates and reefs that preserve the seabed and promote the settlement of oyster larvae is an important goal for restoration.

Many studies have already been conducted on settlement cues and the search by pelagic marine invertebrate larvae for suitable substrates. Since larval settlement constitutes a critical bottleneck in the life cycle of marine invertebrates, more insight into these processes could significantly improve marine ecological restoration efforts. It has been demonstrated that planktonic larvae are sensitive to a wide range of environmental factors over their biological development: water temperature, salinity, gravity and pressure (see reviews by Hidu and Haskin, 1978; Mann et al., 1991; Young, 1995), current and turbulence (e.g., Fuchs et al., 2015; Knights et al., 2006), sound (e.g., Lillis et al., 2016), light (e.g., Vazquez and Young, 1998), chemical molecules (Tamburri et al., 2008, 1996) and, finally, substrate nature at settlement (see review by Hodin et al., 2018). These different cues are used partially or totally by larvae at different ontogenic stages to assist them in feeding, protecting themselves, swimming or diving, but also for settlement and habitat selection. Obviously, these cues differ according to species: for instance, bivalves that adhere via a byssal thread tend to be less discerning in their substrate preference compared with those that adhere with a cement glue (Tamburri et al., 2008). In some cases, settlement can be very highly specific, e.g., *Ostrea denselamellosa* larvae only use veneriid clam shells as their settlement substrate (Noseworthy et al., 2016).

In the mid 20th century many studies were conducted on *O. edulis* settlement in an attempt to sustain oyster culture (e.g., Korringa, 1941, and see Bayne, 2017, for a recent exhaustive review). It was clearly shown that *O. edulis* larvae are able to attach themselves to a wide range of hard substrates, but that they have a strong preference for shells or coralline algae (e.g., Smyth et al., 2018). The reasons for this preference are thought to include surface roughness and chemical composition (calcium carbonate) (e.g., Cuadrado-Rica et al., 2016). Limestone, tiles or plates coated with lime and other calcareous materials (sand and shell fragments), therefore constitute efficient alternative substrates (e.g., Lok and Acarli, 2006). In contrast, smooth surfaces such as very smooth pebbles, glass or seaweed are intrinsically unsuitable for this species (Cole and Jones, 1939). Chemical cues are also involved in settlement, especially molecules emitted by conspecific or prey species (Bayne, 1969; Rodriguez-Perez et al., 2019) or by biofilms composed of bacteria, micro-algae and exopolysaccharides (Hadfield, 2011; Tritar et al., 1992). Concerning artificial substrates, Graham et al. (2017) showed that concrete is the most effective substrate for *Crassostrea virginica*, being better than limestone rocks, oyster shells and river pebbles. Anderson (1995) explained that the phenomenon of calcium hydroxide leaching, which increases the surface alkalinity of concrete, combined with the presence of a biofilm, would allow better recruitment of oyster larvae, while this alkalinity is detrimental to the development of other invertebrate organisms (Lukens and Selberg, 2004). In addition, Nguyen et al. (2013) and Cuadrado-Rica et al. (2016) emphasised the potentially useful role of seashells in the conception of artificial substrates.

In this context and with the aim of restoring native oyster beds, the goal of our study was to find an optimal artificial substrate that would be both very attractive to *O. edulis* larvae and easy to produce (e.g., Theuerkauf et al., 2015). Considering the settlement preferences of the species for shell and calcareous material, it was decided to use limestone concretes into which different amounts and grain sizes of oyster shells were incorporated.

Then, taking the most attractive concrete-shell formulation, different moulds were tested to identify larval preferences in terms of surface texture. Ultimately, the optimal concrete formulation and surface texture will be used for artificial reef building, taking into account that concrete also makes it possible to build massive and complex shapes (Baine, 2001) without significant environmental consequences that other materials may cause (Risso-de Faverney et al., 2010).

2. Materials and Methods

Our study was based on two complementary experiments carried out in 2018 and 2019 in two natural sites (bays of Brest and Quiberon, see 2.1) where there are still living flat oyster beds. The first experiment (summer 2018) was designed to characterise the most attractive concrete formulation for *O. edulis* larvae. It was conducted in both sites, by submerging pavers composed of different formulations of shell concrete. The second experiment (summer 2019) was designed to characterise the most attractive surface texture for *O. edulis* larvae. It was conducted in the bay of Brest only (where flat oyster recruitment is higher), by exposing concrete pavers moulded with different surface textures. The same overall testing method was used in both cases: (1) fabrication of pavers to be tested in spring, (2) testing pavers on-site during the flat oyster reproductive season (which occurs in July) using a random experimental design operated by scientific divers and (3) evaluating the performance of each paver by counting larvae settled on the surface after 15 days of immersion (this value is called 'recruitment density').

2.1. Experimental sites

Experiments to test substrates were conducted in two coastal environments in Brittany (France) where *Ostrea edulis* is still present and reproduces each year in summer: the bay of Brest (North Brittany) and the bay of Quiberon (South Brittany).

Bay of Brest: Located in north-western France, the bay of Brest is a semi-enclosed macrotidal coastal ecosystem influenced by both freshwater inputs from rivers to the east and fast-mixing exchanges with the Atlantic Ocean to the west. One of the most productive areas within the bay of Brest is Daoulas bay, a very shallow embayment (maximum depth 8 m) that is home to various aquaculture and fishing activities and is also a protected marine area managed by Natura 2000. In the south-east part of this bay, there is still a residual wild population of *Ostrea edulis* (Le Roz oyster bed) at 3–4 m depth, which covers less than 10 hectares at low density (< 5 individuals/m²). Experiments for the bay of Brest were conducted in the heart of this bed.

Bay of Quiberon: Quiberon bay, which is located on the south coast of Brittany, France, is a semi-circular bay bordered on the west and south-west by a peninsula extending approximately 12 km offshore. This bay is an important location for shellfish aquaculture (around 80 shellfish growers with about 2500 hectares of shellfish farms). Oyster production (6000 t Pacific oysters and 500 t flat oysters) is carried out in deep water. On the west side of this bay, there are two beds of flat oysters managed by the oyster farming profession to produce the spat necessary for farming. In these two beds, oysters cover around 20 hectares at 2–6 m depth, at densities between 0 and 10 oysters/m^2.

2.2. Raw materials

Two families of formulations were used for the substrates: concretes in experiment 1 and a mortar (the formulation chosen based on the results of experiment 1) in experiment 2. All the formulations were made up of the following compounds. The base material was cement CEM III/A 52.5N, adapted to the marine environment, with a slag content of more than 60%. Four types of aggregate were used: a limestone sand, a siliceous sand, a limestone gravel and, as a substitute for some gravel and sand, oyster shells (*Crassostrea gigas*) of different grain sizes. The density and water absorption (at 24 hours; WA_{24}) of the various aggregates were determined according to standard NF EN 1097-6 (2014) (see Table 1).

Table 1. Characteristics of the main materials

Aggregates	Density (ton/m^3)	WA_{24} (%)
Limestone sand < 4 mm	2.52 ± 0.02	2.80 ± 0.05
Siliceous sand < 4 mm	2.64 ± 0.05	2.50 ± 0.05

Limestone gravel 4–10 mm	2.49 ± 0.03	3.00 ± 0.03
Oyster Shell < 1 mm	2.27 ± 0.02	20.94 ± 0.06
Oyster Shell < 6 mm	2.56 ± 0.03	14.70 ± 0.04
Oyster Shell 6–10 mm	2.74 ± 0.04	4.50 ± 0.04

2.3. Experiment 1: concrete formulation comparison

In this first experiment, nine concrete formulations were developed and tested by adjusting the proportions of cement, sand, gravel and shell. The mixtures were made in a mixer with volume of 50 L and then poured into rectangular moulds of 28 x 24 x 3 cm. A vibrating table ensured the distribution and compaction of the concrete. A diamond saw was then used to cut the samples into 9 cm squares. All formulations are summarised in Table 2 and described below.

- ***Reference (without shell):*** A reference concrete (called REF) with only limestone sand (60%, grain size < 4 mm) and gravel (40%, grain size 4–10 mm) and without shell was formulated to obtain an optimal granular skeleton (with a water/cement ratio of 0.5).
- ***Influence of oyster shell (grain size and proportion):*** Based on this reference, oyster shells were incorporated by partly replacing the limestone sand and gravel. Three grain sizes of shell pieces were selected to replace the limestone aggregates: size class S1 consisted of powdered shells, with particles up to 1 mm, size class S2 consisted of shells fragments up to 6 mm and finally, size class S3 consisted of large shell fragments between 6 and 10 mm. They were incorporated at a fixed proportion of 20% by replacing the limestone (sand and gravel) according to the grain size, thus obtaining the formulations 20Shell-S1, 20Shell-S2 and

20Shell-S3, respectively. In order to determine the influence of shell proportion, three substitution rates were also compared: 20%, 35% and 50%. These formulations were made with the S2 size class (up to 6 mm), which gave the formulations 20Shell-S2, 35Shell-S2 and 50Shell-S2, respectively.

– ***Influence of siliceous sand:*** It was also decided to test whether the presence of silica in the formulation could have an impact on recruitment, since it is known that this compound plays a role during larval adhesion and fixation (in *Crassostrea virginica*, 6% of the adhesive is composed of silicon; Metzler et al., 2016). To study the effects of silica, a common siliceous sand (up to 3 mm) was used at a proportion of 15% by replacing the limestone sand in the reference concrete, giving the formulation REF-15Si, and in the formulation containing the greatest proportion of shell (50Shell-S2), giving the formulation 50Shell-15Si.

Table 2. List of cement formulations tested in Experiment 1; W/C corresponds to the water/cement ratio for each formulation

Study of:	Formulation name	Oyster shell (various granulometry)			W/C ratio
		S1 <1 mm	S2 <6 mm	S3 6–10 mm	
Reference	REF	-	-	-	0.5
Variation of proportion and oyster shell grain size	20Shell-S1[*]	20%	-	-	0.5
	20Shell-S2	-	20%	-	0.5
	20Shell-S3	-	-	20%	0.5
	35Shell-S2	-	35%	-	0.5
	50Shell-S2	-	50%	-	0.5
	50Shell	-	-	50%	0.5
Influence of siliceous sand	REF-15Si	-	-	-	0.5
	50Shell-15Si	-	50%	-	0.5

[*]20Shell-S1: '20Shell' correspond to the 20% proportion of shell and S1 to the shell grain size

These different formulations created a roughness gradient. Overall roughness measurements were performed on an area of 8 x 8 cm^2 of each sample. A Keyence VHX-6000 optical microscope equipped with a confocal measurement system was used, allowing the reconstruction of relief images. Several parameters of the microscope were calibrated: in-plane resolution, depth fields, brightness, numerical aperture and wavelength for white light. To calibrate these parameters, we did a repeatability test four times, with variation under 1%. Roughness was expressed by the arithmetic mean height (Sa; eq.1), calculated as follows:

$$Sa = \frac{1}{A} \iint Z\,(x\,y) dx\,dy \qquad \text{(Eq.1)}$$

With Sa: Total surface area analysed; x, y = length and width of part of the study area; A: area of the analysed surface with dimensions (x, y), and Z: mean height of the analysed surface with dimensions (x, y).

For each concrete formulation obtained, the physical (water porosity and gas permeability) and mechanical (compressive quality) properties were first tested in triplicate. The water porosity test was realised according to EN 206 NF P18-459 (2010). Gas permeability tests were realised with a Cembureau cell device according to EN 206 XP P18-463 (2011). Compression tests (results expressed as compressive strength, σ_c) were realised according to EN 206 NF EN 12390-3 (2019).

These nine concrete formulations were then tested in the field for their biological efficiency in terms of larval settlement. Testing was performed from 10 to 23 July 2018 in the bay of Brest and from 28 June to the 13 July 2018 in bay of Quiberon. On each site, the 9 tested formulations (pavers of 9 x 9 x 2 cm^3) were randomly positioned on duplicate grids. See section 2.5 for details of the recruitment density assessment.

A relationship between roughness and recruitment was observed visually. It was therefore decided to carry out a detailed roughness analysis on the 50Shell formulation which had the highest recruitment density (see 3.1.3) and was also the roughest (supplementary data 1). To this end, the sample was divided into four zones measuring 4 x 4 cm^2 called Qi (n = 4). Each Qi zone was then divided into four sub-areas of 2 x 2 cm, called qi (n = 16). Finally, the qi sub-areas were also divided into four fractions of 1 x 1 cm^2 called pi (n = 64; Figure 1). Settled larvae were then counted in these pre-defined areas. Roughness measurements (expressed as Sa) were made using the Keyence microscope to reconstruct a 3D image by moving the working distance of the lens. Repeatability tests were performed with different lens zooms by varying the brightness and analysis step. The x50 zoom was chosen to obtain sufficient accuracy and repeatability despite variations in light intensity. In order to avoid edge effects, the periphery of the concrete paver samples was excluded from the analysis.

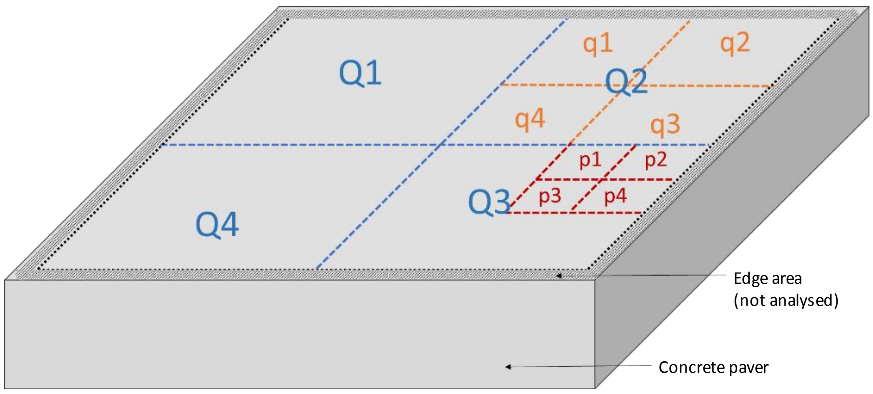


Figure 1. Scheme representing the different scales of roughness analysis on the surface of a sample

2.4. Experiment 2: surface texture preference

In the second experiment (conducted in 2019), it was decided to focus on surface texture of one specific formulation. Based on the results of experiment 1, the chosen formulation was composed of a cement base (587 kg/m^3) to which two aggregates were added: 1057 kg/m^3 of limestone sand and 762 kg/m^3 of crushed oyster shell sieved at 500 µm (these two ingredients represented 58% and 42% of the aggregates, respectively). The formulation was made in a 5-L mixer, to reach a water/cement ratio (W/C) = 0.5, and then poured into moulds measuring 7 x 7 x 2 cm^3.

These moulds were then covered with ten different matrices, selected for their differences in surface texture, with a gradient from the smoothest to the waviest. These reusable elastic structural matrices are manufactured by Reckli® (https://www.reckli.com/fr/). The ten selected matrices and their structural features, as well as the substrates obtained with them are presented in Figure 2. The resulting substrates made it possible to test larval settlement preferences for (1) more or less smooth surface textures and (2) positions relative to the surface texture micro-topology (on the tops, valleys or sidewalls).

1	**2/81 Plafond:** a *smooth formliner without a pattern to create an absolutely smooth concrete surface.*
2	**2/200 Sodingen:** t*he functional formliner creates a smooth concrete surface with a fine sand look without noticeable elevations.*
3	**2/157 Fichtelberg:** a *rock pattern with slight irregularities. Viewed as a whole, it still appears very even though.*
4	**2/69 Marne:** a *pattern with a surface reminiscent of coarse roughcast. The granulation amounts to 16 mm.*
5	**2/104 Sambesi:** a *pattern with a coarse roughcast look with a granulation of up to 8 mm.*
6	**2/108 B Indus:** v*ertically running ribs with a rough fractured pattern. Separated by u-shaped joints with a diameter of 10 mm.*
7	**2/63 Wisla:** v*ertically aligned u-shaped elevations with a smooth surface and a diameter of 10 mm.*
8	**2/190 Ardenne:** a*n abstract pattern with indented small plates that are stacked behind each other, creating a scale-like texture.*
9	**2/94 Orinoco:** a *wave pattern with a wavy steel roof look, which is enlivened by the interplay of light and shadows. The distance between the elevations is 35 mm.*
10	**1/36 RIB Type H:** a *clear and smooth rib pattern with vertically aligned ribs, separated by 15-millimeter wide joints.*

Figure 2. Selected matrices for Experiment 2. Each matrix (left picture) and the resulting paver (right picture) are shown. Each matrix is then described, using the designation and features mentioned on the manufacturer's website.

For each of the ten textures, nine square pavers were made. These were placed in seawater for 3 days to release any impurities, then fixed *in situ* on the grids by scientific divers. This experiment was conducted in the Bay of Brest only, with substrates left in place from 9 to 23 July, 2019. The substrates were randomly positioned on three grids, each grid supporting 30 pavers (3 pavers for each texture per grid). To resume, on each grid there were n = 3 pavers for a given texture, and n = 9 all grids combined.

In section 3.2.2, we paid particular attention to the geographical distribution of settlement at two scales: between two juxtaposed pavers and within each individual paver (micro-topography effects).

Besides classical recruitment, we also looked at the distribution of settled larvae on textures with marked relief (n°7, 9 and 10). Here, three zone types were defined: top (for the tops of the bumps), pit (for the valleys) and sidewall (for the slopes). The number of settled larvae was assessed relative to the area of each of these zone types on the pavers.

2.5. Recruitment, larval counting and statistics

Recruitment of flat oysters is known to be greatly affected by larval supply, environmental conditions, transport processes, habitat selectivity and substrate characteristics (Rodriguez et al., 1993), but also by competition, predation and other post-settlement processes (Michener and Kenny, 1991).

In our study, we paid specific attention to standardising the protocol for assessing recruitment. For both experiments, the tested substrates were handled by scientific scuba divers who systematically placed them horizontally, just above the sea floor and near adult oyster beds within each site (bays of Brest and Quiberon). In both experiments, the pavers were randomly positioned on underwater grids using the R 'sample' function (Figure 3). Concerning exposure time, it is known that biogenic modification of the substratum surface (especially the build-up of bacterial films) occurs on new substrates within some days of their immersion and this step is a preliminary phase essential for oyster settlement. Conversely, after a long period (> 1 month) substrates accumulate too much biofouling and sediment deposits and thus become progressively less suitable for oyster settlement. A period of 15 days of immersion therefore constitutes an optimum compromise, allowing enough recruitment to evaluate substrate differences. Substrates were immerged at the time when larval concentration was around its maximum (from the end of June to the end of July, depending on location and year; Pouvreau et al., in press) and seawater temperature was around 20 °C (+/- 1 °C).

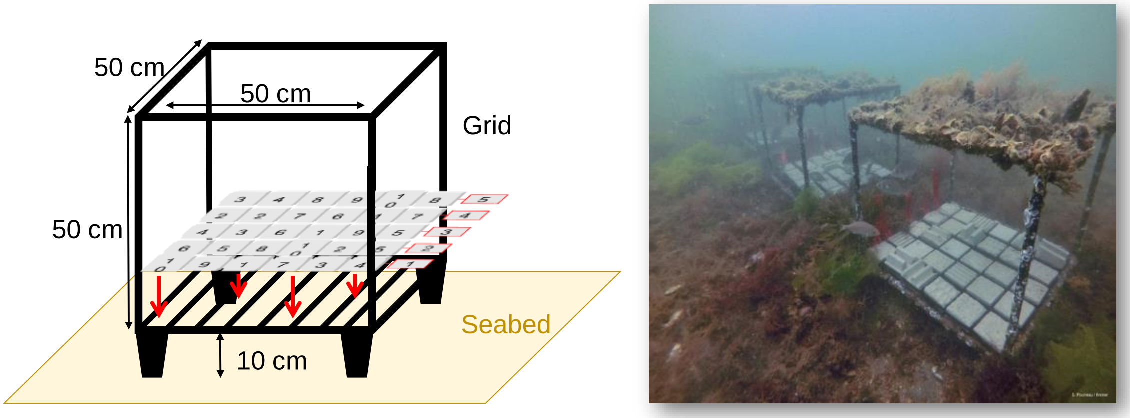


Figure 3. Experimental design with pavers positioned on underwater grids

After 15 days of immersion, the substrates were collected by scientific divers, gently rinsed and dried at ambient temperature at the laboratory. Newly-settled recruits were identified, counted and marked on the top sides of each paver by visual inspection under the microscope (Keyence VHX 6000). This species is easily distinguishable and confusion with other bivalve species can be avoided using morphological criteria. *Ostrea edulis* post-larvae have a symmetrical umbo with two nearly identical shells. The potential confusion with *Crassostrea gigas* post-larvae can be avoided because the umbo of this other species has a 'twisted' asymmetry (Trimble et al., 2009). Confusion with *Anomia ephippium* can be avoided because the inferior shell of this species is almost glued on the substrate and thus invisible. For data analysis, recruitment values were standardised to the number of individuals settled per cm^2 (total area) within 15 days of exposure, which we refer to in this study as 'recruitment density'.

After checking normality and homoscedasticity, the effect of experimental factors was tested using permutational multivariate analysis of variance (PERMANOVA) or Kruskal Wallis tests, depending on homoscedasticity and the number of experimental factors considered (site and/or formulation in experiment 1; texture and/or grid/zone type in experiment 2). Comparisons between conditions were made using pairwise Student t test; *p-values* were corrected using the False Discovery Rate (Benjamini & Hochberg method) and a *p-value* < 0.05 considered significant. The relationship between roughness and recruitment was tested using linear models (adjusted R^2). Statistical analyses were made using R (R Development Core Team, 2005) software version 3.5.3.

3. Results

3.1. Experiment 1: concrete formulation comparison

3.1.1. Microstructural characterisation

The different formulations created a roughness gradient (see Supplementary Data 1). Roughness varied from Sa 362 to 1417 µm depending on formulation.

Incorporation of shell material is thought to significantly modify the properties of concrete. The influence of shell grain size and proportion on porosity is shown in Supplementary Data 2 for the six formulations containing shell.

Firstly, at the same rate of substitution (20%), we can observe a decrease in porosity as the shell grain size increases from S1 (< 1 mm), through S2 (< 6 mm) to S3 (6–10 mm) (Supp. Data 2a). This phenomenon is due to the granular arrangement. The compactness of the formulation increases with decreasing shell size, the finest particles filling the porosity. Organisation of the aggregates of the largest shells does not seem optimal and generates porosity within the materials.

Secondly, we studied the influence of shell substitution rate at 20%, 35% and 50% for the same shell grain size (S2). We chose S2 because it was the intermediate aggregate size. The porosity increases with increasing shell substitution rate (Supp. Data 2b).

After studying the porosity, we also examined the gas permeability of the six shell-containing formulations (Supplementary Data 3). The shell grain size seems to have an impact on porosity as air permeability is ten times higher for the larger shell grain sizes, S2 and S3 (Supp. Data 3a). The air permeability value for the smallest grain size (S1), was the same as that of ordinary concrete. The greater the proportion of shell incorporated into the formulation, the higher the permeability becomes (Supp. Data 3b).

Supp. Data 2b shows how porosity increases with the proportion of shell in the concrete, and Supp Data 5b suggests that increased porosity leads to higher permeability of the material.

3.1.2. Mechanical properties

Addition of shells to concrete significantly reduces its mechanical strength (Supplementary Data 4). This fall appears to occur quite linearly with the increase in shell proportion. It can be seen that with 50% of Shell-S2, the compressive strength of the concrete is divided by almost three compared with the reference without shell.

The grain size of the shells also affected mechanical strength. While there was no change in resistance between the S1 and S2 shell classes ($\sigma_c \approx 25$ MPa), the strength with S3 was about 34 MPa, representing a relative gain of more than 30%.

3.1.3. Formulation effect on larval settlement

Flat oyster recruitment was systematically higher in the bay of Brest than in the bay of Quiberon (PERMANOVA, $p = 0.001$; F = 604.5; df = 1), with a four-fold difference between sites (Figure 4). However, there was no significant impact of formulation ($p = 0.19$; F = 1.69; df = 8), or of the interaction between site and formulation ($p = 0.38$; F = 1.16; df = 8) on recruitment.

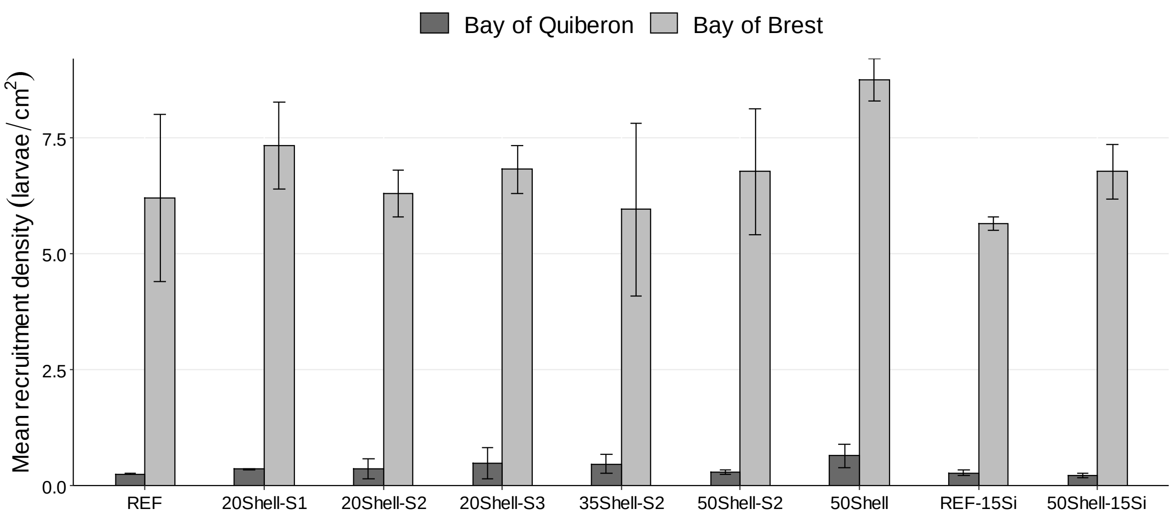


Figure 4. Recruitment results (n° of newly settled flat oysters per cm²) after 15 days of immersion at the sites (mean ± SD, n = 2)

Whatever the source of these differences, the results per site are the same for the overall ranking of the formulations despite significant variation between replicates. The impact of concrete formulation on recruitment was non-significant according to Kruskal-Wallis tests, whether we consider both sites simultaneously ($p = 0.88$; $\chi^2 = 3.7$, df = 8), bay of Brest only ($p = 0.36$; $\chi^2 = 8.8$, df = 8) or bay of Quiberon only ($p = 0.38$;

χ^2 = 8.6, df = 8). Shell grain size had no apparent effect on recruitment for 20Shell-S1, 20Shell-S2 and 20Shell-S3 and there was no significant relationship between the proportion of shell in the concrete (20Shell-S2, 35Shell-S2 and 50Shell-S2) and the recruitment observed. The REF and REF-15Si samples had similar recruitment, so the presence of siliceous sand did not appear to have a significant influence. Finally, 50Shell stood out among the formulations, showing the highest recruitment values at both sites.

3.1.4. Small scale roughness

A gradual roughness analysis was performed on the 50Shell sample by analysing the first the four Q_i zones (largest scale), then the 16 q_i zones (intermediate scale) and 64 p_i zones (smallest scale; Figure 5). We chose the 50Shell formulation to perform this analysis because it showed the highest roughness values (Sa = 1417 µm) and reached the greatest recruitment values (Figure 4). For the largest areas (Qi), all the values are located randomly in the centre of the scatterplot, suggesting an absence of any trend in the relationship between roughness and recruitment at this largest scale (adjusted R^2 = -0.05). The values corresponding to the qi zones are also very clustered, with a roughness between 650 and 1100 µm and a corresponding recruitment density between 8 and 12 individuals/cm² (adjusted R^2 = 0.003). However, the values corresponding to the p_i zones form an extended scatterplot both in terms of roughness (from 200 to 1600 µm) and in terms of recruitment (from 2 to 17 individuals/cm²), suggesting an effect of roughness on recruitment that becomes observable at this small scale as we approach dimensions similar to those of larvae (adjusted R^2 = 0.36). This key result highlights that the surface texture of the substratum is probably a major factor in settlement and which we then made the focus of our second experiment.

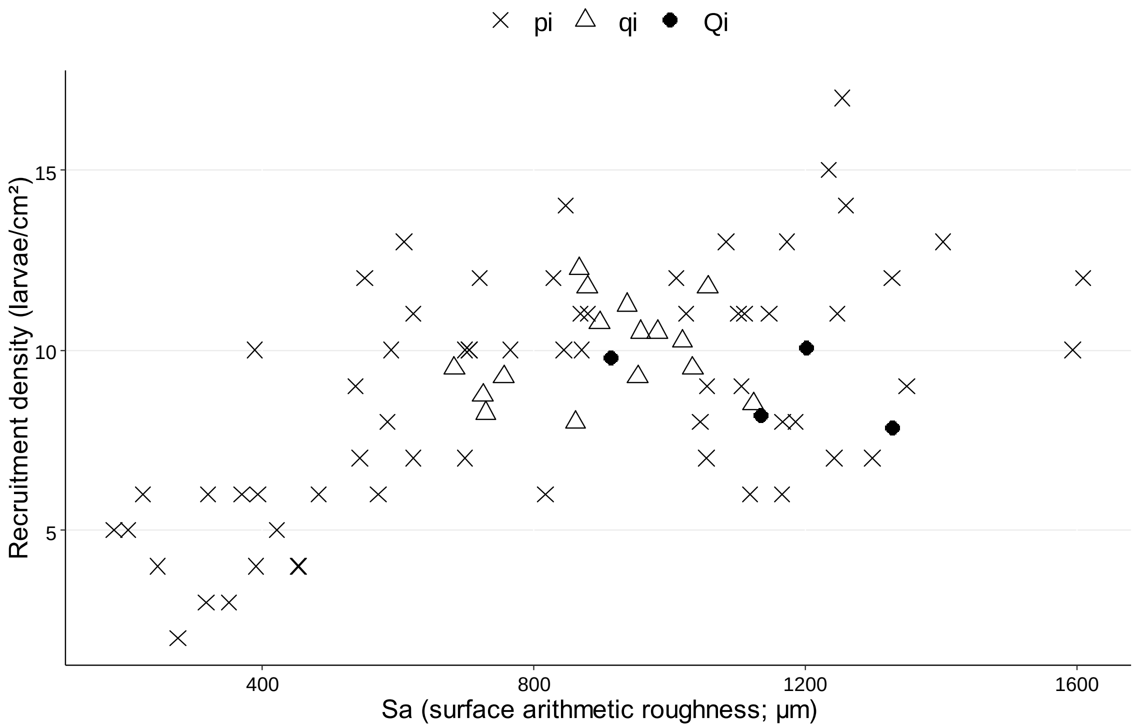


Figure 5. Recruitment density as a function of roughness (Sa) on the 50Shell sample at different analytical scales (pi = 1 x 1 cm²; qi = 2 x 2 cm²; Qi = 4 x 4 cm²)

3.2. Experiment 2: surface texture preference

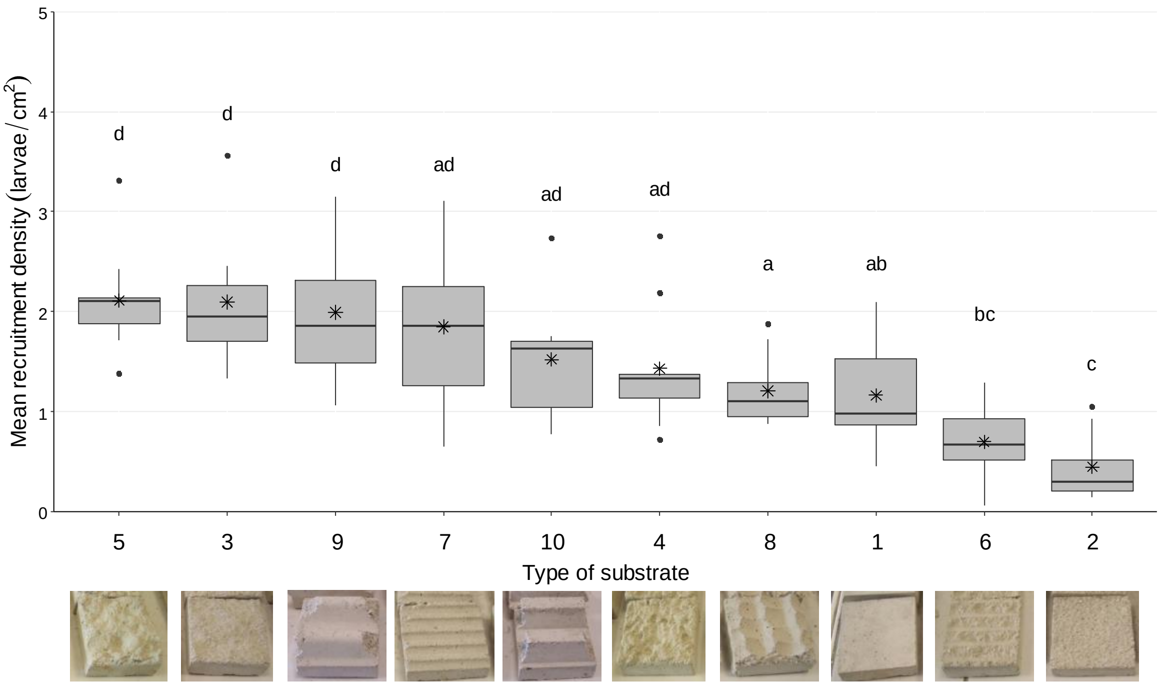
In view of the results from experiment 1, we sought to understand the preferences of the larvae by reasoning on their scale by providing substrates with different moulded surface textures in experiment 2. The formulation used for this second experiment was only mortar, although the raw materials were the same than for experiment 1. This mortar formulation was used to assess the surface texture preferences for larval settlement.

3.2.1. Recruitment: general effects

Surface texture had a significant effect on larval settlement, as shown by the comparative recruitment density (Figure 6; p = 0.001; F = 9.43; df = 9; all grids combined). The highest recruitment density values were obtained on textures n°5 and n°3, with 2.1 larvae/cm^2, and the lowest on texture n°2 with 0.45 larvae/cm^2. The effect of surface texture alone could result in differences of up to a factor of 5.

Some of the textures (n°9, 7, 1 and 6) showed a high variance compared with the others (n°5, 3, 10, 4, 8 and 2).

The more wavy textures (n°9, 7 and 10) had a similar mean recruitment density (2, 1.8 and 1.5 larvae/cm^2, respectively) and were grouped, as were the flattest textures (n°1, 6 and 2), which showed the lowest densities (1.2, 0.7 and 0.45 larvae/cm^2, respectively).



<u>Figure 6. Boxplot representing recruitment density for each texture (n = 9). The mean for each substrate is shown by a black star (n = 9). Outliers are indicated by circles. Substrates that do not share a common letter have a significantly different recruitment (*p* < 0.05; PERMANOVA followed by a pairwise Student t test for multiple comparisons)</u>

Figure 7 shows the results obtained by grid, for which there was no effect (p = 0.13; F = 2.15; df = 2) and no interactive effect of texture*grid (p = 0.79; F 0.71; df = 18). Thus, there were no significant differences in recruitment density between experimental grids, even though grid B seems to have slightly (though not significantly) higher values.

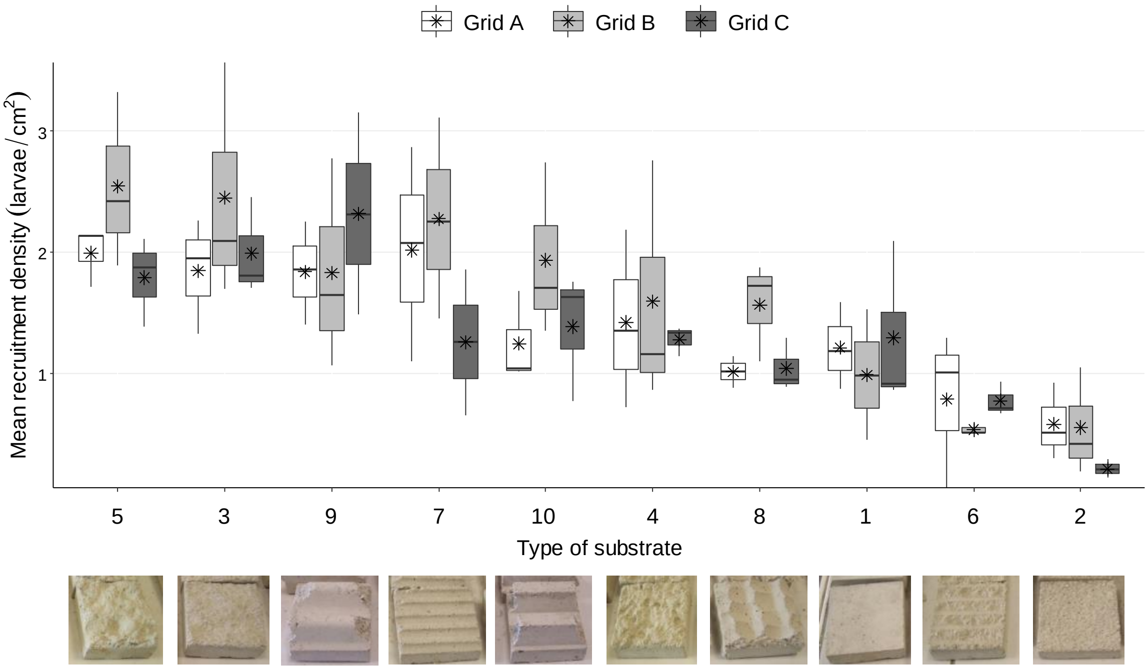


Figure 7. Boxplot representing recruitment density for each texture (n = 3) on each grid (A in white, B in light grey, or C in dark grey). The mean value for each substrate is indicated by a black star

3.2.2. Recruitment: larval distribution at different scales

Figure 8a shows strong inter-paver variability: two neighbouring pavers could have huge difference in recruitment density. When considering the position of the pavers on the grids (Figure 8b), no differences were observed between the pavers positioned on the perimeter and those positioned in the centre of the grids (Student t-test; $p = 0.98$; t = -0.02; df = 88). If both texture and paver position factors were integrated simultaneously, PERMANOVA indicated no influence of the position ($p = 0.18$, F = 1.84, df = 1), but a significant effect of texture ($p = 6.3910\text{-}11$, F = 11.6, df = 9) and of the interaction of both factors ($p = 0.01$, F = 2.76, df = 9). When looking at these combinations in detail, there was only a significant interaction between position and texture n° 7 (p = 0.04), with a higher recruitment density when this texture was positioned in the centre of the grid. This interaction did not appear with the other textures.

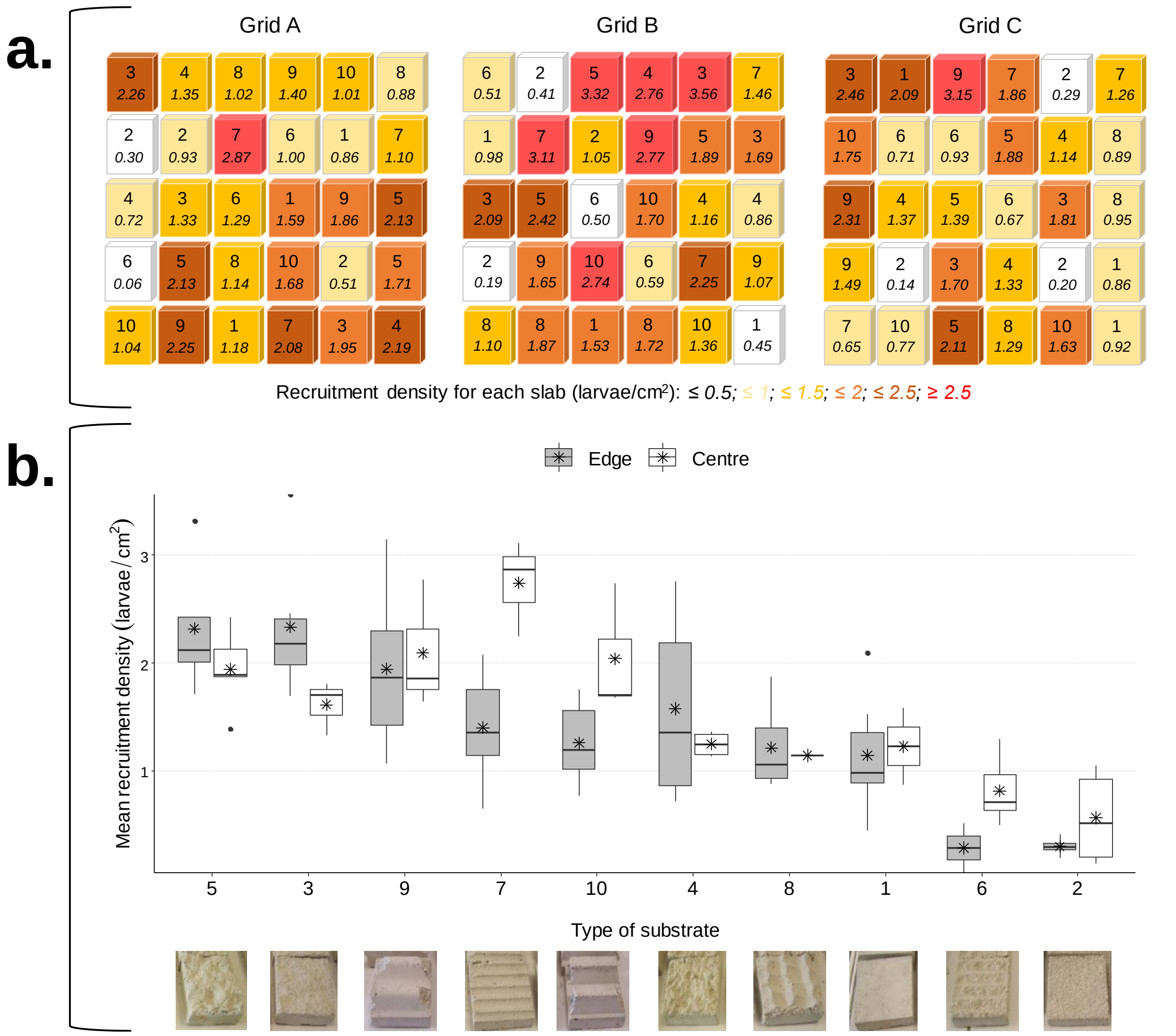
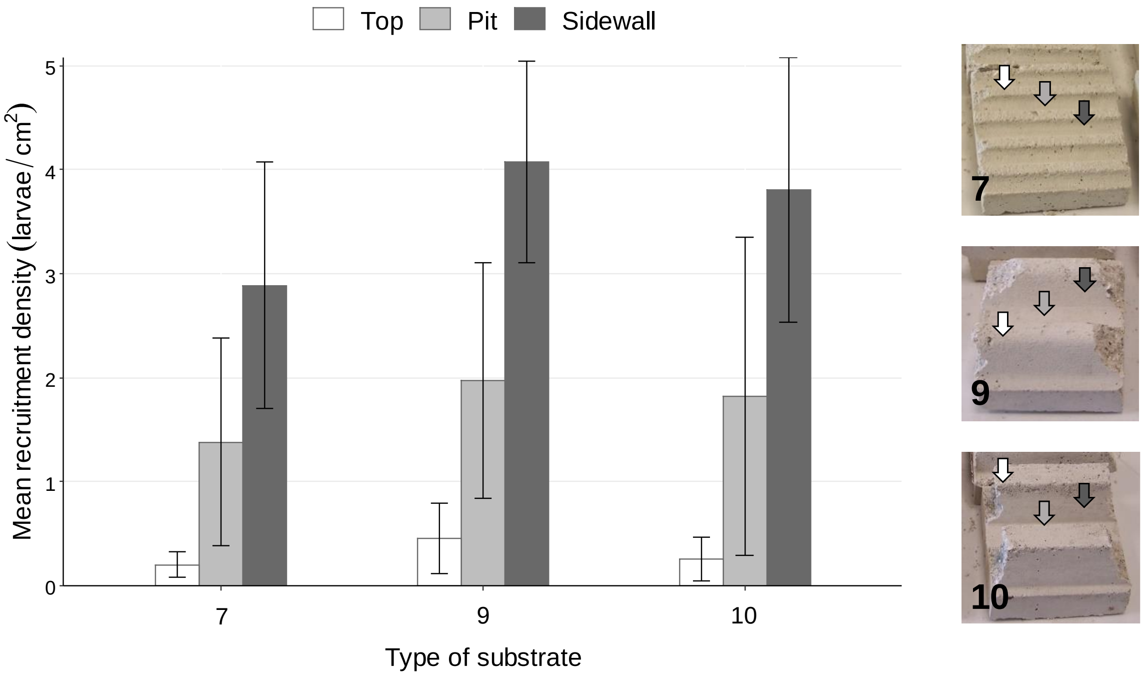


Figure 8. a) Random distribution of pavers on experimental grids. For a given paver, the top number is the texture reference and the bottom number the recruitment density. b) boxplot representing, for each texture, mean recruitment for pavers that were positioned on the edge (the perimeter) or in the centre of the grids

The results also revealed intra-texture variability, with both high and low recruitment values for some textures, which explains the higher variance observed for some of them, especially for pavers with a highly wavy texture (n° 7, 9 and 10). For these textures, a specific analysis was conducted on the repartition of larvae among three pre-defined zone types: top, pit and sidewall. Figure 9 shows this repartition: there is a significant effect of zone type on recruitment density (*p* = 0.001; F = 75.38; df = 2), with a strong larval preference for settlement on sidewalls (3.6 larvae/cm²) and in pits or valley bottoms (1.7 larvae/cm²), whereas settlement on exposed flat areas on the tops is very low (0.3 larvae/cm²). These results again demonstrate the effect of texture at a small scale (closer to oyster larva size).



<u>Figure 9. Barplot representing recruitment density (mean ± SD; n = 3) on each defined zone type (top, pit or sidewall) for pavers with highly wavy textures (n°7, 9, 10)</u>

Figure 10 illustrates this key observation. When larvae settle, they obviously avoid light exposition by fixing on more shadowed and protected areas, even with non-wavy textures such as n°5 and 3.

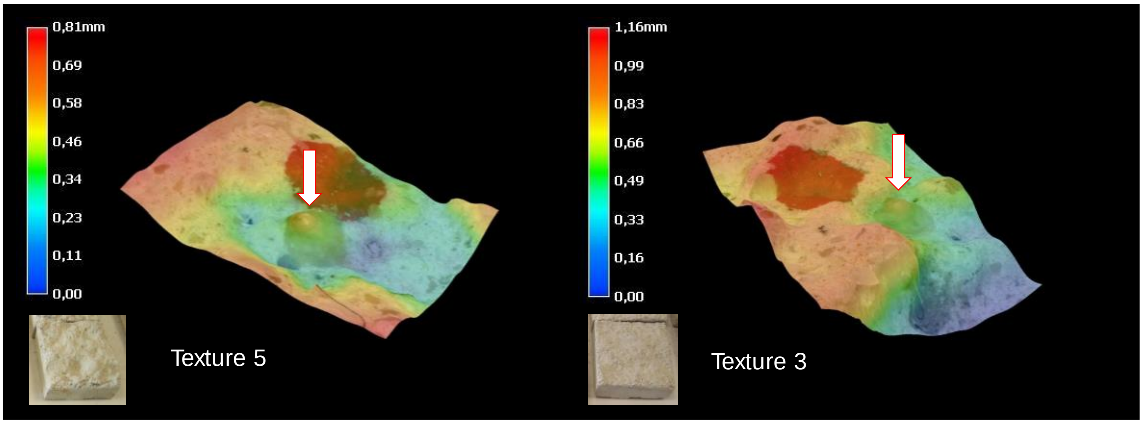


Figure 10. 3D pictures made with a Keyence VHX-6000, showing larval preference for 'hiding in a hole'. The arrow indicates the position of the larva and the colour gradient indicates the depth (in mm) within the sample. Two textures were chosen for this illustration: texture n°5 (on the left) and texture n°3 (on the right)

4. Discussion

Oyster bed restoration is mainly dependent on the availability of natural hard substrates and the creation of artificial ones (Kerckhof et al., 2018; Pogoda et al., 2019; Smyth et al., 2018). The selection of an efficient substrate is important both for larval fixation in open water and for commercial oyster culture (zu Ermgassen et al., 2020).

Oyster shells are used extensively as substrates for restoration but are in limited supply, so a variety of alternative substrates have been tested (George et al., 2015; Manning et al., 2019). For instance, Graham et al. (2017) evaluated recruitment efficiency on different materials for *Crassostrea virginica* larvae. They showed that concrete is the most efficient, ahead of limestone, oyster shells and finally river rocks (granite). A similar study was recently conducted for *O. edulis* (Colsoul et al., 2020) in the field and showed that settlement rates were significantly higher on baked clay, followed by slaked lime and bivalve shells, whereas wooden materials did not perform.

4.1. Weak effects of formulation but strong effects of micro-topography

The formulations we tested were all based on a standard cement, to which different grain sizes of aggregates (limestone sand, limestone gravel, siliceous sand) and/or oyster shells were incorporated in variable proportions (20%, 35% and 50%).

Due to their different compositions, the nine formulations tested showed differences in terms of porosity, air permeability and mechanical strength. We observed that porosity and air permeability increased with the proportion of shell incorporated into the concrete. As shell grain size increased, porosity decreased, whereas air permeability increased. Shell proportion and grain size may thus influence the ability of a concrete substrate to retain water and minerals, and to release compounds into its surrounding environment. A higher granulometry provides a better mechanical resistance, whereas a higher shell proportion tends to decrease mechanical resistance.

Although testing differences between sites was not an objective of this study, one explanation for the difference in recruitment between sites could be related to the preparation of the samples. For the bay of Brest, the samples were 'conditioned' in seawater by immersion in a tank for 7 days, while those from Quiberon bay were directly tested at the site, due to time constraints. The significant differences in recruitment between the two sites could partly be linked to differences in the release of repellent substances by the concrete substrates over the first days of immersion and/or the colonisation of their surfaces by favourable microorganisms (biofilm effects, see below). Another explanation could be the reduced flushing time in the bay of

Quiberon, which could explain the lower recruitment despite higher larval densities for this site.

Despite these differences in mechanical properties, the impact of formulation on larval settlement was quite limited in our study, irrespective of the location of the test.

Several previous studies (e.g., Graham et al., 2017) have, in contrast, shown more of an impact of substrate type on recruitment. Theuerkauf et al. (2015) showed that juvenile recruitment of *C. virginica* was three times higher on Oyster Castles® made with concrete and unconsolidated shell than on embedded shell. For *O. edulis* in the field, Colsoul et al. (2020) showed a settlement preference for baked clay and slaked lime over shell and wood substrates. In some other cases, results differed less among substrates, e.g., George et al. (2015), who found no significant difference in spat density between concrete, porcelain, limestone, river rock, oyster shell and bare sediment, and Lok and Acarli (2006), who found no differences in recruitment between oyster and mussel shell collectors.

In our case, the different formulations did not differ significantly from one another. Some trends can nevertheless be observed (see Figure 4), and the lack of significance might be due to the low number of replicates (n = 2). We must also recall that our formulations all had the same cementitious base. Differences in terms of settlement preference were consequently very low compared with those of Colsoul et al. (2020), for example, who tested shell vs river rock or wood. In our study, even without incorporating oyster shells (e.g., REF-15SI), recruitment remained high. The most important compound appears to be cement, rather than the proportion of incorporated shells or their granulometry. The work done here on concrete formulation can be useful for human-made artificial substrates. It clearly shows that artificial substrates made of concrete are suitable for flat oyster recruitment, even if the precise formulation varies a little. The integration of limestone and oyster shells in variable proportions and grain sizes into the cement should rather be considered in terms of mechanical characteristics and practical optimisation (3D design, weight, durability, resistance, etc.).

Previous studies have mostly used limestone aggregates and shells, but some have also been done with siliceous materials. Metzler et al. (2016) showed a very high silica content (6%) in the adhesive of *Crassostrea virginica* oysters compared with their shell composition. However, in our case, we found that silica in the concrete formulation had no influence on *O. edulis* larval fixation and so we did not consider this compound further.

Of the formulations tested, best compromise seemed to be the 50Shell, for which the recruitment density was the highest. Furthermore, this formulation offers good mechanical resistance, which is a useful property for reef building. It is also one of the roughest formulations, a parameter which is known to increase larval fixation in many marine invertebrates (Kohler et al., 1999; Skinner and Coutinho, 2005; Su et al., 2007). Surface characteristics, i.e., topography at a microscale, can play an important role in larval settlement (Coombes et al., 2015; Hanlon et al., 2018) and we revealed a positive correlation between roughness over 1 cm² (millimetre-scale) and recruitment for this specific formulation. From a biological point of view, this roughness effect on settlement demonstrated at a small scale (closer to the oyster larvae size) is presumably linked to a 'protection' mechanism, i.e., camouflage, against predators during the sensitive period of metamorphosis (George et al., 2015; O'Beirn et al., 2000).

The 50Shell formulation was then modified slightly to maximise both recruitment and mechanical properties before being used in the texture preference experiment

(experiment 2) and for future artificial reef building. The proportion of concrete was increased to enhance surface alkalinity which is also known to play a role in recruitment based on Anderson's work (1996). The proportion of shell was slightly reduced to approximately 40% and grain size was reduced (< 500 µm); the remaining aggregate was composed of limestone sand. This formulation made it possible to maximise both porosity and mechanical resistance.

Contrary to the small differences observed for recruitment between formulations, our second experiment revealed a significant impact of surface texture on recruitment, demonstrating a micro-topography effect. Indeed, substrate texture produced up to 5-fold increased settlement (n°5 = 2.1 larvae/cm^2; n°2 = 0.45 larvae/cm^2). Differences in recruitment among the different surface textures demonstrate high larval selectivity, a conclusion that is also supported by the heterogeneous distribution on experimental grids: although the pavers were arranged very close to each other, differences could be highly pronounced between neighbouring pavers. The preferred surface texture, with a low variability, was n°5. It is also interesting to note that this texture is the one that looks most like natural stone.

The repartition of larvae on the waviest textures (n°7, 9 and 10) revealed that larvae prefer to settle on the sidewalls, but systematically avoid the tops. Being on the tops would expose them to stronger water currents and presumably to predation; in the pits and on sidewalls they are more protected. Moreover, several studies have shown a negative phototaxis for oysters (Cole and Jones, 1939) and other invertebrate larvae (Ells et al., 2016), which is in total agreement with our results.

Another explanation lies in the hydrodynamical properties of the substrate. The shape, orientation and texture of the substrate are known to influence water flows over its surface and thus larval attachment (Whitman and Reidenbach, 2012). Johnson (2017) showed that the best profile for *C. virginica* larval settlement is intermediate concavity. On the one hand, if the profile is too flat, water flows smoothly over it and particles (i.e., larvae) are not retained. This might explain why, in our case, the flattest substrates (n°2, 6 and 1) were also those avoided by settling larvae. On the other hand, if the profile is highly concave, particles make several circuits before being ejected. Rough textured substrates, such as concrete, create more surface turbulence and increase settlement compared with smooth pebbles, for example (Fuchs and Reidenbach, 2013). This difference highlights the interest of such materials for artificial reef building.

It is also known that larval fixation is enhanced when substrates are vertical. For instance, when collector lines are laid horizontally, so that the collectors are in a vertical position, the attached *C. gigas* spat is significantly greater than in other orientations (Lagarde et al., 2016). Oyster reefs tend to form vertically, which might increase long-term survival of oysters by protecting them from predation and sedimentation (Soniat et al., 2004) and could explain the preference for vertical settlement observed in the present study.

In conclusion, we can say that the effects of water movement and larval behaviour combine to influence settlement on different substrates. Besides chemotaxis, larvae may also be subject to phototaxis and geotaxis, since they prefer to settle vertically in sheltered concavities. All of these microscale properties are important to consider in restoration. In our study, changing only the surface texture could increase *O. edulis* settlement by up to five times.

4.2. Perspectives for further research

Although some major optimal characteristics for artificial reef building were identified in this study (i.e., formulation and texture), other aspects could also be improved in the future. Many questions on larval preferences at settlement remain to be answered that would help to develop more efficient and ecologically-friendly artificial substrates for oyster restoration. Nevertheless, it has been shown that the larvae actively select their substrate by testing different areas before finding the right place to settle (Fuchs et al., 2015; Zimmer-Faust and Tamburri, 1994), a finding also supported by our study.

Close relationships exist between the materials used for substrate formulation, surface reactivity, biofilm formation and larval fixation (Anderson, 1996; Hadfield, 2011). As the aim of this study was to see to what extent different concrete formulations and textures were favourable to oyster settlement, we did not evaluate the biofilm communities here. We thus cannot conclude whether the larval preferences resulted from (1) direct influences (Anderson and Underwood, 1994; Bavestrello et al., 2000) such as chemical cues released from the formulations (experiment 1) or the paths followed by larvae on different surface textures (experiment 2), or (2) effects mediated via the development of biofilm communities (Keough and Raimondi, 1995), which may also have differed depending on formulations (different components) or surface textures (hollows, bumps, areas more or less exposed to light or water movement). This aspect could be examined in greater depth in a future study.

Beyond the nature and micro-topography of the substrate, *O. edulis* larvae have gregarious behaviour and settle according to cues representative of their habitat requirements as adults (Rodriguez-Perez et al., 2019). Larval fixation particularly occurs in response to chemical signals, which can be compounds that will induce a specific behaviour or metamorphosis (reviewed by Pawlik, 1992). Signals can also be linked to a response to the presence of conspecifics, with chemical signals inducing fixation close to adults (for review see Burke, 1986; Zimmer-Faust and Tamburri, 1994). As mentioned above, bacterial and algal biofilms are also settlement cues

(Tamburri et al., 1992) and presumably aid settlement physically. Larvae using these signals show active habitat selection. There is much evidence of the influence of chemical signals on larvae from laboratory studies, but relatively little from the field, and results can be contradictory between laboratory and field (Anderson, 1996). It would be useful to clarify such behaviours in the field in order to identify optimal restoration conditions, particularly concerning the choice of site locations.

Concerning chemical formulation, Manning et al. (2019) recently tested a new formulation called NEC (Nutrient Enriched Concrete) on *Crassostrea virginica* larvae. They integrated nutrients into concrete to stimulate biofilm and larval growth; they also added pine sawdust, which helps to control predation. Such material is very attractive to larvae, although attention must be paid to the proportion of each compound to avoid weakening the structure.

Substrate colour also plays a role in larval fixation. Studies have shown larval phototaxis and a preference for darker substrates (Wang et al., 2017) and for less bright areas (Ells et al., 2016). This aspect should also therefore be tested in further studies as it is an easy feature to modify on artificial substrates. We should perhaps consider using natural dyes, e.g., cuttlefish ink, carbonised wood or even oyster-shell ashes that can replace lime and simultaneously enhance the substrate's mechanical strength (Li et al., 2015).

Another aspect that should be addressed concerns the 3D shape, form, size and height of an artificial reef. It is known that *O. edulis* physiological performances increase when their distance from the seabed increases (Sawusdee et al., 2015), related to increased food availability and water renewal increase with height due to boundary layer properties, which improve growth and reproduction. Moreover, height could also increase avoidance from predation, sedimentation and disease. Although concrete substrates are a good alternative to the use of oyster shells, they are generally large and heavy structures that require specialist transport, installation by barge, and which occupy a large area on the seabed. To facilitate the widespread distribution and installation of artificial substrates in coming years, it would be interesting to conceive smaller supports that could just be thrown in the water, and would work regardless of the way in which they fell. These would have a low footprint on the bed, and could be made from eco-friendly materials through the development of 3D printing, another promising avenue for native oyster restoration (e.g. Li et al., 2020).

Conclusion

This study demonstrates that *Ostrea edulis* larval recruitment is influenced more by substrate surface texture and micro-topography than by substrate formulation (within the ranges tested here). The best formulation of the nine formulations tested was that containing the highest proportion of shell (50%), although this trend was not significant. This concrete provides good mechanical resistance and is also rougher than the other formulations. Our results suggest that, overall, to enhance recruitment, the ideal surface texture must have irregularities and slight concavities at a microscale. Substrates should also be oriented so that the larvae can settle vertically. The optimal support to maximise both larval recruitment and mechanical properties would be made from a formulation containing aggregate made up of 60% limestone sand and 40% crushed oyster shells sieved at 500 µm, with an irregular and rough texture, and should be fixed vertically.

The formation of a natural reef, capable of resisting natural environmental stresses, takes several years. For the moment, solitary oysters, fixed on small stones, are easily displaced by currents, which precludes or slows down the reformation of dense banks.

Setting up protected areas in which a large number of small, stable, eco-friendly supports could be deployed could significantly help to rebuild more resilient populations. Restoration efforts must be maximised by deploying such substrates at the right time and on the most favourable sites in terms of parental density, but also considering hydrodynamic and physico-chemical parameters.

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