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Patterns of bacterial diversity in the marine planktonic particulate matter continuum

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Depending on their relationship with the pelagic particulate matter, planktonic prokaryotes have traditionally been classified into two types of communities: free-living (FL) or attached (ATT) to particles, and are generally separated using only one pore-size filter in a differential filtration. Nonetheless, particulate matter in the oceans appears in a continuum of sizes. Here we separated this continuum into six discrete size-fractions, from 0.2 to 200 μm , and described the prokaryotes associated to each of them. Each size-fraction presented different bacterial communities, with a range of 23–42% of unique (OTUs) in each size-fraction, supporting the idea that they contained distinct types of particles. An increase in richness was observed from the smallest to the largest size-fractions, suggesting that increasingly larger particles contributed new niches. Our results show that a multiple size-fractionation provides a more exhaustive description of the bacterial diversity and community structure than the use of only one filter. In addition, and based on our results, we propose an alternative to the dichotomy of FL or ATT lifestyles, in which we differentiate the taxonomic groups with preference for the smaller fractions, those that do not show preferences for small or large fractions, and those that preferentially appear in larger fractions.

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Introduction

Particulate matter in the oceans appears in a high variety of types, in a continuum of sizes from truly dissolved to visible macroaggregates (Azam *et al.*, 1993; Azam, 1998; Simon *et al.*, 2002; Verdugo *et al.*, 2004). This continuum is dynamic and patchy (Long and Azam, 2001) and can present hot spots of microbial activity (Alldredge *et al.*, 1986; Azam *et al.*, 1993; Seymour *et al.*, 2004). Depending on their relation with the particulate matter present in the environment, planktonic microorganisms have traditionally been classified into two types of communities: free-living (FL) or attached (ATT). It is well known that ATT pelagic prokaryotes can develop dense communities of cells (Simon *et al.*, 2002), and present specialized metabolisms characterized by high rates of extracellular enzyme activity (Karner and Herndl, 1992; Smith *et al.*, 1992), prokaryotic production (Kirchman and Mitchell, 1982) and respiration (Grossart *et al.*, 2007). In contrast, FL microorganisms tend to have smaller genomes (Smith *et al.*, 2013) adapted to low substrate

concentrations, with high expression of membrane transporter genes (Satinsky *et al.*, 2014), and tend to exhibit motility (Mitchell *et al.*, 1995; Fenchel, 2001; Grossart *et al.*, 2001).

To analyze both communities, microbial ecologists use differential filtration, so that the first filter retains the ATT communities while the FL prokaryotes go through that filter and are collected by a second (typically 0.2 μm) filter. There is a wide range of filters used to distinguish between FL and ATT fractions: 0.8 μm (Schapira *et al.*, 2012), 1.6 μm (Ganesh *et al.*, 2014), 3.0 μm (Eloe *et al.*, 2011), 5.0 μm (Lapoussiere *et al.*, 2011), or even 30 μm (Fuchsman *et al.*, 2011) pore sizes. But most of these studies have used only one size to separate ATT from FL and have thus missed the possibility of detecting, if they exist, diverse ATT communities associated to distinct sizes. A few studies have focused on two size-fractions (for example, 0.8 and 10 μm (Dang and Lovell, 2002), 1.0 and 60 μm (Kellogg and Deming, 2009), 0.8 and 3.0 μm (Smith *et al.*, 2013 and Wilkins *et al.*, 2013) and 3.0 and 8.0 μm (Milici *et al.*, 2016)), yet no one has systematically used a set of different sized filters to size fractionate bacterioplankton samples. The lack of consensus on the pore-size used to separate both types of communities makes a comparison among studies very difficult or even impossible. The choice of the filter may bias the results as distinct filters might retain distinct particles with distinct bacterial communities.

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Moreover, the use of various size-fractions might reveal a more comprehensive view of bacterioplankton complexity by contemplating a wider variety of habitats where planktonic bacteria develop.

Considering that the biogeochemical role of prokaryotes in the microbial food web depends strongly on the size, quantity and quality of the suspended particles (Malfatti and Azam, 2009; Grossart, 2010a), current studies should perhaps deviate from the traditional dichotomy of ATT vs FL communities and take into account the variety of sizes and complexity of organic and inorganic structures that can be found in the water column and that might serve as prokaryotic niches. To test this idea, we analyzed marine bacterial communities in different size-fractions, ranging from the purely FL to particles of 200 μm , in an oligotrophic coastal station of the Mediterranean Sea, and all along a year. The microbes were collected by serial filtration on six filters of decreasing pore sizes, to test whether the composition of the bacterial community differs among the size-fractions and to explore whether a multiple size-fractionation of the samples provides a more complete description of the whole bacterial community than the use of only one filter to separate FL from ATT communities. The null hypothesis would be that microbial community composition shows no relationship with the pore-size of the filter and, by extension, to the sizes of the particles.

Materials and methods

Study area, sampling and basic parameters

Samples were collected monthly from June 2012 to June 2013 from the Blanes Bay Microbial Observatory (BBMO, www.icm.csic.es/bio/projects/icmicrobis/bbmo/) a coastal station (20 m depth) placed at 0.5 miles offshore (41°40'N, 002°48'E) in the NW Mediterranean Sea which has regularly been sampled for microbial ecology studies during the last decades (Gasol *et al.*, 2012). Surface water (0.5 m depth) was taken and pre-filtered through a 200 μm mesh net and transported to the laboratory in darkness. For DNA analysis, a total of 10 l were filtered sequentially through 20, 10, 5, 3, 0.8 and 0.2 μm pore-size polycarbonate filters (20 μm pore-size filter from GE Water and Process Technologies (Trevose, PA, USA) and the rest of the filters from Millipore (Billerica, MA, USA)) of 47 mm diameter, using a peristaltic pump at very low speed and pressure. To prevent clogging we changed the filters when the flow slowed down (usually the 0.2 and 0.8 μm pore-size filters were replaced at least once per filtration). All the filters of the same pore-size were pooled as one sample. The filters were stored immediately at -80°C until extraction. The size-fractions were defined as: 0.2–0.8; 0.8–3.0; 3.0–5.0; 5.0–10; 10–20; and 20–200 μm , and in order to simplify the nomenclature, they will also be referred by the lowest size (that is, '0.8 fraction' indicates

from 0.8 to 3 μm) along the manuscript. A description of the measurement of environmental parameters and the abundance of prokaryotes on those filter sizes can be found in Supplementary Methods 1.

DNA extraction, sequencing and sequences processing

The DNA was extracted as described in (Massana *et al.*, 1997). Hypervariable V1–V3 16 S tags were PCR amplified and 454 GS FLX+ pyrosequenced with primers 28 F/519 R by Research and Testing Laboratory (Lubbock, TX, USA; <http://www.researchandtesting.com/>). A total of 495 897 amplicon fragments were produced. Reads from 150 to 600 bp were quality checked (Phred quality average > 25) by using a 50 bp sliding window in QIIME (Caporaso *et al.*, 2010). Pyrosequencing errors were reduced with Denoiser in QIIME. Reads were clustered into Operational Taxonomic Units (OTUs) with a 97% similarity threshold with UCLUST in QIIME. Chimeras were detected with ChimeraSlayer (Haas *et al.*, 2011) and SILVA108 as a reference database, in MOTHUR (Schloss *et al.*, 2009). Taxonomy assignment was done using SILVA Incremental Aligner (SINA v1.2.11). Unwanted OTUs (eukaryotes, chloroplast, mitochondria or OTUs with less than five sequences in total) were removed. The months with at least one size-fraction that could not be amplified were discarded. A total of 48 samples were selected, representing 8 months: September, October, November and December of 2012 and January, March, April and June of 2013. The samples were randomly subsampled to the lowest number of reads present in the data set. A summary of sequence information can be found in Supplementary Table 1.

Data analysis

Statistical analyses and graphs were done in R (www.r-project.org) and JMP software (www.jmp.com). The OTU table was square-root transformed and a Bray–Curtis distance matrix was generated. The environmental database was normalized and an Euclidean distance matrix was generated. Non-metric multidimensional scaling analysis was used to visualize the distances between communities. A PERMANOVA (Adonis test, vegan-Package) was performed to discern statistically significant differences due to the factors size-fraction and month. Bray–Curtis distances were calculated between samples of September 2012 and the respective size-fractions of the following months. The diversity of each size-fraction was calculated using the Shannon Index (H') and the True Alpha, Beta and Gamma Diversity (Tuomisto, 2010) with R package Simba. Similarities among size-fractions were explored with the average clustering method (Unweighted Pair Group Method with Arithmetic Mean: UPGMA), and a SIMPROF analysis was performed to detect the significant clusters (at $P < 0.05$). Rank-abundance curves for each size-fraction were plotted in log–log

scales. Indicator OTUs (Dufréne and Legendre, 1997) of a given size-fraction were obtained using the INDVAL analysis (R package lavdsv, INDVAL values >0.3 and $P<0.05$). Niche Breadth (B_j ; Levins, 1968) of each OTU was calculated and OTUs with $B_j<3$ were considered specialists, OTUs with $B_j=[3-4]$ were considered Intermediate and OTUs with $B_j>4$ were considered generalists. OTUs with relative abundance $>1\%$ were considered abundant, those at $1-0.1\%$ were considered intermediate, and those $<0.01\%$ were considered rare.

The OTUs were grouped at phylum, class and genera level. The high-rank taxonomic groups which represented $>1\%$ of the total abundance in at least one size-fraction, were selected for further analyses. The rest were classified as 'other bacteria'. With these criteria, a total of 17 taxonomic groups were selected. To assess differences in the relative abundances of individual taxonomic groups among size-fractions, analysis of variance ($P<0.05$) and Tukey's *post-hoc* tests were conducted. Relative abundances of the taxonomic groups were drawn in a heatmap and were clustered hierarchically by the UPGMA method.

Results

The environmental parameters of the eight sampled dates presented elevated variability (the CVs were on average 57%; Supplementary Table 2). The non-metric multidimensional scaling plot (Figure 1) indicated that the samples clustered by month and by size-fraction. A PERMANOVA test (Supplementary Table 3) confirmed that differences between 'size-fractions' were significant ($P<0.001$) and differences between 'months' were also significant ($P<0.001$). Bray–Curtis distances between distinct months (Supplementary Figure 1) showed annual periodicity, being the community composition of nearby months more similar than those of distant

months. The communities of smaller size-fractions remained rather constant over the year, compared with the communities of the larger size-fractions. Most (94% on average) bacteria were in the $>0.2\ \mu\text{m}$ filter, while 4.9% were in the $0.8\ \mu\text{m}$ filter, and $<0.5\%$ were present in the remaining filters (Supplementary Figure 2).

Bacterial diversity increased with increasing size-fraction at each month as indicated by the Shannon Index and species richness (Figures 2a and b). The $20-200\ \mu\text{m}$ size-fraction presented the most diverse community, whereas the $0.2-0.8\ \mu\text{m}$ size-fraction had the lowest diversity. The average α -diversity (diversity within a size-fraction) was 179.8, the global β (rate of community differentiation among size-fractions) was 3.3 and the global γ (total diversity within the six size-fractions) was 595.6 (Supplementary Table 4). The accumulated number of species in the range of the size-fractions (species discovery curve or species-accumulation curve) presented a logarithmic form, which reached a 'plateau' and was close to saturation (Figure 3). A clustering dendrogram (Figure 4) revealed that every fraction shared OTUs preferentially with the closest size-fractions. Lower levels of dissimilarity were found in the larger size-fractions (that is, they were more similar among them). A SIMPROF analysis to detect significant clustering ($P<0.05$) separated primarily the smallest fraction ($0.2-0.8\ \mu\text{m}$) from the rest of fractions, and secondly the $0.8-3.0\ \mu\text{m}$ size-fraction from the larger fractions. The larger fractions clustered by pairs: $3.0-5.0\ \mu\text{m}$ with $5.0-10\ \mu\text{m}$ and $10-20\ \mu\text{m}$ with $20-200\ \mu\text{m}$. The rank-abundance curves (Figure 5) indicated that each size-fraction presented a strong dominance generated by a few OTUs, yet the size-fractions with higher dominance (that is, the smaller fractions) presented less diversity, as can be observed by the steeper slope in this representation.

On an average ($\pm$ s.d.), the percentage of unique OTUs in each size-fraction ranged from

Figure 1 Non-metric multidimensional scaling (nMDS) representing the distance between samples by size-fraction (a) and month (b). The nMDS was created with a Bray–Curtis distance matrix derived from the OTU table.

Figure 2 (a) Box-plot of the Shannon diversity Indices for each size-fraction. Upper and lower lines correspond to the first and third quartile of the distribution of values. The median values are shown with horizontal black wide lines. Outliers are displayed as dots. (b) Richness standardized to that of 0.2 µm for each size-fraction and for each month. The lines correspond to the log-linear regressions between the size of the fraction and richness. The thick line is the log-linear regression of the average values.

Figure 3 Species-accumulation curve, representing the number of OTUs (species) accumulated from the smallest to the largest size-fraction. The curve was constructed with the median values of the 8 months, with its s.d. The equation of the curve: $y = 97.7 \ln(x) + 152$. $R^2 = 0.95$.

23 ± 4.5% (3.0–5.0 µm) to 42.6 ± 7.9% (20–200 µm) (Supplementary Table 5), and the percentage of shared OTUs by all size-fractions (that is, ubiquitous, with global co-occurrence) accounted for 3.3 ± 1.1% of the total OTUs (Supplementary Table 6). The percentage of shared OTUs between two size-fractions (co-occurrence between two size-fractions) ranged from 23.7 ± 6.8 to 44.7 ± 6.7% (Supplementary Table 7), where every fraction shared species preferentially with the closest size-fractions and the highest percentages were found in the intermediate fractions. A total of 49 Indicator OTUs were identified (INDVAL > 0.3, $P < 0.05$) (Supplementary Table 8). The size-fractions with higher numbers of indicator OTUs were the size-fraction 0.2–0.8 µm and the 20–200 µm (24 and 18, respectively).

When separating the values of Niche Breadth (Bj) into three ranks of abundances (Supplementary Table 9), specialists were predominantly 'rare' (< 0.01% abundant) and generalist OTUs were predominantly 'abundant' (> 1% abundant). The

number of specialist OTUs increased with the size of the fraction, while the number of generalist OTUs decreased with the size of the fraction (Figure 6a). The relative abundances of the specialists decreased from 0.2 to 3.0 µm and increased from 3.0 to 20 µm. The contrary was observed for the relative abundances of the generalist organisms (Figure 6b).

Analysis of variance tests showed that 16 of the 17 selected (that is, relatively dominant) taxonomic groups presented statistically significant differences in relative abundances among size-fractions (Supplementary Table 10). Considering the differential presence of each group in the six particle-sizes (Figure 7) and the samples clustering (Figure 8), a total of four categories could be differentiated. The first category (A) encompassed the taxonomic groups that were enriched in the smallest size-fractions: for example, SAR11 and SAR116. The second category (B) comprised the taxonomic groups that were enriched in the smaller size-fractions, but depleted or absent in the smallest size-fraction (0.2–0.8 µm): for example, *Synechococcus* sp. The third category

Figure 4 Clustering dendrograms of the six size-fractions calculated by average clustering criteria (Unweighted Pair Group Method with Arithmetic Mean, UPGMA). Data used is the average of the distance between two size-fractions of the 8 months, with: (a) relative abundance data; and (b) presence-absence (binary) data. Thick lines indicate significant differences (determined by SIMPROF).

Figure 5 Rank-abundance curve for the six size-fractions, represented as a log-log plot. The data represent the average values in each of the 8 months.

(C) included the taxonomic groups that did not present enrichment when increasing or decreasing the size-fraction: for example, *Deferribacteriales*, *Oceanospirillales*, *Actinobacteria*. The fourth category (D) was composed of taxonomic groups that were enriched in the larger size-fractions: for example, *Rhodobacterales*, *Cytophagia*, *Alteromonadales*, *Verrucomicrobia*, *Vibrionales*, *Spingobacteriia*, *Firmicutes*, *Planctomycetes*, *Deltaproteobacteria*, *Flavobacteria* and *Rhizobiales*.

Discussion

We describe here the diversity of a coastal bacterioplankton community by using a serial filtration system

designed to separate the continuum of sizes of the plankton particulate matter into six discrete size-fractions. Our approach is unique because most studies that have analyzed the diversity of FL vs PA communities have done so by using only one or two filter sizes. In addition, we have inspected a broader range of sizes (from 0.2 to 200 µm). Our choice of the filters was based on the most common filters used to separate the various types of communities found in the literature. Many studies have considered as 'FL fraction' all bacteria that passed through 0.8 µm filters, yet in quite some studies the size limit chosen was the 3 µm as they intended to recover also the very large bacteria. Others considered that the 'ATT fraction' starts at 5.0 µm or even at larger sizes. Here, by characterizing the different bacterial communities present in various size-fractions we obtained information on the size dependence of bacterial community structure, a protocol that reveals a more comprehensive view of the pelagic microorganisms in the plankton. The data comprised here covers almost a year-round data set of the Blanes Bay Microbial Observatory including high variability in terms of environmental parameters and bacterial community composition (Figure 1, Supplementary Table 2 and 3, Supplementary Figure 1). Despite this high seasonal variability, we describe patterns of bacterial diversity in the size-fractions that were conserved all along the year. We consider that these patterns are strong, conserved with time, and have the potential to be present also in other aquatic systems. From our data we cannot state that this is a general trend across all aquatic ecosystems yet the patterns derived from the analysis of the six size-fractions in our samples is a null hypothesis to be tested in further studies.

A common question addressed in FL vs ATT bacterial studies is to determine which size-fraction contains more diversity. Previous studies performed

Figure 6 Specialist, Intermediate and Generalist OTUs (see Material and Methods for definition) in each size-fraction, represented as: (a) number of OTUs; and (b) their relative abundances. The boxplots were constructed with the upper and lower lines corresponding to the first and third quartile of the distribution. The median values are shown with horizontal black wide lines. Outliers are displayed as dots.

in marine areas close to our sampling site have shown contradictory trends: some found more diversity in the FL fraction (Acinas *et al.*, 1999; Ghiglione *et al.*, 2007), whereas other studies found more diversity in the ATT fraction (Crespo *et al.*, 2013). The same occurs in other marine ecosystems: some authors found that FL communities were richer than ATT communities (Hollibaugh *et al.*, 2000; Moeseneder *et al.*, 2001), whereas other authors found the opposite (Zhang *et al.*, 2007; Eloie *et al.*, 2011; Fuchsmann *et al.*, 2011; Ortega-Retuerta *et al.*, 2013; Bižić-Ionescu *et al.*, 2015). Still, others found a gradient, with more diversity in the smallest size-fractions (Kellogg and Deming, 2009). A recent study across a latitudinal gradient in the Atlantic found that in some of the sampled stations the small size-fraction was the richest, whereas in other stations the larger size-fraction presented more diversity (Milici *et al.*, 2016). This highly contrasting conclusions could be produced by the different filters used in the various studies or because of the distinct kinds of particles present in each environment. Here, we covered a broader range of sizes and we analyzed more size-fractions. Our data reflect that the diversity is variable in each size-fraction depending on the season, but there is always an increase of diversity from the smallest to the largest size-fractions. Diversity tripled from the commonly considered FL sample (that is, 0.2–0.8 µm) to the largest size-fraction (>200 µm, Figure 2a and b). Moreover, the larger fractions presented lower levels of dominance compared with the smallest fractions, being dominance and diversity inversely proportional in all size-fractions (Figure 5). Our results indicate that there is a high decrease in the abundance of bacteria per unit of volume in the larger fractions (on average 94% bacteria were in the 0.2 µm fraction, 4.9% bacteria were in the 0.8 µm fraction, and less than 0.5% were present in the remaining size-fractions in these samples, Supplementary Figure 2). Thus,

according to our results, the larger the size-fraction, the more bacterial diversity is contained, even though there are fewer bacteria per unit of volume.

We also observed that the % of unique OTUs in each filter ranged from 23 to 42% (Supplementary Table 5), indicating that the six size-fractions analyzed contained different environments (that is, types of particles) that created distinct niches and that contributed to the global differentiation of bacterial communities. Interestingly, we observed more specialist organisms (in number of OTUs and in relative abundance) with increasing size-fraction, which could be understood as an increase in the number of distinct niches with the increase of the size-fraction. The existence of these niches could be assigned to the development in larger particles of chemical gradients including low levels of oxygen or even anoxia (Alldredge and Cohen, 1987), and where biogeochemical processes such as denitrification (Karl *et al.*, 1984) or methanogenesis could be present. In fact, in the large size-fractions we detected *Tenacibaculum* sp. that are known to carry out nitrate reduction (Suzuki *et al.*, 2001) and thus develop in microaerophilic conditions; *Blastopirella* sp. that are known to perform nitrate reduction under anoxic conditions (Anammox; Schlesner *et al.*, 2004); facultative anaerobes such as *Vibrio* sp. (Baumann *et al.*, 1980), and strict anaerobes such as *Propionigenium* sp. (Schink and Pfennig, 1982). In particular, these organisms were identified as size-fraction indicator species according to our analysis (Supplementary Table 8). In addition, the size-fractionation scheme would also separate the phytoplankton and zooplankton present in the nano- and micro-sized fractions, and thus the distinct bacterial communities that they harbor. The phytoplankton community in Blanes Bay is generally dominated throughout the year by Prymnesiophyceae (~5 µm) and episodically by Bacillariophyta (2–200 µm) (Gutiérrez-Rodríguez *et al.*, 2011), and the

Figure 7 Selected taxonomic groups and their distribution (in relative abundance) among the six size-fractions: SAR11, *Synechococcus* sp., Bacteroidetes, Planctomycetes, Verrucomicrobia, Actinobacteria, Alteromonadales, Rhodobacterales. Boxplots were constructed with the upper and lower lines corresponding to the first and third quartile of the distribution. The median values are shown with horizontal black wide lines. Outliers are displayed as dots.

zooplankton community is dominated by nauplii and copepodites, in the 53–200 µm and 20–200 µm size-fractions respectively (Calbet *et al.*, 2001; Almeda *et al.*, 2011). Several studies have shown that certain bacterial OTUs can be associated to certain phytoplankton taxa (for example, Pinhassi *et al.*, 2004; Sala *et al.*, 2005; Sison-Mangus *et al.*, 2016) and, to a lesser extent, also to zooplankton (Grossart *et al.*, 2010b; Bickel *et al.*, 2014). Thus, the higher diversity and the higher percentage of unique OTUs in the larger size-fractions can also presumably be related, at least in part, to the specific bacterial communities attached to phytoplankton and <200 µm zooplankton.

There is not a strict separation between ATT and FL, and it likely exists a dynamic exchange between these categories (Grossart, 2010a). The presence of commonly considered FL groups in large particles can be explained if they have the potential to also live attached to particles, they search refuge from predation in particles, they form filaments or cellular aggregates, they are parasites or symbionts of protists or phytoplankton, or they are hitchhiking on protists or zooplankton (Grossart *et al.*, 2010b). By contrast, the presence of commonly considered ATT bacteria in small size-fractions could be explained if they can also live as FL or if they are individual cells dispersing from an aggregate. It may be argued that filtration, the most common method to separate FL and ATT lifestyles, may cause clogging and disaggregation. With clogging, FL would be retained in larger size-fractions. And by disaggregation, ATT bacteria could pass through the filter to smaller size-

fractions. We minimized both processes by prefiltering all the sample through 200 µm, filtering a reduced water volume (10 l in total), using very low vacuum pressure, and changing the filters when the flow slowed down. Our data indicate that several taxonomic groups can be found in more size-fractions than one. Moreover, we can associate various preferences to some of the high-rank taxonomic groups: some were enriched when increasing the size-fraction, some were enriched when decreasing the size-fraction, some did not enrich when increasing or decreasing the size-fraction, and some were enriched when decreasing the size-fraction but were depleted or absent in the smallest size one (Figures 7 and 8). We believe this is a new perspective that might allow a better understanding of the natural history of the different bacterial taxonomic groups in relation with the particulate matter present in the environment.

Various remarkable organisms could be assigned to each of the categories cited above. 'SAR11' were present all along the continuum of size-fractions but were enriched in the smallest size-fraction (Figure 8: category 'a'). They were ~70% of the community in the 0.2 µm fraction, and 5–15% in the remaining size-fractions. Their elevated presence in the smallest size-fraction is understandable since isolates of this group are known to have a very reduced size, and genomic studies of the first cultured member of this clade (*Pelagibacter ubique*) indicate adaptation to a mostly FL lifestyle (Giovannoni *et al.*, 2005). Yet, some SAR11 have also been observed in larger size-fractions, and have been considered as ATT ecotypes

Figure 8 Heatmap representing the relative abundances of individual taxonomic groups among size-fractions. The taxonomic groups were clustered hierarchically by the UPGMA method. A total of four categories were differentiated: (a) taxonomic groups enriched in the small size-fractions; (b) taxonomic groups enriched in increasing size-fractions, but depleted or absent in the smallest one (0.2–0.8 µm); (c) taxonomic groups that do not present enrichment in relation with the size-fraction; and (d) taxonomic groups enriched in increasing size-fractions. The data presented are the average of the 8 months.

that may occupy a niche in association with larger bacterioplankton and phytoplankton (Allen *et al.*, 2012). The relatively large presence of SAR11 in larger particles occurred in 2 months in particular, suggesting that this particle-attachment could appear under specific environmental conditions only. This would be consistent with the extensively described microdiversity within the SAR11 clade (García-Martínez and Rodríguez-Valera, 2000; Brown and Fuhrman, 2005).

Other relevant bacterial groups that presented the same pattern as SAR11 (that is, were enriched mainly in the 0.2–0.8 µm fraction: category ‘a’, Figure 8) were the ‘SAR116’ (>1% of the 0.2 µm community and <1% in the largest size-fractions). It is interesting to point out that in a previous 16S rDNA pyrotag sequencing study of the composition of sorted high-nucleic acid containing (HNA) and low-nucleic acid containing (LNA) bacteria from Blanes Bay done a few years before the current study, SAR11 were enriched in the LNA cell fraction while SAR116 were enriched in the HNA fraction (Vila-Costa *et al.*, 2012). This same pattern was observed also for the Acidobacteria, the Betaproteobacteria and for the Gammaproteobacteria (enriched in the LNA in the study by Vila-Costa *et al.*, but present in the higher size-fractions in our study).

Other groups enriched in the HNA cells in that study (such as the Rhodobacterales and the Bacteroidetes) presented higher contributions to community structure in the larger sizes in our current work (see below). We take these contrasting observations as evidence that association to particles (and thus, to large size-fractions) is not directly a size (and genome content) -related feature of the different organisms, but goes beyond that feature in what has been considered a ‘lifestyle’, that is known to present a phylogenetically defined signal (that is, Salazar *et al.*, 2015).

Cyanobacteria of genus *Synechococcus* sp. were also enriched in small size-fractions with relative abundance maxima in the 0.8–3.0 µm size-fraction (Figure 8: category ‘b’; 50% of the community) and almost non-existent in the smallest size-fraction (0.2–0.8 µm, <2% of the community). The average size of *Synechococcus* sp. is ~1 µm (for example, Morel *et al.*, 1993), which is larger than that of most FL bacteria, so they are large enough to be retained by the 0.8 µm filter and not contribute to the smaller fraction. In fact, the absence of *Synechococcus* sp. from the 0.2–0.8 µm fraction and the relatively low contribution to the community in the size-fractions >3 µm indicate that the filtration system used was successful and bias-free.

If *Synechococcus* had been found in the $<0.8\ \mu\text{m}$ fraction or abundantly in the $>3\ \mu\text{m}$, it would have raised concerns on the quality of the filtration. Their absence supports our size-fractionation protocol.

In contrast with the patterns observed in SAR11 and *Synechococcus* sp., some taxonomic groups were enriched in the largest size-fractions (Figure 8: category 'd'). Generally the taxonomic groups in category 'd' had been found in the ATT bacterial fraction in previous studies in the Mediterranean. Planctomycetes together with Bacteroidetes and Alpha- and Proteobacteria were found enriched in particles in the Adriatic Sea (Bižić-Ionescu *et al.*, 2015); In the NW Mediterranean, Bacteroidetes was the most important group in the ATT fraction, although as in our study, also Firmicutes or Verrucomicrobia were abundant phyla (Crespo *et al.*, 2013). In other contrasting environments, such as at 6000 m in the Puerto Rico Trench (Eloe *et al.*, 2011) or in the Black Sea suboxic zone (Fuchsman *et al.*, 2011) other groups were found in the ATT fraction, but Planctomycetes was always found enriched in particles. The phylum 'Planctomycetes' has been described as able to attach to surfaces (Bauld and Staley, 1976) such as macroalgae (Bengtsson and Øvreås, 2010; Lage and Bondoso, 2011), invertebrates (Fuerst *et al.*, 1997) or macroscopic detrital aggregates (Delong *et al.*, 1993; Crump *et al.*, 1999), where they contribute to biopolymer degradation (Woebken *et al.*, 2007). They were $>3\%$ of the communities of sizes $>3\ \mu\text{m}$, but $\sim 1\%$ in the smallest size-fractions (Figure 3).

Phylum 'Bacteroidetes' is here represented by classes Flavobacteria, Cytophagia and Sphingobacteriia, and all of them were enriched in the large size-fractions. They were $\sim 20\text{--}30\%$ of the communities of sizes $>3\ \mu\text{m}$, but $<10\%$ in the smallest size-fractions. Phylum Bacteroidetes has been known to contain heterotrophic bacteria with capacity for adhesion to particles (Williams *et al.*, 2012) and production of extracellular enzymes with degradative capabilities (Kirchman, 2002), which allow them to have an important role during algal blooms (Buchan *et al.*, 2014). Moreover some bacteroidetes have the capacity to survive as FL cells in situations of low levels of nutrients and the presence of light, thanks to the proteorhodopsin gene (González *et al.*, 2008). This might explain their presence also in the smallest size-fraction.

The phylum 'Verrucomicrobia' was also enriched when increasing the size-fraction. This group has been observed in marine snow (Rath *et al.*, 1998), where they are very efficient biopolymer degraders (Martinez-Garcia *et al.*, 2012), and they have also been observed in association with nanoeukaryotic cells (Petroni *et al.*, 2000). The association of Verrucomicrobia and eukaryotes is that close that it has even been speculated that Verrucomicrobia was the origin of the eukaryotic flagella (Li and Wu, 2005). The possible association with nanoeukaryotes would explain the observed elevated

values of Verrucomicrobia in the $3.0\text{--}5.0\ \mu\text{m}$ size-fraction ($\sim 2\%$).

Finally, some groups did not present a gradient of enrichment when increasing or decreasing the size-fraction (Figure 8: category 'c'). 'Actinobacteria' were enriched in the smallest size-fraction but presented also enrichment in the largest size-fractions. As SAR11, they have been described as small FL cells with streamlined genome and with rhodopsins that allows a photoheterotrophic metabolism (Ghai *et al.*, 2013). And as in SAR11, we can interpret the enrichment in large size-fractions as caused by the presence of different ecotypes. These ecotypes might be expressing light-capturing proteorhodopsin in the particle microenvironment, as has already been described in a river plume (Satinsky *et al.*, 2014).

Following the Sieburth *et al.* (1978) nomenclature, the size spectra sampled by our multiple size-fractionation comprises from the pico- ($0.2\ \mu\text{m}$) to the microplankton sizes ($200\ \mu\text{m}$), where we observed a saturation of the species-accumulation curve. This defines the border between the classic 'microbial environment', spatially structured by pico/nanostructures, and a 'non-microbial environment', characterized by larger structures. This information might be useful in the design of multi-scale studies focusing on the holistic description of the community (Pinel-Alloul and Ghadouani, 2007). Therefore, the knowledge of the dynamics at small scales and their implications for larger scales would allow us to improve our understanding of global ocean biogeochemistry (Azam and Malfatti, 2007).

Concluding, we show that the size of the particle is crucial for determining prokaryotic community structure, and the use of various size-fractions reveals a more comprehensive view of the pelagic microorganisms in the plankton. Moreover, and in the same way as the dichotomy of particulate organic matter vs dissolved organic matter is more accurately regarded as a continuum of sizes (Azam *et al.*, 1993; Verdugo *et al.*, 2004), the dichotomy of FL vs ATT bacteria should be better regarded as gradients of enrichment in larger or smaller size-fractions. This approach provides a more integrated perspective of the relations between the ecology of microbes and the chemical substrates presents in the ocean.

Conflict of Interest

The authors declare no conflict of interest.

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