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Addressing critical limitations of oyster (*Ostrea edulis*) restoration: Identification of nature-based substrates for hatchery production and recruitment in the field

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Abstract

1. The European flat oyster (*Ostrea edulis*) is an ecosystem engineer that provides important biogenic reef habitat with associated ecosystem functions and services. Most stocks have been commercially exploited and degraded; some are functionally extinct. Ecological restoration now aims to recover these degraded, damaged or destroyed ecosystems.
2. Availability of seed oysters and substrate for successful larval recruitment has been identified as a major limiting factor for restoration projects in Europe. In substrate-limited areas, restoration approaches have to involve the restoration of suitable substrates.
3. The present study provides an evaluation of such potential substrate types. Various categories were investigated through hatchery and/or field experiments: (1) marine bivalve shells; (2) inorganic materials; (3) sandy sediment; (4) 3D sandstone reefs; (5) wood materials; and (6) limed materials. The respective settlement rates (settled larvae per cm^2) indicate settlement preferences.
4. Hatchery experiments showed significant preferences for bivalve shells and inorganic materials. Best settlement rates were observed on *Mytilus edulis* shells, followed by *O. edulis* shells as well as on slaked lime and on baked clay. Settlement was significantly higher on bottom-oriented areas of bivalve shells and 3D reefs in laboratory experiments; however, this was not substantiated in the field experiments.
5. Field experiments showed significant settlement preferences between substrate categories (bivalve shells, inorganic materials and wood materials). Best settlement rates were observed on baked clay, followed by slaked lime and bivalve shells. Wooden materials did not perform.
6. Settlement rates and substrate preferences of larvae in controlled environments (laboratory, hatchery) differed from rates in the natural environment (field). This

study provides a list of substrate types considering these specific environments. The relevance of these results for ecological restoration in the field and potential applications in seed oyster production are discussed.

KEYWORDS
coastal, invertebrates, restoration, settlement, substrates

1 | INTRODUCTION

The European flat oyster (*Ostrea edulis*) is an ecosystem engineer, forming biogenic reef habitats and thus providing various ecosystem functions and services (Pogoda et al., 2019). Its natural distribution ranges from Norway to Morocco, where it was once abundant not only along the coast, but also in sublittoral waters (Kerckhof, Coolen, Rumes, & Degraer, 2018). The species has been used as a food source for more than 3,000 years and has been exploited extensively since the eighteenth century all over Europe, resulting in severe population declines in many European regions (Thurstan, Hawkins, Raby, & Roberts, 2013; Voultsiadou, Koutsoubas, & Achparaki, 2010). In Germany, the species is listed as functionally extinct since the 1950s. With the extirpation of this habitat builder, the ecological key-functions of a living species-rich oyster habitat were also lost (Pogoda, 2019).

Today, the ecological restoration of *O. edulis* habitats is being addressed by a number of projects in Europe (Pogoda et al., 2019). The restoration of this species and of both oyster habitats and biogenic reefs contributes to the achievement of objectives defined under the OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic, under the EU Habitats Directive (Directive 92/43/EEC) and under the EU Marine Strategy Framework Directive (Directive 2008/56/EC); (Pogoda, Merk, et al., 2020). It is also part of a more general approach to the conservation and restoration of biodiversity as such in response to the current global crisis (Vogel, 2017).

Restoration areas can be either recruitment limited or substrate limited, or both (Westby, Geselbracht, & Pogoda, 2019). The number of larvae ready for settlement and the availability of appropriate substrates are among the main factors determining recruitment success in oyster populations (Abbe, 1988; Korringa, 1946a; MacKenzie, 1970). Accordingly, successful restoration of biogenic oyster reefs will clearly depend on detailed knowledge of larval settlement mechanisms and preferences, and on the availability of suitable substrates (Cole & Knight Jones, 1939; Korringa, 1946b; Rodriguez-Perez et al., 2019; Smyth, Mahon, Roberts, & Kregting, 2018). *Ostrea edulis* larvae are pelagic for a period of 6–14 days (depending mainly on water temperature), after which settlement occurs with larvae selecting and attaching to a solid substratum and consequently metamorphosing into spat (Bayne, 2017).

The settlement mechanism of *O. edulis* larvae is influenced by a number of physiological and environmental (abiotic and biotic) factors. Several relevant factors according to the literature are listed here in no particular order of importance: (1) general physiological status of larvae (Cranfield, 1973; Robert, Vignier, & Petton, 2017); (2) temperature (Davis & Calabrese, 1969; Marteil, 1976); (3) pH (Carbonnier et al., 1990; Cole & Knight Jones, 1949); (4) light (Bayne, 1969; Bracke & Polk, 1969; Walne, 1974); (5) hydrodynamics (Helm & Spencer, 1972; Korringa, 1940); (6) substrate type and composition (Cole & Knight Jones, 1949; Guesdon, Le Bec, Mazurie, & Lassale, 1989; Korringa, 1976); (7) orientation angles and shape of the substrate (Carbonnier et al., 1990; Cole & Knight Jones, 1949; Guesdon et al., 1989; Korringa, 1976); (8) colour and transparency of substrates (Cole & Knight Jones, 1949; Herman, 1937; Walne, 1974); (9) biofilm and fouling (Carbonnier et al., 1990; Cole & Knight Jones, 1949; Korringa, 1940; Walne, 1958); and (10) presence of conspecifics (Cole & Knight Jones, 1949; Rodriguez-Perez et al., 2019).

Substrate characteristics are in the focus of this study as they play an important role in practical ecological restoration (Fitzsimons et al., 2020). Abiotic factors are usually considered within the process of restoration site selection (Kamermans et al., 2018; Pogoda, Merk, et al., 2020). Substrate-limited areas lack natural reef structure to which oyster larvae can attach and restoration will include the selection and supply of optimal substrates (Westby et al., 2019). Focusing on the quality and suitability of substrate for ecological restoration, this study addresses open questions related to factors (6) substrate types and composition and (7) orientation angles and shape of substrate. Biotic factors, e.g. biofilm, fouling and conspecifics, were not addressed in this study.

Previous studies, focusing on substrate suitability, were carried out under different conditions (laboratory vs. field), at different locations, scales and times (Cole & Knight Jones, 1949; Coste, 1861; Smyth et al., 2018), which limits comparison between them. Additionally, these studies mainly addressed the needs of aquaculture production (spat collection) and investigated traditional local substrates such as bivalve shells or plant-based substrates (Benovic, 1997; Gaarder & Bjerkan, 1934; Korringa, 1976) and easy-to-use settlement supports such as artificial collectors (Coatanea, Oheix, Mazzara, & Hamon, 1992; Guesdon et al., 1989; Hidu, Chapman, & Soule, 1975; Korringa, 1976; Locard, 1900; Naas, 1991) instead of nature-based materials appropriate for restoration.

In the new context of ecological restoration, the suitability of different substrate types for *O. edulis* settlement needs to be re-evaluated altogether, under both laboratory and field conditions. Furthermore, substrates used in the past did not take into account relevant modern sustainability criteria such as the prevention of spread of invasive species, diseases or pathogens via substrate transfer, or the environmentally responsible sourcing of substrates. Accordingly, within this study, these additional criteria were considered to address the needs of sustainable and large-scale restoration efforts. The objective of this study was to investigate the settlement preferences of *O. edulis* larvae through combined laboratory (hatchery) and field approaches. Six substrate categories comprising 20 substrate types were examined, from historically used wood and abundantly available shell material to highly innovative 3D-printed sandstone structures. The results provide practical information for the selection of substrate for (1) ecological restoration of *O. edulis* in substrate-limited areas and (2) systematic hatchery-based seed oyster production for ecological restoration of *O. edulis* in recruitment-limited areas.

2 | METHODS

Assessments of substrate preferences for the settlement of *O. edulis* larvae were conducted through three separate experiments. The first two were performed in a hatchery under controlled experimental conditions and aimed at comparing settlement preferences among three categories of substrates (empty marine bivalve shells, inorganic materials and sandy sediments) and assessing the applicability of innovative 3D-printed structures as a settlement substrate under similar conditions. The third experiment consisted of deploying potential settlement substrates at suitable field sites during the natural swarming season of *O. edulis* larvae.

Substrate types were selected based on the following criteria, reflecting the focus of ecological restoration against the background of nature conservation measures:

1. Natural materials – artificial materials, e.g. plastics or concrete, with potential negative effects on the environment (marine litter, microplastic, chemical pollution) were not considered and not tested in the study. Only natural or nature-based materials were selected (shells, lime, clay, stone). Furthermore, existing substrates at designated restoration sites were tested (sandy sediments, granite).
2. Sustainably sourced – abundant bivalve shells, available from aquaculture or fisheries and industrially processed in many areas in Europe, were selected, allowing for sustainable sourcing of substrates without negative impacts on natural substrates. Furthermore, comparing settlement preferences between the shells of *O. edulis* and other bivalve species may provide information supporting the possible spread of *O. edulis* reefs. Inorganic materials (lime and clay) also offer a quantitative (stable and substantial supply) and qualitative alternative without negative impacts on natural substrates.
3. Knowledge transfer and common sense – different collector types successfully used in aquaculture production for seed collection were selected (bivalve shells, lime). Furthermore, wood was tested as historical records document successful settlement (Coste, 1861; Gaarder & Bjerkan, 1934; Korringa, 1976).
4. Technical innovation potential – 3D-ReefVival-Experimental-Reefs® made from sandstone (dolomite) were selected to test environmentally friendly reef ball structures, avoiding the further input of concrete (i.e. containing adjuvants) structures into the marine environment. At offshore sites, e.g. the designated oyster restoration area Borkum Reef Ground, sediment movements, including silt and sand waves, may affect future spat recruitment (Cole & Knight Jones, 1949; Kamermans et al., 2018; Pogoda, Merk, et al., 2020). Elevated massive 3D-structures would decrease the potential negative effects of sediment dynamics (Sawusdee, Jensen, Collins, & Hauton, 2015). Electrolytic mineral accretion (EMA) was selected as an additional innovative substrate type, already successfully implemented in coral reef restoration (Goreau, 2012; Goreau & Trench, 2012; van Treeck & Schuhmacher, 1997). The deposition of natural $CaCO_3$ on steel structures allows the formation of complex 3D structures as settlement surfaces.

2.1 | Experiment 1 (hatchery)

2.1.1 | Larval origin

Eye-spotted larvae (7 days post-swarming with mean size of 264.60 ± 13.43 μm) of *O. edulis* were purchased in July 2017 from a commercial hatchery (Ferme Marine de l'île d'Arun EARL, Hanvec, France) and transferred to the research hatchery of Ifremer, Argenton en Landunvez (France) for experiments.

2.1.2 | Substrate types

Three categories of substrates were investigated: empty marine bivalve shells, inorganic materials and sandy sediment. The first category c-shells included shells of four species: *Crassostrea gigas*, *Mytilus edulis*, *O. edulis* and *Pecten maximus*. Prior to experimentation, shells were cleaned and sterilized in a chlorine bath in order to study the effects of the substrate and not of the potential biofilms growing on them. The second category c-inorganics included four inorganic substrates: EMA as commonly used in coral reef restoration; baked clay and slaked lime as natural products commonly used in mariculture; and granite as an abundant natural stone material in the marine environment. Electro-mineral accretion grid plates were manufactured according to the process described by Taylor (2011). Baked clay was produced by Korallenwelt®, Germany (composition detailed in Table S1). Slaked lime produced from magnesium–calcite hydrated lime powder (Figure S1) supplied by Lhoist France Ouest SASU (Neau, France) and seawater was applied to a tile surface. Granite pieces were collected on the Argenton en Landunvez foreshore. The third category c-sediments included sandy sediments of three different size classes (International scale ISO 14688-1:2002): fine and medium sand (>0.063 to ≤0.63 mm), coarse sand (>0.63 to ≤2.0 mm) and fine gravel (>2.0 to ≤6.3 mm), collected from the marine protected area Borkum Reef Ground (53°52′59″N 6°25′08″E), an important target area for European flat oyster restoration pilots in the German Bight (Pogoda et al., 2019; Pogoda, Merk, et al., 2020). These sandy sediments were dried and glued to PVC sheets.

2.1.3 | Experimental setup

For each category, all substrate types were placed in sieves (44 × 35 × 14 cm, mesh size 150 μm). Sieves from each category were placed in rectangular tanks (depth 20 cm, Figure 1) with a flow-through system (down-welling), and supplied with natural seawater taken directly from the sea (filtered to 1 μm and UV sterilized) at a rate of 8.60 ± 0.85 L h^{-1}. Experiments were run in triplicate in three individual tanks. All substrates were positioned and trimmed to cover a surface area of 212 cm^2 each. For shells, upper-surface (all outer-shells here) and bottom-side (all inner-shells here) were examined. In c-shells and c-inorganics, *N* = 35,000 larvae (from the same batch of larvae) were placed all at once and randomly in each sieve at a density of ~2,273 larvae L^{-1}. In c-sediments, *N* = 10,500 larvae (from the same batch) were applied at a lower density of ~682 larvae L^{-1} (owing to a logistical issue in larval supply). Larvae were added immediately after their arrival and fed continuously by peristaltic pumps that mixed the algae with filtered seawater at the inlet of each tank, with a bispecific diet (1:1) consisting of *Tisochrysis lutea* and *Chaetoceros muelleri* with a food density of 1,000 μm^3 μl^{-1}. Seawater at the inlet and outlet of each experimental tank was sampled twice a day (morning and afternoon) and microalgae counts were performed using an electronic particle counter (Multisizer™3 equipped with a 100 μm aperture). Adjustments were then made to the feeding rate to keep the algal cell density constant. Temperature (20.87 ± 0.07°C), pH (8.40 ± 0.06), salinity (35.75 ± 0.08) and dissolved oxygen (89.88 ± 11.51 %) were monitored and adjusted to optimal conditions twice a day. The experiment was ended after a settlement period of one week by carefully removing the substrates from the water, gently cleaning with fresh water, drying and storing them (in independent plastic bags between air bubble films at 18°C) for the counting of settled larvae.

2.2 | Experiment 2 (hatchery)

2.2.1 | Larval origin

Eye-spotted larvae (6 days post-swarming) of *O. edulis* were produced in the period from July to August 2018 in a commercial hatchery (Novostrea Bretagne SAS, Sarzeau, France) from local broodstock.

2.2.2 | Substrate type

The 3D-sandstone reefs (3D-ReefVival-Experimental-Reefs® designed by Reef Design Lab®) were printed by Boskalis Nederland BV using the following ingredients: dolomite sand, trass flour (Tubag™), white cement (Standard EN 197-1:2011, CEM I/II) and fresh tap water (Table S2). The reefs consisted of four round and horizontal platforms supported by pillars (Figure 2). The dimensions of the reefs were 50 cm in height and 50 cm in diameter.

2.2.3 | Experimental setup

Settlement experiments were carried out using two structurally identical reefs. Each reef was placed in a cylindrical tank (400 L)

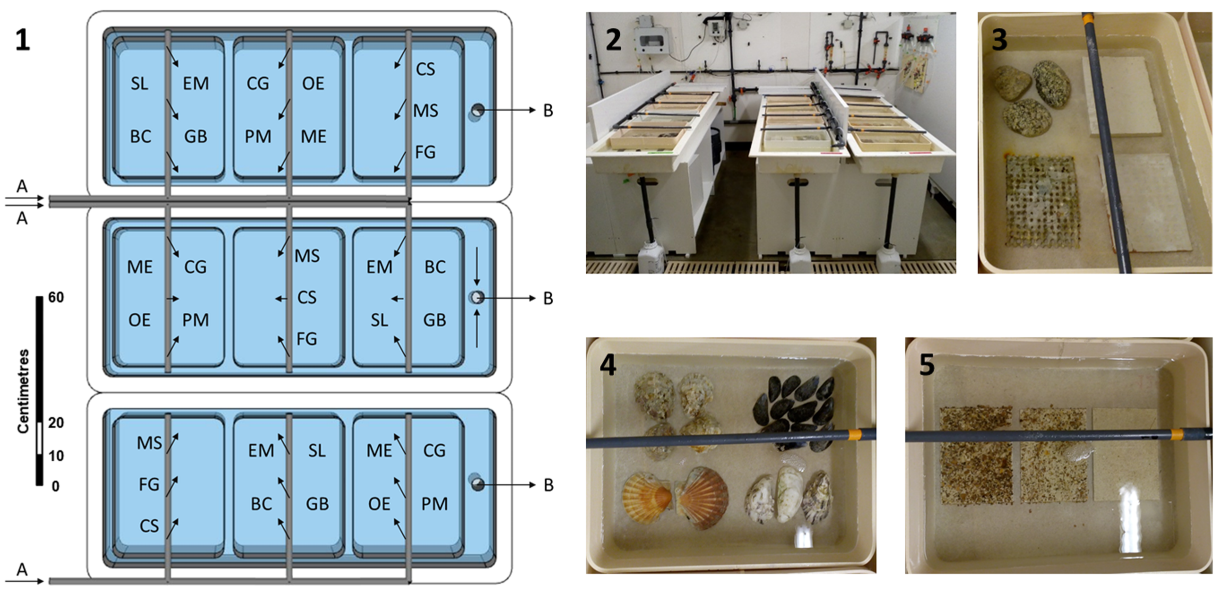


FIGURE 1 Experimental design and set up of settlement experiments for *Ostrea edulis* larvae in the hatchery. (1) Schematic view of the basins and sieves from above, including the layout of the substrate types; (2) profile photograph of the experimental basins and sieves; sieves with (3) inorganic substrates; (4) bivalve shells; and (5) sandy sediments. Abbreviations: A = Inlet of water and feed; B = water outlet by overflow; BC = baked clay; CG = *Crassostrea gigas* shells; CS = coarse sand; EM = electrolytic mineral accretion; FG = fine gravel; GB = granite; ME = *Mytilus edulis* shells; MS = medium/fine sand; OE = *O. edulis* shells; PM = *Pecten maximus* shells; SL = slaked lime on tile. See Section 2.1.3 for more details regarding the dimensions of the setup

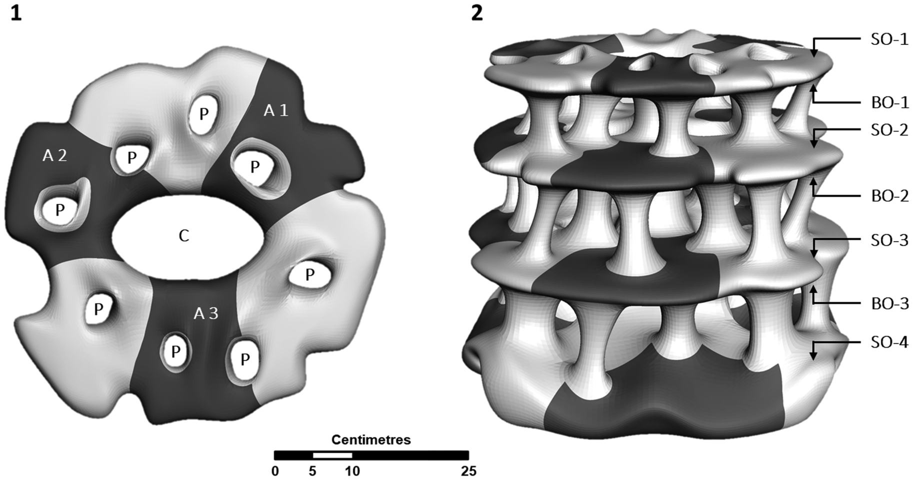


FIGURE 2 Schematic views of 3D-ReefVival-Experimental-Reefs® tested as settlement substrate for *O. edulis* in hatchery experiment 2: (1) horizontal section of one tray and (2) a profile view of the whole reef. Dark areas represent examined substrate surface (only in this scheme); tested reefs were all white. Abbreviations: A = Data acquisition area; BO = bottom-oriented area (bottom-side); C = hollow centre of the reef; P = pillars located between the different strata; SO = surface-oriented area (upper-surface)

with flow-through systems. In each tank *N* = 500,000 larvae were placed at a density of ~1,250 larvae L^{-1}. Food composition, food concentration and settlement duration were consistent with experiment 1. Temperature, pH and salinity were monitored daily and were in the ranges 21–23°C, 7.5–8.5 and 34–36, respectively. Dissolved oxygen was not monitored. The flow-through system (downwelling) was supplied with natural seawater filtered to 1 μm and UV sterilized at a rate of 5 L h^{-1}.

2.3 | Experiment 3 (field)

As the field study was carried out after the hatchery experiments, the selection of substrates was adapted accordingly. The low settlement response of *O. edulis* larvae on *P. maximus* shells, EMA and granite in the laboratory led to their exclusion from the third experiment. Sandy sediments and 3D-sandstone reefs were not included for logistical reasons. Nevertheless, considering historical information, wood materials were tested in the field (Coste, 1861; Gaarder & Bjerkan, 1934; Korringa, 1976). Additionally, and in order to determine whether substrate shape affects settlement response, five different substrate types were coated with slaked lime.

2.3.1 | Study area and larval origin

In situ tests were carried out at Roz Bank, Daoulas Cove (48°19′29″ N 4°19′26″ W), a natural *O. edulis* bed in the Bay of Brest, France. The experimental structures were installed at 5.8 m water depth. Larval abundance at Roz Bank has been monitored since 2012 using the protocol presented by Pouvreau (2015). Based on results and observations of previous years, the maximum larval abundance and recruitment period were estimated for mid-July 2018, and settlement substrates were deployed in that period. Chlorophyll concentration, salinity, temperature and turbidity were monitored daily.

2.3.2 | Substrate types

Four substrate categories were tested: c-shells (*C. gigas*, *M. edulis*, and *O. edulis*), c-inorganics (slaked lime on tile, baked clay), c-woods (*Juniperus communis*, *Picea abies* and *Phyllostachys edulis*) and c-limed, shells (*C. gigas*, *M. edulis* and *O. edulis*) and woods (*P. edulis* and *P. abies*) coated with slaked lime.

2.3.3 | Experimental setup

Field experiments were started at the larval peak and were carried out in three supports (50 × 50 cm) moored 10 cm above the seafloor. Each support had 13 horizontal experimental positions (9.5 × 9.5 cm) for attaching different substrate types (Figure 3). Triplicates were prepared for each substrate type and placed randomly in each support. Shells were glued on tiles (9.5 × 9.5 cm): upper-surface and bottom-side settlement preferences of larvae were investigated by attaching shells to both sides of the tiles (Figure 4). For *C. gigas* and *O. edulis*, two shell valves were attached on each tile side. For *M. edulis*, six shell valves were used with three placed with the convex side facing the tile (outer surface of the shell) and three facing up (inner surface of the shell) on each side of the tiles. Inorganic substrates and wood (*P. abies* and *J. communis*) were cut to size (9.5 × 9.5 cm). Tiles were coated with slaked lime and dried prior to deployment. *Phyllostachys edulis* was vertically cut in half and four halves were fitted into each of the designated experimental positions, two facing with the convex side up and two facing down. Limed materials were positioned in the same way as the non-limed material, but coated with slaked lime and dried prior to deployment.

After the experimental period of 14 days, corresponding to the maximum swarm peak period in July 2018 (Pouvreau, Cochet, Gachelin, Chaudemanche, & Fabien, 2019), substrates were brought to the surface, gently cleaned with fresh water, dried and stored (in independent plastic bags between air bubble films at 18°C) for the counting of settled larvae.

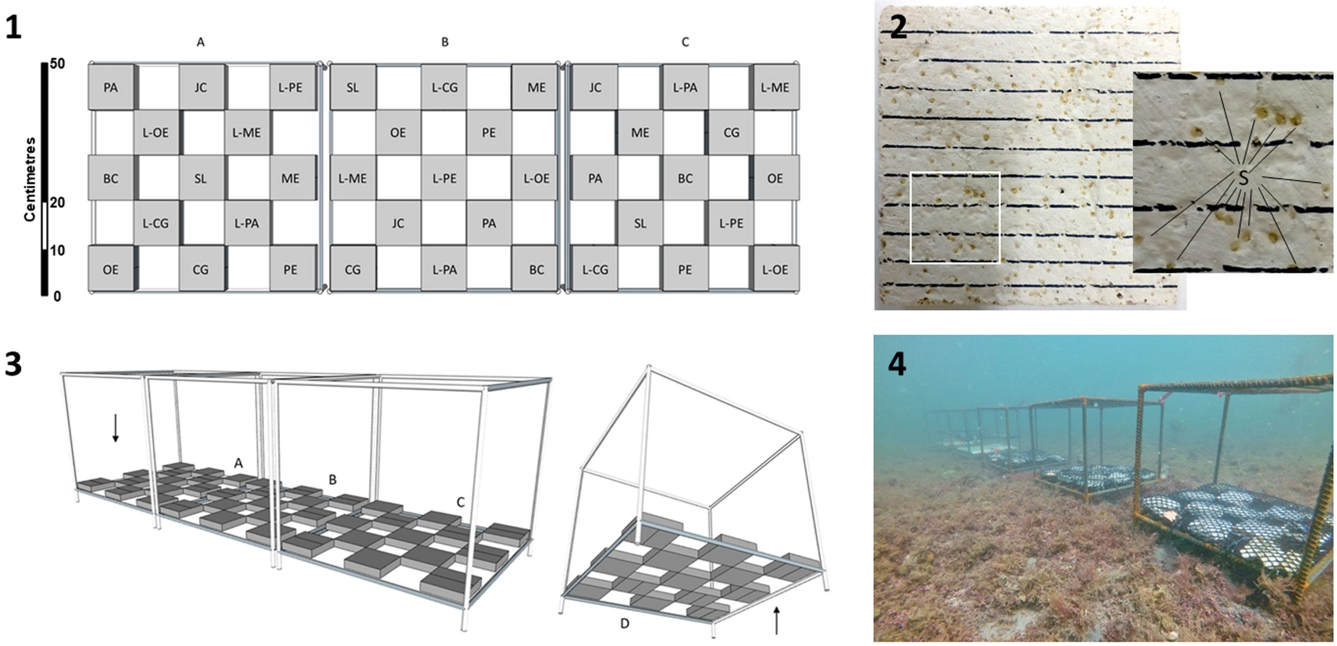


FIGURE 3 Experimental design and set up of settlement experiments for *O. edulis* larvae in the field. (1) Schematic view of the layout of the substrates tested in supports. (2) Picture illustrating an example of one substrate (here baked clay) tested with an enlargement highlighting several larvae (in yellow) settled on a white background (baked clay) between black lines added after the test for counting. (3) Two schematic profile views of the supports used, including the substrates, highlighting the two orientations (i.e. surface and bottom) of each substrate. (4) Underwater photography of experimental structures. Abbreviations: A–C = surface-oriented areas of the three replicates; BC = baked clay; CG = *C. gigas* shells; D = bottom-oriented areas of one of the replicates; JC = *Juniperus communis*; L-CG = coated *C. gigas* shells with slaked lime; L-ME = coated *M. edulis* shells with slaked lime; L-OE = coated *O. edulis* shells with slaked lime; L-PA = coated *Picea abies* with slaked lime; L-PE = coated *Phyllostachys edulis* with slaked lime; ME = *M. edulis* shells; OE = *O. edulis* shells; PA = *P. abies*; PE = *P. edulis*; S = settled larvae; SL = slaked lime. See Section 2.3.3 for more details regarding the dimensions of the setup

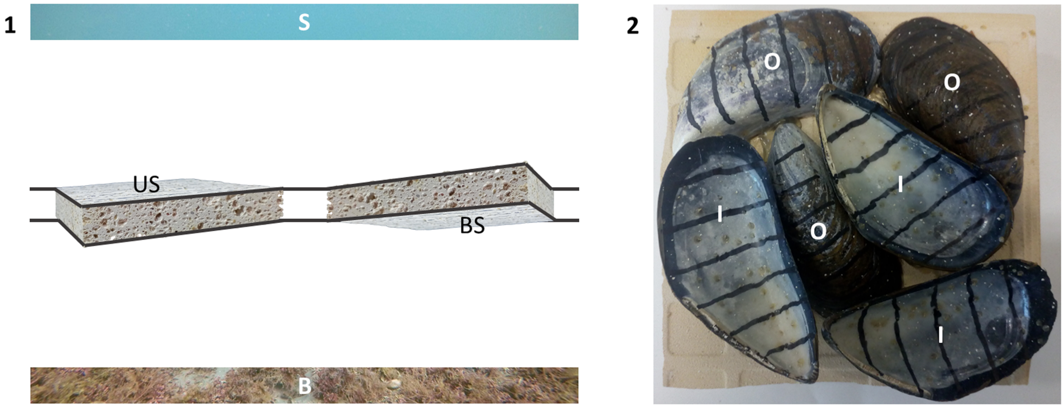


FIGURE 4 Illustrations of the different orientations and surfaces tested. (1) Schematic view of the two orientations on an example substrate (here baked clay). (2) Schematic view of the two types of shell surfaces (here *M. edulis*). Abbreviations: B = bottom; BS = bottom-side/bottom-oriented; I = inner-shell/Inside of the valve; O = outer-shell/outside of the valve; S = water surface; US = upper-surface/surface oriented

2.4 | Data collection and treatment

2.4.1 | Counting of settled larvae

For experiments 1 and 3, the total number of settled larvae on the tested substrates was counted using a stereomicroscope (Zeiss™ Stemi™ DV4) with a magnification of 32× (Figure 3(2)). For experiment 2, photographs were taken with an ultra-high definition (4K) camera (Nikon™ Coolpix™ W300), with a positioned scale bar. Owing to the specific shape of 3D-ReefVival-Experimental-Reefs®, 780 ± 4.97 cm^2 of each replicate reef, corresponding to 40% of each horizontal settlement area (Figure 2), was counted. All data from each of the experiments are reported in larvae per cm^2 (Table 1). Larval losses during experiments 1 and 2 were determined by subtracting the total number of settled larvae from the initial number of seeded larvae.

2.4.2 | Statistical analysis

For experiment 1, the differences between the total numbers of settled larvae on all of the tested substrates were determined within each category. Each substrate category was studied in a separate tank and statistical comparisons were only done within each substrate category. For each substrate category (e.g. c-shells, c-inorganics and c-sediments), count data were analysed using negative binomial generalized linear models (Poirier et al., 2019). Negative binomial generalized linear models (selected based on the lowest AIC values) were fitted for each substrate category. For all substrate categories, model structure included substrate type as a fixed effect, and the inclusion of the shell orientation (bottom-side or upper-surface) was added as an additional fixed effect for c-shells. All statistical analyses were performed using R version 3.5.2 (R-Development-Core-Team, 2018) using the 'lme4' package and *post-hoc* tests were completed using the 'emmeans' package (Bates, Mächler, Bolker, & Walker, 2015; Lenth et al., 2018; Lüdecke, 2018). *Post-hoc* test results were fitted with the log scale.

For experiment 3 (field study), independent data were tested for normality (Shapiro's test) and homogeneity of variances (Levene's test). One-way ANOVAs followed by Tukey's tests were then used to determine significant differences among the different substrate types. Effects of substrate orientation (upper-surface or bottom-side orientation) were tested using a one-way ANOVA (c-shells and c-woods) and a Student's t-test (c-inorganics). In c-shells, the effects of shell surface (inner or outer) were tested using a one-way ANOVA followed by Tukey's tests. The level of significance for statistical analyses was always set at $\alpha = 0.05$.

TABLE 1 Results of the second hatchery experiment on settlement preference of *Ostrea edulis* larvae on 3D-ReefVival-Experimental-Reefs®

Orientation and layers (see Figure 2)	Reef 1 (settled larvae per cm^2)	Reef 2 (settled larvae per cm^2)
SO-1	1.54 ± 0.48	1.59 ± 0.61
BO-1	7.63 ± 2.41	4.53 ± 1.70
SO-2	1.28 ± 0.46	1.14 ± 0.71
BO-2	5.10 ± 2.13	5.51 ± 2.55
SO-3	1.04 ± 0.59	1.51 ± 0.97
BO-3	2.91 ± 1.03	9.21 ± 2.38
SO-4	1.30 ± 0.50	0.41 ± 0.08

Abbreviations: BO = bottom oriented (bottom-side); SO = surface oriented (upper-surface).

3 | RESULTS

3.1 | Hatchery experiments

Settlement preferences differed significantly for the tested substrate types within c-shells and c-inorganics (Table 2 and Figure 5). In c-shells, *M. edulis* shells (29.1 ± 3.3 larvae per cm^2; mean ± SD) and, to a lesser extent, *O. edulis* shells (17.7 ± 3.6 larvae per cm^2) were significantly preferred by *O. edulis* larvae while lower settlement was observed on *C. gigas* shells (10.0 ± 3.0 larvae per cm^2) and on *P. maximus* shells (9.5 ± 3.3 larvae per cm^2) (Figure 5). In c-inorganics, the highest settlement was observed on slaked lime (30.3 ± 10.7 larvae per cm^2) and baked clay (15.3 ± 6.3 larvae per cm^2), with no significant differences between those two substrate types (z value = −2.485, $P = 0.062$). Settlement was very low on EMA (3.4 ± 2.9 larvae per cm^2) and on granite (0.5 ± 0.4 larvae per cm^2). In c-sediments, the mean number of settled larvae per cm^2 was lower than for inorganic and bivalve shell substrates (Figure 5). Furthermore, no significant difference was observed for the different grain size classes (Table 2 and Figure 5) within this category. The larvae mainly settled on the shell fragments, whereas sand and gravel grains were less attractive for settlement (Figure S2). The average number of settled larvae in c-sediments (i.e. initial larvae density of ~682 larvae L^{-1}) ranged from 3.4 ± 0.8 to 6.3 ± 6.3 larvae per cm^2, while the settlement ranged from 9.5 ± 3.3 to 29.1 ± 3.3 larvae per cm^2 and from 0.5 ± 0.4 to 30.3 ± 10.7 larvae per cm^2 in c-shells and c-inorganics (i.e. initial larvae density of ~2,273 larvae L^{-1}), respectively. The proportions of non-settled larvae in experiment 1 were 59.7 ± 5.5% (mean ± SD) in c-shells, 70.0 ± 6.2%, in c-inorganics and 70.9 ± 18.7% in c-sediments, respectively.

In addition to the effects of substrate types on settlement preference of larvae, the effects of shell orientation were also assessed within c-shells (upper-surface vs. bottom-side orientation, irrespective of inner or outer surface of the valves) (Table 2). A significant preference for bottom-oriented shells (z value = −9.098, $P < 0.0001$) was observed for all shell types (*C. gigas*, *M. edulis*, *O. edulis* and *P. maximus*). This effect was particularly pronounced for *M. edulis* shells, where 28.1 ± 2.8 larvae per cm^2 settled on bottom-oriented shells, while only 1.0 ± 0.6 larvae per cm^2 settled on the upper-surface of the shells (Figure 6).

In experiment 2, the average settlement on the reefs ranged from 0.41 ± 0.08 to 9.22 ± 2.38 larvae per cm^2 (Table 1). Only 40% of the horizontal reef surface was examined and as no swimming larvae and no settlement were observed on the experimental tanks (visual observations), larvae were obviously attracted by this type of substrate. Furthermore, the positions of the settled larvae (upper-surface vs. bottom-side) clearly indicate the preference for bottom orientation (5.7 ± 2.4 larvae per cm^2) compared with the upper surface (1.2 ± 0.4 larvae per cm^2). As experiment 2 was conducted in duplicate, no statistical analysis was performed on the data gathered from artificial reefs.

TABLE 2 Results of negative binomial generalized linear models for counts of *O. edulis* spat for the first laboratory experiment assessing the settlement substrate preferences

Response: Counts	Estimate	SE	*z* Value	*P*-Value
Substrate category: shells				
Intercept	2.1746	0.1842	11.807	<0.0001
M. edulis	1.0660	0.2113	5.045	<0.0001
O. edulis	0.5683	0.2281	2.492	0.0127
P. maximus	−0.0582	0.2616	−0.223	0.8239
Shell faces	−1.9600	0.2154	−9.098	<0.0001
Contrasts				
C. gigas–M. edulis	−1.0660	0.211	−5.045	<0.0001
C. gigas–O. edulis	−0.5683	0.228	−2.492	0.0612
C. gigas–P. maximus	0.0582	0.262	0.223	0.9961
M. edulis–O. edulis	0.4976	0.174	2.861	0.0220
M. edulis–P. maximus	1.1242	0.216	5.205	<0.0001
O. edulis–P. maximus	0.6265	0.232	2.696	0.0354
Substrate category: inorganic				
Intercept	2.7270	0.2082	13.098	<0.0001
EMA	−1.5090	0.4043	−3.732	0.0002
Granite	−3.4942	0.8848	−3.949	<0.0001
Slaked lime	0.6845	0.2755	2.485	0.0130
Contrasts				
Baked clay–EMA	1.509	0.404	3.372	0.0011
Baked clay–granite	3.494	0.885	3.949	0.0005
Baked clay–slaked lime	−0.685	0.275	−2.485	0.0623
EMA–granite	1.985	0.927	2.141	0.1402
EMA–slaked lime	−2.194	0.391	−5.614	<0.0001
Granite–slaked lime	−4.179	0.879	−4.756	<0.0001
Substrate category: sediments				
Intercept	1.8377	0.3502	5.247	<0.0001
Fine gravel	−0.3037	0.5139	−0.591	0.555
Medium/fine sand	−0.6018	0.5377	−1.119	0.263
Contrasts				
Coarse sand–fine gravel	0.304	0.514	0.591	0.8250
Coarse sand–medium/fine sand	0.602	0.538	1.119	0.5021
Fine gravel–medium/fine sand	0.298	0.555	0.537	0.8530

Note: The reference (intercept) category/substrates are *Crassostrea gigas*/bottom, baked clay, coarse sand for bivalve shells, inorganic substrates and sedimentary substrates, respectively. Marginal contrasts are provided. Results are given on the log scale.

3.2 | Field experiment

In the field, salinity and temperature were stable over the entire period, with mean values of 34.40 ± 0.14 and 19.93 ± 0.12°C, respectively. The chlorophyll concentration was on average 1.24 ± 0.07 μg L^{-1} and turbidity was 0.62 ± 0.07 NTU. As all substrate types were tested in one experimental setup at the same time, a direct comparison of settlement response of all respective substrate types was possible (Figure 7). Wild *O. edulis* larvae preferred substrates from c-inorganics. A significant effect of substrate type was found (F = 48.44, P < 0.0001) for all inorganic substrates, especially baked clay (4.1 ± 0.5 larvae per cm^2), compared with c-shells (2.4 ± 0.5 larvae per cm^2) and c-woods (0.2 ± 0.1 larvae per cm^2) (Figure 7). Within all three substrate categories, Tukey's test revealed no significant difference among the substrate types within the category tested (Figure 7). c-Woods shows by far the lowest rate of larval settlement with differences of ∼12- and ∼20-fold lower than c-shells and c-inorganics, respectively.

In contrast to the laboratory experiments, no significant effect of substrate orientation (upper-surface and bottom-side) was observed in the field (F = 1.872, P = 0.173 for bivalve shells; F = 2.626, P = 0.126 for inorganic materials and F = 1.619, P = 0.229 for wooden substrates; Figure 8). Settlement preference may also be related (irrespective of upper-surface and bottom-side orientation) to inner and outer shell surface (F = 5.14, P = 0.002, Figure 9): *O. edulis* settling on *M. edulis* shells showed a significantly higher settlement on the inner surface (P < 0.0001). Nevertheless, no difference was observed for *C. gigas* shells (P = 0.919) and *O. edulis* shells (P = 0.952) (Figure 9).

Settlement results of *O. edulis* on wooden substrates coated with slaked lime were not reliable enough to be analysed. The slaked lime did not attach itself sufficiently to the material. However, no significant differences in settled larvae rates were observed between the other substrate types within c-limed, nor with the slaked lime on tile for c-inorganics (Figure S3).

4 | DISCUSSION

In 2017, the Native Oyster Restoration Alliance was founded to support and facilitate the ecological restoration of biogenic oyster reefs throughout Europe. The network identified several critical issues which currently limit sustainable large-scale restoration operations and outlined recommendations for ecological oyster restoration (Pogoda et al., 2019), e.g. to provide suitable substrates for successful recruitment (recommendation 3), as adding or introducing suitable substrates in restoration sites will increase recruitment success. Accordingly, this study focused on the identification of suitable substrate types, either for practical restoration in the field or to produce sufficient oysters for restoration of oyster reefs (recommendation 1).

For the first time, an experimental setup was created to observe settlement preferences of *O. edulis* in both controlled and natural environments with different substrate types. The results confirm that *O. edulis* larvae show settlement preferences depending on the type of substrate, and unexpectedly, these preferences differ between controlled and natural conditions.

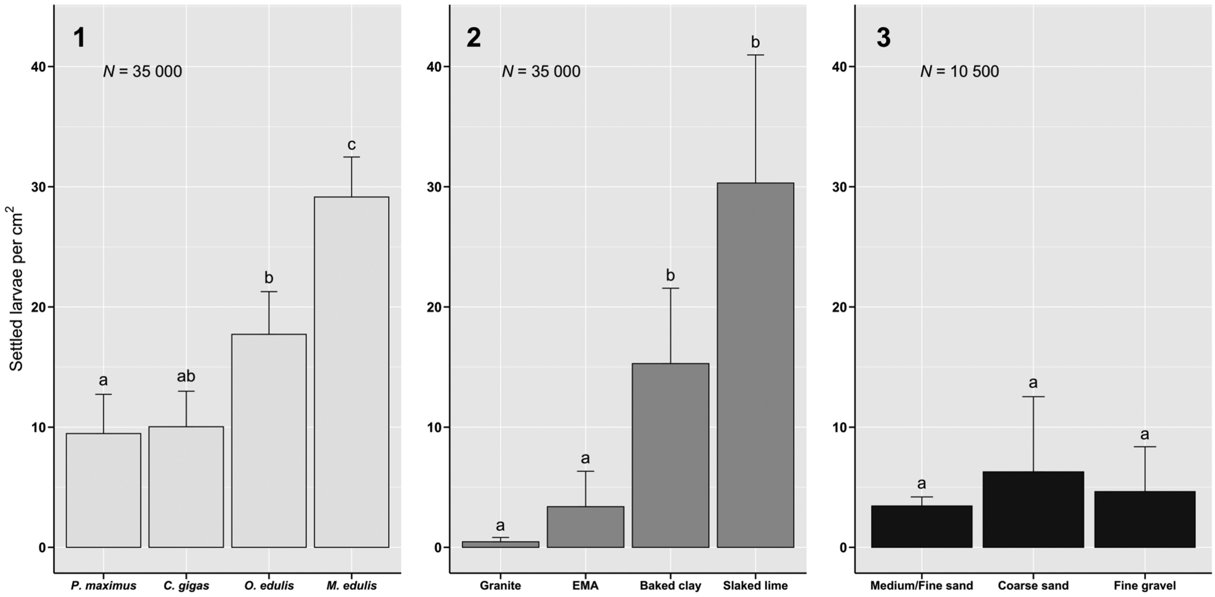
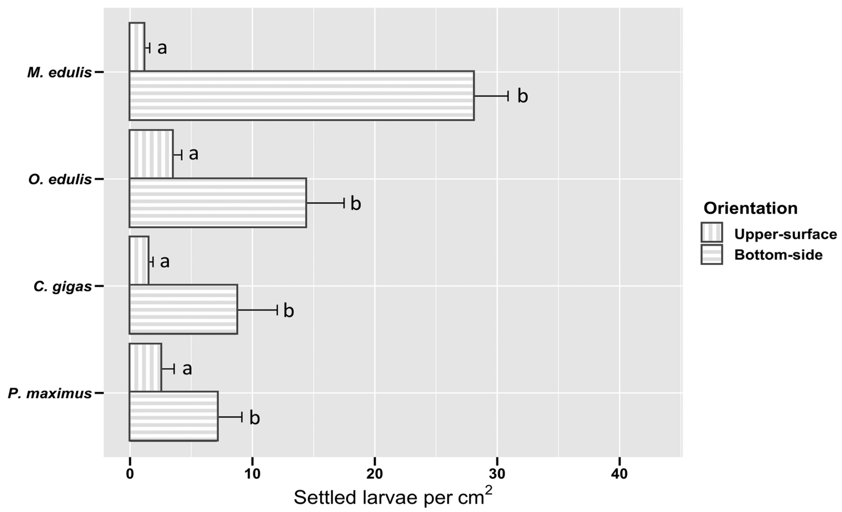


FIGURE 5 Settlement preference of *O. edulis* larvae on different substrate types in experiment 1 (hatchery): 1 = category of bivalve shells; 2 = category of inorganic materials; 3 = category of sandy sediments. Homogenous groups are marked with similar letter (Table 2 for details). Different larval densities (*N*) were used between the categories 1–2 and category 3 (see Section 2.1.3) and no statistical comparisons were done between categories

FIGURE 6 Effect of bivalve shell orientation (upper-surface and bottom-side) on larval settlement of *O. edulis* in experiment 1 (hatchery). Letters indicate significant differences between upper-surface and bottom side for each shell species



4.1 | Settlement preferences of hatched *Ostrea edulis* pediveligers

High larval concentrations were chosen for the hatchery experiments based on their relevance for commercial aquaculture production. This was designed to compensate for the potential loss of larvae owing to the high mortality of early life stages in hatchery production, but it also facilitated the identification of settlement preferences in controlled environments.

Key finding 1: *M. edulis* and *O. edulis* shells are the most preferred substrate types to produce *O. edulis* seed oysters in hatcheries.

In this study, a direct comparison of the larval settlement response to different shell substrates of species harvested in large volumes (hundreds of tonnes per year worldwide) was evaluated in a hatchery setting. Interestingly, settlement on *M. edulis* shells, and to a lesser extent on *O. edulis* shells, was significantly higher than on other shells. Recent laboratory experiments indicate a high settlement preference of *O. edulis* larvae on shells of life conspecifics in comparison with *C. gigas* shells (Rodriguez-Perez et al., 2019), which is consistent with the results here. However, further investigations on the settlement preference of *O. edulis* larvae between different substrates in the presence of live individuals should be carried out in order to dissociate substrate preferences and settlement cues. As an example, habitat-associated underwater sounds are a cue for *Crassostrea*

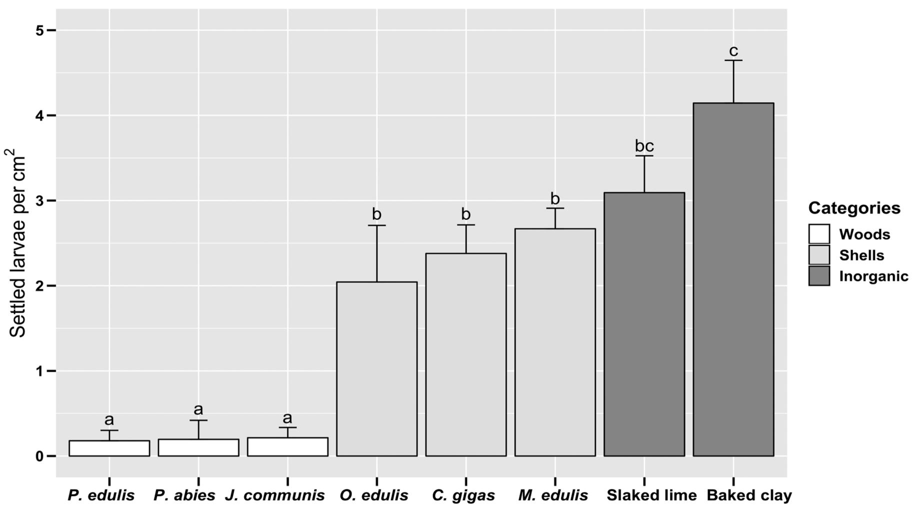


FIGURE 7 Results of field experiment on settlement preferences of *O. edulis* larvae on different substrate categories and types (orientations combined): wood materials (white), bivalve shells (light grey) and inorganic substrates (dark grey). All results presented exclude the limed substrate category; the comparison between hydrated lime and limed shells is provided in Figure S1. Homogenous groups are marked with the same letters

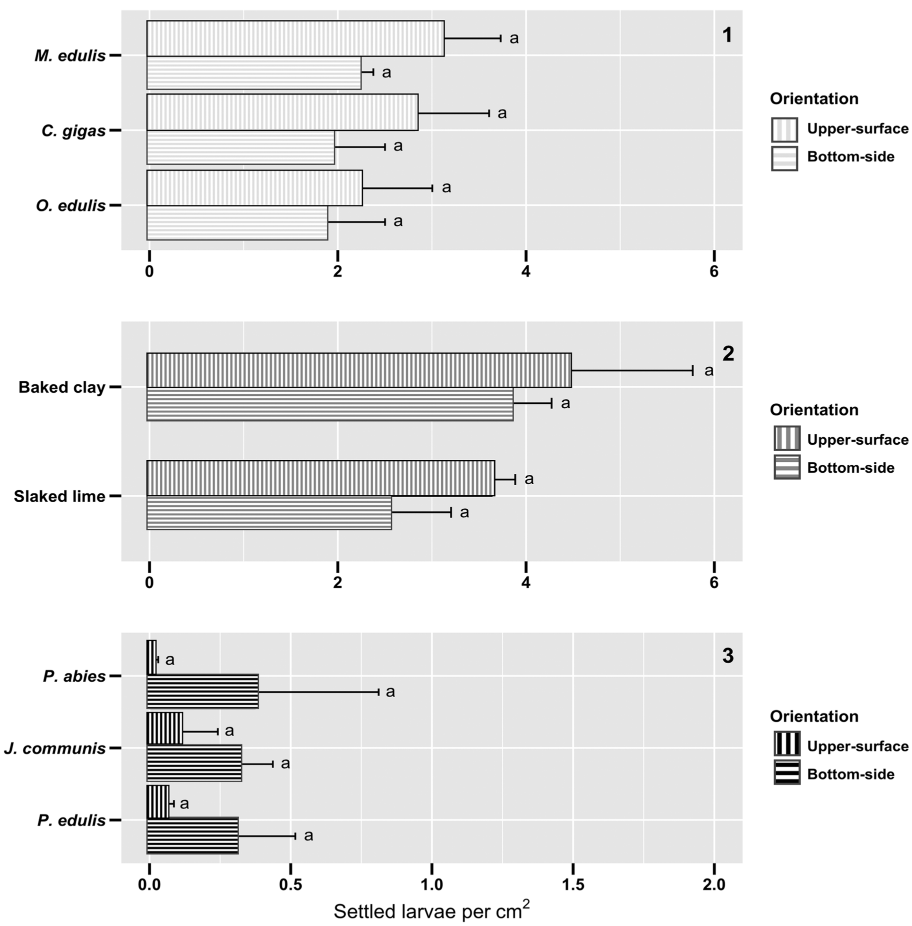


FIGURE 8 Effect of substrate orientation (upper-surface and bottom-side; irrespective of inner or outer surface of the valves) on the settlement of *O. edulis* larvae in the field: 1 = category of bivalve shells; 2 = category of inorganic materials; 3 = substrate category of wood materials. Homogenous groups are marked with the same letters

virginica (Lillis, Eggleston, & Bohnenstiehl, 2013). The significant attraction of *O. edulis* larvae to *M. edulis* shells may be related to shell colour and composition. According to Cole and Knight Jones (1949), the eventual blackening of oyster shells influences the settlement rate of *O. edulis* larvae and dark faces of the substrates seem to increase the settlement rate (Walne, 1974), possibly related to negative phototropism of the late larval stages at the time of settlement (Bracke & Polk, 1969), which merits further investigation. No direct comparison is possible between c-shells and c-inorganics in experiment 1 owing to the isolation of categories within the experimental design. However, we can conclude that the shell of recently bleached *M. edulis* is a very attractive substrate for the hatchery production of

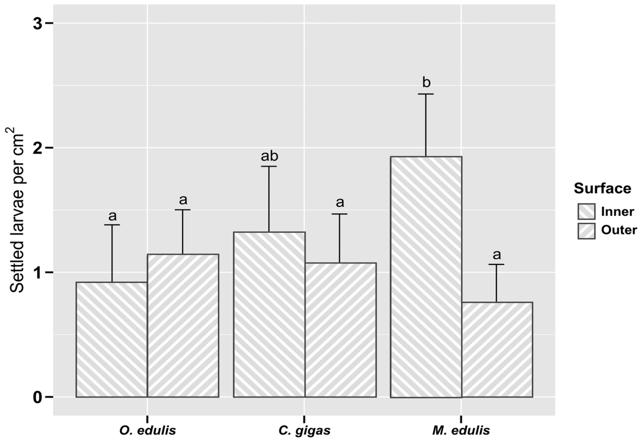


FIGURE 9 Settlement preferences of *O. edulis* larvae in the field between inner and outer shell surface (irrespective of upper-surface and bottom-side orientation). Inner surface of the shells here corresponds to their concave surface. Homogenous groups are marked with the same letters

spat-on-shell. The greater attractiveness of a substrate limits the loss of larvae by their settlement on tank walls or mortality during the search for an appropriate substrate.

Key finding 2: Lime and clay are ideal inorganic materials to use for *O. edulis* seed production in hatcheries. EMA was not identified as successful.

The significant preference for slaked lime and baked clay over EMA and granite is possibly related to its respective composition and/or surface texture, as the colours of all of these substrate types were similar: bright and whitish. The compositions of slaked lime, baked clay and granite are known (Figure S1 and Table S1); no analysis was performed for EMA. Slaked lime is calcium based and may resemble the composition of oyster shells. The clay has high silicate as well as high calcium and magnesium contents, again, similar to oyster shells (Medaković, Traverso, Bottino, & Popović, 2006; Yonge, 1960). Granite is made from quartz and feldspar and has a much coarser structure than clay and lime. In summary, clay and lime, whose composition is close to that of oyster shells, are adequate as components of nature-based reef structures used in hatchery production of oyster spat for ecological restoration.

Key finding 3: *O. edulis* larvae settle on shell fragments of sandy sediments.

No settlement preference was observed among the different size classes of the tested sediment types. The aim was to determine whether an increased settlement of *O. edulis* larvae could be expected on sediment, as a function of a grain size gradient. However, we did observe that larvae were fixed on the small shell pieces rather than the stone grains (Figure S2). These findings confirm the poor settlement rate of *O. edulis* larvae on granite and, furthermore, that high proportions of shell detritus in soft sediments may contribute to high substrate suitability for European flat oyster restoration in the field.

Key finding 4: Innovative 3D-sandstone reef structures show high settlement response of *O. edulis* larvae.

Three-dimensional-sandstone reefs (3D-ReefVival-Experimental-Reefs®) were produced specifically for the ecological restoration of *O. edulis* in the North Sea sublittoral. Producing spat on artificial reefs in a hatchery for recruitment-limited and substrate-limited areas is a promising approach to introduce certified disease-free young oysters on structures that are massive enough to potentially withstand the prevailing sediment dynamics. The settlement response on 3D-sandstone reefs was investigated for the first time and clearly showed successful results: settlement rates of *O. edulis* larvae on examined areas were confirmed while no settlement was observed on the tank walls, which indicates that larvae clearly preferred the substrate provided by the sandstone reefs. Living reefs were kept in tanks and will be used for further field studies. The counting of settled larvae was limited to accessible areas for visual inspection. In a next step, the applicability of 3D-sandstone reefs for *O. edulis* statement needs to be tested in the field.

4.2 | Settlement preferences of *Ostrea edulis* larvae in the field

Key finding 5: Clay, lime and bivalve shells are suitable substrate types to enhance recruitment in the field. Limed materials are attractive for larvae regardless of which shell material is coated.

Key finding 6: No significant differences in settlement preference were observed within the categories of bivalve shells or inorganic materials in the environment.

This finding clearly differs from results obtained in controlled conditions and confirms the importance of natural biofilms (Korringa, 1940; Rodriguez-Perez et al., 2019). It is possible that the formation of biofilms on the substrates in the field would successfully mask the differences in the original settlement response of the respective substrate types. Smyth et al. (2018) also found no difference in settlement rates on different shell types tested in field conditions. We assumed that marine biofilm development on these substrates may override the differences in *O. edulis* larvae settlement observed in the hatchery experiment and may play a major role in settlement response.

Key finding 7: *Ostrea edulis* larvae do not settle successfully on the tested wood materials.

Friele (1899) and Korringa (1976) described dried branches of common juniper as very good collectors for *O. edulis* larvae in breeding polls (Norway, Colsoul et al., submitted). In this study, cut and dried *J. communis* wood was used, which showed only a poor settlement response. This is assumed to be unrelated to the flat surface of the wooden substrates, as it is similar to inorganic substrates that showed the highest settlement rates. However, the structure of the surface may delay the development of a biofilm owing to the less pronounced roughness. Coating the wood materials with slaked lime failed, as it did not adhere to these materials: the smooth surfaces of *P. edulis* and *P. abies* are not suitable for retaining slaked lime.

4.3 | Orientation and surface

Following up on different settlement responses of upper-surface and of bottom-side shell areas obtained in the hatchery, both orientations were investigated in the field, including the potential effects of inner and outer shell surfaces.

Key finding 8: Differences in settlement preference regarding substrate orientation were significant in hatchery experiments, but not in the field.

In experiments 1 and 2, the majority of the larvae settled on the bottom-oriented surfaces of bivalve shells and of the 3D-sandstone reefs. Cole and Knight Jones (1939, 1949) also observed a significant number of settled larvae on bottom-oriented surfaces, which can be connected to the shadow that the bottom-sides provide and the negative phototropism identified for oyster larvae in their late stages by Bracke and Polk (1969) and Walne (1974). The larvae of *O. edulis* are active swimmers until their final settlement and move through the water column, driven by food availability and ideal stream layers for dispersal and settlement (Cranfield, 1973; Waller, 1981). In experiment 2 (Table 1) settlement occurred not only near the bottom, but over the entire reef height and with significantly greater settlement preference for the bottom-oriented areas in each layer. In contrast, no significant differences were observed between upper-surface and bottom-side surfaces in the field (experiment 3), where Korringa (1940) observed higher numbers of settled *O. edulis* larvae on upper- than bottom-side substrate surfaces. This could be related to potential effects of turbulent hydrodynamic conditions, in particular under laboratory conditions (down-welling systems) and should be included in future studies, especially in high-energy environments of designated oyster restoration sites in the open North Sea.

Key finding 9: Effects of inner and outer surface only apply for *M. edulis*.

Ostrea edulis larvae showed no significant settlement preferences for inner or outer shell surfaces of substrates, except for the concave surface of *M. edulis*. The preference for the inner shell surface of *M. edulis* may further indicate the influence of the surrounding hydrodynamics, as other tested shell types did not have the same hump shape.

4.4 | Implications and applications

Considering the requirements for ecological restoration of the European flat oyster, this study provides suggestions for the selection of sustainable, environmentally friendly and nature-based substrates, both for hatchery production and for implementation in the field. Existing studies have so far not addressed the direct comparison of similar substrates, nature, texture, composition, orientation and shape under hatchery and field conditions. Different settlement preferences of *O. edulis* larvae assessed in this study, in both hatchery experiments and in the field, provide some explanations for the contrasting results from the literature, which indicate that substrate factors influencing larval behaviour are still not well understood (Cole & Knight Jones, 1949; Korringa, 1940; Rodriguez-Perez et al., 2019; Smyth et al., 2018; Walne, 1974).

A clear and extremely relevant outcome of this study is the scientific confirmation that natural and native substrate types (*M. edulis* shells, *O. edulis* shells) as well as commonly used nature-based (lime) and innovative nature-based (clay, 3D printed sandstone) materials are useful for the ecological restoration of *O. edulis*. Accordingly, the implementation of artificial (e.g. concrete, plastics) substrate types can be avoided. This will minimize potential negative side-effects of active restoration measures and decrease biosecurity risks at the same time, as the introduction or translocation of non-native shell material (if not sterilized) may bring hitch-hiking, invasive species or diseases (Jeffs, 1999).

The high settlement response in *M. edulis* found in hatchery experiments is a key finding for hatchery production of seed oysters. Accordingly, hatchery production of single seeds could consider a similar composition of micro-cultch to increase larval settlement. *M. edulis* shells and *O. edulis* shells are appropriate substrates for the production of spat-on-shell in Europe. Furthermore, the high settlement response of bottom-side surfaces can be relevant for the production of spat-on-shell and spat-on-reef. For ecological restoration of *O. edulis*, it may be relevant that *M. edulis* shell disintegrates relatively quickly – less quickly than the slaked lime but faster than the shells of *O. edulis* and *C. gigas* (Korringa, 1976). Slaked lime also showed a high settlement response and can be applied to many substrates to increase larval settlement in hatcheries. However, high concentrations of slaked lime in recirculation systems can increase pH values, which may cause malformations of the larvae (Carbonnier et al., 1990). Baked clay also showed a high settlement response. As it can be produced in any 3D structure, its application as a reef structure, to be seeded with young oysters in the hatchery, is a relevant approach for future implementation of nature-based oyster reefs in the field.

As baked clay was proven to be the most attractive substrate in the field, a Europe-wide monitoring system of settlement rates could be established with clay plates. The need for common monitoring protocols to assess the success and ecological effects of oyster restoration is formulated in recommendation 5 of the Berlin Oyster Recommendation (Pogoda et al., 2019; Pogoda, Boudry, et al., 2020). Slaked lime and *M. edulis, O. edulis* and *C. gigas* shells all showed similar good settlement responses and are appropriate substrate types for enhancing recruitment in the field. With reference to the results of this study, substrates of wooden materials are not recommended for ecological restoration.

In conclusion, these results provide a comprehensive list and a scientifically established comparison of suitable substrates. The use of sustainable, environmentally friendly and nature-based substrates for the ecological restoration of *O. edulis*, which are presented here, is key to future developments in hatcheries and for restoration practitioners in the field. The identified substrates will on the one hand increase a sustainable and successful hatchery production of spat-on-shell and of three-dimensional reef structures for recruitment-limited areas, and on the other hand enhance the recruitment of spat in substrate-limited areas.

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