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Seasonality combined with the orientation of surfaces influences the microbial community structure of biofilms in the deep Mediterranean Sea

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Abstract
Attachment to surfaces represents an important life strategy for microbial communities as indicated by the rapid colonization of biotic and abiotic surfaces in marine waters. However, little attention has been paid to the development of biofilm-associated microbial communities and the environmental parameters influencing biofilm development in the deep sea. In this study, a deep-sea experimental setup was used to follow the development of the microbial community colonizing solid surfaces deployed at 4500m depth at the deepest point of the Mediterranean Sea. The experiment was performed during summer (May to October 2007) and winter (October 2007 – May 2008), each lasting for 155 d. The phylogenetic composition of the biofilm community was determined by tag sequencing of the 16S rRNA gene. We investigated whether the composition of the deep-sea microbial biofilms is influenced by seasonality. Based on tag sequencing, operational taxonomic units were identified and diversity indices calculated. Seasonality combined with the orientation of the solid surface on which the biofilms were growing was the main factor influencing the structure of the microbial community. The most abundant phyla of deep-sea biofilm communities attached to the solid surfaces were Gammaproteobacteria (range: 10.8% to 92.6%), Alphaproteobacteria (range: 34.9% to 92.6%) and Betaproteobacteria (range: 0.3% to 2.1%), irrespective of the variables (surface, orientation, season). Flavobacteria and Epsilonbacteria show a clear preference with respect to the orientation of the deployed surfaces during the winter, however, they were essentially absent at the surfaces during the summer. Some bacterial classes such as Campylobacterales and Rhodobacterales showed distinct preferences for specific seasons or orientation of the substrate. Taken together, we conclude that even on deep-sea biofilms, there is to some extent seasonality detectable in the composition of the surface associated prokaryotic community, despite the fact that the deep-sea is, in terms of physico-chemical parameters, a fairly stable environment.

1. Introduction
All biotic and abiotic surfaces present in marine waters are rapidly colonized by microorganisms, also known as biofilms or microfilms (Loeb et al. 1975, Dang and Lovell 2015). Biofilm formation is typically initiated by the adsorption of organics to solid surfaces forming a primary organic coating (extracellular polymeric substances) onto which microbes from the surrounding water attach (Qian et al. 2007). Typically, microbes attached to surfaces are exhibiting higher growth rates than their free-living counterparts (Karner & Herndl 1992). Also, the composition of the microbial community attached to solid surfaces is different from that of the surrounding water indicating life-style preferences among members of the microbial community, i.e., surface-attached versus free-living (DeLong et al. 1993; Moeseneder et al. 2001; Salazar et al. 2015).

Surface attachment of microbes involves the expression of pili with which they attach to surfaces (Tuson & Weibel 2013). Since a number of the surface-associated microbes, mainly bacteria, produces copious amounts of extracellular polymers (Garrett et al. 2008), the biofilm grows into a three dimensional structure with redox-gradients potentially developing and generating ecological niches for e.g. anaerobes even in an otherwise oxygenated environment. Furthermore, in low-nutrient environments, microbial activity is higher in particle-attached and biofilm-associated microbes than in their free-living counterparts (Ellwood et al. 1982). This indicates that surface colonization and biofilm development provide advantages to bacteria (Dang and Lovell 2015).

Biofilm development has been intensively studied in medical and sanitary microbiology but less in natural environments, especially in deep sea environments. The first in situ studies on deep-sea biofilms used culture-dependent methods to describe microbially induced corrosion (Venkatesan et al. 2002) and biofilm formation
on structural material (Venkatesan et al. 2003; ANTARES Collaboration et al. 2003). A culture-independent fingerprinting method was used to describe the community composition of bacterial biofilms growing on solid surfaces in the deep Mediterranean Sea (Bellou et al. 2012). The advent of high throughput sequencing allowed for the characterization of the microbial communities with much higher resolution, recovering a large number of taxa with very low relative abundances, coined the ‘rare biosphere’ (Sogin et al. 2006). Also the dynamics of microbial communities were revealed (Zhang et al. 2014, Lee et al. 2014, Ding et al. 2019, Zhang et al. 2019). The microbial community composition in the deep Mediterranean, however, has not been as extensively investigated as that of the Atlantic and Pacific (Brian-Jaisson et al. 2014).

The aim of this study was to follow the succession of microbes associated with biofilms on solid surfaces in the deep Mediterranean Sea using amplicon sequencing and thereby achieving a higher phylogenetic resolution than fingerprinting methods applied in a previous study (Bellou et al. 2012). The hypothesis was tested whether seasonality influences the composition of deep-sea bacterial biofilm communities as it has been shown for the surface waters (Munteanu & Maly 1981, Underwood 1984, Lau et al. 2005). Specific focus was put on the role of substrate type and orientation on the composition of the prokaryotic community. Furthermore, as the deep Mediterranean is an oligotrophic system, we hypothesized that under these conditions it is particularly relevant for microbes to attach to surfaces and develop in a biofilm where nutrient concentrations are orders of magnitude higher than in the surrounding seawater. The results of this study are compared with studies on free-living versus particle-attached bacteria.

2. Material and methods

2.1. Study site description

The study site Nestor 4.5 is located in the Ionian Sea (Eastern Mediterranean Sea) representing the deepest part of the Mediterranean Sea. The Eastern Mediterranean Sea is characterized by extremely oligotrophic conditions (NESTOR Collaboration et al. 2006) with rather stable depth profiles in temperature (mean: 13.468°C) and salinity (mean: 38.76) from 1500 m downwards (Kontoyiannis and Lykoysis 2011). The Ionian deep-sea basin represents one of the largest areas of warm bathypelagic waters (Roether et al. 1996) compared to the typical bathypelagic water temperature of 1–3°C in the global ocean (Thistle 2014).

2.2. Experimental Setup

To test whether season, surface type and orientation influence the prokaryotic community composition of biofilms, an experimental platform was deployed at the Nestor 4.5 study site at 4500 m depth. A detailed description of the experimental setup is given elsewhere (Bellou et al. 2011, 2012) and images of the setup of the platform are provided in Supplementary Material. Briefly, the experimental platform hosted five different surfaces (aluminum, titanium, glass, limestone and shale), each in two orientations (horizontal and vertical) during two deployment seasons: summer (May to October 2007) and winter (October 2007 – May 2008). The total duration of the deployment for each of the two seasons was 155 d. The R/V Aegaeo was used for servicing and sampling the experiment.

2.3. DNA extraction and amplicon pyrosequencing

The methods used to collect the biofilm for molecular analyses are described in detail by Bellou et al. (2012). In short, after retrieving the experimental platform, samples were taken with a sterile cotton swab (Schwartz et al. 1998, Park et al. 2001) from each surface, placed into a sterile Eppendorf tube and immediately stored at -20°C until further processing in the laboratory. In the laboratory, DNA was extracted from each sample with the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc.) following the manufacturer’s recommendations. The integrity of DNA was checked on a 1% (wt/vol) agarose gel and DNA concentrations were determined fluorometrically on a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) following the manufacturer’s recommendations. The bacterial specific primers used for PCR were 6-carboxyfluorescein (6-FAM)-labelled primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACC T-3') (Lane 1991). DNA concentrations ranged between 3 and 16 ng/µl. Samples were sequenced using the bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) method performed by the Research and Testing Laboratories (Lubbock, TX; www.researchandtesting.com) (Dowd et al. 2008, Wolcott et al. 2009). This method has been shown to be effective in assessing the microbial community structure and used for bacterial communities in the marine environment (Sogin et al. 2006, Sundarakrishnan et al. 2012, Huang et al. 2013, Koo et al. 2015).

2.4. Data analyses
For each of the five surface types and the two orientations, one sample was analyzed per season and for each orientation resulting in a total of 10 samples per season. The raw DNA sequencing data were processed with QIIME version 1.7, an open-source bioinformatics pipeline for performing microbiome analysis of high-throughput sequencing data including quality filtering, operational taxonomic unit (OTU) picking, taxonomic assignment, and diversity analyses (Caporaso et al. 2010; http://qiime.org). The first step in the pipeline consisted of quality filtering to remove low quality and short reads. The minimum average quality score and minimum read length allowed in reads were 25 and 100, respectively, followed by demultiplexing the raw reads for assigning them to samples based on their barcode. The primer usage was disabled for the following analysis. The reads from all of the samples were clustered into OTUs based on 97% sequence similarity using uclust. Data retrieved via QIIME were manually quality controlled and whenever necessary adjusted/merged, e.g. summarizing all “unknown bacteria.”

A representative sequence was picked from each OTU for taxonomic identification and phylogenetic alignment followed by the assignment to each representative sequence using uclust and the database Greengenes version 12.10. A table of OTU abundances in each sample with taxonomic assignments was assembled. Those OTU abundances were used as the input table in the Vegan R package to calculate the Richness (S), Shannon (H) and Simpson diversity indices. Bray-Curtis matrix, ANOSIM, and SIMPER statistical analyses were also performed using the Vegan R package and the table of OTU abundances. For testing the influence of the factors season and orientation on the composition of deep-sea biofilm communities, principal component analyses (PCA) were performed based on the presence/absence data of phyla and taxa recorded in deep-sea biofilms using the software PRIMERv6 (Clarke & Gorley 2006).

3. Results

Rarefaction curves of all the deep-sea microbial communities are presented in Figure 1. For each sample separately, the total sequence number, total number of OTUs, as well as the indices richness (S), Shannon index (H) and Simpson index are presented in Table 1. The total number of sequences ranged between 1997 to 22821 in both seasons. Both the highest and lowest numbers of sequences were observed during the summer deployment reflected in the total number of OTUs ranging between 1666 and 18222. During summer, 4 out of 10 samples exhibited a total number of OTUs of > 8000 while in winter only 2 out of 10.

In total, 34 different classes and 64 different bacterial phyla were detected in the deep-sea prokaryotic biofilm communities (Figure 2, 3). Out of the 34 different classes detected, 21 of them exhibited at least in one sample an abundance > 1% of total community DNA (Figure 2A). More specifically, the bacterial phylum Proteobacteria, Bacteroidetes, Planctomycetes, Actinobacteria, Firmicutes, Cyanobacteria, Gemmatimonadetes, Verrucomicrobia, Thermotogae, Thermi, Tenericutes, Nitrospirae, Lentisphaerae, Fusobacteria, Chloroflexi, Chlororobi, Caldithrix, Armatimonadetes, Acidobacteria along with unknown Bacteria and other Bacteria were present in at least one variable (surface, orientation and season) with an abundance > 1% (Figure 2A, Table 1). Out of the 64 different bacterial phyla detected in the deep-sea biofilms, 11 of them (Gamma-, Alpha-, Beta-, Delta-, Epsilon-Proteobacteria, Flavobacteria, Planctomyceae, Sphingobacteria, Phycisphaerae, unclassified Proteobacteria and unknown Bacteria) had at least in one sample an abundance > 3% of total DNA (Figure 3A, Table 2).

Preferences in the deployment orientation were not observed within the bacterial phyla of deep-sea biofilms (Figure 2A). In contrast, the bacterial class Epsilonbacteria showed a clear preference for the winter and for colonizing horizontal surfaces. Although Flavobacteria were present on all surfaces and in both seasons, they showed a preference for the summer and for vertically oriented surfaces. Gammaproteobacteria was the most abundant class during the winter and on vertically oriented surfaces (Figure 3A, Table 2).

The most abundant phyla of deep-sea biofilm communities irrespective of surface, orientation or season were Gammaproteobacteria (range: 10.8% to 92.6%), Alphaproteobacteria (range: 34.9% to 92.6%) and Betaproteobacteria (range: 0.3% to 2.1%) (Figure 2B). Flavobacteria showed a clear preference to grow on vertically oriented surfaces during the summer and to horizontal surfaces during the winter (Figure 2A). Epsilonbacteria showed a clear preference to settle on horizontal surfaces and were almost absent in the biofilm community on vertical surfaces during the winter (Figure 2A). Epsilonbacteria were essentially absent during the summer (Table 2). Furthermore, when combining at least two of the tested variables only Proteobacteria, Bacteroidetes, unknown Bacteria, other Bacteria, Planctomyceae, Actinobacteria, Firmicutes, Cyanobacteria, and Gemmatimonadetes had an average relative abundance > 1% (Figure 2A, B, Table 1). The most abundant class on all surfaces, orientation and season was Proteobacteria (range: 41% - 97%), followed by Bacteroidetes (range: 0.1% to 45%) and unknown Bacteria (range: 2% to 14%) (Figure 3A, B). Campylobacteriales, an order of Proteobacteria, which makes up the epsilon subdivision together with
Rhodobacterales, an order of the Alphaproteobacteria, was present in high relative abundance only on the horizontally oriented surfaces. Rhodobacterales, an order of the Alphaproteobacteria, showed a shift in their preference of orientation between summer and winter, as their highest relative abundance during the summer was in biofilms growing on vertical surfaces. In contrast, Rhodobacterales was more abundant on horizontal than on vertical surfaces in the winter. The main contributing family or genus was the Rhodobacteraceae, a family of Proteobacteria of the order Rhodobacterales within the Alphaproteobacteria subgroup, and Sulfurimonas, as well as the Sulfurovum genus within the Epsilonproteobacteria class. The order Oceanospirillales was the only order of the Gammaproteobacteria present during the summer months.

Comparing average relative abundances of the bacterial phyla present in deep-sea biofilms over the two seasons indicated that Proteobacteria were the most abundant group both, in summer and winter. During the summer, the abundance of Proteobacteria was lower than in winter. Bacteroidetes dominated over Proteobacteria in relative abundance in winter followed by Planctomycetes, unknown Bacteria and other Bacteria (Figure 2B, Table 1).

Comparing average relative abundances of the bacterial classes present in deep-sea biofilms over the two seasons indicated that during the winter Gammaproteobacteria, Epsilonproteobacteria and Sphingobacteria exhibited the highest percentages (54; 15; 2%, respectively) (Figure 3B, Table 2). In contrast, Flavobacteria, Betaproteobacteria, Deltaproteobacteria, Phycisphaerae, Sphingobacteria, unclassified Firmicutes, unclassified Proteobacteria exhibited the highest abundances during the summer (Figure 3B, Table 2).

Figure 1. Rarefaction curves using the Vegan R package. Red curves are summer samples and blue ones, winter samples of the deep-sea biofilm communities.

Table 1. Total number of sequences, the total number of OTUs and the indices richness (S), Shannon index (H) and Simpson index in the deep-sea biofilms.

| Season | Sample ID   | total sequences | OTUs total | Richness (S) | Shannon index (H) | Simpson index |
|--------|-------------|-----------------|------------|--------------|-------------------|---------------|
|        | 4500.A.H.S1 | 10752           | 8813       | 1090         | 5.077             | 0.942         |
|        | 4500.A.V.S1 | 15071           | 10813      | 1390         | 5.272             | 0.968         |
|        | 4500.G.H.S1 | 1997            | 1666       | 225          | 3.851             | 0.917         |
|        | 4500.G.V.S1 | 22882           | 18222      | 1121         | 4.273             | 0.934         |
|        | 4500.L.H.S1 | 14745           | 11959      | 718          | 3.728             | 0.893         |
|        | 4500.L.V.S1 | 9353            | 7399       | 962          | 5.478             | 0.987         |
|        | 4500.S.H.S1 | 9139            | 7555       | 787          | 4.763             | 0.953         |
Figure 2. Relative abundances (as percentages) of bacterial phyla present in biofilms grown at different surfaces, seasons and orientation at 4500m depth at site Nestor 4.5 in the Eastern Mediterranean Sea; only phyla with at least 1% of total DNA in at least one of the tested variables (surface, season, orientation) are shown; (A) relative abundance for each variable separately — (A1) all phyla; (A2) all phyla except Proteobacteria; (B) relative abundances in each season — (B1) all phyla; (B2) all phyla except Proteobacteria.
Figure 3. Relative abundances (as percentages) of bacteria classes present in deep-sea biofilms according to the tested variables season, substrate type and orientation at 4500m depth at site Nestor 4.5 in the Eastern Mediterranean Sea; only classes with above 3% of total DNA in at least one of the tested variables (surface, season, orientation) are shown; (A) relative abundance for each variable separately – (A1) all classes (A2) all classes except Gammaproteobacteria; (B) average relative abundance grouped per season – (B1) all classes (B2) all classes except of Gammaproteobacteria.

Table 2. Average relative abundance (as percentage) of bacterial phyla present in deep-sea biofilms according to the tested variables (season and orientation) (> 1% in at least one of the three variables (season, surface, orientation). In bold the highest abundance as percentage for each bacterial phylum is given according to the variable tested.

| Season | Orientation | values per orientation & season separately |
|--------|-------------|-----------------------------------------------|
|        | horizontal | vertical                                       |
|        | horizontal | vertical                                       |
|        | horizontal | vertical                                       |
|        | horizontal | vertical                                       |
| Proteobacteria | 60.0 | 80.9 | 67.3 | 73.7 | 58.4 | 61.6 | 76.2 | 85.7 |
| Bacteroidetes | 13.3 | 5.8 | 8.4 | 10.7 | 8.7 | 17.9 | 8.1 | 3.5 |
| Bacteria unknown | 8.3 | 7.0 | 8.7 | 6.7 | 9.5 | 7.1 | 7.9 | 6.3 |
| Bacteria others | 10.0 | 8.1 | 10.2 | 7.9 | 11.1 | 8.9 | 9.2 | 6.9 |
| Planctomycetes | 9.4 | 2.7 | 8.6 | 3.5 | 13.6 | 5.2 | 3.6 | 1.8 |
| Actinobacteria | 2.6 | 1.3 | 1.8 | 2.1 | 1.80 | 3.4 | 1.8 | 0.7 |
| Firmicutes | 2.6 | 0.4 | 2.0 | 1.0 | 3.8 | 1.50 | 0.3 | 0.5 |
| Gemmatimonadetes | 0.0 | 0.0 | 1.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

Table 3. Average relative abundance (as percentages) of bacterial classes present in deep-sea biofilms according to the tested factors season and orientation (> 3% in at least one of the three variables season, surface, orientation). In bold the highest abundance as percentage is given for each bacterial phylum according to the variable tested.

| Season | Orientation | values per orientation & season separately |
|--------|-------------|-----------------------------------------------|
|        | horizontal | vertical                                       |
|        | horizontal | vertical                                       |
|        | horizontal | vertical                                       |
|        | horizontal | vertical                                       |
| Gammaproteobacteria | 39.8 | 53.9 | 37.9 | 55.8 | 47.9 | 31.7 | 27.9 | 79.9 |
| Alphaproteobacteria | 16.3 | 10.0 | 14.6 | 11.7 | 14.1 | 18.4 | 15.2 | 4.9 |
| Betaproteobacteria | 0.7 | 0.1 | 0.5 | 0.3 | 0.9 | 0.5 | 0.1 | 0.2 |
| Deltaproteobacteria | 1.2 | 0.3 | 1.0 | 0.6 | 1.5 | 1.0 | 0.6 | 0.10 |
| Epsilonproteobacteria | 0.3 | 15.2 | 14.8 | 0.7 | 0.1 | 0.5 | 29.5 | 0.9 |
| Unknown | 8.4 | 7.2 | 8.9 | 6.6 | 9.2 | 7.5 | 8.7 | 5.6 |
The PERMANOVA test (significance level set at a p-value of < 0.01) showed that there were no significant differences between deep-sea biofilm communities grown on different surfaces (p = 0.12991) and orientation (p = 0.031). Significant differences, however, were found in the composition of the deep-sea biofilm communities growing in the two different seasons, summer and winter (p < 0.001). These results were confirmed by ANOSIM based on the Bray Curtis index of the deep-sea biofilm communities grown on different surfaces (p = 0.97602), on the orientation of surfaces (p = 0.17782) and in different seasons (p = 0.000999).

Using the presence – absence data on the phylum- (Figure 4A1, A2) and class- (Figure 4B1, B2) level, a principal component analysis (PCA) showed that the prokaryotic community composition of the biofilms grown on surfaces was influenced less by the season alone, or by the orientation of the surface, but more by the combined effect of season and orientation of the surface. The substrate type, as expected from the PERMANOVA test, had only a minor influence on the bacterial community composition of the biofilms (data not shown).

Based on ANOSIM analysis, the phyla contributing most to the dissimilarities between biofilms grown on substrates with different orientation were Proteobacteria (67.2%), Bacteroidetes (16.9%), Planctomycetes (9.7%) and Actinobacteria (2.1%). The same phyla contributed to the seasonal differences between biofilms grown in winter and summer, as well as Proteobacteria (72.5%), Bacteroidetes (14.4%), Planctomycetes (7.6%) and Actinobacteria (1.7%). At the class level, Gammaproteobacteria (37.5%), Alphaproteobacteria (16.0%), Flavobacteria (16%), Epsilonproteobacteria (12.5%) and Phycisphaerae (8.6%) contributed the most to bacterial dissimilarities between biofilms grown on substrates with different orientations. The same classes but with a different percentage contributed as well to the bacterial dissimilarities between biofilms that were detected in different season: Gammaproteobacteria (47.9%), Alphaproteobacteria (13.4%), Flavobacteria (13.2%), Epsilonproteobacteria (10.3%), Phycisphaerae (6.4%), Actinobacteria (1.8%) and Sphingobacteria (1.2%). The top 10 OTUs that contributed to the differences in the bacterial community structure of biofilms grown on substrates with different orientations and of biofilms grown in different seasons are listed in Table 4.
Figure 4 PCA based on the presence/absence of bacterial phyla (A) and bacterial classes (B) present in deep-sea biofilms according to the parameters season (1) and combined season vs. orientation of the surfaces (2).

Table 4. The top 10 OTUs based on ANOSIM analyses contributed most to the dissimilarities of the bacterial community structure of biofilms grown on substrates with different orientations and of biofilms grown in different seasons. (Accession numbers from the NCBI)

| Orientation                      | Accession No. | OTU  | Genus             | Class                   | Phylum         |
|----------------------------------|---------------|------|-------------------|-------------------------|----------------|
| 13976 5213                       | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria | 14609 |                      |                         |                |
| 23173 5361                       | Root; k_Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__ | 129 |                      |                         |                |
| 16675 22                         | Root; k_Bacteria; p__Bacteroidetes; c__Flavobacteria; o__Flavobacteriales; f__Flavobacteriaceae | 4887 |                      |                         |                |
| 13498 2263                       | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__flavobacteria | 6022 |                      |                         |                |
| 18227 3594                       | Root; k_Bacteria; p__Planctomycetes; c__Phycisphaerae; o__Phycisphaerales; f__ | 329 |                      |                         |                |
| 121743109                       | Root; k_Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Helicobacteraceae | 31 |                      |                         |                |
| 2565 4215                        | Root; k_Bacteria; p__Proteobacteria | 1237 |                      |                         |                |
| 14569 140                        | Root; k_Bacteria; p__Bacteroidetes; c__Flavobacteria; o__Flavobacteriales; f__Flavobacteriaceae | 2968 |                      |                         |                |
| 16675 4904                       | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__flavobacteria | 5404 |                      |                         |                |
| 12174 0                          | Root; k_Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Helicobacteraceae | 3140 |                      |                         |                |
| 14589 249                        | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Oceanospirillales | 194 |                      |                         |                |
| 2069 188                         | Root; k_Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae | 1921 |                      |                         |                |

| Season                           | Accession No. | OTU  | Genus             | Class                   | Phylum         |
|----------------------------------|---------------|------|-------------------|-------------------------|----------------|
| 13976 645                        | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria | 19177 |                      |                         |                |
| 13498 345                        | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__flavobacteria | 7940 |                      |                         |                |
| 2565 5451                        | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__flavobacteria | 1 |                      |                         |                |
| 23173 27                         | Root; k_Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__ | 5463 |                      |                         |                |
| 16675 4904                       | Root; k_Bacteria; p__Bacteroidetes; c__Flavobacteria; o__Flavobacteriales; f__Flavobacteriaceae | 5 |                      |                         |                |
| 12174 0                          | Root; k_Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Helicobacteraceae | 3140 |                      |                         |                |
| 14569 3084                       | Root; k_Bacteria; p__Bacteroidetes; c__Flavobacteria; o__Flavobacteriales; f__Flavobacteriaceae | 24 |                      |                         |                |
| 14589 3067                       | Root; k_Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Oceanospirillales | 28 |                      |                         |                |
| 18227 3221                       | Root; k_Bacteria; p__Planctomycetes; c__Phycisphaerae; o__Phycisphaerales; f__ | 702 |                      |                         |                |
| 853 2403                        | Root; k_Bacteria; p__Proteobacteria; c__Alphaproteobacteria | 140 |                      |                         |                |

4. Discussion and Conclusions

So far, only a few studies have been devoted to biofilm development on submerged surfaces in the deep-sea. The number of studies describing the factors influencing the bacterial community composition of deep-sea biofilms is even less.

In total, 64 different bacterial phyla were detected in our deep-sea biofilms and thus, a higher microbial richness in these biofilms were obtained under conditions present in the deep Mediterranean Sea than in a cold seep system where 39 phyla were detected (Lee et. Al. 2014). Overall, the most abundant phyla in our biofilm communities attached to the solid surfaces were Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria irrespective of the variables surface, orientation or season. These results confirm the global predominance of Proteobacteria in marine biofilms (Salta et al. 2013) but in deep-sea biofilms, Gammaproteobacteria exhibit a higher abundance compared to Alphaproteobacteria, the latter reported to dominate the bacterial communities in biofilms of coastal waters (Salta et al. 2013, Zang et al. 2019).
In coastal waters, factors such as surface type, depth, exposition of the deployment and season influence the formation of bacterial biofilms (Head et al. 2004, Jones et al. 2007, Pasarelli et al. 2015). In this study, we hypothesized that the composition of deep-sea biofilms is more influenced by season than by surface type and its orientation to the prevailing current. Although in coastal waters, surface type has an impact on biofilms, this was not found in the present study. At least at the tested deployment depth, surface type seems to have less influence on the composition of the deep-sea biofilm compared to orientation and seasonality. This confirms the results of the limited influence of surface type compared to other variables on shaping microbial composition of deep-sea biofilms both in the open Mediterranean (Bellou et. al. 2012) as well as in brine pools and cold seeps in the Red Sea (Lee et al. 2014).

The results of this study showed that seasonality combined with the orientation of the solid surface was identified to influence the structure of the microbial community of deep-sea biofilms more than orientation and season separately. This is caused by a few bacterial classes showing distinct preferences for a specific season or orientation. At the same time the same phyla (Proteobacteria, Bacteroidetes, Plantomycetes and Actinobacteria) and classes (Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Epsilonproteobacteria and Phycisphaerae) contributed to the dissimilarities between these two tested factors. Bacteroidetes (mainly the Flavobacteria group) are frequently associated with surfaces (Salta et al. 2013). The dissimilarities of bacterial communities in the deep sea between particle-attached and free-living bacterial communities was also caused by different relative abundances of the "shared taxa" in the two types of communities (Liu et al 2018). While in this study we show that the dominant bacterial deep-sea biofilm classes are Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Epsilonproteobacteria and Phycisphaerae, in particle-attached and free-living bacteria communities Gamma-, Alpha-, Delta-, and Beta-proteobacteria are the dominant classes of Proteobacteria, without clear differences between the two lifestyles (Liu et al 2018).

A clear preference regarding the orientation of the deployed surfaces was evident for the two phyla Flavobacteria and Epsilonbacteria. Furthermore, the bacterial class Campylobacteriales exhibited a high relative abundance only on horizontally oriented surfaces, while Rhodobacteriales showed a shift in their preference between summer and winter. The influence of orientation in deep-sea biofilms has been described in an in situ study in the Eastern Mediterranean Sea (Bellou et. al. 2012). Using a fingerprinting method to characterize the richness of the bacterial community, the richness of the bacterial community was significantly higher in biofilms grown on horizontally oriented surfaces deployed in deep-waters between 1500m and 3500m, while at 4500m depth, the richness of the bacterial community was not significantly different between horizontally and vertically oriented surfaces (Bellou et. al. 2012). Studies in coastal seas have shown that settling of the benthic fauna is triggered by bacteria in biofilms (Huggett et al. 2006, Qian et al. 2007, Zardus et al. 2008, Sneed et al. 2014) and that recruitment patterns are affected by sediment deposition and organic biofilm present on horizontal and vertical surfaces (Sokolowski et al. 2017). The largest difference in bacterial community composition in biofilms has been observed between the surface orientation facing down- or upwards (Pia et al. 2017). This ‘preference’ of some bacterial phyla may result from their attachment to sinking particles landing on the upper side of the horizontal surface. It has been suggested that the diversity of particle-attached bacterial communities differs from that of free-living communities (De Long et al. 1993). Thus, particle-associated microbes might be better adapted to the conditions prevailing in biofilms than free-living microbes. Moreover, in a study on ocean gliders the authors observed the highest microbial abundance at the bottom of the glider’s body and the lowest abundance on the glider’s nose (Dobretsov et al. 2019).

A comparison of bacterial communities inhabiting the bathy- and abyssopelagic zones of the New Britain Trench showed that there are differences in the relative abundances of Actinobacteria and Flavobacteria in the particle-attached and free-living community (Liu et al 2018). In temperate waters, Rhodobacterales, especially the marine Roseobacter clade members, has been described to form the most common and dominant primary surface-colonizing bacterial group (Dang et al. 2008), indicating that the surface-colonization process in the deep sea might be slower than in surface and temperate waters. With respect to the season, it was observed that during the winter deployments Gammaproteobacteria, Epsilonproteobacteria, and Sphingobacteria exhibited the highest percentages, while during the summer the highest abundances were recorded for Alphaproteobacteria, Flavobacteria, Betaproteobacteria, Deltaproteobacteria, and Phycisphaerae, unclassified Firmicutes and unclassified Proteobacteria. Deep-sea particles and the surrounding water constitute two highly distinct niches for bacteria (Salazar et al. 2015).

The reasons for the observed seasonal differences in bacterial community composition in biofilm-associated microbial communities remain obscure since seasonal variability in physical and chemical parameters are essentially absent at the depth where the surfaces were deployed. Also, the variability of input of organic matter from surface waters should be rather minor and constant in these oligotrophic waters and at this depth
layer. While the composition of marine microbial biofilms is generally distinct from the surrounding seawater (Zang et al. 2014), it can be influenced by the location (Lee et al. 2014, Zang et al. 2019) and by the initial bacterial colonization (Gregorio et al. 2018). In coastal waters, the composition of the bacterial community of biofilms can change rapidly with changing environmental conditions such as temperature and salinity and seasonal bloom conditions (Lau et al. 2005, Bourne and Webster 2013). In contrast, the Eastern Mediterranean Deep Water is the bottom water of the Eastern Mediterranean filled with waters of Adriatic origin (Bensi et al. 2013) and dominated by bottom stratification (Rubino and Hainbucher 2007). Stable conditions are characteristic for these Mediterranean deep waters. Thus, seasonal changes in deep-sea surface-associated microbial communities are not expected. A study on bacteria exhibiting bioluminescence performed in the Eastern Mediterranean Sea also did not indicate seasonal variability in their abundance (Craig et al. 2011). Thus, seasonal variation in the community composition of deep-sea biofilms at 4500 m depth is rather surprising. The extent of variation in the community composition on these surfaces, however, is much lower than the variations observed in surface waters of the Eastern Mediterranean Sea. In coastal waters, studies showed significant seasonal changes in the composition and abundance of biofilm-associated microbes (Munteanu & Maly 1981, Underwood 1984, Lau et al. 2005). In a more recent study, a clear relation was found between bacterial growth, community composition and phytoplankton blooms (Luria et al. 2016). Specifically in the Ionian Sea, primary productivity exhibits a high seasonal variability with highest primary production observed during the winter/spring convective mixing period (Bosc et al. 2004, D’Ortenzio and Ribera d’Alcala 2009). Although seasonality in the deep-sea biofilm community composition is surprising, a study on downward fluxes of sinking particulate matter in the same study area where this experiment took place showed seasonal and interannual variability (Stavrakakis et al. 2013). The total mass flux of particles collected by sediment traps showed a strong seasonal signal with higher fluxes during the summer (May to October 2007) and lower fluxes during the winter (October 2007 – May 2008). Furthermore, regarding the vertical distribution of mean total mass fluxes, the results of the latter study showed relatively increased fluxes and organic carbon concentrations are present at 4300m as compared to the upper water layers. Specific bacterial communities are associated with hydrographically distinct water masses (De Corté et al., 2009, Galand et. al 2009, Yokohama et al., 2010, Agogué et al. 2011), which exhibit qualitative and quantitative variations in their organic substrate contents (Meador et al., 2010).

In summary, we have shown that deep-sea artificial surfaces are colonized by bacteria in a similar way as surfaces in the euphotic layer. There are indications that the process of biofilm development takes more time in deep-sea environments compared to coastal waters. The community composition of deep-sea biofilms differs from that described for coastal waters. Seasonality influenced the structure of the microbial community of deep-sea biofilms more than surface type and orientation. There are indications that the combination of the orientation of the solid surface together with season is mainly determining the structure of the bacterial community of deep-sea biofilms. Furthermore, a clear preference of specific phyla was detectable for the orientation of the solid surface. Although the deep-sea community structure in the biofilms changed within season, specific bacteria were detected for each of the two seasons.

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7. Competing interests statement
No competing interests
Dear Editors,

We are enclosing herewith a manuscript entitled “Seasonality combined with the orientation of substrata influences the microbial community structure of biofilms in the deep open sea” for publication in “Deep-Sea Research II – SI: Recent Med Status”.

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript (see table Contributions of all authors to the work and table Responsible authorship). Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the.

For the Editors, we would like to disclose the following information about the project:
The research project was conducted under the supervision of Prof. Colijn, Franciscus, Christian-Albrechts-Universität zu Kiel, Forschungs- und Technologiezentrum Westküste, Büsum, Germany and Helmholtz-Zentrum Geesthacht, Zentrum für Material und Küstenforschung, as the Ph.D. supervisor of Dr. N. Bellou. The data were then analyzed and processed after the fulfillment of my Ph.D. and during my short-term visit at Prof. Herndl Gerhard lab at the University of Vienna, Austria, specifically at the Faculty of Life Sciences and the Division Bio-Oceanography, Dept. of Limnology & Bio-Oceanography and in cooperation with Mr. Juan Antonio Garcia.

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From the same project the following manuscripts have been published.

Bellou N, Colijn F, Papanathanassiou E. 2011. Experimental settlement study in the Eastern Mediterranean deep-sea (Ionian Sea). Nuclear instruments and methods in physics research Section A: Accelerators, spectrometers, detectors and associated equipment 626–627 (1): S102–S105.

Bellou N, Papanathanassiou E, Dobretsov S, Lykousis V & Colijn F. 2012. The effect of substratum type, orientation and depth on the development of bacterial deep-sea biofilm communities grown on artificial substrata deployed in the Eastern Mediterranean, Biofouling, 28(2): 199-213.

Contributions of all authors to the work

Dr. Nikoleta Bellou is a Research Scientist at the Institute of Oceanography at the HCMR. Her research foci are benthic ecosystem ecology with focus on biofouling and on impacts of marine litter on these ecosystems. Her PhD research topic was “Biofouling in different habitats, from coastal to deep sea” under the Framework of the European Project KM3NeT. Results from this latter study are being submitted. The experimental design, the fieldwork during ship-cruises (deployment, retrieve, and sampling) as well as the laboratory work was made by her. Statistical analyses, graphs as well as the preparation of the manuscript down to the final format were done by her. The use of bibliographic
Information and references are mainly her responsibility with the collaboration with the co-author.

Juan Antonio Garcia is a bioinformatician at department marine biology of the University of Vienna. Together with the first author, he processed the raw DNA sequencing data for the diversity analyses of the bacterial communities, supported the manual quality control and data interpretation.

Prof. Herndl Gerhard hosted Nikoleta Bellou at his lab at the University of Vienna during her visit as a Visiting Scientists. He supported the Data interpretation, bibliographic information and references and the manuscript preparation, as well as evaluated critically the current manuscript before submission.

Prof. Colijn Franciscus, supervised the work of Nikoleta Bellou during her PhD at the Christian-Albrechts-Universität zu Kiel, Forschungs- und Technologiezentrum Westküste, Büsum, Germany and Helmholtz-Zentrum Geesthacht, Zentrum für Material und Küstenforschung. He supported the, bibliographic information and references and the manuscript preparation and evaluation before submission.

**Responsible authorship:**

| Title of Manuscript: | “Seasonality combined with the orientation of substrata influences the microbial community structure of biofilms in the deep open sea” |
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| Cross (X) used to denote each individual author’s involvement in the following aspects of the research: | Framing hypotheses and experimental design | Laboratory or field work | Data analysis and interpretation | Manuscript preparation |
|                       | X | X | X | X | |

**Significant findings of submitted articles and novelty of work**

So far, only a few studies have been devoted to biofilm development on submerged surfaces in deep-sea environment. The number of studies describing the factors that are influencing the bacterial community composition of deep-sea biofilms are even less.

In this study, in situ experiments took place at the Hellenic Trench, the deepest point of the Mediterranean Sea for not only testing the variables substrate, orientation and season on their influence on bacterial community composition of deep-sea biofilms but as well to describe the bacteria that are forming biofilms in deep-open sea using tag sequencing of the 16S rRNA gene.

Results showed that seasonality combined with the orientation of solid surface was identified to influence the microbial community structure of deep-sea biofilms and to a lesser extend orientation and season separately. The bacterial community composition of deep-sea biofilms is being presented.

To the authors knowledge this is the first study, testing and presenting data on the influence of the tested variables on deep-sea biofilm communities with emphasis on the seasonality, as well as describing the most abundant bacterial class and phyla contributing the biofilm communities that are growing on substrata deployed in the deep sea. Considering that in situ experiments in deep-sea are not only costly but as well difficult to perform data and results are unique and give insights on the settling processes that take place in deep-open sea environments.
Conflict of Interest

In accordance with Elsevier policy and the ethical obligation as researchers, we would like to declare that there are no potential conflicts of interest in relation to our submitted manuscript.