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Assessing the Potential of Innovative Clay Designs for Enhancing Oyster Reef Restoration

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Abstract

The restoration of European flat oyster (*Ostrea edulis*) population is critical for biodiversity and ecosystem services recovery. This study evaluates the potential of innovative clay substrates, developed and patented by Oyster Heaven, as cost-effective and degradable alternatives for large-scale oyster reef restoration. Field trials conducted in an oyster aquaculture site and hatchery experiments under controlled conditions assessed the larvae settlement efficiency, microbial biofilm composition, and environmental influences. Results showed significantly lower larvae recruitment on clay prototypes and spat collectors used as control, in wild conditions, with environmental factors such as temperature and hypoxia playing a key role. In the hatchery, larvae preferred oyster shells (17,174 ± 659 spats/m^2) over clay prototypes (2917 ± 111 spats/m^2) and spat collectors (1451 ± 160 spats/m^2). Microbial analysis revealed a shift towards Cyanobacteria and *Woeseia* species, with *Pseudoalteromonadaceae*, a genus previously linked to both stimulating and inhibiting larvae settlement, detected only on clay surfaces. Despite challenges, the clay prototypes demonstrated potential as sustainable substrates for restoration, particularly in managed "spats-on-reefs" strategies. This research highlights the importance of environmental monitoring, microbial interactions, and substrate optimization in enhancing restoration success for *O. edulis* populations. Further investigations are recommended to refine clay designs and assess their broader applicability under variable environmental conditions.

Keywords

Oyster Restoration, Larvae Settlement, Artificial Substrate, Microbial Biofilm, *Ostrea edulis*

1. Introduction

Biogenic reefs built by bivalves such as oysters provide ecosystem goods and services such as food and revenues for humans, habitat, shelter, spawning ground, settlement substrate, and food provisioning for a variety of marine species, including algae, invertebrates, fish and fish larvae, and crustaceans. Most importantly, oyster beds or reefs help maintain the water quality as bivalves are effective filters in the marine ecosystem. They feed on suspended particles, reducing turbidity and facilitating sunlight penetration for the growth of primary producers such as macroalgae and microalgae [1] [2].

About 100 years ago, the population of European flat oysters, *Ostrea edulis*, expanded over 25,000 km^2 of the North Sea floor in The Netherlands as reef structures [3]. However, a combination of overfishing, habitat degradation and diseases such as *bonamiosis*, have brought these native oysters to near extinction [4] [5]. It has been reported that sudden changes in an ecosystem, inducing loss in natural biomes, lead to the reduction of biodiversity accompanied by loss of ecosystem functions and services. This loss is referred to as a "recovery debt" and the more the lost ecosystem functions and services are accrued, the more tenuous the natural recovery pathway will be [6] [7]. Thus, the need for large scale restoration to minimize the associated ongoing damage [8].

Learnings from successful oyster restoration projects in the Chesapeake Bay, Pamlico Sound (North Carolina, USA), Strangford Lough (Northern Ireland, UK) and a few sites in Australia [9]-[14], suggest the need to aim for large scale restoration, high investments, an immediate source of competent larvae and the availability of suitable hard substrate for oyster settlement. Most studies confirmed oyster shells as the most effective substrate for promoting oyster growth. Unfortunately, oyster restoration efforts, in the USA and Australia have shown that the use of dead shells is not always sustainable [15]-[17]. Availability is a major limitation; shucked shells are in limited supply, and dredged shells are often unavailable, restricting their widespread use. One explanation is that the existing competing markets for the use of empty oyster shells, considered a rich source of calcium carbonate, include products such as artificial stone tables, construction materials, food supplements, pharmaceuticals, detergents, and animal feed [18] [19].

Generally, other shell types, such as hard and surf clam shells are less effective due to their insufficient interstitial space, which impacts oyster survival by limiting protective spaces against predators [16]. Interestingly, observation of the settlement behavior of *O. edulis* in the wild has shown that they can use shell fragments of other bivalves including Pacific oysters (*Magallena. gigas*), blue mussels (*Mytilus edulis*), clams (e.g. *Spisula solida*) and cockles (*Cerastoderma edule*) [13] [14]. Nevertheless, in addition to low availability, shells used for restoration require strict processing to avoid biosecurity issues, which is quite costly [20].

In order to support large scale restoration, scientists have turned to alternative substrates, both natural and artificial ones. An artificial substrate is considered efficient when larvae settle and develop onto it. It has been reported that larvae

prefer surfaces that provide good attachment points, often favoring rough or textured surfaces over smooth ones. As for composition, substrates similar to oyster shells, which are naturally calcified, often attract more larvae compared to other materials like plastic or metal [16]. Also, the presence and composition of microbial biofilms on the substrate are believed to play a critical role. Several microbial species, including Shewanella colwellia, have been linked with the settlement of marine invertebrates, including oysters, by emitting cues that help larvae locate the settlement site and facilitate their attachment and metamorphosis [21] [22].

Artificial materials such as concrete, porcelain, limestone, noncalcium stone and baked clay have been tested [14] [23] [24]. Concrete was reported to be more effective, supporting good oyster settlement and growth due to its interstitial spaces and stable structure. However, there are concerns about potential chemical leaching, especially with repurposed materials, which may limit its acceptability. Porcelain offered a habitat similar to natural reefs but resulted in smaller oyster growth compared to other substrates, which could restrict its effectiveness for achieving larger populations. Limestone performed well biologically, as its calcium content promotes oyster settlement, but its long-term benefits appear to plateau after several years, reducing its effectiveness over time. Noncalcium stones, such as granite, supported moderate oyster recruitment but lacked the calcium-based chemical properties that optimize oyster growth, making it a less ideal substrate [14] [16] [25].

Stone was moderately available and of medium performance but provided limited predator protection, which could affect long-term oyster survival. Crushed concrete and marl are rated highly in performance, with good surface area and protective features for oyster larvae, though economic and availability factors may influence their practicality. Finally, reef balls provide substantial surface area and durability for long-term reef construction, but higher costs associated with these engineered options could be a barrier for large-scale projects [16]. Colsoul *et al.* (2020) proposed baked clay as a substrate for European flat oyster larvae settlement, following field trials where it outperformed materials like slaked lime and wood.

The material's adaptability to be shaped into diverse 3D structures enhances its potential for building reef habitats that provide both protection and ample surface area for larvae. This makes baked clay a strong candidate for large-scale restoration projects, offering an eco-friendly alternative to concrete and reducing biosecurity risks linked to non-native shell materials. In addition, clay substrate has been used in the aquaculture industry to immobilize microorganisms supporting nitrification and denitrification, which are key processes in the marine nitrogen cycle and can further enhance the ability of oyster reefs to manage excess nitrogen [26].

In this study, we investigated the possibility of using a patented clay design, through the use of prototypes, to collect *O. edulis* larvae in the wild and under controlled conditions in a hatchery. We also monitored any shift in the microbial

biofilm on the surface of the clay substrate over time. The aim of this investigation was to evaluate the efficiency of using clay bricks as a substitute, artificial substrate for large scale restoration of the declining oyster population worldwide.

2. Materials and Methods

In this experiment, the possibility of using a cheaper, clay substrate to facilitate the settlement and growth of oyster larvae and achieved restoration at larger scale was tested.

2.1. Clay Prototypes

The prototypes were based on a clay structure developed by Oyster Heaven (https://oysterheaven.org/) and patented under the number 2032634, in The Netherlands [27]. Clay was molded into tiles of 20 cm × 26 cm, with a thickness of 2 cm. The top consisted of a rough surface achieved via an alternate inner and outer surface wave pattern, while the bottom remained smooth. These tiles were fired at 900˚C by a brick manufacturer (Wienerberger, Brunssum, The Netherlands). Once fired, the bricks were immediately wrapped and boxed to minimize contamination before delivery to the Danish Shellfish Centre (DTU Aqua, Nykøbing Mors, Denmark**).**

2.2. Biofilm Formation

Sixteen bricks, 16 oyster shells and 16 stacks of 22 spat collectors each, were placed in a 6 m × 0.6 m × 0.6 m raceway, containing flow through locally filtered seawater at 80 µm to reduce the presence of large plankton. These were left for 8 days in the raceways to allow for surface biofilm formation before being used for larvae settlement in the field study.

2.3. Field Study

In this experiment, clay substrate to facilitate settlement and growth in nature was tested, with spat collectors as the control substrate. The selected subtidal plot located at 56˚35'02.8"N 8˚17'49.0"E, within an oyster aquaculture site, to ensure suitability for oyster growth in terms of physiographic preference, food availability, seabed mobility, sedimentation, current velocity, tides and wave exposure. The study consisted of cages fitted with stacks of spat collectors (**Figure 1A**), one tile with the rough surface facing up (**Figure 1A(i)**) and the other reversed (**Figure 1A(ii)**). Each cage was fitted with a labelled buoy and a concrete anchor, and deployed randomly in the experimental plot, on the 4th week of June 2022, to limit the impact of independent variables. Monitoring was done weekly until the second week of September 2022. Depending on the weather forecast, the cages were lifted for visual observations and spat counts.

2.4. Hatchery Settlement Tests

The hatchery experiment was set-up in tanks. The tiles were cut into quarters.

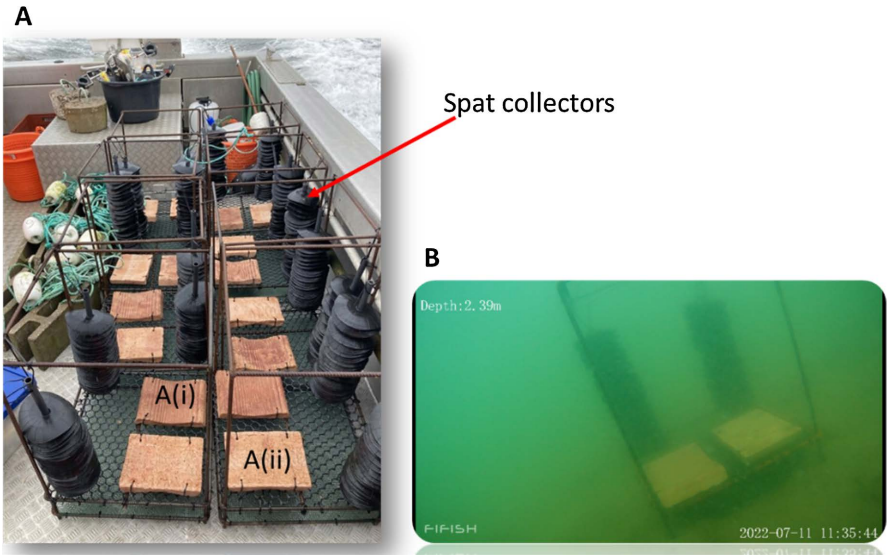

Figure 1. Experimental layout for the investigation of wild larvae settlement on clay prototypes with spat collectors as the control substrates, showing (A) cages prior to deployment each with 2 stacks of 22 spat collectors each and 2 clay prototypes secured with either (i) the rough surface facing up or (ii) the reverse. These were deployed in a subtidal plot (B) for wild settlement.

Empty oyster shells and spat collectors were both used as control substrates. To ensure that the 3 substrates have comparable surface area for settlement, each tank contained 4 oyster shells, cut spat collectors to the equivalent surface area, a quarter of the tiles with the rough surface facing up and the second quarter with the smooth surface facing up. The tanks were filled with UV 1 µm filtered seawater at 25˚C. Approximately 30,000 larvae were added per tanks and these were fed continuously with live microalgae (*Chaetoceros* sp., *Tisocrysis lutea* and *Pavlova gyrans*) cultured by the Danish Shellfish Centre (DTU Aqua, Nykøbing Mors, Denmark).

2.5. Water Quality Monitoring

A self-logger multiparameter sonde (AquaTROLL 600, In-Situ US), was deployed on site, 50 cm above the bottom, to measure pH, dissolved oxygen, salinity, turbidity and total dissolved solids (TDS) every 15minutes. Data was downloaded remotely and compared with reference data, where available (**Table 1**) [28]. To monitor water quality, the concentration of ammoniacal nitrogen, nitrite, nitrate, orthophosphate ions (bioavailable form of phosphate) and sulfate in the seawater was measured weekly using the methods described in the Hach Lange (DR 3900) manual.

2.6. Sample Collection for DNA Analysis

Samples were collected as previously described by Juste-Poinapen *et al.* (2024). Sterile cotton swabs were used to collect the biofilm from the surface of the tiles, oyster shells and spat collectors. The swabs were transferred to a 5 ml sterile

Table 1. Environmental variables, key to the reproduction, growth and survival of *O. edulis* adapted to Danish conditions. Where available optimum references have been included [28].

Parameters	Details
Salinity	Spawning: ≥20 PSU
	Larvae: ≥20 PSU
	Adult: ≥16 PSU
	Ideally approximately 25 - 35 PSU
Dissolved Oxygen	3.5 mg/L
Temperature	Reproduction: Denmark: optimal > 20˚C, range 18˚C - 23˚C
	Survival during summer: 26˚C - 30˚C
	Survival during winter: −1.5˚C
	Growth and normal metabolic function: 5˚C - 9˚C
pH	>6.9

cryogenic tube containing 3 ml of RNA later solution [29]. All tubes containing biofilm samples were kept at 4˚C overnight before being stored at −20˚C until they were sent to the Department of Molecular Medicine (Aarhus University Hospital, Denmark), for DNA analysis.

2.7. DNA Extraction & Sequence Processing

Replicates were submitted with code names to prevent analytical bias. All genomic DNA extraction and sequencing was performed by the staff of the Department of Molecular Medicine (Aarhus University Hospital, Denmark), The samples were thawed on ice and vortexed for 2 × 5 seconds. 200 µl were used for DNA-extraction using the Qiagen DNeasy PowerBiofilm Kit. The DNA extraction was performed as per Manufacturer's instructions, with bead beading using TissueLyser II for 2 × 5 min at 25 Hz. The DNA concentration was measured by Qubit HS (Thermo Fisher Scientific).

Amplicon libraries were prepared with a first PCR using the V4 16S SSU rRNA primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTAC-NVGGGTWTCTAAT-3') with overhangs matching the Nextera XT-indices [29] [30]. The PCR mix contained 14 µl DNA-extract, 0.5 µl 10 µM forward and reverse primer and 10 µl 2 × KAPA HiFi Hot Start Ready-mix. The PCR was done with 3 min denaturation at 94˚C, 35 cycles of 45 sec denaturation at 94˚C, 60 sec annealing at 50˚C and 90 sec elongations at 72˚C, and a final elongation for 10 min at 72˚C. The PCR products were cleaned up with Ampure XP beads at 0.8× ratio. Following cleanup and QC, we prepared an equimolar pool of the amplicon libraries which was sequenced on an MiSeq Nano V2 flow cell 2 × 150 bp PE. The raw sequencing data was processed using cutadapt for adaptor removal and DADA2 (Version 1.28.0) for quality filtering and taxonomic characterisation. The

SILVA-database as reference for the creation of ASV-tables. It is important to note that such identifications may change over time if new sequences with closer relationships are uploaded to the database.

2.8. Data Analysis

Mean and standard error at 95% Confidence Interval (CI) for biological repeats and bar charts were generated in Microsoft Excel. Sequencing data ASV-tables received from the Department of Molecular Medicine (Aarhus University Hospital, Denmark) were further analyzed for statistical significance using the RStudio IDE (version 2022.7.1.554) running R version 4.1.0 (2021-05-18). Heatmaps were generated with the "tidyverse" (version 2.0.0) and "pheatmap" (version 1.0.12) packages using RStudio (2023.12.1+402). Abundance data was first normalized by Hellinger transformation and the top 50 operational taxonomic units were then selected for heatmap generation.

3. Results and Discussion

3.1. Larvae Recruitment at Sea

The cages with clay prototypes and spat collectors were deployed at the oyster farm in summertime, when spawning was expected by oyster farmers. The European flat oyster (*Ostrea edulis*) typically spawns from June to September, with peak activity during the warmer months. The number of spats recruited on each substrate was quite low with an estimated 1.56 spats/m^2 for the clay prototype and 6.00 spats/m^2 for the spat collectors. In **Figure 2**, we observed that both the clay prototype (**Figure 2A**) and the spat collectors (**Figure 2B**) were colonised by seaworms (mainly the tubeworm *Spirobranchus triqueter*) and different species of ascidians (mainly European sea squirt *Ascidiella aspersa*), with only one settled juvenile *O. edulis* (spats). One explanation can be linked to the fact that the site was characterised by significant spatial and temporal variation in larvae density across the sampling stations and time periods (**Table 2**).

At KFOI (2.7 m), larvae density peaked in July (106.1 larvae/m^3 in Week 4) after moderate levels in June (11.8 larvae/m^3 in Week 2), with no larvae detected in August and September. KFO1 (3.7 m) exhibited generally low densities, with notable increases in late June (8.6 larvae/m^3) and July (34.4 larvae/m^3 in Week 4), while GSA East Port (4.5 m) showed moderate larvae densities in June (7.1 larvae/m^3 in Week 2) but no measurements were taken in July and August due to bad weather conditions. By August and September, larvae density was consistently zero across all stations, indicating a seasonal decline. KFOI consistently had the highest densities, suggesting it may provide more favorable conditions for larvae. These trends highlight the combined impact of environmental variability and logistical challenges on data collection. The low density associated with spawning this year, could also explain the excessive biofouling observed on both the clay prototypes and the spat collectors. Biofouling on artificial substrates would limit the space for larvae settlement [31].

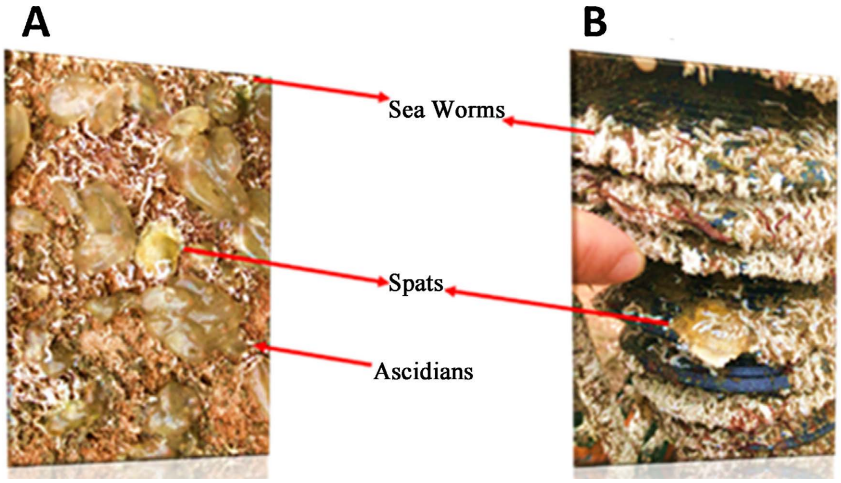

Figure 2. Comparative images of settlement and growth on one submerged (A) clay prototype and (B) one spat collector, 6 weeks after deployment, including one juvenile oyster (spat) and associated biofouling layers composed of worms and sea squirts.

Table 2. *O. edulis* larvae density measured, once a week (Wk), at 3 different locations and depth, around the deployment area, before and after deployment of substrates at sea. Some measurements were not recorded due to bad weather conditions.

Sampling Dates	Larvae Density (Number of Larvae/m³ of Seawater) at Each Sampling Station, Depth		
	KFOI, 2.7 m	KFO1, 3.7 m	GSA East Port, 4.5 m
June-Wk 2	11.8	0	7.1
June-Wk 3	0	0	0
June-Wk 4	0	8.6	0
July-Wk 1	35.4	0	Not measured
July-Wk 2	23.6	0	Not measured
July-Wk 3	0	0	Not measured
July-Wk 4	106.1	34.4	Not measured
Aug-Wk 1	0	Not measured	Not measured
Aug-Wk 2	0	0	0
Aug-Wk 3	0	0	0
Sep-Wk 2	0	0	0

3.2. Changes in Selected Abiotic Variables

Universal environmental variables (abiotic) are known to impact oyster growth, reproduction and survival [1] [32]. The analysed, online readings recorded at Lemvig in 2022 shows temporal variations in environmental parameters, including temperature (˚C), dissolved oxygen (DO) (mg/L), pH, and salinity (PSU).

Temperature fluctuated seasonally, peaking in July and gradually declining towards September, while brief gaps in data correspond to probe maintenance.

Dissolved oxygen levels were relatively stable, between 7.5 - 8.5 mg/L but display minor fluctuations, likely linked to changes in temperature and biological activity. The pH remained consistently between 7.8 and 8.2, indicating stable water chemistry suitable for oysters, with minor interruptions also related to probe maintenance. Salinity varied between 30.0 - 32.5 PSU, with a gradual increase from late June to September, reflecting seasonal influences such as reduced freshwater input or increased evaporation.

While, the dataset highlights stable environmental conditions interspersed with maintenance-related data gaps, low larvae density was still observed. There have been reports of a general reduction in the Ostrea edulis population and broodstock in the Limfjorden which led to a fishing ban in 2022 but this was later eased.

The above results would suggest an impact from temperature. Spawning is closely linked to water temperatures, generally commencing when temperatures reach around 15˚C - 20˚C [30]. Previous studies have reported that the reproduction of O. edulis is stimulated at 15˚C - 20˚C. However, the onset of spawning in the Limfjorden (Denmark) is triggered around 20˚C [32]-[34]. The variation in temperature remained below or close to 20˚C all throughout this experiment (**Figure 3**).

3.3. Sea Water Quality Parameters

To assess the water quality at the study site, the trend for the concentration of

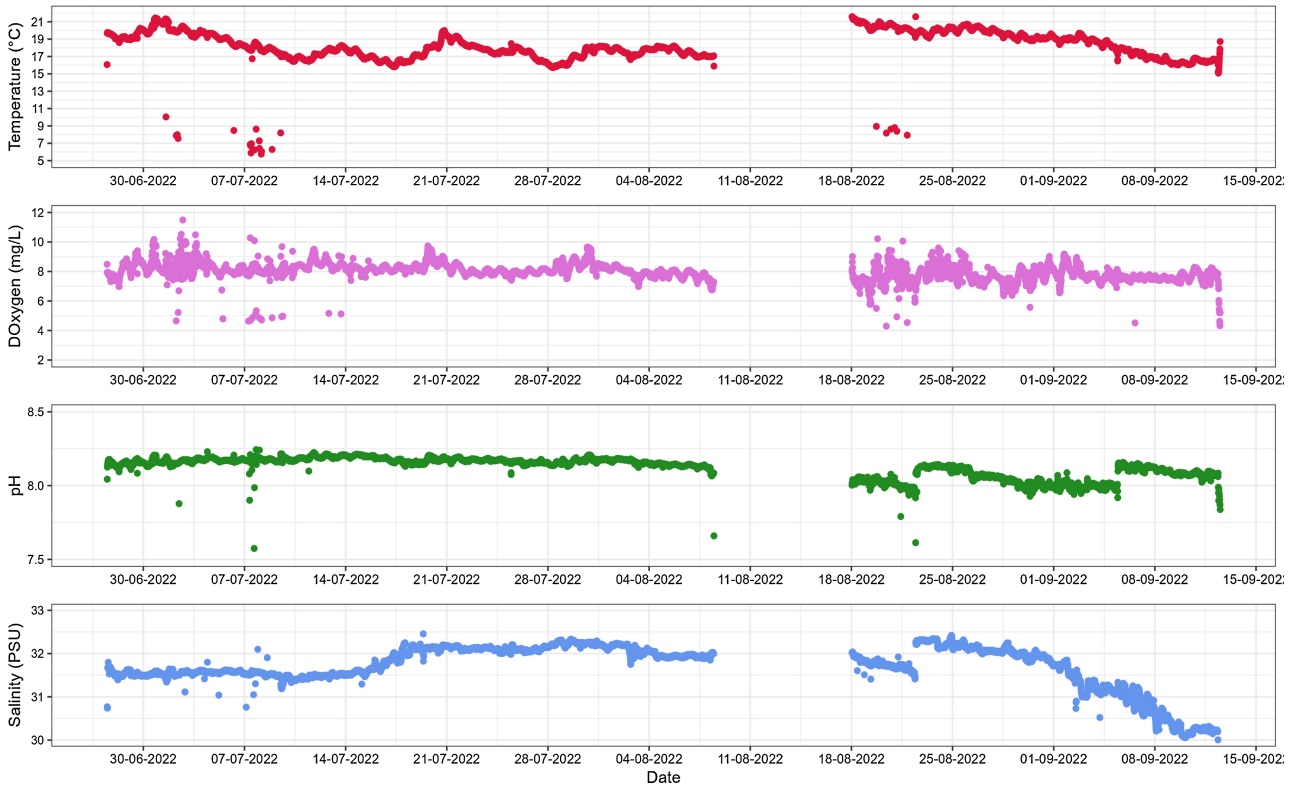

Figure 3. Changes in the average weekly seawater abiotic variables at the test site in the Limfjorden (56˚35'02.8"N 8˚17'49.0"E), from the 27 of June 2022 (June-Wk 4) to the 12 of September 2022 (Sep-Wk 2). The parameters measured include turbidity, salinity, pH, DO and temperature. All of which have been shown to influence spawning in *O. edulis*.

inorganic nutrients, such as dissolved inorganic nitrogen (DIN-sum of ammonia, nitrates and nitrites), sulfates and phosphates (orthophosphate ions), was recorded (**Figures 4A-C**) [35]. For the first 3 weeks, the concentration of DIN (μM/L) was mostly measured around 3.30, with a higher value of 3.100 ± 0.385 in the 3rd week of July, and the lowest at 1.31 ± 0.13, in the first week of August (**Figure 4A**). According to the European Environment Agency (EEA), DIN concentration less than 5 μM/L is considered as 'very low'. Due to their filter-feeding ability, oysters can mitigate both the amount of nutrient and phytoplankton in the water [1] [2]. As such, variations in the concentration of DIN have not been shown to affect native oysters [36] [37].

With regards to sulfate and orthophosphate ions, both can reach the ocean through runoffs. With an expected average concentration of 28mM sulfate in seawater [38], all the values registered at the test site in the Limfjorden was below 20mM (**Figure 4B**). Orthophosphate ion is another key element for growth of marine species, meaning that excess availability can result in high uptake by phytoplankton, favoring outgrowth and algal bloom [39]. In this experiment, the concentration measured ranged between 15 - 20 μg/L, peaking at 22.030 ± 0.210 μg/L during the first week of July before dropping down to 15.610 ± 0.080 the following week. The lowest value was recorded on the final sampling day at 11.650 ± 0.580 (**Figure 4C**). According to the EEA, if the concentration of orthophosphate ions in seawater is within the range of 10.0 - 20.0 μM/L, it is termed as

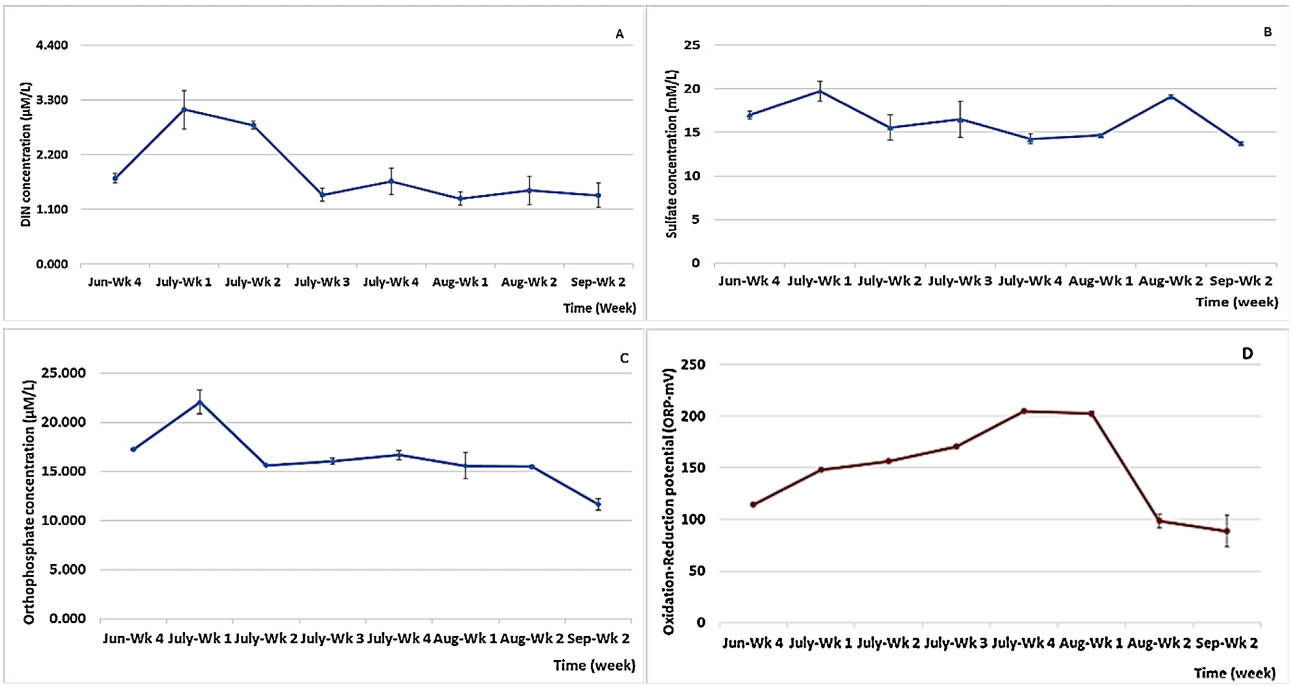

Figure 4. Changes in the average weekly concentration of selected inorganic nutrients (mg/L) at the test site in the Limfjorden (56˚35'02.8"N 8˚17'49.0"E), from the 27 of June 2022 (June-Wk 4) to the 12 of September 2022 (Sep-Wk 2), including (A) DIN (dissolved inorganic nitrogen, including ammonia, nitrates and nitrites) in μM/L, (B) sulfates in mM/L and (C) orthophosphate ions in μM/L. Also included is the trend in the oxidation-reduction potential (mV) of the test site over the same period of time. The error bars represent the standard error at a 95% confidence interval.

'moderate' and between 20.0 - 40.0 µM/L is regarded as high [40].

The moderate to high amount of soluble phosphate in the seawater could be the result of the release of active phosphate, otherwise buried in the sediment. The ORP values recorded, (mostly below +100 mV, **Figure 4D**) seem to support this explanation, since the ORP of a healthy marine ecosystem typically ranges between +200 mV and +400 mV, indicating a well-oxygenated water condition, crucial for supporting diverse marine life and maintaining overall ecosystem health. Results below +200 mV indicate reducing conditions, often associated with hypoxia (low oxygen) or anoxia (no oxygen) and the release of harmful substances like hydrogen sulfide, with the mobilization of toxic metals, and release of phosphate into the water column [41]-[43].

3.4. Changes in the Associated Microbial Biofilm

The microbial composition on both substrates underwent significant changes over 12 weeks, with a notable increase in Cyanobacterial species by Week 12. **Table 2**

Table 2. Summary of the presence of various microbial taxa, in descending order of percentage relative abundance, at different time points (T0, Week 4, Week 12) on the clay prototypes. The microbial taxa are identified by their taxonomic classification (e.g., genus, family, order, class) and operational taxonomic units (OTUs).

Clay Prototypes		
T0	**Week 4**	**Week 12**
g_Candidatus Nitrosopumillus (OTU 24)	c_Gammaproteobacteria (OTU 10)	c_Cyanobacteria (OTU 15)
o_BD7-8 (OTU 13)	*f_Desulfocapsaceae* (OTU 11)	*g_Ferrimonas* (OTU 64)
g_Candidatus Nitrosopumillus (OTU 53)	*g_Pseudoheliea* (OTU 28)	*g_Synechoccus* CC9902 (OTU 62)
c_Gammaproteobacteria (OTU 10)	c_Cyanobacteria (OTU 21)	*g_Cyanobium* PCC-6307 (OTU 89)
f_Sva1033 (OTU 14)	f_Sva1033 (OTU 14)	*f_Desulfocapsaceae* (OTU 11)
g_Cycloclasticus (OTU 68)	*g_Synechococcus CC9902* (OTU 36)	c_Cyanobacteria (OTU 109)
f_Desulfocapsaceae (OTU 11)	*g_Woeseia* (OTU 19)	c_Cyanobacteria (OTU 3)
g_Woeseia (OTU 20)	*c_Cyanobacteria* (OTU 12)	*g_Cyanobium* PCC-6307 (OTU 92)
o_B2M28 (OTU 22)	*c_Cyanobacteria* (OTU 23)	c_Cyanobacteria (OTU 12)
g_*Candidatus Nitrosopumillus* (OTU 67)	*c_Cyanobacteria* (OTU 51)	*f_Flavobavteriaceae* (OTU 125)
g_Poseidonibacter (OTU 94)	o_BD7-8 (OTU 13)	c_Cyanobacteria (OTU 101)
g_Woeseia (OTU 31)	*f_Flavobavteriaceae* (OTU 26)	c_Gammaproteobacteria (OTU 10)
g_Arcticiflavibacter (OTU 16)	o_B2M28 (OTU 22)	*g_Thalassotalea* (OTU 185)
f_Kangiellaceae (OTU 26)	*g_Vibrio* (OTU 112)	c_Cyanobacteria (OTU 23)
g_Woeseia (OTU 19)	*c_Cyanobacteria* (OTU 96)	*g_Halioglobus* (OTU 39)
o_BD7-8 (OTU 35)	*g_Woeseia* (OTU 43)	*g_Synechoccus* CC9902 (OTU 36)
g_Aliivibrio (OTU 58)	c_Cyanobacteria (OTU 3)	f_Sva1033 (OTU 14)
g_Thiothrix (OTU 153)	*g_Halioglobus* (OTU 55)	*g_Lutibacter* (OTU 140)
g_Maritimimonas (OTU 46)	*g_Woeseia* (OTU 20)	*g_Flavicella* (OTU 173)
c_Cyanobacteria (OTU 23)	*c_Cyanobacteria* (OTU 50)	o_B2M28 (OTU 22)

shows the evolution of microbial communities over time, at T0, Week 4, and Week 12. The presence of different microbial taxa varies, indicating changes in the microbial composition of the clay prototypes over these time periods. T0 shows a variety of microbes including different species of Candidatus Nitrosopumillus and Woeseia, while Week 4 sees an increased presence of various Cyanobacteria species and Woeseia, among others. Week 12 features a dominance of Cyanobacteria and various other genera such as *Ferrimonas*, *Synechococcus*, and *Halioglobus*.

In comparison, Table 3 shows the microbial community dynamics over the three time points for the biofilm collected on the spat collectors. T0 was characterised by the dominance of Gammaproteobacteria, *Desulfocapsaceae*, and various other taxa such as *Arcticiflavibacter*, *Colwellia*, and *Woeseia*. Similar to the clay prototypes, an emergence of various Cyanobacteria species, was seen as from

Table 3. Summary of the presence of various microbial taxa, in descending order of percentage relative abundance, at different time points (T0, Week 4, Week 12) on the spat collectors. The microbial taxa are identified by their taxonomic classification (e.g., genus, family, order, class) and operational taxonomic units (OTUs).

Spat Collectors		
T0	**Week 4**	**Week 12**
c_Gammaproteobacteria (OTU 10)	*c_Cyanobacteria* (OTU 21)	*f_Desulfocapsaceae* (OTU 11)
f_Desulfocapsaceae (OTU 11)	*g_Coxiella* (OTU 60)	*c_Cyanobacteria (OTU 15)*
g_Arcticiflavibacter (OTU 16)	*g_Pseudoheliea* (OTU 28)	*c_Cyanobacteria* (OTU 23)
g_Colwellia (OTU 34)	*f_Flavobavteriaceae* (OTU 145)	*c_Cyanobacteria* (OTU 41)
g_Woeseia (OTU 31)	*c_Cyanobacteria* (OTU 96)	*f_Sva1033* (OTU 14)
g_Woeseia (OTU 20)	*c_Cyanobacteria* (OTU 187)	*c_Cyanobacteria* (OTU 12)
g_Poseidonibacter (OTU 94)	c_Gammaproteobacteria (OTU 10)	*c_Cyanobacteria* (OTU 3)
f_Kangiellaceae (OTU 26)	*g_Synechoccus* CC9902 (OTU 36)	c_Gammaproteobacteria (OTU 10)
o_BD7-8 (OTU 13)	*c_Cyanobacteria* (OTU 23)	*c_Cyanobacteria* (OTU 12)
g_Woeseia (OTU 19)	*g_Colwellia* (OTU 52)	*c_Cyanobacteria* (OTU 50)
f_Sva0081 sediment group (OTU 80)	*f_Desulfocapsaceae* (OTU 11)	o_B2M28 (OTU 22)
o_B2M28 (OTU 22)	o_BD7-8 (OTU 13)	*g_Synechoccus* CC9902 (OTU 62)
f_Sva1033 (OTU 14)	*g_Woeseia* (OTU 19)	*f_Thiotrichaceae* (OTU 85)
g_Lutimonas (OTU 38)	*g_Arcticiflavibacter* (*OTU 16*)	*f_Sandaracinaceae* (OTU 66)
g_Maricaulis (OTU 285)	*g_Woeseia* (OTU 43)	*g_Halioglobus* (OTU 39)
g_Woeseia (OTU 69)	*f_Sva1033* (OTU 14)	*g_Woeseia* (OTU 43)
g_Halioglobus (OTU 102)	*c_Cyanobacteria* (OTU 48)	*c_Cyanobacteria* (OTU 93)
c_Gammaproteobacteria (OTU 57)	*g_Thalassomonas* (OTU 110)	*f_Pseudoalteromonadaceae* (OTU 157)
g_Halioglobus (OTU 55)	c_Cyanobacteria (OTU 50)	*g_Cyanobium* PCC-6307 (OTU 92)
g_Cycloclaticus (OTU 68)	*g_Woeseia* (OTU 20)	o_B2M28 (OTU 61)

week 4, indicating a shift towards photosynthetic microbes, in addition to the presence of *Coxiella*, *Pseudoheliea*, and an increase in *Woeseia* and *Synechococcus* species. There was a further increase in Cyanobacteria species at week 12, coupled with the continued presence of *Desulfocapsaceae*, indicating stable anoxic conditions.

The introduction of *Thiotrichaceae*, *Sandaracinaceae*, and *Pseudoalteromonadaceae* might be indicators of an evolving environmental conditions or nutrient availability. The first OTU related to the genus *Shewanella* (OTU 252), previously associated with stimulating larvae settlement, was detected at only after 12 weeks at a relative abundance of 0.5 ± 0.2.

The changes in microbial communities over these periods, driven mainly by *Cyanobacteria* and *Woeseia* species, suggest shifts possibly due to environmental conditions, the type of substrate, or possibly the microbial interactions within the natural clay material. Cyanobacteria is a common bacterium in sea water, where they use the energy from the sun to use part of the nitrogen produced during denitrification to produce Ammonium (NH_4^+) compounds through a process known as nitrogen fixation. They are also known indicators of algal bloom and some species, such as Synechococcus, have a symbiotic relationship with ascidians [44]-[46].

The presence and abundance of *Woeseia* in marine sediments are indicators of specific environmental conditions, such as organic matter availability and redox potential since these bacteria contribute to the decomposition of organic matter in marine sediments, breaking down complex organic compounds into simpler molecules, making nutrients available to other marine organisms, and are involved in the cycling of essential nutrients, such as carbon, nitrogen, and sulfur [47]. For example, some *Woeseia* species are known to carry out sulfate reduction which is crucial for the sulfur cycle in marine sediments, where it influences the availability of sulfur compounds for other microorganisms. They can also switch to nitrite reduction [47] [48].

3.5. Interaction between Measured Parameters

Often in nature, different species or environmental factors influence each other's presence, abundance, and behavior. In this experiment, the recorded ORP value below +200 mV, moderate concentrations of soluble phosphate, and the increasing abundance of Cyanobacteria and *Woeseia* could be interconnected.

Ideally, high nutrient levels increase primary production, providing more food (phytoplankton) for filter-feeding oysters. If managed properly and without leading to harmful hypoxia, this could support better growth and potentially more robust spawning, unlike low ORP conditions leading to hypoxia that can stress or kill adult oysters, impair their ability to spawn, and adversely affect developing larvae. Hypoxic conditions can also reduce the availability of suitable habitats for oysters and limit their food supply, as filter feeders rely on oxygen-rich waters for optimal feeding [52]. Reducing conditions, also facilitates the release of nutrients

like phosphate, which promotes the growth of Cyanobacteria and support anaerobic bacteria like *Woeseia*, which thrive in low-oxygen environments [49].

In addition, a low ORP would favor anaerobic or facultative anaerobic bacteria, including iron-reducing (*Ferrimonas*), sulfate-reducing (*Desulfocapsaceae*), and sulfur-oxidizing bacteria (*Thiotrichaceae*). Nutrient-rich conditions, with soluble phosphate availability, would explain the growth of heterotrophic and nutrient-cycling bacteria such as *Halioglobus*, *Arcticiflavibacter*, *Colwellia*, *Pseudoheliea*, *Sandaracinaceae* and *Pseudoalteromonadaceae* [50]-[52].

Nutrient cycling microorganisms can play beneficial roles in maintaining a balanced environment that supports oyster health. For instance, bacteria involved in organic matter decomposition, such as *Halioglobus* and *Pseudoalteromonadaceae* can help recycle nutrients in a form usable by phytoplankton, which in turn supports the oyster food web. Furthermore, increased bacterial activity in reducing conditions can also lead to the production of other harmful metabolites that can negatively impact oyster health. For example, *Desulfocapsaceae*, produce hydrogen sulfide (H_2S) as a byproduct, toxic to marine life, including oysters and their larvae, affecting their survival and development [53]. Low oxygen and high nutrient environments can also promote the growth of pathogenic bacteria, such as some species of *Coxiella* and other opportunistic pathogens. These bacteria can cause diseases in oysters, reducing their reproductive success and increasing mortality rates among larvae [54].

These findings suggest that the observed environmental conditions, including low ORP values, nutrient-rich but oxygen-depleted waters, and the proliferation of anaerobic and pathogenic bacteria, likely contributed to the low oyster settlement rates in this experiment. The potential interplay between hypoxia, harmful metabolites like hydrogen sulfide, and the presence of opportunistic pathogens may have created an environment unsuitable for oyster larvae survival and settlement, highlighting the need for targeted mitigation strategies to improve conditions for successful restoration efforts.

3.6. Hatchery Settlement Test

Settlement tests under controlled conditions were also carried out on the clay tiles, with oyster shells and spat collectors as controls and an average of 27,333 ± 302 oyster larvae dispersed in each of the 6 tanks. After 2 weeks, healthy spats were observed on all substrates with a clear preference for the oyster shells (**Figure 5**). This was expected, since oyster larvae have been shown to favor shells structures for settlement in the wild [13]. The average percentage settlements per substrate were 0.41% ± 0.04%, 0.64% ± 0.02% and 3.15% ± 0.12% for spats collectors, clay prototypes and oyster shells respectively, and the highest number of spats was observed on oyster shells at 17,174 ± 659 spats/m^2, followed by the clay prototypes with 2917 ± 111 spats/m^2, while spat collectors had the least number of spats at 1451 ± 160 spats/m^2 (**Figure 5**).

These results highlight the potential of clay prototypes as a viable alternative

substrate for oyster larvae settlement. While oyster shells showed the highest settlement rates and spat density, the clay prototypes outperformed the spat collectors. This demonstrates that the clay prototypes provide a superior settlement surface compared to spat collectors, which are less natural in composition. Additionally, the clay prototypes offer a significant surface area advantage over oyster shells (Figure 6A and Figure 6B), which are limited by their shape and availability in large-scale restoration projects. As a biodegradable and scalable material, clay prototypes combine the ecological benefits of a natural substrate with the practicality of a manufactured material.

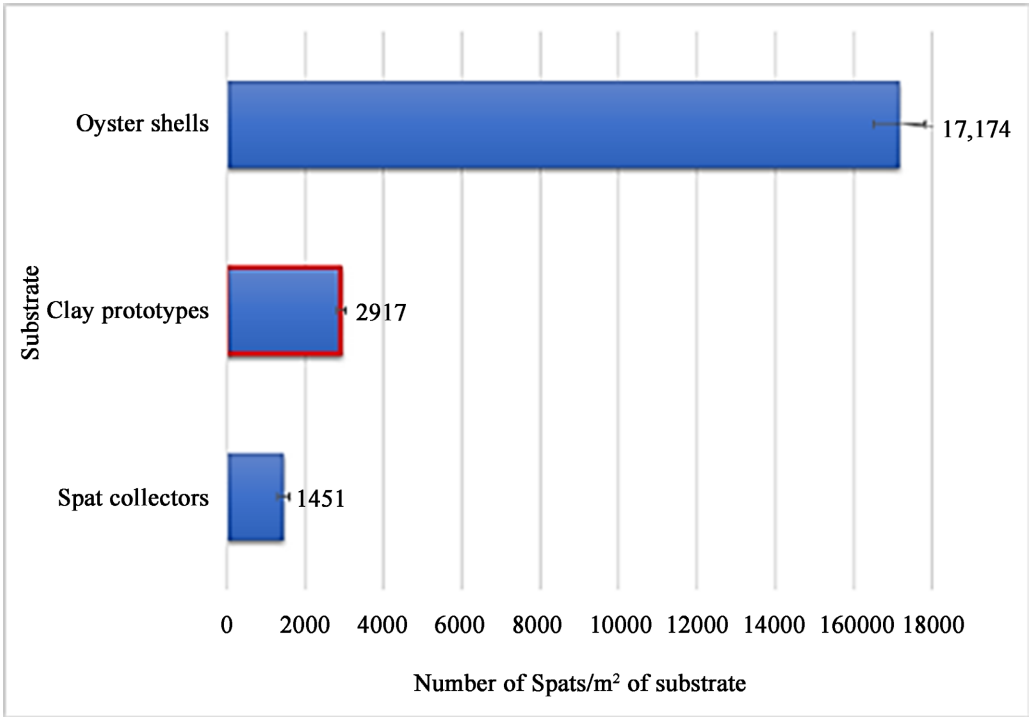

Figure 5. Average numbers of spats per m^2 of surface area of substrate used including oyster shells (area = 506 cm^2), clay prototypes (area = 600 cm^2) and spat collectors (area = 382 cm^2).

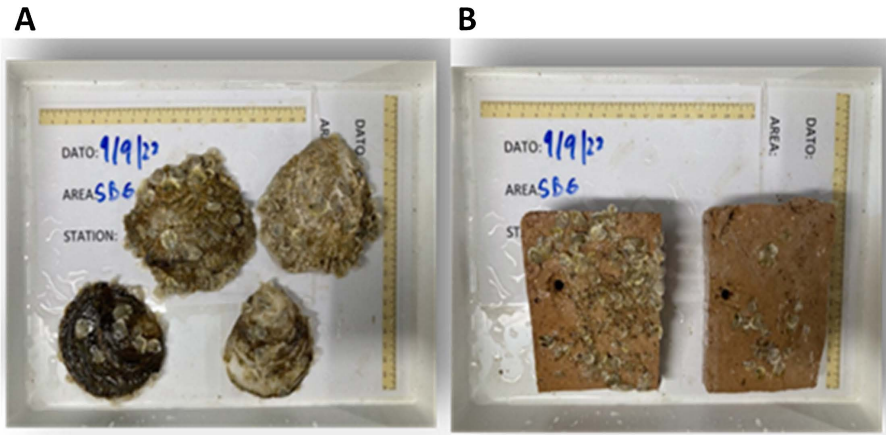

Figure 6. Comparison of the spat settlement matrix on the (A) oyster shells with that of (B) clay prototypes from tank number 6 of the preference settlement experiment.

3.7. Comparing the Microbial Biofilm on Both Substrates

Previous research mentioned the important role of microbial biofilm in facilitating larvae settlement, possibly through the emission of chemical cues [21] [22] [55]. Before the experiment, the biofilm from both oyster shells and clay prototypes was swabbed and characterised by DNA analysis. After analysis, 818 Operational taxonomic units (OTUs) were identified at the closest taxonomic ID. Separate heat maps for each substrate were generated to showcase any difference in both composition and relative abundance of the microbial population (**Figure 7** and **Figure 8**). Since the water in the raceway was 80 µm drum filtered and the bricks were sterile, it can be assumed that the microorganism for the biofilm was contributed from both the water in the raceway and the oyster shells from the wild. Differences in dominance and abundance could be observed.

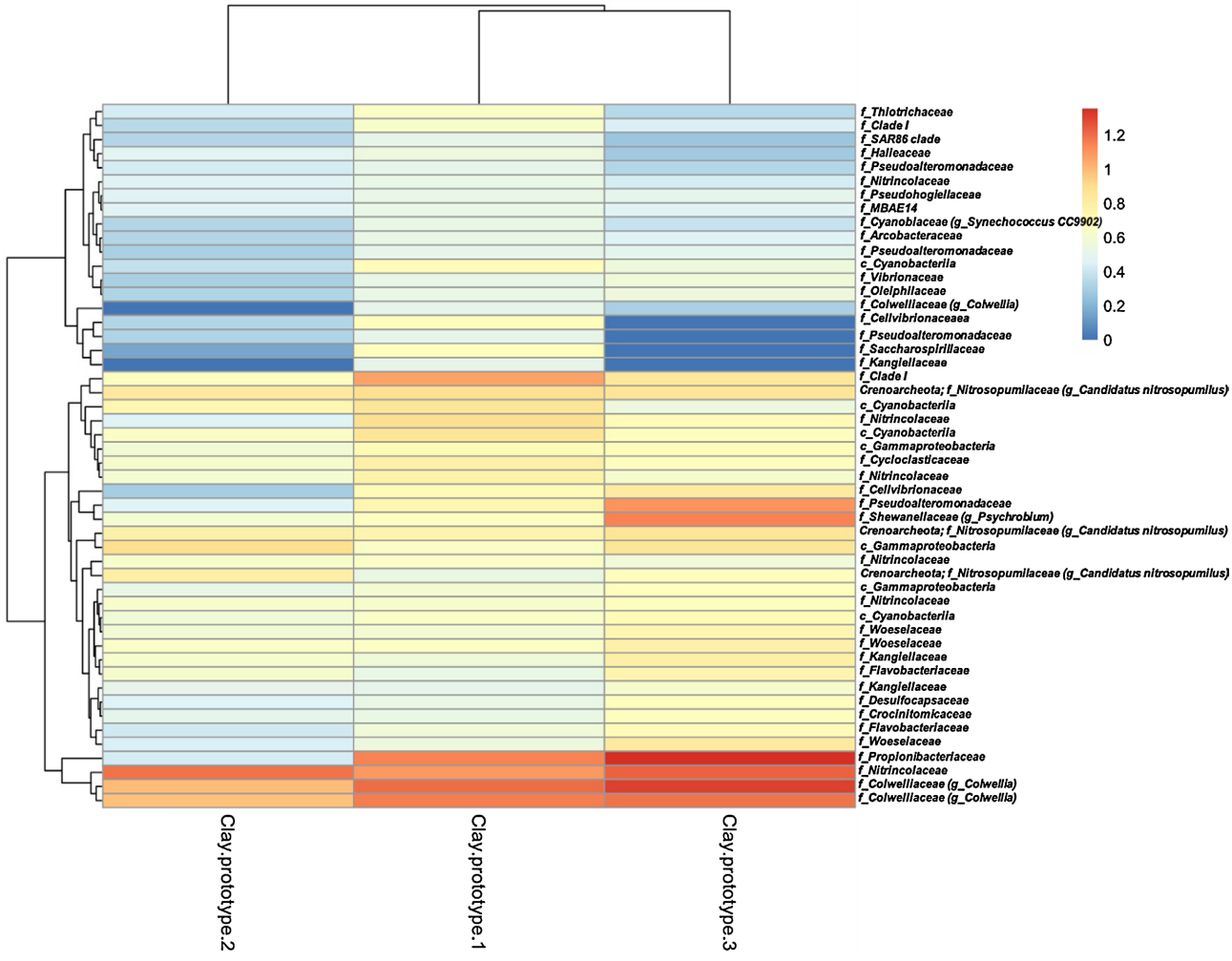

Figure 7. Heatmap representing the relative abundance of the top 50 most abundant Operational Taxonomic Units (OTUs) associated with bacterial and archaeal taxa across three clay prototypes. Rows represent different taxa, while columns correspond to the prototypes (Clay-prototype 1, Clay-prototype 2, and Clay-prototype 3). Colors indicate relative abundance, with blue representing low abundance, yellow moderate abundance, and red high abundance, as shown in the color scale. Clustering dendrograms on the axes indicate similarities in taxa composition and distribution across prototypes. This figure highlights the variation in community structure among the prototypes. The values represent the percentage of the normalized fraction of total sequences.

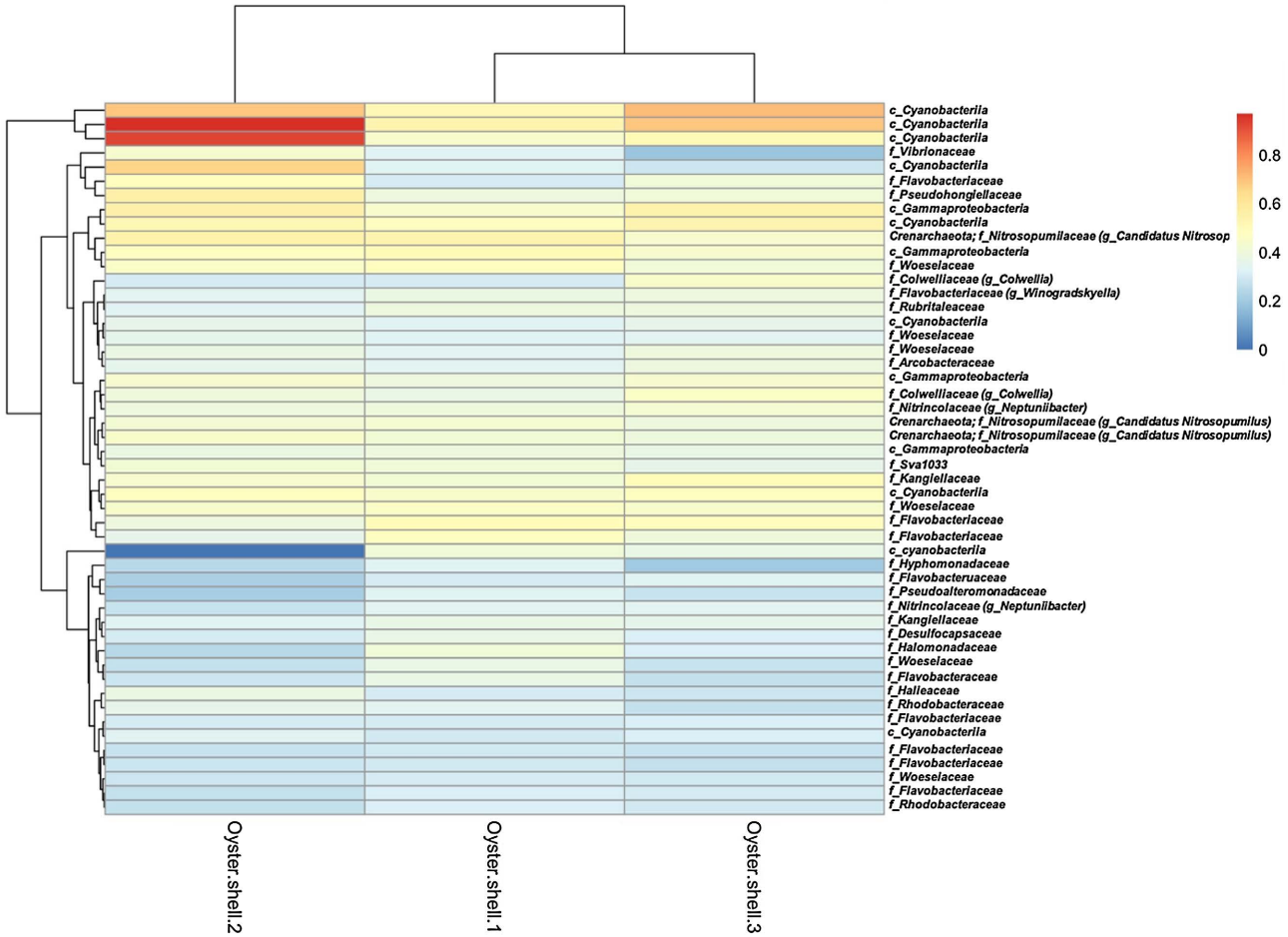

Figure 8. Heatmap representing the relative abundance of the top 50 most abundant Operational Taxonomic Units (OTUs) associated with bacterial and archaeal taxa across three oyster shell samples (Oyster-shell 1, Oyster-shell 2, and Oyster-shell 3). Rows correspond to the OTUs, and columns represent the oyster shell samples. The color gradient indicates relative abundance, with blue representing low abundance, yellow moderate abundance, and red high abundance, as depicted in the color scale. Hierarchical clustering dendrograms along the axes illustrate similarities in community composition among the OTUs and oyster shell samples, highlighting the variation in microbial communities linked to bacterial and archaeal taxa across the samples. The values represent the percentage of the normalized fraction of total sequences.

Four bacterial strains recorded the highest relative abundance (%) on the clay prototypes (**Figure 7**), with 2 from the *Colwelliaceae* family (OTU 17: 1.2 ± 0.2 and OTU 150: 1.2 ± 0.1), one f_*Nitrincolaceae* (OTU 8: 1.1 ± 0.1) and one f_*Propionibacteraceae* (OTU 42: 1.0 ± 0.6). Also abundant were *Psychrobium* from the *Shewanellaceae* family (OTU 82: 0.8 ± 0.3), a strain of *Pseudoalteromonadaceae* (OTU 213) and another OTU related to Clade I (OTU 37). Microorganisms belonging to the *Colwelliaceae* and *Shewanellaceae* families, including *Psychrobium* species, are common to marine environment and have been previously associated with wild oysters [29]. Similar to *Propionibacteraceae* and *Pseudoalteromonadaceae*, they can produce exopolysaccharides, assisting in biofilm maintenance [56]-[58]. *Nitrincolaceae* are also common in sea water with high carbon availability due to its diverse carbon utilisation capacity, making it a good indicator of phytoplankton blooms [59] [60].

In contrast, the most abundant OTUs from the oyster shells biofilm (**Figure 8**) belonged to the class Cyanobacteria with OTUs 65, 47, 21 and 3 at relative abundance 0.7 ± 0.3, 0.6 ± 0.1, 0.6 ± 0.3 and 0.5 ± 0.0, respectively. A previous study at the Danish Shellfish Centre (DTU Aqua, Nykøbing Mors, Denmark; 56.78855˚N, 8.8775˚E) identified Cyanobacteria, *Colwelliaceae*, and Clade Ia species (capable of denitrification) associated with the microbial population on shells immersed at the research platform [29], with one OTU related to the archaea *Candidatus Nitrosopumilus*, a well-known marine ammonium oxidizing archaea (AOA), under aerobic conditions [29] [61]. Currently, at least 3 OTUs linked to this archaeon were identified in the top 50 most abundant microorganisms from both biofilms, including OTU 24 (clay prototype: 0.9 ± 0.1 and oyster shells: 0.5 ± 0.1), OTU 67 (clay prototype: 0.8 ± 0.1 and oyster shells: 0.4 ± 0.) and OTU 53 (clay prototype: 0.7 ± 0.1 and oyster shells: 0.4 ± 0.1). The abundance of Crenarchaeota in the North Sea has previously been documented [61], and the current findings suggest that they successfully transferred onto the clay prototype and adapted to the hatchery conditions.

The analysis did not reveal any clear link between the OTUs and bacterial strains known to induce settlement in oysters like the Shewanella sp. [62]. OTU 213 associated with a *Pseudoalteromonadaceae* is shown in the heat map as abundant on the clay prototypes (0.8 ± 0.2, **Figure 8A**) but absent on the oyster shell surface. *Pseudoalteromonas* are common inhabitant of marine biofilms, capable of both inducing and inhibiting settlement [55] [63]. Whether the presence of this bacteria could explain the lower percentage settlement of the clay prototypes, compared to the oyster shells (**Figure 5**) will require further investigation.

4. Conclusion

This study demonstrated the potential of clay-based structures as substrates for oyster larvae settlement in large-scale restoration efforts for European flat oysters (*Ostrea edulis*), but also highlighted the risks of simply deploying these substrates into the sea to attract wild larvae. Success depends heavily on the presence of larvae and environmental factors such as temperature and hypoxic events that affect larvae recruitment. To better assist in the regeneration of oyster reefs, the strategy of deploying "spats on reefs" appears more promising. The timing of larvae settlement is also critical. The findings emphasize the need for ongoing research to refine these conditions and deepen our understanding of microbial community interactions during larvae settlement. Shifts in microbial biofilms can be influenced by environmental changes, nutrient availability, and microbial interactions. Understanding these dynamics is crucial for optimizing substrate conditions. Monitoring ORP levels and bacterial populations can help predict and mitigate adverse conditions for oyster reef restoration. Additionally, restoring oyster reefs in areas with good water circulation can maintain oxygen levels and reduce hypoxia risks.

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Data Availability Statement

The datasets generated during and/or analysed during the current study are available in the PRJNA1129081 repository.
https://www.ncbi.nlm.nih.gov/sra/PRJNA1129081.

Author Contributions

N. J., C.S. and G. B. designed the project that was funded by Oyster Heaven B. V and partly by the Kyeema Foundation. N. J, Q.G and C. S carried out the experiment and performed chemical analyses, N.J and N.H undertook all bioinformatic analysis. All co-authors reviewed and contributed to the writing of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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