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Abstract

Despite clear advances in characterizing marine biofilms, details on their formation and species succession remain scarce particularly during the early stage of development. We investigated the microbial community composition and succession in coastal marine biofilms on plastic. Samples were collected over 75 days of immersion with strengthened samplings during the early stages of biofilm establishment. Biofilm composition was estimated using Illumina Miseq and microbial community interactions were assessed through microbial association network analysis. *In silico* analyses showed that primers used in most of previous studies considerably underestimated marine biofilm diversity. Unintentionally ignored so far, we showed that Flavobacteriia might be key actors in the functioning of marine biofilms. Gamma-proteobacteria from the genus *Oleibacter* strongly dominated microbial communities during the first hours of biofilm formation. These pioneer communities were quickly replaced by alpha-proteobacteria and Flavobacteriia. Bacterial communities exhibited fast temporal structure dynamics with taxa displaying rapid increases and declines. 90% of OTUs were intermittent or ephemeral reinforcing the conclusion that marine biofilms are highly dynamics. With 2/3 of positive significant connections between bacterial OTUs, microbial biofilm communities appear to be more inclined to develop inter-specific cooperation rather than competition and might thus form sets of functional guilds with mutual metabolic exchanges.

Key words: marine biofilm, dynamics, Flavobacteriia, network, pioneer bacteria, artificial surface

Introduction

The past 20 years of research in aquatic microbial ecology have seen advances in understanding the dynamics of microbial communities in marine systems and have revealed the importance of environmental forcing in determining general features of microbial community dynamics and composition (e.g. (Fuhrman, 2009, Galand *et al.*, 2009, Ghiglione *et al.*, 2012, Gilbert *et al.*, 2012, Giovannoni & Vergin, 2012, Teeling *et al.*, 2012, Chow *et al.*, 2013, Fuhrman *et al.*, 2015, Suh *et al.*, 2015, Berdjeb *et al.*, in review)). It becomes now obvious that inter-and intra-specific microbial interactions also represent remarkable drivers of microbial communities in aquatic ecosystems (Fuhrman *et al.*, 2015). It is thus critical to identify and characterize these interactions to better understand the dynamics of microbial communities in these ecosystems.

Marine biofilms are fascinating ecosystems. They are complex microbial aggregations that ubiquitously develop on substrates in seawater and are composed of thousands different microbial species that potentially interact together. Biofilms are known to significantly influence the productivity and functioning of coastal ecosystems by contributing to fundamental microbial processes such as degradation of organic matter and environmental pollutants, photosynthesis and cycling of nitrogen (Lock *et al.*, 1984, Davey & O'Toole, 2000, Egan *et al.*, 2008). It has also been suggested that they are both inductive (Huang & Hadfield, 2003, Qian *et al.*, 2003, Webster & Negri, 2006, Harder *et al.*, 2012, Chen *et al.*, 2013) and inhibitive mediators (Maki *et al.*, 1988, Wieczorek & Todd, 1997) of larval settlement for a number of fouling benthic species. Biofilms are also commonly known for their detrimental impacts on human activities as their development negatively influences the efficiency of ships by reducing speed and increasing fuel consumption (e.g. (Townsin, 2003, Schultz, 2007)), or promote the corrosion of metallic structures (Paisse *et al.*, 2013, Kip & van Veen, 2015). They are also actors of the plastic degradation as key members of the plastisphere, that become a prominent marine environmental issue (eg (Zettler *et al.*, 2013,

Debroas *et al.*, 2017). In these ecological and economical contexts, information on biofilm microbial ecology and functioning are crucial to protect these delicate ecosystems and develop successful natural control strategies to prevent their development on the hull of boats.

During the past ten years, and conversely to planktonic communities, microbial structure, biodiversity and composition in marine biofilms only started to be described (e.g. (Lee *et al.*, 2008, Briand *et al.*, 2012, Toupoint *et al.*, 2012, Lee *et al.*, 2014, Zhang *et al.*, 2014, Lawes *et al.*, 2016, Yang *et al.*, 2016, Briand *et al.*, 2017, Sathe *et al.*, 2017). As summarized in latest reviews (Salta *et al.*, 2013) (Dang & Lovell, 2016), bacteria in the *Alteromonas* (*γ-Proteobacteria*) and *Roseobacter* (*α-Proteobacteria*) genus would be the main primary substrate colonizers whereas Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, *β-*, *δ-* and *ε-Proteobacteria* and Verrucomicrobia have been identified as minor groups during the first stage of colonization. After several days of immersion, the predominance of *Proteobacteria* (especially *α-Proteobacteria*) was observed in many studies. However, most of these previous studies used primer pairs with a non-optimal coverage for prokaryote communities (e.g. (Dang & Lovell, 2000, Lee *et al.*, 2008, Lee *et al.*, 2014, Lee *et al.*, 2014, Muthukrishnan *et al.*, 2014, Zhang *et al.*, 2014, Lawes *et al.*, 2016, Briand *et al.*, 2017). This could have considerably biased results on both microbial diversity and composition and overshadow potential interesting interactions. The choice of 16S primers is crucial to obtain high resolution and the most accurate estimation of microbial diversity and composition in biofilms ecosystems. In addition, details on species succession and interaction remain scarce and poorly understood. Finally, no information is currently available on the co-occurrence of microbial taxa and factors that drive the biofilm formation and dynamics.

We investigated marine biofilm development and potential interactions within microbial communities in a coastal Mediterranean site for 1 to 75 days. 16S rDNA amplifications were

performed using a generalist primer pair (515F-Y/926R, (Parada *et al.*, 2015)) which presented, according to *in silico* analysis, the most important coverage for several Bacteria and Archaea groups (between 83% and 88%). We used Miseq Illumina chemistry to characterize both microbial community composition and dynamics and identify the main OTUs actors in marine biofilms. Microbial succession and co-occurrence patterns were characterized using multivariate approaches and network analysis

Materials and methods

Experimental design, immersion site and environmental variables

We used 42 Polyvinyl Chloride (PVC) panels as substrate to study the biofilm formation. Panels were previously sandblasted to promote microbial adhesion. All PVC panels have been immersed for one to 75 days. Seven sampling time points have been chosen: 1, 4, 8, 12, 20, 28, and 75 days. The immersion site was located in the Toulon Bay (France, Mediterranean Sea). A static permanent raft allowed the immersion of panels at one meter depth.

For each sampling time points, water temperature, pH and salinity were measured using a Hydrolab® DS5X probe (Hatch Hydromet, USA) Dissolved organic carbon (DOC) and total nitrogen (TN) were analyzed on a TOC-VCSH analyzer (Shimadzu) (Oursel *et al.*, 2013). Nutrients (NO_3^-, PO_4^{3-}, $Si(OH)_4$) were analyzed using standard colorimetric methods for seawater (Coclet *et al.*, 2017). Because the Toulon Bay is known to be highly contaminated by various trace metals (Cd, Cu, Pb, Zn), their concentrations were determined by voltammetry on fully automated Metrohm/Ecochemie system (Cindric *et al.*, 2015).

Microbial community analysis

Flow cytometry analyses

For each sampling time points, a set of three panels were used to estimate microbial community abundances by flow cytometry. Panels were totally scraped using sterile scalpel and the collected biofilms were fixed with 4 ml of a 2% formaldehyde–sterile artificial seawater (ASW). Samples were quickly frozen in liquid nitrogen and maintained at -80°C until analysis. Abundances of both prokaryotic and autotrophic communities were estimated using a BD AccuriTM C6 flow cytometer (BD Biosciences) (Camps *et al.*, 2014). Data were acquired using BD Accuri CFlow Plus software and abundances of each group were expressed as number of cells per cm^{-2}.

Nucleic acids extraction (DNA) and PCR amplifications

For each sampling time points, three PVC panels were used to DNA extraction. Panels were totally scraped and biofilms were immediately dropped into liquid nitrogen and maintained at -80°C. DNA were extracted using the PowerBiofilm DNA isolation Kits (Mobio) following the supplier's instructions.

In silico primer coverage for multiple available primer pairs was analyzed with 0 mismatch using Silva TestPrime 1.0. We analyzed 6 different primer pairs usually used to amplify the 16S rRNA gene and study biofilm microbial community diversity and composition (Table 1) and tested a primer pair recently developed in marine waters, 515F-Y/926R (Parada *et al.*, 2015). Based on these results we chose the primer 515F-Y/926R.

The PCR reaction (50 µL) contained 10 µL of 5x HotStar HiFidelity PCR buffer, 1 µM of each primer, 2.5 U of HotStar High Fidelity DNA polymerase and approximately 2 ng of DNA. The following thermal cycling scheme was used: initial denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 1min, followed by extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 min. Negative controls were performed by using the reaction mixture without template. PCR products were checked on a 1% agarose gel.

Amplicons were cleaned and concentrated using 1X magnetic Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Concentrated DNA was quantified by PicoGreen fluorescence assay (Quant-iT™ PicoGreen® dsDNA Assay Kit, ThermoFischer Scientific) and pooled at equimolar concentrations.

Sequencing and data processing

The equimolar mix was sequenced by GENOSCREEN (Lille, France) using Miseq Illumina 2 x 250 pb chemistry. Sequences were processed following the MiSeq SOP (Kozich *et al.*, 2013) including alignment against the SILVA v119 database, and trimming to include only the overlapping regions. Sequences were then clustered to form operational taxonomic units (OTUs) at 97% similarity and pre-clustered at 2 bases similarity to reduce the effects of sequencing errors. Chimera detection was performed with UCHIME (Edgar *et al.,* 2011) and classified with the default mothur classifier (Wang *et al.*, 2007) using the SILVA v119 database at an 80% confidence cut-off (Quast *et al.*, 2013). Data sets were resampled down to equal number of 5000 sequences. Samples with fewer than 5000 sequences were not included in the analyses. Four samples were thus excluded from the analysis because they had too few sequences (T1R2, T4R1, T4R2 and T75 R3). The samples were normalized by analysing the relative abundance for each OTU as the proportion of all sequences in a sample.

Hierarchical agglomerative clustering of Bray-Curtis similarities was performed on the 350 most abundant OTUs (> 1% of the total number of sequences) to discriminate different clusters and identify potential succession in the temporal formation of biofilms (PRIMER software version 6.1.18). To test the null hypothesis, that there was no significant difference between the groups discriminated according to the agglomerative clustering analysis, we

conducted an analysis of similarities with the subroutine ANOSIM on PRIMER (6.1.18). Among the 350 OTUs, the temporal dynamics of the 31 most abundant, displaying a relative abundance > 2% in at least one sample was visualized as bubble chart using 'bubble.pl.program'. A network analysis was finally performed. Extended Local Similarity Analysis (eLSA) (Xia *et al.*, 2011) was used to assess temporal covariation between the 350 most abundant bacterial OTUs. P-value was estimated using "mixed" approach (Xia *et al.*, 2013). Q-value was calculated to control false positives (Storey 2002). eLSA network was visualized using Cytoscape v2.8.3 (Shannon *et al.*, 2003), with $P<0.01$ and $Q<0.05$. Because the sampling was not evenly spaced, the time-lagged was not considered. The cluster detection was performed using AllegroMCODE (Bader & Hogue, 2003). In each cluster, the betweenness centrality (BC) and the closeness centrality (CC) of each node (OTU) were estimated (Bauer *et al.*, 2010).

Results

Environmental characteristics

Whereas temperature increase from T0 (22.3°C) to T20 (25.8°C) before decreasing (23.4°C for T75), , salinity (36.8 to 38.5), pH (7.98 to 8.18), low values of TN, nitrates or DOC slightly varied (Table S1) Trace metals levels remained weak for Cd (average factor of 3) when Pb, Cu and Zn exhibited much higher levels (maximal factors of 50, 54 and 146, respectively), (Table S1).

Microbial community densities

Heterotrophic prokaryote and autotroph (including *Synechococcus*-like, pico and nanoautotrophs) abundances are presented in Figure 1. We estimated prokaryote abundances between 3.5×10^4 after one day of immersion and 7.2×10^7 cells.cm^{-2} after 75 days of

immersion. Autotroph abundances were lower and ranged between 5.7×10^2 (T_1 day) and 4.9×10^6 cells.cm^{-2} (T_{75} days). Similar trends were observed for both communities with increasing in microbial cell abundances throughout the immersion (approximatively 20.000 and 9000-fold increase for prokaryotes and autotrophs respectively), and a steady-state that seems to be almost reach after 75 days.

Microbial community composition and structure

In Silico analyses

Results of *in silico* primer pair comparisons are presented in the Table 1. Six studies on biofilm diversity used primer pairs with coverage for bacteria ≤ 65% (Table 1A). Most of these primer pairs do not detect archaea. Only the primer pairs U905F/U1492R, and 515F/1390R matched 4 and 32% of archaea sequences respectively. The primer 515F-Y/926R, recently developed to study microbial communities in marine waters, matches the two domains with coverages of 88% and 83% for Bacteria and Archaea respectively. The 515F-Y/926R thus yields more accurate estimates of bacterial and archaeal community diversity and composition.

Considering bacterial phyla usually found in marine biofilms, comparisons of the primer 515F-Y/926R with the others (differences between the percentages found for this primer pair and the smallest and highest percentages found among the six tested primer pairs) showed an increase from 24 to 85.2% in the perfect matches to Bacteroidetes mainly driven by an increased detection of Sphingobacteria and Flavobacteriia from 20 to 90% and 21 to 90% respectively (Table 1B). Comparison also showed an increase from 18 to 92 % in the perfect matches to Alpha-proteobacteria driven by Rhodobacterales and increased from 6 to 92% in the perfect matches of Gamma-proteobacteria driven by greater detection of Alteromonadales. Finally, primers showed an increase from 14 to 88%, 10 to 83%, 18 to 85% and 24 to 87% in the perfect matches to

Cyanobacteria, Firmicutes, Planctomycetes and Verrucomicrobia respectively (Table 1B).

Community composition and structure

After sequence analysis, few sample replicates (T_1R_2, T_4R_1-R_2, $T_{75}R_3$) have been removed due to their low number of sequences (< 5000). Considering all samples, 7012 OTUs have been identified. H' index varied between 4.1 and 6.1 with the lowest and highest values estimated after T1 and T12 days of immersion respectively (Figure S1). No clear temporal patterns were detected except a lowest diversity during the first 24h of immersion. Chao varied between 1302 and 3463 with the lowest and highest values estimated after T_1 and T_4 days of immersion respectively. For all samples, the rarefaction curves did not reach the saturation level which indicates that a higher number of sequences would be required to cover the whole diversity of samples (Figure S2).

Prokaryotic communities were dominated by *Proteobacteria* through the immersion period with relative abundances between 36.6% and 69.9%. *Gamma-proteobacteria* were highly dominant after one day of immersion (T1) representing up to 59.1% (Figure S3, Figure 2). They were mainly represented by Oceanospirillaceae (20 - 30%) and Alteromonadaceae (14%). Their presence strongly decreased from T_4 with percentages estimated between 12 and 19.3%. Alpha-proteobacteria, mainly represented by Rhodobacteraceae, were conversely less present after one day (T_1) of immersion (10%) but became dominant from T_4 up to the end of the immersion (21 – 44%). Delta-proteobacteria that include Sulfate-Reducing Bacteria (SRB) were very low or absent during a large part of the experiment and appeared at 75 days (>1%). Bacteroidetes were the second most represented bacterial phylum with relative abundances estimated between 22.4 and 39.3% without clear temporal patterns. In all studied samples, they were constantly dominated by members of Flavobacteriia particularly Flavobacteriaceae with relative abundances estimated between 14.3 and 31.7%. In contrast, relative abundances of Sphingobacteria ranged between 4.9 and 13.2% which represented, in average, less than 10% of the microbial biofilm

community. Unclassified Archaea were detected in some samples (T_1R_3, T_4R_3, T_{12}R1-R_2, $T_{20}R_2$) but their relative abundances did not exceed 0.4%.

Hierarchical agglomerative clustering of Bray-Curtis similarities was performed on the 350 most abundant OTUs (Figure 3). Five different clusters were discriminated at 50% of Bray-Curtis similarities (ANOSIM: R = 0.952, p = 0.01). This cluster analysis revealed high succession changes with the predominance of Gamma-proteobacteria, Flavobacteriia and Alpha-proteobacteria. Bacterial communities exhibited fast temporal structure dynamics with bacterial taxa that displayed rapid increases and declines. The succession of the most abundant OTUs (i.e. > 2 % in at least in one sample) is presented in Figure 4. We defined these 31 most abundant OTUs as persistent (>75% of sampling dates), intermittent (25-75%) or ephemeral (<25%) (Chow *et al.*, 2013, Berdjeb *et al.*, in review). More than half of considered OTUs (61%) are intermittent and 29% are ephemeral. Only three OTUs (OTUs 7, 8 and 17), all members of Alpha-proteobacteria - Rhodobacteraceae, were persistent.

Both OTUs 1 and 5 strongly dominated prokaryote communities after one day of immersion (T_1) with relative abundances around 13% and 30% in average respectively (Figure 4) before collapsing from T_4. After four days of immersion (T_4), several OTUs members of Flavobacteriia (3, 79 and 75) became dominant as well as some proteobacteria (OTU 311 for gamma-proteobacteria and OTU 8 for Alpha-proteobacteria). These OTUs quickly rarefied and some others became dominant at T_8, mainly represented by Alpha-proteobacteria (OTUs 17, 29, 56), Flavobacteriia (OTU 45) and Sphingobacteria (OTU 27). New dominant OTUs appeared at T_{12} were members of Verrucomicrobia (OTU 46), Gamma-proteobacteria (OTU 61), Alpha-proteobacteria (OTU 7) and Flavobacteriia (OTU 11). From T_{12}, the dynamics slowed down as three of the four most abundant OTUs (46, 7, 11) remained present among the most abundant at

T_{20} (46, 7, 2, 65, 11, 91). Similarly, four of the six OTUs found at T_{20} stay among the most abundant at T_{28} (OTUs 65, 2, 7, 11). Finally, five OTUs (8, 14, 34, 112, 167), most of them rarely present or absent through the immersion, dominated the community at T_{75}. All these OTUs belong to the phylum Proteobacteria, members of Alpha-proteobacteria (OTUs 8 and 14), Gamma-proteobacteria (OTUS 34 and 112) and Delta-proteobacteria (OTU 167).

Network analysis

Among the 350 most abundant OTUs, 303 nodes and 1913 edges were determined considering correlations higher than or equal to 0.8 (SSCC ≥ 0.8; PSSCC < 0.01 and QSSCC < 0.05) (Figure 5). 65% of correlations were positive. Five classes of bacteria were mostly represented (Table 2A), Alpha-proteobacteria (76 nodes), Flavobacteriia (57 nodes), Gamma-proteobacteria (52 nodes), Sphingobacteria (25 nodes) and Planctomycetacia (18 nodes) (Figure 5). Flavobacteriia exhibited the highest number of edges (723). 55 OTUs had a number of edges higher than 20 (Table 2B, Figure 5). Among them, 13 belong to the 31 most abundant OTUs. The remaining OTUs were rare with low temporal dynamics through the immersion period. Among the most abundant OTUs, the OTUs 75 (*Krokinobacter sp*), 65 (*Granulosicoccus sp*) and 91 (unclassified Flavobacteriaceae) presented the higher number of edges (54, 54 and 53 respectively). They were characterized by high closeness centrality (> 0.9).

The network exhibited 12 clusters (Figure 6). 18 of the most abundant OTUs were found among the 12 clusters which presented a total of 105 nodes. The 31 most abundant OTUs represented only 17% of the OTUs identified in the Clusters. Clusters exhibited various structures which consisted of 3 to 21 nodes (Figure 6). For most of clusters and particularly for the four bigger ones, OTUs belonging to Flavobacteriia exhibited the highest BC and CC and thus represented the more central nodes (Figure 6). Six clusters had exclusively positive edges

(clusters III, V, VI, VIII, XI and XII). clusters I, II and IV exhibited much more positive edges than negative. Finally, only 3 clusters (VII, IX and X) displayed more negative edges than positive. Note that the 3 persistent OTUs were absent (OTUs 7 and 8) or detected in the Cluster VII with only 3 edges (OTU 17).

Considering the 350 most abundant OTUs, environmental variables appeared highly connected with bacteria community dynamics as they presented a total of 316 links with OTUs and between 10 and 54 links were observed for each environmental variable (Table 2C, Figure 5). Temperature was the most connected variables (54 edges) when silicate (SiOH) was the less (10 edges). Interestingly, when we considered only the 31 most dynamics OTUs, the total number of edges strongly decrease to 35.

Discussion

Members of Flavobacteriia highly underestimated in coastal marine biofilms

The choice of primer sets to amplify 16S genes is crucial to estimate as accurately as possible microbial diversity and composition. Before developing our study, we tested the theoretical performance of six primer sets recently used to study marine biofilms and we compared results with a primer set (515F-Y/926R) initially developed to improve the coverage of bacterial groups in marine waters (Parada *et al.*, 2015). The six tested primer sets showed contrasting efficiency and considerably underestimated many bacterial phyla as Firmicutes, Planctomycetes, Verrucomicrobia and Bacteroidetes. In this latter phylum, it was particularly the case for Flavobacteriia with coverage sometimes inferior to 1% for some primer sets. The 515F-Y/926R primer pair considerably increases the percentage of detection of numerous bacterial and archaeal taxa. Using this primer set, our work highlighted the great abundance of Bacteroidetes in marine biofilms particularly Flavobacteriia which represented between 14 and 32% of the

bacterial community. While we recently used a less efficient primer set (775F/1103R), Flavobacteriia represented only 1.5% of the taxa found on PVC immersed in Toulon Bay during summer (Briand *et al.*, 2017).. While the large predominance of Alpha- and Gamma-proteobacteria together with Bacteroidetes as a phylum seemed to be definitively established in marine biofilms (e.g. (Jones *et al.*, 2007, Dang *et al.*, 2008, Elifantz *et al.*, 2013, Salta *et al.*, 2013, Dang & Lovell, 2016), we demonstrate using a more efficient primer sets that Flavobacteriia been largely underestimated and represent dominant members in these ecosystems.

Microbial community succession in coastal marine biofilms

Despite clear advances in characterizing marine biofilms, details on their formation and species succession remain scarce particularly during the early stage of biofilm establishment. As previously reported (Jones *et al.*, 2007, Dang *et al.*, 2008, D'Ambrosio *et al.*, 2014, Simon *et al.*, 2014, Briand *et al.*, 2017), marine biofilms were highly diversified from 24h of immersion. The pioneer biofilm was largely dominated by Gamma-proteobacteria particularly members of the genus *Oleibacter*. Succession of bacterial communities in biofilms during the first 36h of substrate immersion has been characterized for the only first time by Lee et al. (Lee *et al.*, 2008). These authors described two stages in this early biofilm formation, i.e. stage 1 during 0-9h highly dominated by Gamma-proteobacteria (e.g. *Acinetobacter*, *Alteromonas*, *Oceanobacter*) and stage 2 during 12-36h dominated by Alpha-proteobacteria (Dang & Lovell, 2000, Jones *et al.*, 2007, Lee *et al.*, 2008). Our findings slightly contrast with this description and it appears that Gamma-proteobacteria (*Oleibacter* sp.) could dominate a bit longer the first hours of biofilm formation as they were still present after 24h of immersion. Note that this might be simply due to dissimilar environmental conditions. However, in accordance to Lee et al. (Lee *et al.*, 2008), our findings support the hypothesis that Gamma-proteobacteria might be considered as the major and genuine

pioneer bacterial group in marine biofilms. Few hypotheses have been proposed to explain this high dominance of Gamma-proteobacteria in the early stages of the biofilm formation. This might depend on the chemical properties of the artificial surfaces used as substrate. Most of these surfaces are partially composed of petroleum derivative products (e.g. PVC ≈ 40% of petroleum compounds). Interestingly, members of the genus *Oleibacter, Oceanobacter, Alteromonas* and *Acinetobacter* are known to be hydrocarbonoclast species and thus involved in the degradation of hydrocarbons (e.g. (Teramoto *et al.*, 2009, Teramoto *et al.*, 2011, Zhang *et al.*, 2014)). Lee et al. (Lee *et al.*, 2008) showed dissimilarity in colonizing communities on materials exhibiting various hydrophobicities. The chemical composition of surfaces, considered as "inert", could finally influence the recruitment of the first micro-organisms in marine biofilms even if community tend to converge with time (Jones *et al.*, 2007).

Beyond 24h of immersion, microbial community structure analysis revealed successional changes with the predominance of Flavobacteriia and Alpha-proteobacteria. These changes are still more obvious looking at the OTU taxonomic level. Bacterial communities exhibited fast temporal structure dynamics at lower taxonomic levels with bacterial taxa that displayed rapid increases and declines without recurrent patterns in microbial community structure and composition. Most of considered OTUs were intermittent or ephemeral accentuating the highly dynamic characteristics of the biofilm structure. Only three OTUs were persistent (OTUs 7, 8 and 17). They were all affiliated with Rhodobacteraceae which supports the recurring presence of these members of Alpha-proteobacteria in marine biofilms (Dang & Lovell, 2002, Jones *et al.*, 2007, Elifantz *et al.*, 2013, D'Alvise *et al.*, 2014, Sathe *et al.*, 2017).

Five main shifts were observed in bacterial communities with a community turnover averaging 64%. Interestingly, time between each shift constantly increase, from 3 days (between T_1 and T_4) to at least 9 days considering the 28 first days of immersion. This suggests an

increasing stability in the biofilm composition which suggest that biofilm might reach a climax state. However, after 75 days of immersion, bacterial communities in biofilms presented more than 65% of dissimilarity with the bacterial communities found during the first month of immersion. Five OTUs dominated the community. Most of them were rarely present or not detected through the immersion. This severe shift in bacterial community composition between T_{28} and T_{75} could question our previous hypothesis. However, bacterial communities found at T_{75} could belong to the biofilm developed at the surface of macro-organisms that colonized the substrate after several weeks. Finally, archaea appear to be rare (< 0.4%) and few diversified in our marine biofilms. Few data on archaeal communities are currently available since few primer sets detected members of this domain. The few studies detecting archaea in biofilms support our findings, with less than 2.5% of total prokaryotes (Webster & Negri, 2006, Zhang *et al.*, 2014)).

Microbial community interactions in coastal marine biofilms

The complexity of marine biofilms makes it difficult to reveal organizational principles of the microbial community. Given the microbial diversity of marine biofilms, interspecies interactions should play important roles in determining the development, structure and function of these biofilms. We used correlation network analyses which represent useful tools to study the organization and microbial interactions in these complex ecosystems. Significant numbers of nodes (303) and edges (1913) were determined (considering the 350 most abundant OTUs, and correlations higher or equal than 0.8). This result suggests very strong connections between bacterial species and reveals that microbial communities in marine biofilms are extremely cohesive. While some studies promote cooperative inter-specific interactions within biofilms (e.g. (Elias & Banin, 2012, Burmolle *et al.*, 2014)), other works suggest that interactions in multispecies communities are mostly competitive, suggesting that adaptation is more likely

achieved by competitive success (Foster & Bell, 2012). Following lab controlled experiments (Rendueles & Ghigo, 2015), and given the inherent complexity of natural ecosystems, the authors hypothesize that competition for space and resources could be harsher and selective pressures stronger in natural ecosystems. We interestingly demonstrate that 65% of connections were positive suggesting efficient cooperation and mutual dependence between bacterial communities in marine biofilms. Positive correlations in multispecies communities have been interpreted as functional guilds of organisms performing similar or complementary functions (Eiler *et al.*, 2012, Chow *et al.*, 2013). The elaborate tri-dimensional architecture of marine biofilms provides the opportunity for metabolic cooperation and interspecies substrate exchange. A biofilm did not constitute a homogenous microhabitat and several internal variations in environmental conditions (nutrient, oxygen, light …) could generate dissimilar niches, allowing members of these communities to form sets of functional guilds conducting interdependent molecular and physiological processes (Davey & O'Toole, 2000).

At a finer scale, modules or clusters represent important microbial associations in the biofilm. As observed at the global network scale, they displayed much more positive correlations too. For most of modules and particularly for the four bigger ones, members of Flavobacteriia exhibited the highest BC and CC and thus represented the more central nodes. Moreover, members of Flavobacteriia exhibited the highest number of edges while they displayed a lower number of nodes than Alpha-proteobacteria. These important findings display that Flavobacteriia would highly influence the functioning of marine biofilms. Combined to the constant increase in autotroph abundances through the kinetics (Figure 1), this finding is highly consistent with the functional role attributed to Flavobacteriia known to thrive during phytoplankton blooms (eg (Buchan *et al.*, 2014, Landa *et al.*, 2016)) and degrade diverse complex organic material (Williams *et al.*, 2013). All the network properties suggest that this class of bacteria could give

resistance to network against random removal of taxa and the absence of its members might fragment some part of the network leading to connectivity failure (Duran-Pinedo *et al.*, 2011). We thus hypothesize with this study that Flavobacteriia represent a keystone bacterial group in the formation and functioning of these ecosystems. Note that the 3 persistent OTUs, members of Rhodobacteraceae, were few connected nodes suggesting that their loss would not alter the organization of microbial consortia and the dynamics and functioning of coastal marine biofilms.

In network analysis, correlations between OTUs and environmental variables describe potential conditions that may favour the formation of specific groups of bacterial OTUs. Temperature and salinity are typical of Mediterranean coastal environment during summer season (Table S1). As expected considering the known oligotrophic status of Mediterranean Sea, TN and nitrates concentrations remain low. DOC values are similar to previously published levels during summer period (Dang *et al.*, 2014). Trace metals levels evidenced the high anthropization of the Toulon bay, similar to the ones earlier observed in the most enclosed parts of the bay (Dang *et al.*, 2015). Indeed, when compared with trace metals concentrations assessed from open Mediterranean Sea (Tankere & Statham, 1996, Morley *et al.*, 1997), Cd contamination remained weak (average factor of 3) when Pb, Cu and Zn exhibited much higher levels demonstrating significant anthropogenic inputs, most probably related to nautical activities (Table S1). We showed that environmental variables were highly associated with the biofilm dynamics. Temperature, salinity, pH are part of variables that are the most connected with bacterial OTUs and thus have the greatest influence on the biofilm formation and evolution. These variables are known to play an important role in shaping marine biofilms (Costerton *et al.*, 1995, Donlan, 2002, Chiu *et al.*, 2006, Briand *et al.*, 2017). Interestingly, our findings also statistically demonstrate that some trace metals such as cadmium and copper would influence the cohesion and temporal structure dynamics of marine biofilms. Cadmium is already known to affect

microbial community abundance and composition in different ecosystems as soils (Chen *et al.*, 2014) and activated sludge (Tsai *et al.*, 2005). Otherwise, recent findings have shown that concentrations of other trace metals as copper remain high in biofilms after a contamination (McElroy *et al.*, 2016). While the Toulon Bay is known to be highly contaminated by various trace metals influencing benthic microbial community structure (Jean *et al.*, 2012, Misson *et al.*, 2016), it is clear that these contaminants are playing an important role in the evolution of the biofilm structure. Interestingly, when we considered only the 31 most abundant OTUs, the total number of edges between environmental variables and OTUs decreased drastically and is close or equal to zero in most of cases. This suggests that environmental factors considered would have few influence on the dynamics of these most abundant OTUs when these latter are not associated with the rest of the community. Even though we cannot rule out that the dynamics of these OTUs could be driven by other factors not estimated in this study, the intrinsic EPS matrix in biofilms, known to reduce the exposition of cells to the external medium, could also limit environmental impacts on the community dynamics. We can finally speculate that the effect of environmental variables on their high dynamics might depend on their links with the other bacterial species in the biofilm.

Conclusion

Unintentionally ignored since many years, we demonstrate using adapted molecular tools that Flavobacteriia represent essential members of marine biofilms and might be key actors in their functioning. Although we cannot declare that we have now a comprehensive and holistic view of interactions within marine biofilms, our network analysis provides information on the microbial associations and their interactions with the environment. Showing that 2/3 of

connections between bacterial species were positives, we logically conclude that members of marine biofilms were more inclined to develop cooperation rather than competition and suppose that they could form sets of functional guilds with mutual metabolic exchanges. Future studies analysing evolution of metabolic compounds through the biofilm formation will have to be performed to validate this hypothesis and improve our understanding of the marine biofilm functioning.

Acknowledgments

We thank Marine Maintenay for her help in biofilm samplings. This work was supported by the Direction Générale de l'Armement, French Government Defense procurement and technology agency (Program DRACONS).

Conflict of interest

The authors declare no conflict of interest

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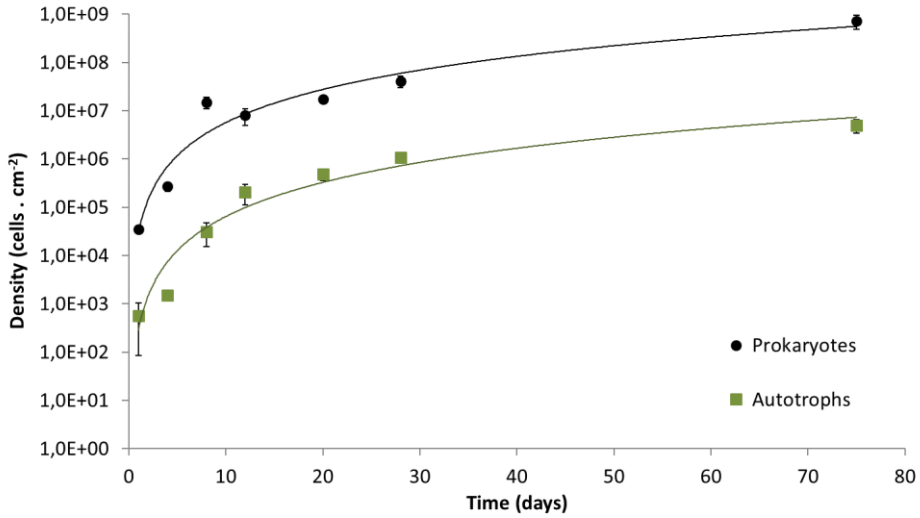

Figure 1: Temporal evolution of heterotrophic prokaryotes and autotrophs densities (cells. cm^{-2}) on the PVC panels immersed from 1 to 75 days.

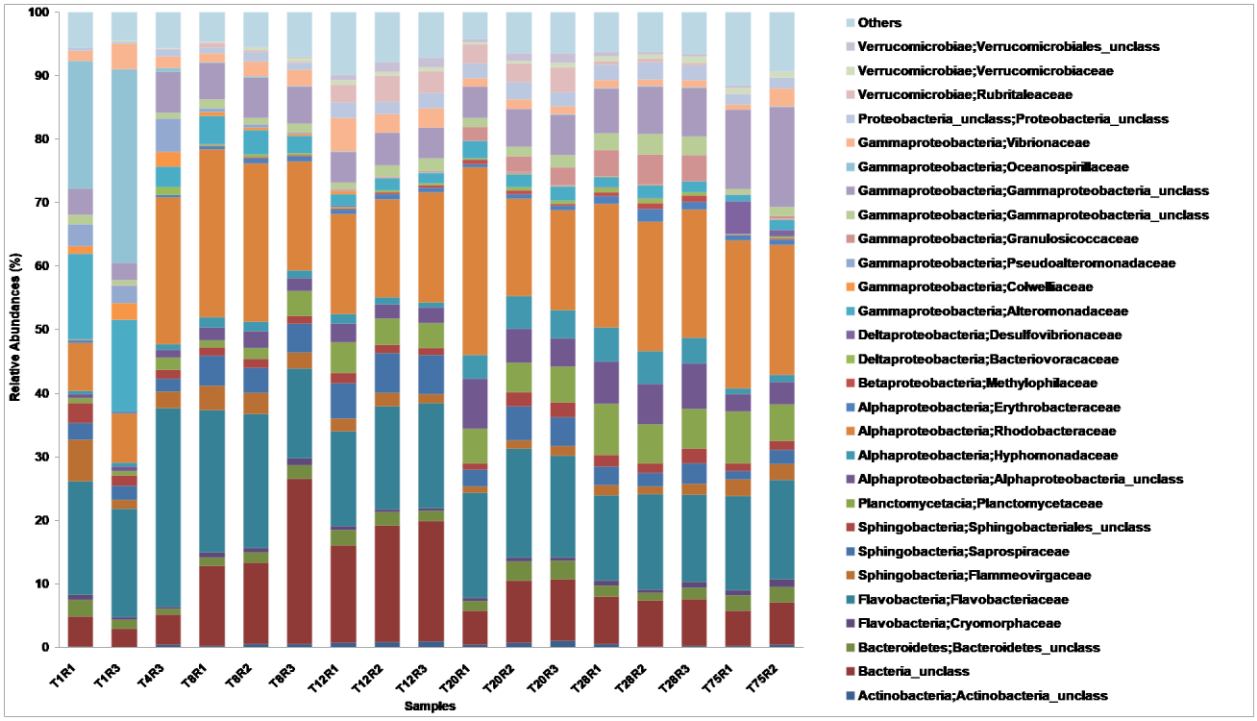

Figure 2. Abundant prokaryote families within biofilm communities. Most abundant prokaryote families present in biofilm communities in sample replicates from T1 to T75 (based on 16S rDNA gene analysis)

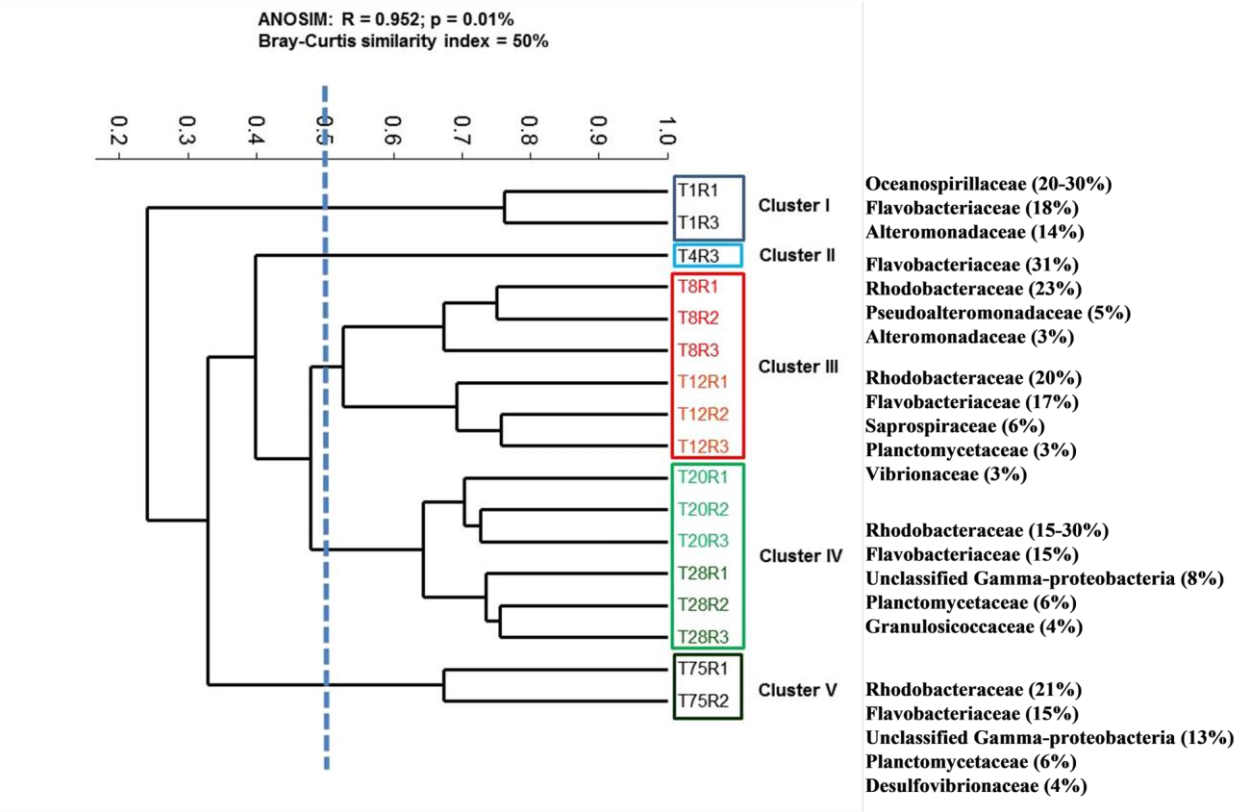

Figure 3. **Cluster analysis of sequences based on the Bray-Curtis index. Scale bars indicated the Bray-Curtis similarity index**. This analysis of similarities was conducted with the subroutine ANOSIM

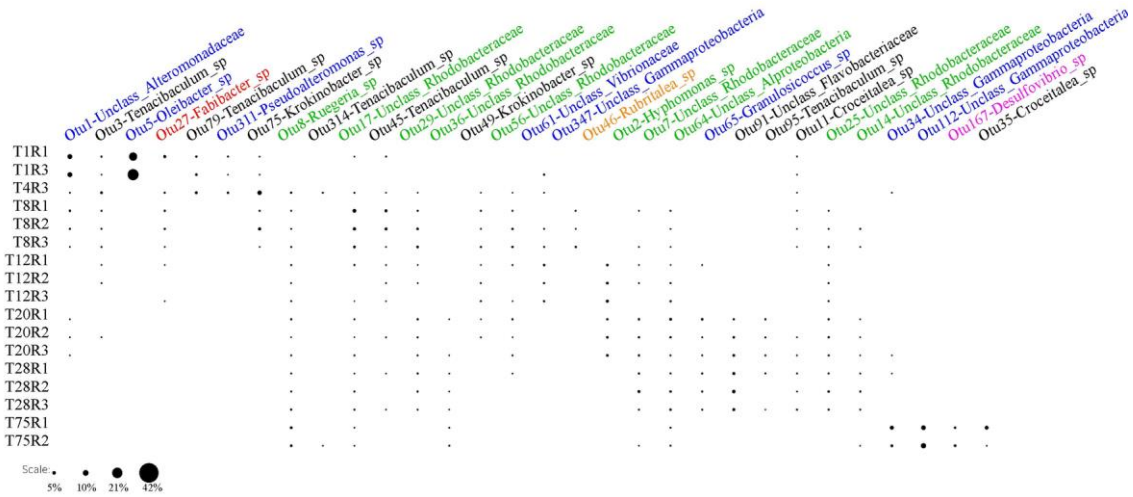

Figure 4. Temporal dot plot showing the most abundant bacteria OTUs, each displaying a relative abundance ≥1.5 % in at least one sample. Blue : Gamma-proteobacteria; Black : Flavobacteriia; Red : Sphingobacteria; Green : Alpha-proteobacteria; Orange : Verrucomicrobia; Purple : Delta-proteobacteria.

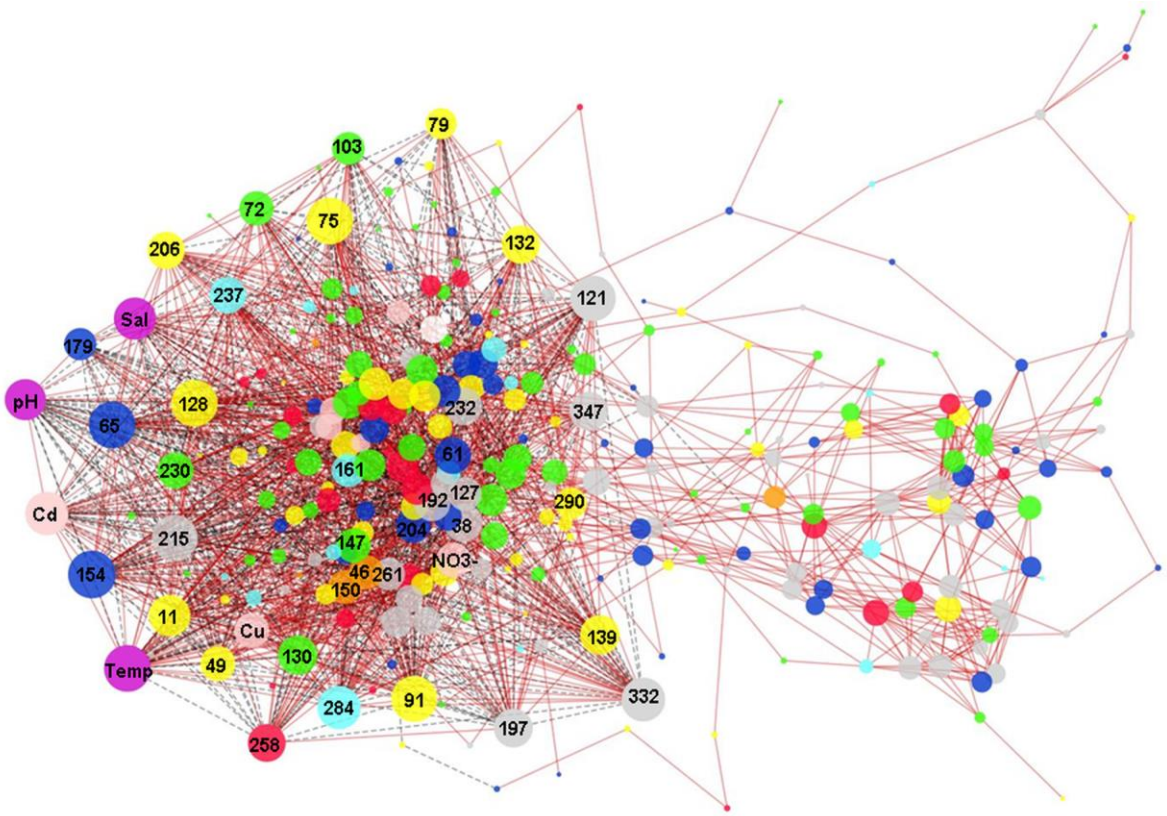

Figure 5. The Network represents system components (nodes) and the relations between those components (edges or links). Each node represents a bacterial OTU and the link between two nodes (edge) represents a relationship. The size of nodes is based on the degree of each node. The red full edges represent the positive interactions and Dotted edges represent the negative ones. Dark blue: Gammaproteobacteria; Yellow : Flavobacteriia; Red : Sphingobacteria; Green : Alphaproteobacteria; Orange : Verrucomicrobia; Light blue: Planctomycetia; Gray: others; Pink: Trace metals and chemical variables; Purple: Physical variables. The taxonomy of OTUs with the highest degree is presented in Table 2B.

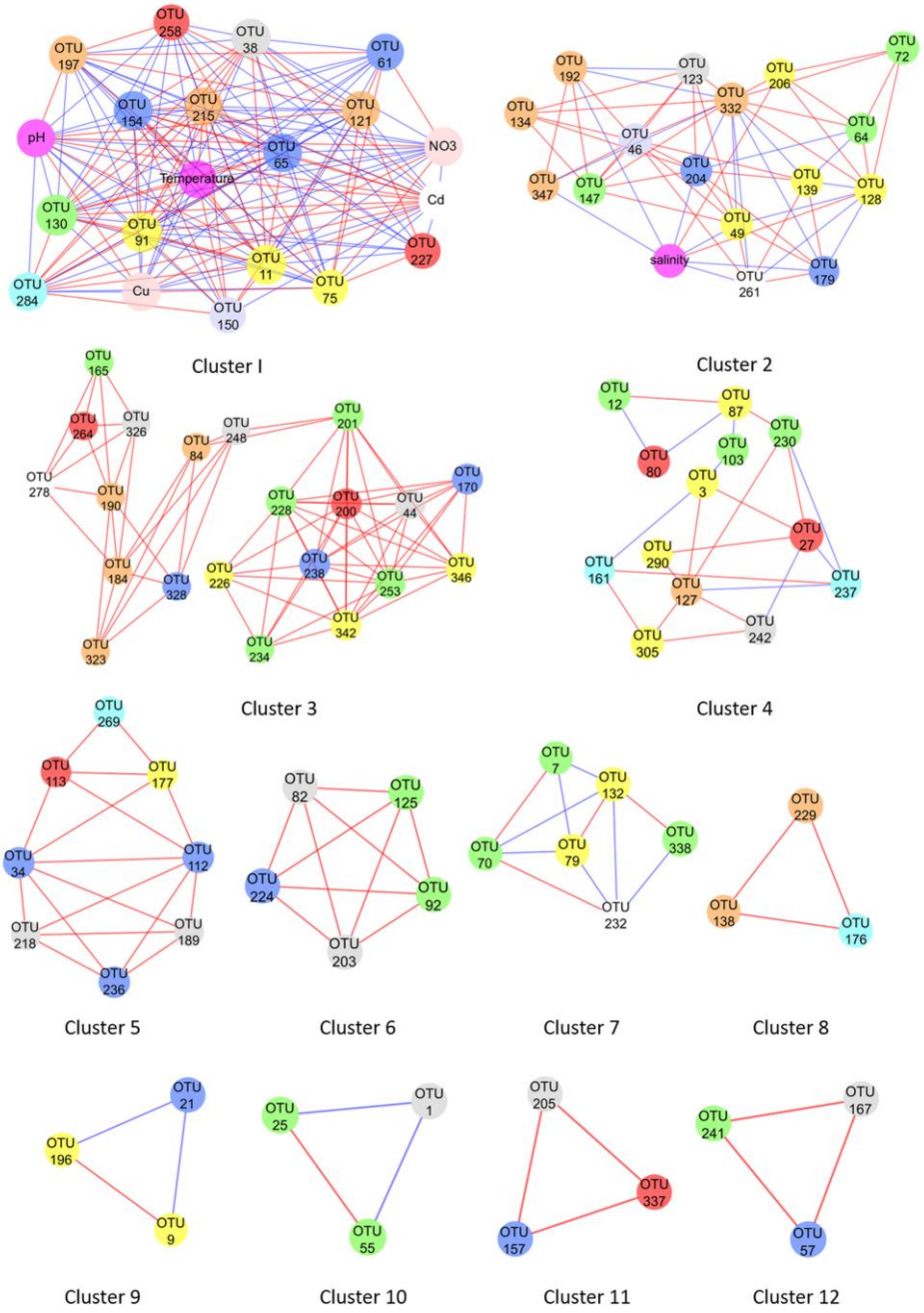

Figure 6. The network has been subdivided into twelve clusters in which nodes are highly connected to each other but weakly connected to nodes outside of their module. The red edges represent the positive interactions while the blue ones represent the negative interactions. Dark blue: Gammaproteobacteria; Yellow : Flavobacteriia; Red : Sphingobacteria; Green : Alphaproteobacteria; Orange : Verrucomicrobia; Light blue: Planctomycetia; Gray: others; Pink: Trace metals and chemical variables; Purple: Physical variables.

A

	0 mismatch		Ecosystems	References
	Bacteria	*Archaea*		
775F/1103R	56%	/	Biofilms	Briand et al 2017
28F/519R	30%	/	Biofilms	Muthukrishnan et al 2014
U341F/685R	24%	/	Biofilms	Lee et al 2014a
104F/519R	65%	/	Biofilms	Lawes et al 2016
U905F/U1492R	4.7%	4%	Biofilms	Lee et al 2014b
515F/1390R	65%	32%	Biofilms	Zhang et al 2014
515F-Y/926R	88%	83%	Marine waters	Parada et al 2015

B

	775F/1103R	28F/519R	U341F/685R	104F/519R	U905F/U1492R	515F/1390R	515F-Y/926R
Bacteroidetes	15%	33%	4.8%	36%	10%	66%	90%
Sphingobacteria	59%	33%	0.84%	16%	16%	71%	91%
Flavobacteria	1.7%	38%	0.12%	5.4%	11%	71%	92%
Alpha-proteobacteria	63%	33%	0.07%	25%	0.38%	75%	93%
Rhodobacterales	7%	35%	0.08%	44%	0.04%	77%	95%
Gamma-proteobacteria	87%	29%	27%	82%	1.2%	71%	93%
Alteromonadales	62%	40%	34%	89%	0.09%	84%	92%
Cyanobacteria	0.87%	29%	36%	75%	14%	74%	89%
Firmicutes	70%	32%	53%	78%	5.2%	58%	88%
Planctomycetes	1.1%	27%	0.54%	7.5%	11%	68%	86%
Verrucomicrobia	14%	34%	0.36%	23%	0.04%	64%	88%

Table 1. (A): *In Silico* analysis performed on 6 primer pairs usually used to study biofilm microbial community diversity and one primer pair recently developed in marine waters. *In silico* primer coverage was analyzed on Bacteria and Archaea with 0 mismatches. Lee et al 2014a, b, Muthukrishnan et al 2014, Zhang et al 2014, Parada et al 2015, Lawes et al 2016, Briand et al 2017. (B): the primer coverage was analyzed at finer scale considering the percentage of matches with the main bacterial groups and classes.

A

	350 OTUs			31 most abundant OTUs		
	Nb of nodes	Nb of edges		Nb of nodes	Nb of edges	
		positives	negatives		positives	negatives
Alphaproteobacteria	76	470	185	42	135	93
Flavobacteriia	57	436	287	35	171	174
Gammaproteobacteria	52	330	185	24	118	96
Planctomycetacia	18	137	63	11	30	24
Sphingobacteria	25	166	114	16	37	45

B

	Nb of edges	
Variables	**350 OTUs**	**31 most abundant OTUs**
NO_3	33	0
Salinity	46	9
Cd	48	0
Pb	17	6
Cu	33	0
TN	18	4
pH	44	8
Temperature	54	6
$Si(OH)_4$	10	1
Zn	13	1

C

OTUs	Edges	Taxonomy	OTUs	Edges	Taxonomy
154	56	*Thioprofundum* sp.	127	31	*Unclass Bact*
75	**54**	*Krokinobacter* sp.	161	30	*Blastopirellula* sp.
128	54	*Muricauda* sp.	150	30	*Unclass Verrucomicrobiales*
65	**54**	*Granulosiccus* sp.	87	29	*Aquimarina* sp.
215	53	*Unclass Bact*	103	29	*Litorimonas* sp.
91	**53**	*Unclass Flavobacteriaceae*	163	28	*Unclass Rhodobacteraceae*
121	52	*Unclass Bact*	227	28	*Lewinella* sp.
332	49	*Unclass Bact*	261	28	*Unclass Bact*
284	48	*Unclass Planctomycetaceae*	179	28	*Unclass Gammaproteo*
11	**45**	*Croceitalea* sp.	68	27	*Unclass Gammaproteo*

258	38	*Unclass Saprospiraceae*	**79**	**26**	*Tenacibaculum* sp.
206	37	*Unclass Flavobacteriaceae*	70	25	*Unclass Alphaproteo*
192	36	*Unclass Bact*	**311**	**23**	*Pseudoalteromonas* sp.
61	**36**	*Unclass Vibrinaceae*	210	22	*Winogradskyella* sp.
237	36	*Unclass Planctomycetaceae*	152	22	*Unclass Gammaproteo*
230	35	*Donghicolas* sp.	80	22	*Ekhidna* sp.
49	**33**	*Krokinobacter* sp.	21	21	*Aestuaribacter* sp.
204	33	*Grimontia* sp.	123	21	*Unclass Actinobacteria*
72	32	*Roseovarius* sp.	104	21	*Arenicella* sp.
147	32	*Unclass Rhodobacteraceae*	229	20	*Unclass Bact*
46	**31**	*Rubritalea* sp.			

Table 2. (A) Number of nodes and edges for the most represented bacterial classes considering the 350 et the 31 most abundant OTUs. The number of positive (+) and negative (-) interactions is indicated for each number of edges. (B) OTUs presenting a number of edges (degree) higher than 20. The taxonomy of each OTU is indicated. Thick OTUs belong to the 31 most abundant OTUs. (C) Number of edges between variables and the total number and the 31 most abundant OTUs.

Supplementary data:

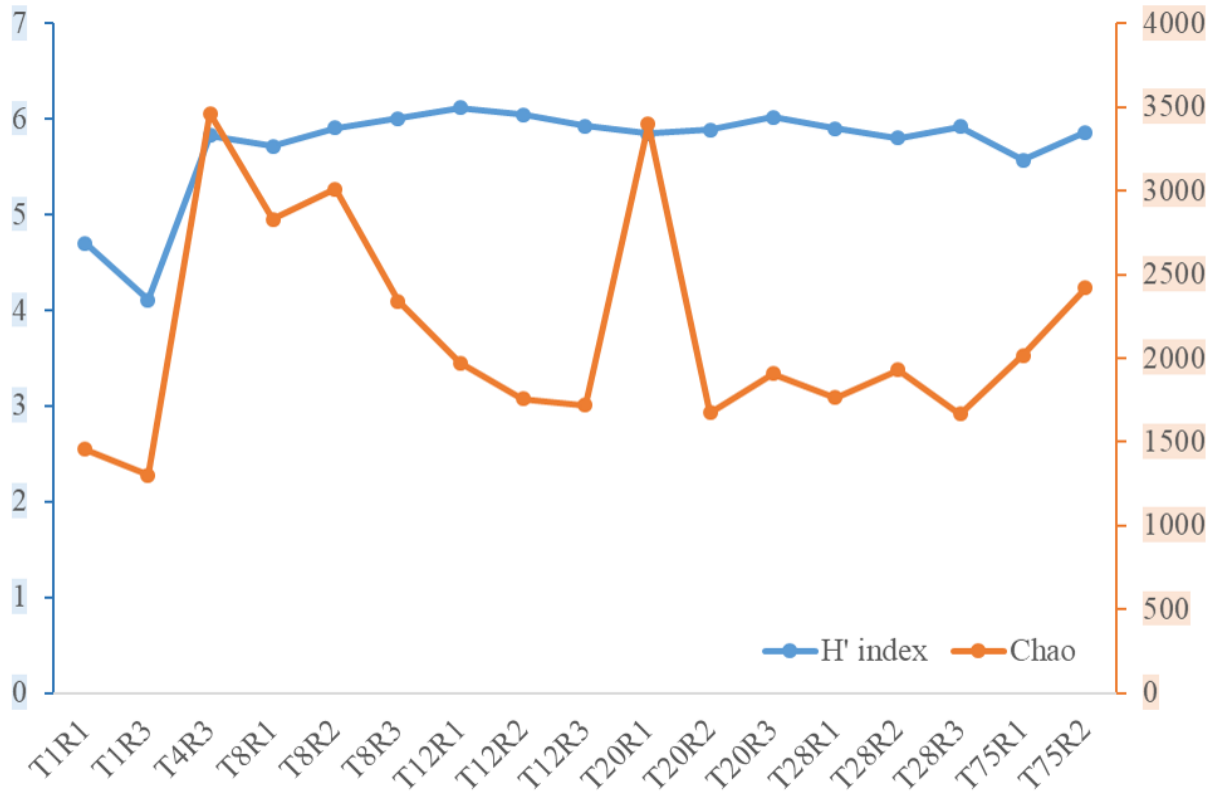

Figure S1. Alpha diversity indices: Shannon (H') and Chao1.

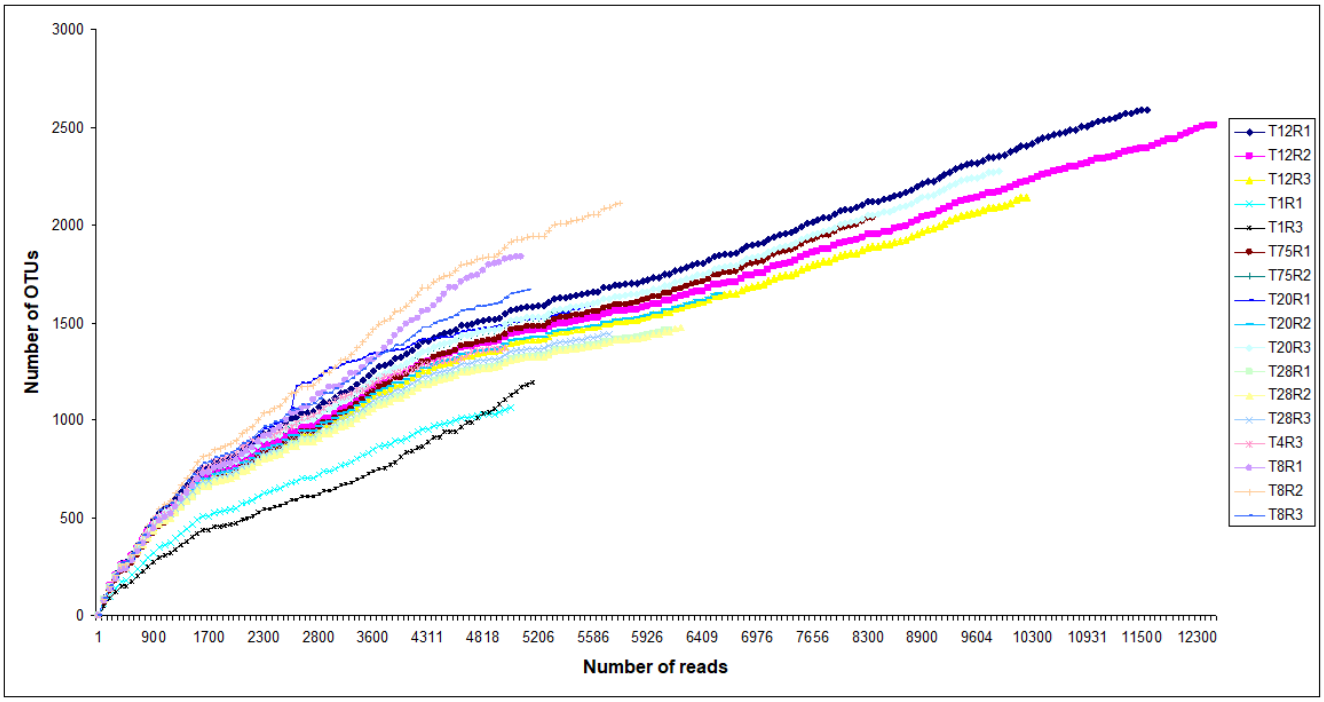

Figure S2. Rarefaction curves before the normalisation of the data.

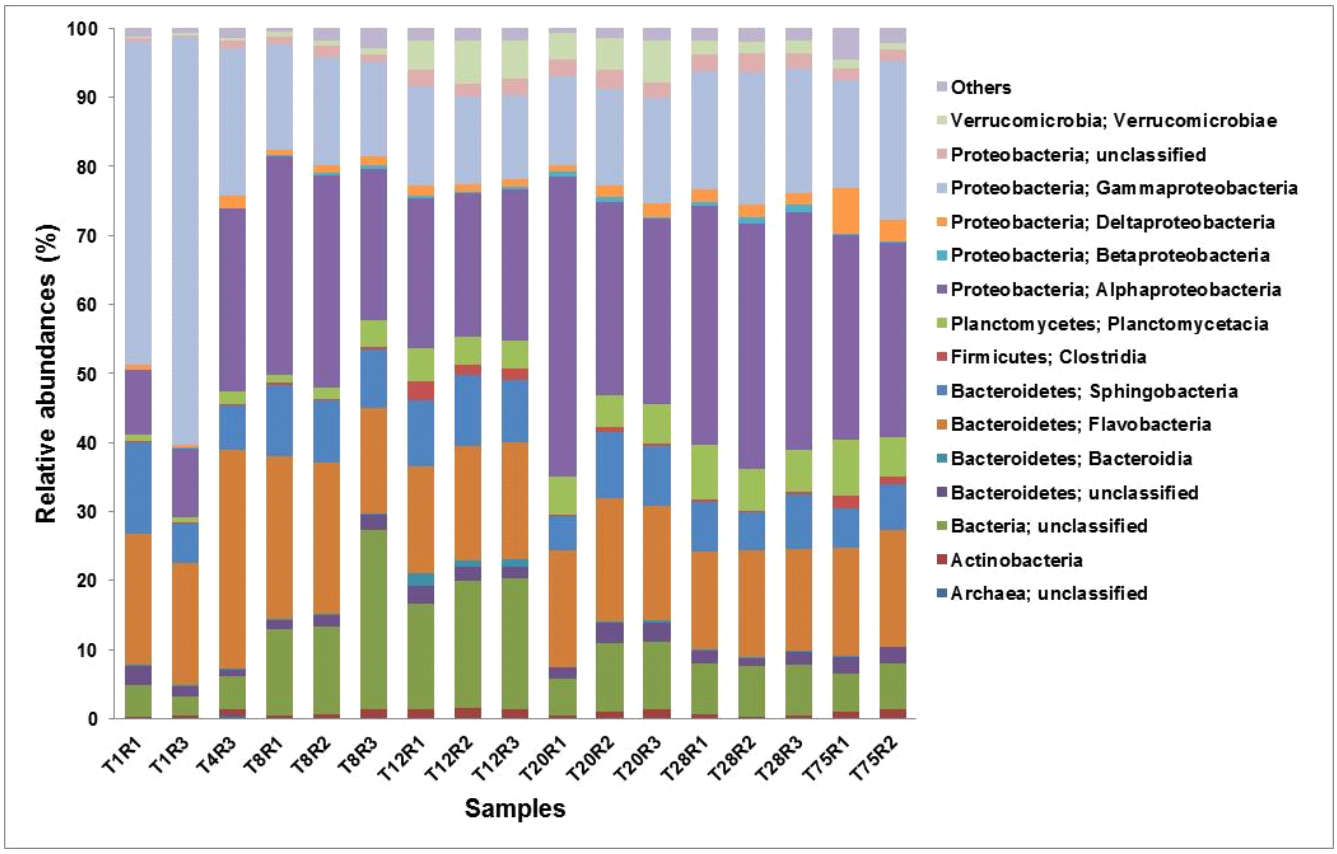

Figure S3. Abundant prokaryote classes within biofilm communities. Most abundant prokaryote classes present in biofilm communities in sample replicates from T1 to T75 (based on 16S rDNA gene analysis)

	Salinity	Temperature (°C)	pH	DOC	TN	NO_3	$Si(OH)_4$	Zn	Pb	Cd	Cu
T0	37.1	23.4	8.13	1.34	10.7	1.67	4.91	173	1.8	0.23	62.9
T1	37.6	23.3	8.18	1.27	5.9	0.79	2.29	187	1.8	0.15	49.7
T4	36.8	22.8	8.09	1.39	12.7	1.98	5.1	289	2.3	0.19	78.5
T8	37.9	22.3	8.07	1.22	9.2	1.15	3.16	308	2.2	0.14	62.3
T12	38.1	23.5	7.99	1.37	14.4	1.41	1.85	318	2.7	0.14	63.2
T20	38.2	25.8	7.98	1.37	15.3	0.89	2.03	290	2.7	0.15	59.9
T29	38.1	25.2	8.01	1.22	10.5	0.58	1.88	347	1.4	0.18	24.2
T75	38.5	23.4	8.07	1.44	12.9	0.84	2.27	395	3.2	0.21	39.3

Table S1: Temporal evolution of environmental variables from 1 to 75 days. Salinity, temperature (°C), pH, dissolved organic carbon (DOC, $mg_C.L^{-1}$), total nitrogen (TN, µM), nitrates (NO_3, µM), silicates ($Si(OH)_4$, µM), zinc (Zn, nM), lead (Pb, nM), cadmium (Cd, nM) and copper (Cu, nM).