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Characterisation of the Bacteria and Archaea Community Associated with Wild Oysters, At Three Possible Restoration Sites in the North Sea

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Abstract

With 85% of the global oyster reefs destroyed, there is an urgent need for large scale restoration to benefit from the ecosystem services provided by biogenic oyster reefs and their associated biodiversity, including microorganisms that drive marine biogeochemical cycles. This experiment established a baseline for the monitoring of the bacterial and archaeal community associated with wild oysters, using samples from their immediate environment of the Voordelta, with cohabiting *Crassostrea gigas* and *Ostrea edulis*, Duikplaats with only *C. gigas* attached to rocks, and the Dansk Skaldyrcentre, with no onsite oysters. The microbial profiling was carried out through DNA analysis of samples collected from the surfaces of oyster shells and their substrate, the sediment and seawater. Following 16S rRNA amplicon sequencing and bioinformatics, alpha indices implied high species abundance and diversity in sediment but low abundance in seawater. As expected, Proteobacteria, Bacteroidetes, Firmicutes and Thaumarchaeota dominated the top 20 OTUs. In the Voordelta, OTUs related to *Colwellia*, *Shewanella* and *Psychrobium* differentiated the oysters collected from a reef with those attached to rocks. Duikplaats were distinct for sulfur-oxidizers *Sulfurimonas* and sulfate-reducers from the Sva 0081 sediment group. Archaea were found mainly in sediments and the oyster associated microbiome, with greater abundance at the reef site, consisting mostly of *Thaumarchaeota* from the family *Nitrosopumilaceae*. The oyster free site displayed archaea in sediments only, and algal bloom indicator microorganisms from the *Rhodobacteraceae*, *Flavobacteriaceae* family and

genus [*Polaribacter*] *huanghezhanensis*, in addition to the ascidian symbiotic partner, *Synechococcus*. This study suggests site specific microbiome shifts, influenced by the presence of oysters and the type of substrate.

Keywords

Oyster Reefs, Microbiome, Marine Bacteria, Marine Archaea, Restoration

1. Introduction

The marine ecosystem is home to the largest microbiome on earth. As such, the oceanic microbial communities are under active study due to their crucial role in the nutrient cycle and their potential to reduce greenhouse gas emissions [1] [2]. Advances in molecular technologies have facilitated the generation and diffusion of much knowledge in this area. Still, there is so much more to be discovered to contribute to this increasing pool of knowledge. For example, the microorganisms associated with oyster reefs are under active study for use as a tool to control nutrient pollution in waters [1] [2].

A decade ago, it was estimated that around 85% of the oyster reefs in the world had been lost [3], among which, the native European flat oyster (*Ostrea edulis*) population. About 100 years ago, there were healthy flat oyster reefs in the subtidal coastal waters and the deeper waters of the Eastern Channel and the North Sea, stretching from the North Norwegian Sea to the South Mediterranean Sea, including Ireland, Britain and Iberian Peninsula waters [3] [4] [5]. Unfortunately, overfishing, habitat degradation, and diseases such as "*Bonamiosis*" have led to the near extinction of these native oysters and subsequent decrease in related marine species [6] [7].

Biogenic reefs built by oysters, and other bivalves, 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 [8]. 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 [8].

Moreover, through the regulating filter feeding services they provide, they manage the discharge of anthropogenic nutrients (C, N, P) from point sources (e.g., sewage treatment plant effluent) and non-point sources (e.g., agricultural runoff of fertilizers and septic tank discharge) as well as atmospheric deposition from fossil fuel combustion, into the marine ecosystem. Some of these nutrients, they accumulate and use for structural growth (flesh-shell), gonadal development and maintenance, and the excess is rejected as wastes, either as excretion or as biodeposits (faeces and pseudofaeces), resulting in sediments rich in nutrients capable of sustaining and diversifying the benthic macroinvertebrate

population and subsequently other marine species higher in the food web [9] [10]. As such, they can be used as mitigation tools to reduce nutrient levels in coastal waters and constitute a cheap, ecosystem friendly solution [8] [11].

Crucial to this process, are the abilities of microbial communities to access these excess nutrients. This is facilitated by the matrix produced by oysters and other bivalves, trapping the nutrients in faeces or mucus as pseudofaeces produced in the stomach [12]. Basically, the microbial community at different sediment shelves, recycles organic N and inorganic N wastes released by bivalves, via interdependent processes of nitrification, denitrification, dissimilatory nitrate reduction (DNRA), anaerobic ammonium oxidation (anammox) and anaerobic methane oxidation (N-Damo and S-Damo). It is interesting to note that, while active denitrification is primarily associated with microorganisms in sediments, these have also been identified in the gut, gills, and shells (alive or dead) of not only oysters, but other species that reside on oyster reefs [2] [13]. The resulting N_2 is partly intercepted by microphytobenthos for use or transformed into Ammonium (NH_4^+) compounds through nitrogen fixation, mainly performed by *Cyanobacteria*, which use their phototrophic ability to capture sunlight and compensate for the invested energy during the day [14].

In addition to utilizing trapped nutrients, recent studies suggest that the environmental and/or host microbial community may play a role in the settlement of marine invertebrates, including oysters and other bivalves, on either oyster shells or other substrates, by emitting cues that help larvae locate the settlement site. One such example is *Shewanella colwelliana*, isolated from biofilm on the surface of oyster shells [15] [16]. Therefore, monitoring and evaluation metrics at restoration sites should include changes in microbial biodiversity.

The purpose of this research is to further elucidate the composition of the microbial population associated with existing oyster reefs (reference sites) to provide a valuable baseline for the monitoring of microbial shifts during oyster reef restoration. This investigation was part of a feasibility study characterizing the microbial community in the surrounding environment at 3 sites; a co-existing wild *C. gigas* and *O. edulis* oyster reefs, a site with only rock oysters and one where the oyster population has been depleted.

2. Materials and Methods

The study was conducted at two wild oyster habitats in the Dutch North Sea and a site from the Danish North Sea located within distance from wild oyster beds. The samples were from oyster shells, substrate, 5 cm deep sediments, and seawater. Next-generation sequencing of 16S rRNA amplicons was used to perform the baseline analysis.

2.1. Study Site

Three sites were selected for the collection of seawater, sediments, and swabs from wild oyster shells and their substrate: 1) Voordelta (The Netherlands:

51.57354˚N 3.51129˚E), where both live *O. edulis* and *C. gigas* coexist either as reef structures or as boulder oysters; 2) Duikplaats (Sas van Goes—The Netherlands: 51.54052˚N 3.92912˚E), with only *C. gigas* attached to rocky structures and 3) a research platform for the Danish Shellfish Centre (DTU Aqua: Nykøbing Mors, Denmark: 56.78855˚N 8.877.5˚E), where samples were collected from sundried, empty oyster shells placed in baskets, suspended to the research platform and submerged in seawater for four weeks to allow for biofilm formation.

2.2. Sterilization

For this procedure, an autoclave (Witeg, WAC-47-PED), a UV (ultra violet) filtration unit (ExpressTM Plus, 0.22 µm, Millipore), a UV lamp and a Biohazard fume hood (Biowizard Xtra, XF 130, Kojair) were used. To avoid contamination of the targeted biofilm with external microorganisms, all materials used were either purchased sterile (Supplier's specifications) or sterilized in the laboratory (BlueCity, Netherlands). Micropipette tips, swabs, and nitrocellulose membrane filters (GVS, USA) were sterilized by autoclaving at 121˚C for 20 minutes. Since the tanks and the UV filtration unit could not be autoclaved, they were sterilized with 70% ethanol. The ethanol was sprayed on both the inside and outside and allowed to sit for 5 minutes [17]. The tanks were then exposed to UV light in a Biohazard fume hood (Biowizard Xtra, XF 130, Kojair). HDPE sampling bottles were sterilized by exposure to UV light in the Biohazard fume hood for 30 minutes using a UV lamp (365 nm).

2.3. Sample Collection

The sterile materials used were cotton swabs, 5 ml sterile tubes, a spatula, 1 L bottles and Millipore ExpressTM Plus filtration unit fitted with 0.22 µm nitrocellulose membrane filters. Sampling was carried out in triplicates, during the Spring season of 2022, at low tide (0.9 m) and sea temperature of 8.74˚C, for locations in the Netherlands and from the suspended baskets at sea temperature of 10.00˚C in Denmark. To avoid sacrificing any oysters, only the biofilm on the shell surface was considered, thus no IACUC (or equivalent) approval was required. For oyster shells and substrates, from all 3 sites, sterile cotton swabs were used to collect the biofilm from the surface of the oyster shells and their attached substrate. The swabs were transferred to a 5 ml sterile cryogenic tube containing 3 ml of RNA later solution. A sterile spatula was used to collect about 2 g of combined sediment samples from a depth range of 0 - 5 cm [18]. These samples were immediately transferred to a 5 ml sterile cryogenic tube containing 3 ml of RNA later solution. UV-sterilized 1 L bottles were filled with seawater from each of the three locations. The outside of the bottles was cleaned with 70% ethanol, placed in an esky with ice, and transported to the laboratory for filtration using the Millipore ExpressTM Plus filtration unit fitted with 0.22 µm nitrocellulose membrane filters (GVS, USA). The nylon filters were then stored in a 15 ml sterile conical tube containing 10 ml of RNA later solution. All

tubes containing biofilm samples were kept on ice during transport and then placed in a fridge at 4˚C overnight before being stored at −20˚C until they were transferred to the DNASense facility (East Aalborg, Denmark) for DNA analysis.

2.4. DNA Extraction & Sequence Processing

Triplicates were submitted with code names to prevent analytical bias. All genomic DNA extraction and sequencing were performed by the staff at the DNASense laboratory in East Aalborg, Denmark, using their own protocols. The FastDNA Spin Kit for Soil (manufactured by MP Biomedicals in the US) was used to extract DNA from the swabs, with an adjusted volume of 980 µl of Sodium Phosphate buffer and 120 µl MT Buffer used for step one. The mechanical shearing was performed by bead beating (bead to sample ratio of 4:5) at 6 $m \cdot s^{-1}$ for 4 × 40 s [19]. Genomic DNA from seawater was extracted using the standard protocol from the DNeasy PowerWater Kit (manufactured by Qiagen in Germany). DNA concentration was measured using the Qubit dsDNA HS/BR Assay kit (manufactured by Thermo Fisher Scientific in the US).

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM, followed by paired-end 16S rRNA sequencing (2 × 300 bp) on the Illumina MiSeq platform (Illumina Inc., USA), targeting the variable region 4 (V4-C) of the 16S rRNA gene in both bacterial and archaeal populations, using specific primers 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGATACNVGGGTWTCTAAT-3'), with the primer tails facilitating attachment of the Illumina Nextera adaptors [20] [21].

The DNA extracted from samples collected from Sites 1 and 2 was further characterized for archaea using Oxford Nanopore Technologies (United Kingdom), targeting the variable regions 1 - 9 (V19-A) of the 16S rRNA gene with archaea-specific primers SSU1ArF (5'-TCCGGTTGATCCYGCBRG-3') and SSU1000ArR (5'-GGCCATGCAMYWCCTCTC-3') [20].

The raw sequencing data were first trimmed for quality using Trimmomatic v.0.32, and merged using FLASH v.1.2.7, before being dereplicated and formatted using the research standard UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered into operational taxonomic units (OTUs) at 97% similarity, and abundances were estimated using search v.11.0.667. The resulting OTUs were assigned with their corresponding taxonomic classification using the Uclust classifier in the Quantitative Insights into Microbial Ecology (QIIME 2) software package and the SILVA database, release 132 [22] [23]. It is important to note that such identifications may change over time if new sequences with closer relationships are uploaded to the database.

2.5. Data Analysis

The polished sequencing data received from DNASense laboratory (Aalborg, Denmark) was further analyzed for statistical significance using the RStudio IDE

(version 2022.7.1.554) running R version 4.1.0 (2021-05-18). After normalization and Hellinger transformation, analysis was carried out using 8723 OTUs across all samples. Principal component analyses (PCAs), based on eigenvector multivariate analysis, were performed on Hellinger-adjusted (square root of total standardized data) operational taxonomic units (OTU) tables, to enhance subdominants [24]. A biplot was generated, with sample type and site as variables, using the "ampvis2" (version 2.7.8) package. Heatmaps were generated with the "tidyverse" (version 1.3.1) package. The data was further processed in QIIME 2 to calculate the alpha indices Chao 1 and Shannon index, using the Bray-Curtis similarity index with 9999 permutations. A higher value of Chao 1 indicates a higher expected species abundance and a higher value of the Shannon index indicates a higher diversity of the biofilm. QIIME 2 was also used to perform differential abundance analysis between samples based on their normalized sequence abundance. Similarities and differences within technical repeats were assessed with one-way analysis of similarities (ANOSIMS). Bar charts were generated in Microsoft Excel to illustrate the changes in the archaea community profiles for each of Sites 1 and 2.

The correlation between samples within each site was assessed by calculating the Pearson coefficient using the multivariate correlation formula in Microsoft Excel. A value of 1 indicates a positive correlation between 2 samples, 0 indicates no relationship while −1 suggests a negative correlation. Since the value for N corresponded to the number of OTUs, which was 8723, the p-value was close to zero.

3. Results and Discussion

3.1. Comparative Analysis of the Site Distribution

The study used ANOSIMS to assess variability among technical repeats and found negligible variability, leading to the use of mean values for each sample (R-value = 1; p-value = 0.0035). The initial PCA analysis revealed differences between the microbial characteristics of seawater and other samples, indicating variations in microbial populations with and without wild oysters (**Figure 1**), with PC 1 accounting for 27.9% of variance attributed mostly to the inverse correlation between the microbial characteristics of seawater and the other samples collected. PC2 suggest a negative correlation at 17.4% variance, with comparable microbial profiles for the Voordelta (squares—Site 1) and Duikplaats, Wilhelminadorp (triangles—Site 2), contrasting with the site in Denmark (circles—Site 3), also implying a difference between the microbial population with and without wild oysters present.

Pearson correlation coefficient (PCC) analysis indicated a strong correlation among most samples, with the sediment microbial characteristics showing varying strengths of correlation, depending on the sample type and location (**Figure S1**, **Figure S3** and **Figure S5**). For instance, while the majority of the *C. gigas* sampled for this study were high up the rock structures to limit the sediment

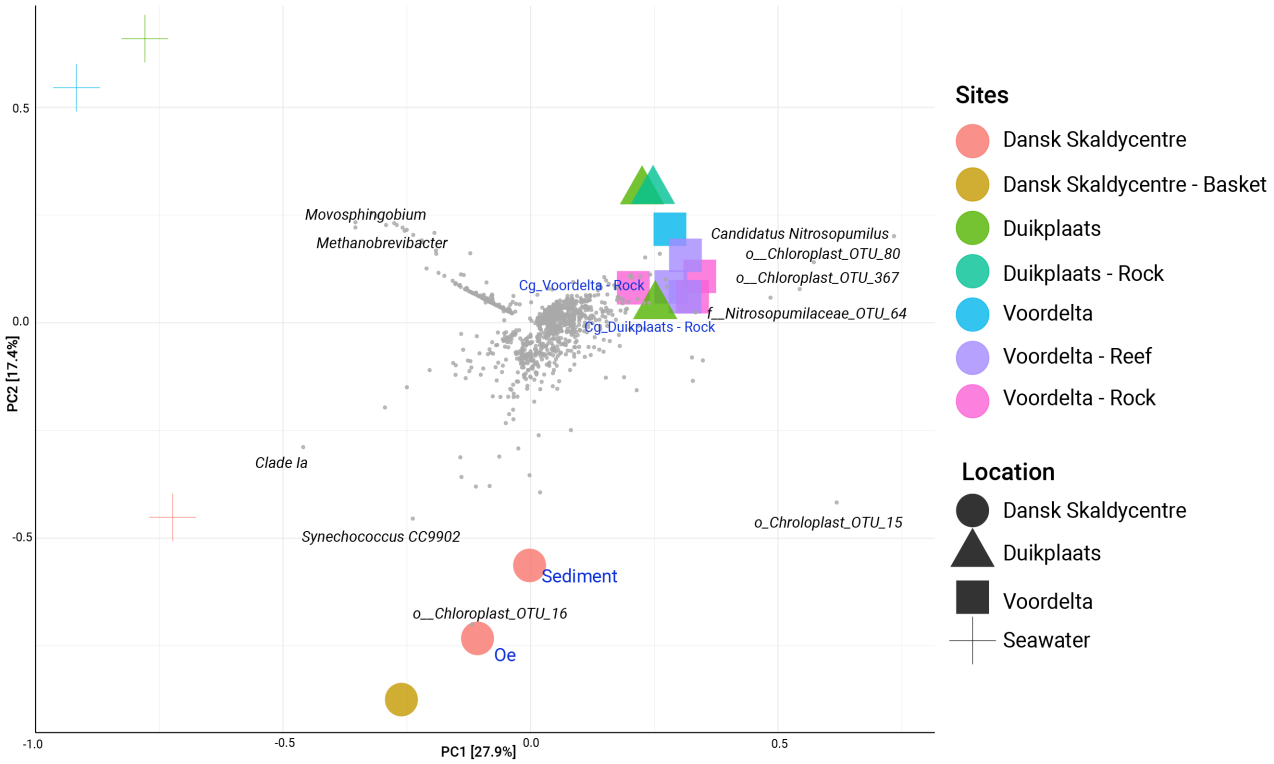

Figure 1. Principal component analysis (PCA) plot showing the migration of samples with respect to their microbial profiles. The samples include oyster shells from *O. edulis* (O. e) and *C.gigas* (C. g), either rocks (boulders), oyster reef or baskets as substrates (Sub), sediments (Sed) and seawater. Each point represents a specific microbial community associated with one of the samples taken from Voordelta (site 1-Orange), Duikplaats, Wilhelminadorp (site 2-Green) and Dansk Skaldyrcenter, NykØbing Mors (site 3-Blue, Denmark). The distance between the sample dots indicates how similar the microbial composition is between the samples, the closer the dots, the more similar the samples.

influence, the *C. gigas* collected from the oyster reef in the Voordelta were implanted in the sediment (PCC = 0.81), as they formed the base of the reef structure, with the cohabiting *O. edulis* (PCC = 0.66) mostly settling on top of the *C. gigas* shells. The lowest PCC was registered between the *C. gigas* collected from the reef structure, and the one attached to the rocky structures (PCC = 0.37), which are intertidal, large boulders. It is believed that the empty shells falling off these boulders could be the origin for the base of the reefs [25].

In contrast, the rock structures in Duikplaats, Wilhelminadorp (The Netherlands), on which the *C. gigas* settled, were similar to stone runs or stone river, at lower elevation with regards to the sediment. For this Site, the strongest PCC (**Figure S3**) was between the sediment and the substrate (PCC = 0.58). A similar trend was observed at the research platform of the Dansk Skaldyrcentre with no onsite oysters (**Figure S5**). Negligible or absence of correlation was noted for all samples against their seawater media. The presence of Chloroplasts in certain samples (OTUs 80 and 367 for Sites 1 and 2, and OTU 16 for Site 3), possibly originating from aquatic vegetation on the oyster shells and substrate in the Netherlands and algae in the seawater in Denmark, posed a challenge in interpreting the results. The identification of Chloroplasts in marine samples is quite

common and often related to aquatic vegetations as a result of cyanobacterial endosymbiosis [26]. When Chloroplast-related OTUs were excluded from the PC analysis (**Figure 2**), more information about the microbial communities contributing to sample differences emerged. OTUs related to families *Prevotellaceae* and *Lachnospiraceae* were attributed to the seawater samples in addition to *Methanobrevibacter* sp., *Novosphingobium* sp., *Synechococcus* sp and Clade Ia.

Excluding the Chloroplast OTUs from site 1 data analysis also resulted in a decrease in the strength of the PCC for the sediment and the *C. gigas* collected from the reef structure (**Figure S1**-PCC = 0.85 to **Figure S2**-PCC = 0.66). In comparison, a stronger correlation was implied between the *C. gigas* and their rock substrate (**Figure S1**-PCC = 0.75 to **Figure S2**-PCC = 0.91) as well as the *O. edulis* and *C. gigas* collected from the boulders (**Figure S1**-PCC = 0.68 to **Figure S2**-PCC = 0.89). For both Sites 2 (**Figure S3** and **Figure S4**) and 3 (**Figure S5** and **Figure S6**), the only difference in PCC without Chloroplast, was between the sediment biofilm and the substrate biofilm, with a stronger relationship for Duikplaats (PCC = 0.58 to 0.74) and a decrease at the Dansk Skaldyrcentre (0.56 to 0.32).

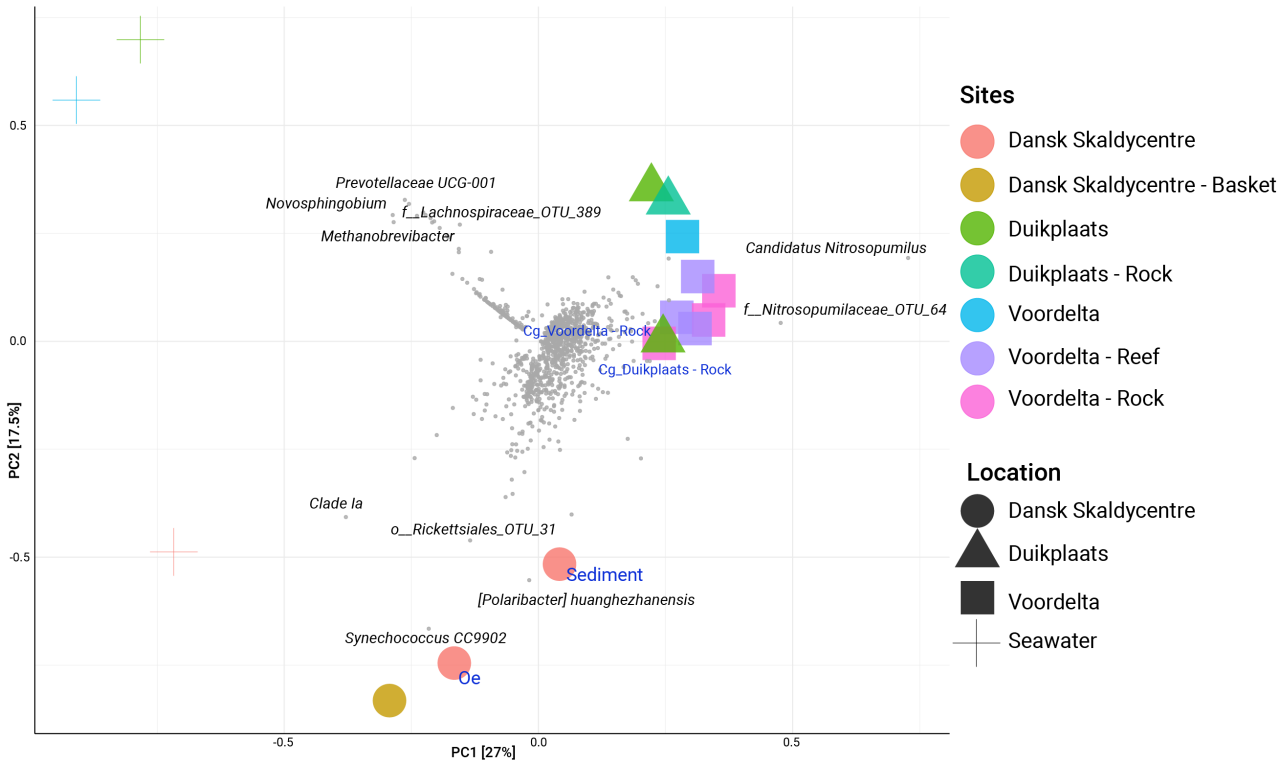

Figure 2. Principal component analysis (PCA 2) plot showing the migration of samples with respect to their microbial profiles that excluded OTUs related to Chloroplasts. The samples include oyster shells from *O. edulis* (O. e) and *C. gigas* (C. g), either rocks (a), oyster reef (b) or baskets (1 and 2) as substrates (Sub), sediments (Sed) and seawater. Each point represents a specific microbial community associated with one of the samples taken from Voordelta (site 1-Orange), Duikplaats, Wilhelminadorp (site 2-Green) and Dansk Skaldyrcenter, NykØbing Mors (site 3-Blue, Denmark). The distance between the sample dots indicates how similar the microbial composition is between the samples, the closer the dots, the more similar the samples.

Alpha diversity indices indicated that sediment samples were richer and more diverse compared to seawater samples (**Table 1**), possibly due to challenges in extracting sufficient DNA from seawater. While 1 L of seawater was filtered in this study, the yield might have been influenced by the presence of exocellular DNA from viruses and vesicles [27] [28] [29]. A new PCA analysis excluding Chloroplast-related OTUs and seawater samples revealed further differences between the Dutch North Sea and the Danish site in terms of microbial composition. The PC plot (**Figure 3**) showed an inverse correlation between Sites 1 & 2, compared to Site 3 along PC1 at 32.1% of variance with a shift from profiles rich in the archaea *Candidatus Nitrosopumilus* and bacteria *Desulfosarcina*, in the Dutch North Sea, to one containing more *Rickettsiales*, *Rhodobacteraceae*, *Synechococcus* and [*Polaribacter*] *huanghezhanensis* at the Danish site. Previous studies related phytoplankton blooms with Cyanobacteria and the family *Rhodobacteraceae*, *Flavobacteriaceae* and [*Polaribacter*] *huanghezhanensis*. Their presence at the research platform at the Dansk Skaldyrcentre, could be related to the observed outgrowth of algae [30].

Variance along PC2 of PCA 3 (**Figure 3**) was at 26% with a negative correlation between most of the samples collected from the Voordelta and the sediment from site 3, against all the other samples. When comparing the two substrates

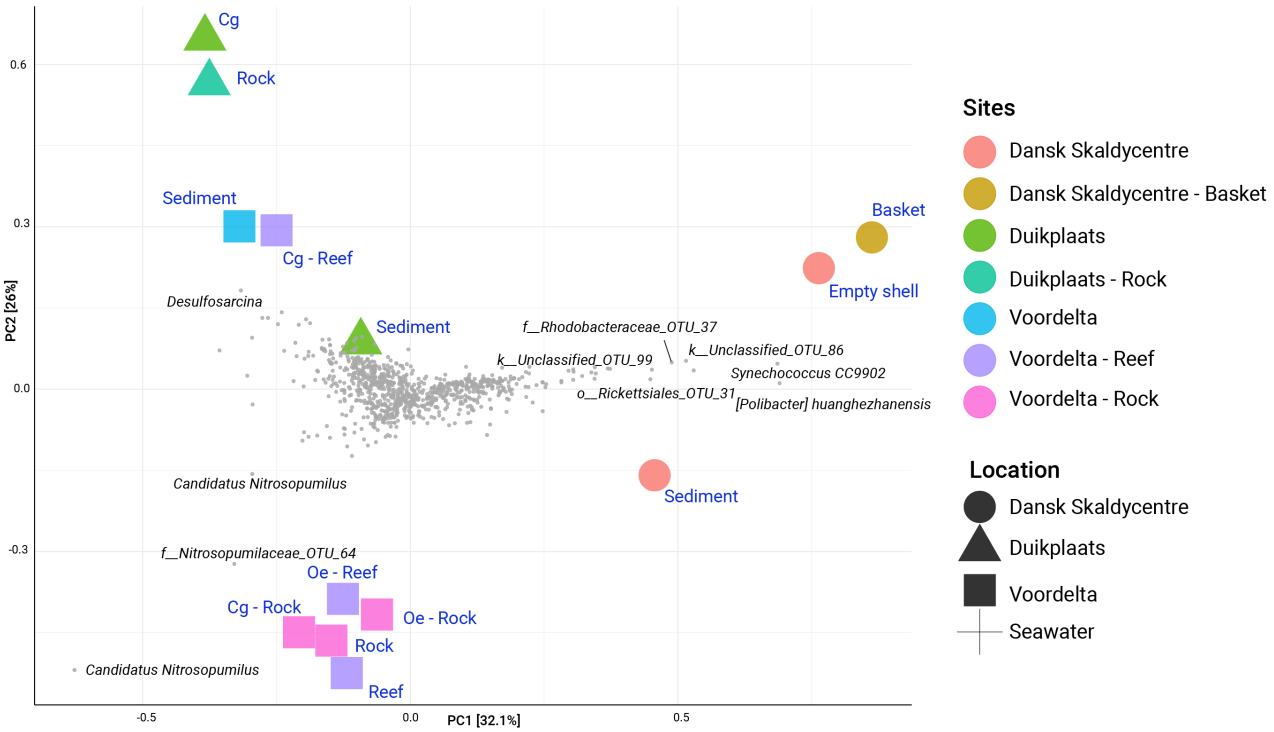

Figure 3. Principal component analysis (PCA 3) plot showing the migration of samples with respect to their microbial profiles that excluded OTUs related to Chloroplasts and seawater samples. The samples include oyster shells from *O. edulis* (O. e) and *C. gigas* (C. g), either rocks (a), oyster reef (b) or baskets (1 and 2) as substrates (Sub), sediments (Sed) and seawater. Each point represents a specific microbial community associated with one of the samples taken from Voordelta (site 1-Orange), Duikplaats, Wilhelminadorp (site 2-Green) and Dansk Skaldyrcenter, Nykøbing Mors (site 3-Blue, Denmark). The distance between the sample dots indicates how similar the microbial composition is between the samples, the closer the dots, the more similar the samples.

Table 1. Summary of 2 diversity indices comparing the abundance of operational taxonomic units (OTUs) (Chao1) and the diversity of OTUs (Shannon) of each sample collected from seawater, oyster shells surface, substrate surface and sediments at Site 1 (Voordelta), Site 2 (Duikplaats) and Site 3 (Danish Shellfish Centre, DTU Aqua, Nykøbing Mors.

Site	Sample name	Shannon (species diversity)	Chao1 (species abundance)
Voordelta	Seawater	**4.60**	**312**
	Rock	5.95	3245
	Reef	5.53	2668
	Sediment	6.26	3097
	O. edulis—Rock	6.05	3451
	O. edulis—Reef	5.98	3061
Duikplaats	Seawater	**3.09**	**85**
	Rock	5.53	2668
	Sediment	6.13	2661
	C. gigas	5.71	2862
Dansk Skaldyrcentre	Seawater	**4.17**	**1256**
	Basket	6.37	3660
	Sediment	6.62	4001
	O. edulis	5.77	3063
Duikplaats	Seawater	**3.09**	**85**

(rocks and oyster reef) in the Voordelta (Site 1), PCA 3 suggests close similarity between most of the samples except for the sediment sample and *C. gigas* surface biofilm collected from the reef, which clustered with the 3 samples collected from Site 2 (Duikplaats). Shannon indices (**Table 1**), suggest that both those samples had more species diversity (sediment = 6.26 and *C. gigas*-reef = 6.30) and were less abundant in the 2 top hits archaeal OTUs, which could explain their position on the PCA 3 plot and assume that *Candidatus Nitrosopumilus* was the main variable in the Voordelta.

3.2. Comparative Analysis of the Taxonomic Distribution

The analysis of microbial communities associated with oyster reefs at different sites, focused on the major microbial groups and their relative abundances. The study used the Uclust classifier and SILVA database to determine the relative abundance of major microbial communities, excluding Chloroplasts, and the data were presented in a heatmap (**Figure 4**) to compare oyster shell biofilm, seawater, substrates, and sediments across the three study sites.

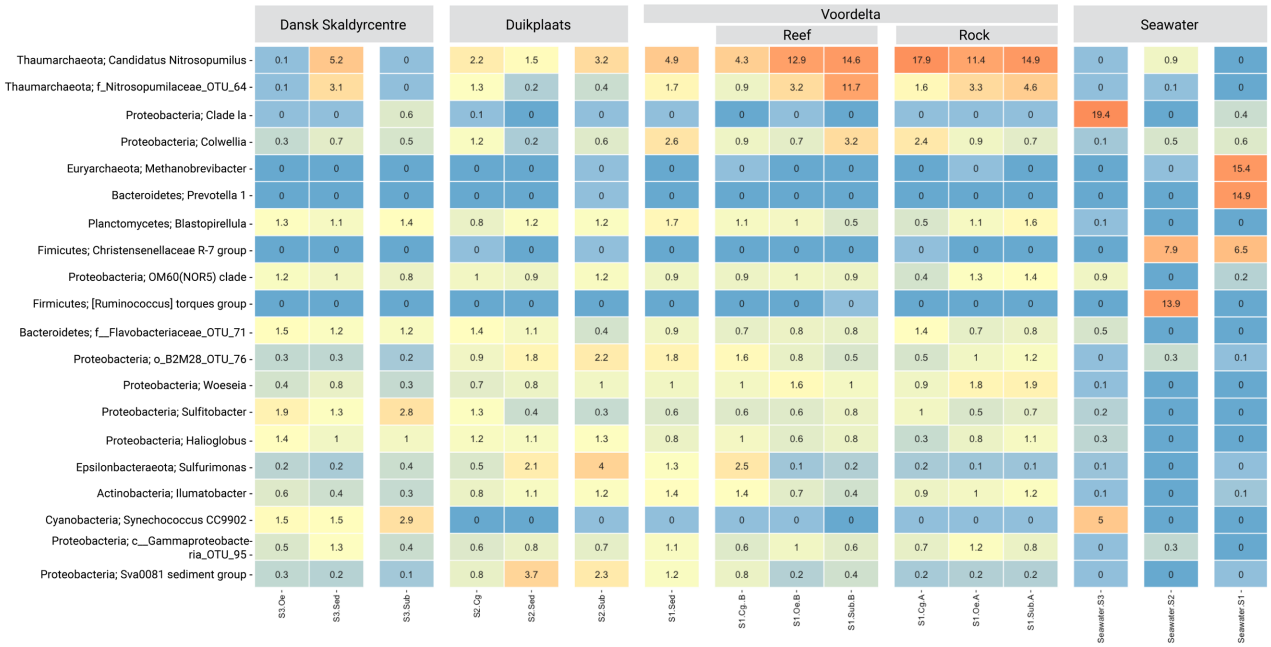

Figure 4. Heatmap of the 20 most abundant bacteria and archaea, at the closest taxonomic classification, and associated with the microbial community on the surface of oyster shells, on their substrate, the sediments with oyster biodeposits and the seawater they are submerged in, for the Voordelta (Site 1), Duikplaats, Wilhelminadorp (Site 2) and the Dansk Skaldyrcentre in NykØbing Mors (Site 3). The values represent the percentage of the normalized fraction of total sequences with blue indicating a 0% relative abundance, increasing in red gradient density with increasing percentage.

Common microbial phyla, such as Thaumarchaeota, Proteobacteria, and Bacteroidetes, were found in all three habitats, similar to previous studies [2]. The top two microorganisms were archaea from the phylum Thaumarchaeota, related to *Candidatus Nitrosopumilus* being highly abundant in the Voordelta, especially on boulders, while another archaeon, from the family *Nitrosopumilaceae* (OTU 64), was prevalent in the Voordelta reef biofilm. The percentage of *Candidatus Nitrosopumilus* in the sediment (4.9%) and on the surface of the *C. gigas* from the reef structure in the Voordelta were comparable to the oyster shell (2.2%) and rock biofilms (3.2%) from Site 2, which further explain why the position of these samples clustered together in the PCA 3 plot (**Figure 3**). In Denmark, this OTU was detected only in the sediment at 5.2% relative abundance. These archaea are ammonia oxidizers, potentially influencing nitrogen cycling [31].

Seawater samples exhibited differences between sites, with the presence of specific Proteobacteria and Cyanobacteria. Observed only in the Dansk Skaldyrcentre seawater was a Proteobacteria belonging to Clade Ia, some of which have been reported to carry out denitrification with subsequent phosphorus uptake [32]. Also detected was *Synechococcus* sp (5.0%), a unicellular Cyanobacteria, known as a great contributor to carbon cycling, with the ability to also utilize NH_4^+, urea, NO_3^-, and N_2 [32] [33]. One possible explanation for their presence could be the thriving population of Ascidians (*Ciona intestinalis*), in the water, with which they have been reported to have a symbiotic relationship [33] [34]

[35].

The seawater microbial communities from the Voordelta had three major OTUs, Euryarchaeota, *Methanobrevibacter* (15.4%), Bacteroidetes, *Prevotella* 1 (14.9%) and Firmicutes, *Christensenellaceae* R-7 group (6.5%), while the water sample collected from Duikplaats were rich in Firmicutes, [*Ruminococcus*] torques group and *Firmicutes*, *Christensenellaceae* R-7 group. The presence of members of the Methanobacteriales capable of methanogenesis and that of Bacteroidetes and Firmicutes, common inhabitants of the gut microbiota, have previously been indicators of fecal pollution [36]. The detection of pollution indicator organisms can be expected since both Sites 1 and 2 are connected to a network of rivers, tributaries and estuaries carrying discharge from agricultural, industrial, recreational and water treatment activities [25]. Also seen were Proteobacteria of genus *Novosphingobium*, along with Cyanobacteria, which are capable of nitrogen fixation [37].

Some phyla and genera were more pronounced at specific sites, indicating site-specific microbial characteristics related to sulfur oxidation, sulfur reduction, and acetate metabolism. These include *Sulfitobacter* sp at the Dansk Skaldyrcentre in Denmark and *Sulfurimonas* (also on the surface of reef *C. gigas* oysters) and Sva0081 sediment group from Duikplaats. The genus *Sulfitobacter* is part of the *Rhodobacteraceae* family common to the marine ecosystem where it undertakes sulfur oxidation [38]. The genus *Sulfurimonas* are known sulfur-oxidizing denitrifying bacteria mostly isolated from marine anoxic environments [39]. The Sva0081 sediment group from the *Desulfobacteraceae* family is natives to the benthic communities, acting as important sinks of acetate and Hydrogen (H_2) and have sulfate reducing capabilities [40].

Differential analysis highlighted significant differences in microbial profiles between the Voordelta and Duikplaats, with specific OTUs more abundant at each site. Proteobacteria, such as *Colwellia*, *Shewanella*, and *Psychrobium* (**Table 2**), sometimes characterised as piezophiles, were more prevalent in the Voordelta reef structure. These microorganisms are often involved in biofilm formation by producing exopolysaccharides that facilitate adhesion to surfaces. *Shewanella* sp. can also reduce nitrate to ammonia during DNRA, as does

Table 2. Summary of the differential abundance analysis comparing the microbial community associated with the oyster reefs as a substrate, to the rock substrate at the Voordelta: The results were filtered for relative abundance below 0.5% and had a p-value adjusted (padj) of 0.05% significance threshold. A negative Log2 fold change value indicates higher abundance of the OTU in the reef-associated samples.

OTU	Phylum	Identity	padj	Log2FC	Reef	Rock
8	Proteobacteria	*Colwellia*	0.017	−5.4	1.239	0.038
229	Proteobacteria	*Shewanella*	0.034	−4.9	6.057	0.249
5495	Proteobacteria	*Psychrobium (f_Shewanella)*	0.034	−7	0.903	0.011

Persicirhabdus sp, with some species also promoting oyster larvae settlement through melanogenesis [41] [42].

The higher frequency of archaea *Candidatus Nitrosopumilus* and Proteobacteria from the genus *Shewanella* for the Voordelta, and *Desulfosarcina* for Duikplaats was confirmed (**Table 3**). This analysis also revealed an archaeon (OTU 496) from Duikplaats (Site 2) closely related to the Euryarchaeota, *Methanosaeta*

Table 3. Summary of the differential abundance analysis comparing the microbial communities associated with site 1 (S1-Voordelta) and site 2 (S2-Duikplaats, Wilhelminadorp). The results were filtered based on relative abundance below 0.5% and p-value adjusted (padj) at a significance threshold of 0.05%. A negative Log2 fold change value indicates a higher abundance of the OTU in samples collected from the first site.

OTU	Phylum	Identity	Padj	Log2FC	S1	S2
1837	Thaumarchaeota	*Candidatus Nitrosopumilus*	0.019	−3	1.191	0.155
229	Proteobacteria	*Shewanella*	0.049	−3.2	0.949	0.097
137	Proteobacteria	*Novosphingobium*	0.038	8.3	0.788	0.234
437	Cyanobacteria	c_Oxyphotobacteria	0.0041	−5.2	0.756	0.015
238	Cyanobacteria	c_Oxyphotobacteria	0.022	−3.3	0.631	0.049
2696	Cyanobacteria	c_Oxyphotobacteria	0.016	−3.5	0.621	0.054
338	Proteobacteria	*Desulfosarcina*	0.038	3.1	0.368	2.463
610	Bacteroidetes	*Alistipes*	4E−16	27	0	2.077
632	Firmicutes	*Christensenellaceae*	4E−16	27	0	1.999
389	Firmicutes	*Lachnospiraceae*	5E−16	27	0.03	1.838
234	Firmicutes	[Ruminococcus] torques group	6E−16	27	0	1.77
745	Acidobacteria	c_Subgroup 6	0.0004	12	0.002	1.765
480	Bacteroidetes	*Prevotellaceae* UCG-001	3E−18	27	0.068	1.663
680	Firmicutes	[Ruminococcus] torques group	0.0028	11	0.003	1.593
2323	Proteobacteria	*Thalassotalea*	0.0007	7.9	0.025	1.447
690	Firmicutes	*Mogibacterium*	0.003	9.5	0.004	1.395
768	Firmicutes	*Phascolarctobacterium*	3E−19	26	0.061	1.312
496	Euryarchaeota	*Methanosaeta*	0.018	9.4	0.004	1.062
48	Firmicutes	*Ruminococcaceae* UCG-010	0.0085	10	0.016	1.036
1807	Bacteroidetes	*Algitalea*	4E−15	26	0	0.994
547	Bacteroidetes	Bacteroidetes BD2-2	0.003	5.6	0.015	0.972
497	Proteobacteria	f__*Nitrincolaceae*	0.037	3	0.161	0.966
6373	Planctomycetes	c__OM190	0.0003	6.2	0.038	0.805
406	Fusobacteria	f_*Fusobacteriaceae*	0.036	3.8	0.075	0.677

(padj = 0.018). *Methanosaeta* sp. are believed to be the predominant methane (CH_4) producers on earth and their incidence in marine sediments, considered to be the largest, global reservoir of methane, is similar to previous studies [43] [44]. The microbial community from the Voordelta, was more abundant in Cyanobacteria, primarily from the c_Oxytophotobacteria. In marine environments Cyanobacteria, have phototrophic ability to capture sunlight and compensate for the energy invested during the day for many processes, including nitrogen fixation [14]. The presence of AOA on the boulder side of the Voordelta and piezophiles on the reef side suggested a shift in microbial profiles related to oyster attachment structures. The study also noted the presence of algal bloom indicators at the site with no oysters, hinting at the potential role of oysters in nitrogen balance maintenance.

3.3. Comparative Analysis of the Archaea Population

The samples were further analyzed for archaeal communities in oyster-related environments and their relative abundances. Previous studies often observed a low relative abundance of archaea in sequenced samples, potentially due to limitations in techniques or the capability of a small community to perform diverse functions. In general, archaea are native to shallow and deep-sea anaerobic sediments, participating in nutrient cycling as methanogens, sulfate reducers and ammonium oxidizers [45]. The study attempted to examine DNA samples more closely using specific archaeal primers to amplify the 16S rRNA variable regions 1 - 9 (aV19-A) with Oxford Nanopore Technologies (UK). Unfortunately, seawater samples produced low-quality DNA reads and were excluded from analysis by the service providers (DNASense, Aalborg, Denmark), leaving only data from oyster shells, substrates, and sediments (**Figure 5**). OTUs associated with the most abundant family or lowest assigned taxonomic classification, as a percentage of normalized fraction of total sequences, were used with a threshold of ≥0.5.

In the samples from cohabiting *C. gigas* and *O. edulis* in the Voordelta, *Candidatus Nitrosopumilus* archaea dominated, with a higher relative abundance (95%) on the rocky side of the delta. The archaeal population in oyster reef and sediments showed more diversity, including various families and classes such as *Nitrosopumilaceae*, *Methanomassiliicoccaceae*, *Methanosarcinaceae*, *Bathyarchaeia*, *Thermoplasmatota*, and *Lokiarchaeia* (**Figure 5(a)**). Similarly, samples from Wilhelminadorp, with only *C. gigas* oysters, had high relative abundances of *Candidatus Nitrosopumilus* archaea on rock substrates (99.44%), oyster shell surfaces (91.11%), and lower in sediments (61.40%) (**Figure 5(b)**). The sediment samples also displayed a variety of archaeal groups, including *Thermoplasmata*, *Bathyarchaeia*, *Woeserchaeales*, *Lokiarchaeia*, genus *Methanococcoides*, and *Candidatus Diapherotrites*.

Archaea are commonly found in shallow and deep-sea anaerobic sediments, participating in nutrient cycling processes such as methanogenesis, sulfate reduction, and ammonium oxidation [2] [46]. Ammonia-oxidizing archaea (AOA)

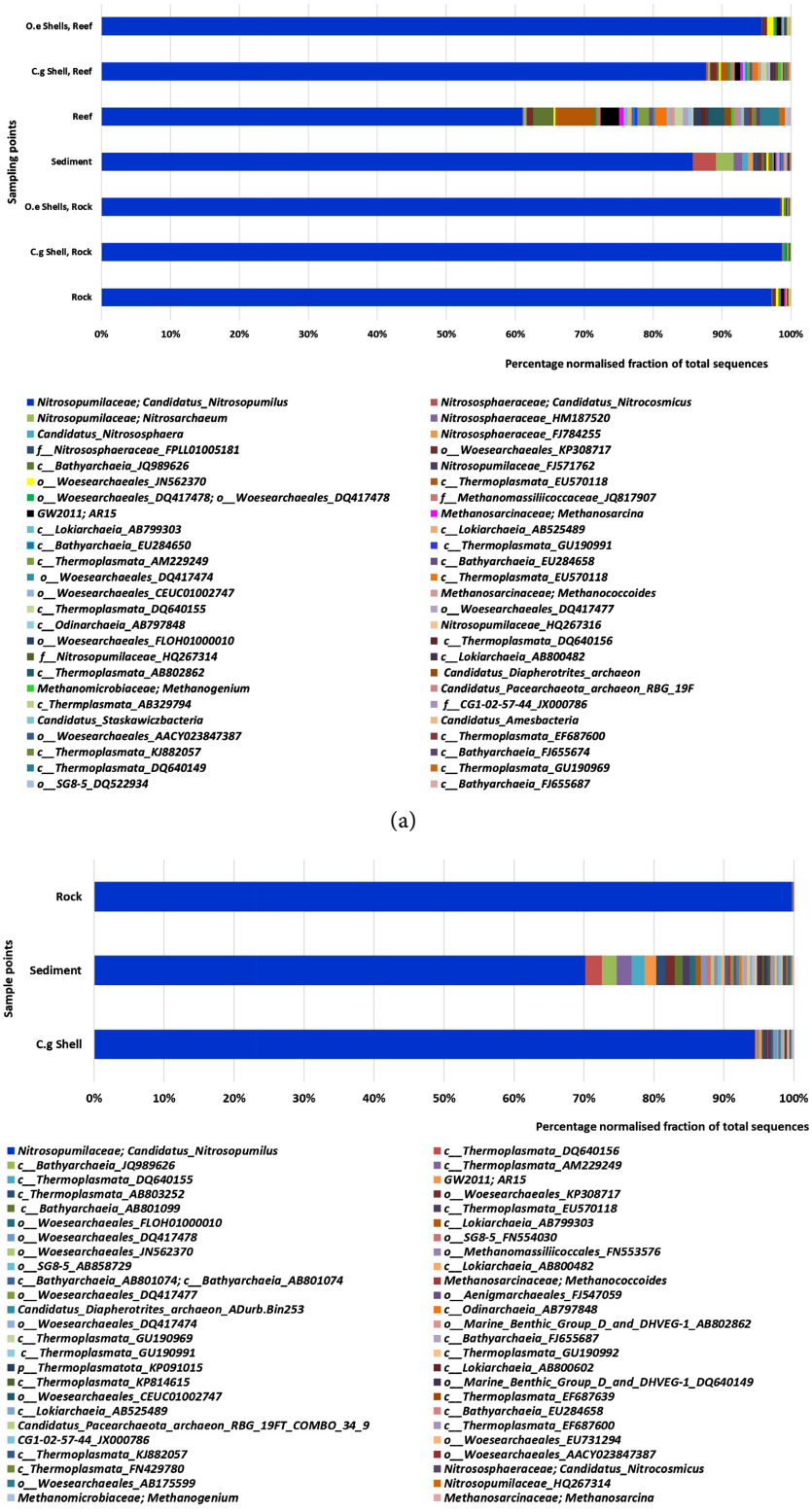

Figure 5. Graphical representation of the 50 most abundant archaea associated OTU arranged for each sample points from (a) Site 1 (Voordelta) and (b) Site 2 (Duikplaats, Wilhelminadorp), excluding the seawater sample. All identifications include the phylum, the closest assigned taxonomic classification and the taxonomic ID where available. The values represent the percentage of the normalized fraction of total sequences at ≥0.5 threshold.

dominated the archaeal populations in oyster shell biofilms and rock substrates, suggesting their adaptation to oxygen exposure. These AOA were categorized within genera like *Candidatus Nitrocosmicus*, *Nitrosarchaeum*, and within the families *Nitrosphaeraceae* and *Nitrosopumilaceae* indicating their role in nitrification processes [47]. Sediments generally exhibited a richer diversity of archaea due to their anoxic nature, with various archaeal groups present [46] [48]. The diversity observed in reef samples may result from proximity to sediments and possibly from archaea originating from oyster gut environments [2] [46]. Unique archaeal species in oyster shell biofilms could also be explored further using advanced molecular techniques.

4. Conclusion

This study used a simple molecular approach to generate preliminary data and insight on the microbiomes associated with wild oysters, including archaea. The results indicate site-specific microbial compositions with microbial groups that could potentially be linked to nitrogen cycling and biofilm formation in oyster reef ecosystems. The study also suggests variations in microbial communities associated with oyster reefs at different sites in the North Sea, with sediment microbial characteristics playing a significant role. Moreover, the presence of Chloroplasts and lower diversity in seawater samples added complexity to the analysis. The detection of methanogens and bacteria associated with ruminants suggests that some sites are impacted by agricultural run-off which would make them unsuitable as reference sites for restoration projects in more pristine oyster habitats. Due to limitations in the method used and limited information in the existing 16S rRNA database for marine microorganisms, further characterisation of the contribution of the oyster reef microbiome to marine bio-geochemical as well as existing interaction between bacteria and archaea, will require more advanced and expensive molecular techniques such as metagenomics, metatranscriptomics and quantitative polymerase chain reaction (qPCR).

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Data Availability Statement

The datasets generated during and analyzed during the current study are available in the PRJNA965952 repository,
https://www.ncbi.nlm.nih.gov/sra/PRJNA965952.

Author Contributions

N. J. and G. B. designed the project and wrote the grant proposals which was co-

funded by Kyeema Foundation and Oyster Heaven B. V. N. J, B. B, G. B and C. S organized and sampled all specimens. N. J performed analyses, Y. L undertook all bioinformatic analysis and B. B did all images and formatting. All co-authors reviewed and contributed to the writing of the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

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Supplementary Materials

	Seawater	Sediment	Rock	Reef	Rock *O. edulis*	Reef *O. edulis*	Rock *C. gigas*	Reef *C. gigas*
Seawater	1.00							
Sediment	0.00	1.00						
Rock	0.00	0.71	1.00					
Reef	0.00	0.60	0.84	1.00				
Rock *O. edulis*	0.00	0.76	0.96	0.79	1.00			
Reef *O. edulis*	0.00	0.66	0.95	0.82	0.93	1.00		
Rock *C. gigas*	0.00	0.42	0.75	0.71	0.68	0.74	1.00	
Reef *C. gigas*	0.00	0.85	0.68	0.55	0.74	0.64	0.37	1.00

Figure S1. Pearson coefficient of correlation among samples from the Voordelta, Netherlands, with blue indicating a positive correlation (between 0 - 1), white indicating no correlation (0) and red indicating a negative correlation (≥0).

	Seawater S1	*S1 Sed*	*Rock*	*Reef*	*Rock O. edulis*	*Reef O. edulis*	*Rock C. gigas*	*Reef C. gigas*
Seawater S1	1.00							
S1 Sed	0.00	1.00						
Rock	0.00	0.61	1.00					
Reef	0.00	0.55	0.87	1.00				
Rock O. edulis	-0.01	0.61	0.97	0.83	1.00			
Reef O. edulis	0.00	0.57	0.95	0.81	0.96	1.00		
Rock C. gigas	0.00	0.51	0.91	0.74	0.89	0.89	1.00	
Reef C. gigas	-0.01	0.66	0.53	0.45	0.56	0.51	0.47	1.00

Figure S2. Pearson coefficient of correlation among samples from the Voordelta, Netherlands, excluding samples from the order Chloroplast, with blue indicating a positive correlation (between 0 - 1), white indicating no correlation (0) and red indicating a negative correlation (≥0).

	Seawater	Sediment	Substrate	*C. gigas*
Seawater	1.00			
Sediment	0.01	1.00		
Substrate	0.01	0.58	1.00	
C. gigas	0.02	0.37	0.31	1.00

Figure S3. Pearson coefficient of correlation among samples from the Duikplaats, Netherlands, with blue indicating a positive correlation (between 0 - 1), white indicating no correlation (0) and red indicating a negative correlation (≥0).

	Seawater	Sediment	Substrate	*C. gigas*
Seawater	1.00			
Sediment	0.00	1.00		
Substrate	0.01	0.74	1.00	
C. gigas	0.01	0.36	0.40	1.00

Figure S4. Pearson coefficient of correlation among samples from the Duikplaats, Netherlands, excluding samples from the order Chloroplast, with blue indicating a positive correlation (between 0 - 1), white indicating no correlation (0) and red indicating a negative correlation (≥0).

	Seawater	Sediment	Substrate	Empty shells
Seawater	1.00			
Sediment	0.06	1.00		
Substrate	0.07	0.56	1.00	
Empty shells	0.03	0.48	0.57	1.00

Figure S5. Pearson coefficient of correlation among samples from the Dansk Skaldyrcentre, Denmark, with blue indicating a positive correlation (between 0 - 1), white indicating no correlation (0) and red indicating a negative correlation (≥0).

	Seawater	Sediment	Substrate	Empty shell
Seawater	1.00			
Sediment	0.07	1.00		
Substrate	0.17	0.32	1.00	
Empty shell	0.08	0.37	0.61	1.00

Figure S6. Pearson coefficient of correlation among samples from the Dansk Skaldyrcentre, Denmark, excluding samples from the order Chloroplast, with blue indicating a positive correlation (between 0 - 1), white indicating no correlation (0) and red indicating a negative correlation (≥0).