UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Facultad de Ciencias del Mar

ANEXO I

Dª MARÍA ISABEL PADILLA LEÓN, SECRETARIA DE LA FACULTAD DE CIENCIAS DEL MAR, ÓRGANO RESPONSABLE DEL PROGRAMA DE DOCTORADO EN OCEANOGRAFÍA, DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

CERTIFICA

Que el Consejo de Doctores del Programa de Doctorado en Oceanografía, en su sesión de fecha 25 de mayo de 2017, tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada: "*Spatial and temporal patterns of marine prokaryotic diversity along the particulate matter continuum*", presentada por la doctoranda: **Dª Mireia Mestre Martín** y dirigida por los Doctores: D. Josep M. Gasol i Piqué y Dª M. Montserrat Sala.

Asimismo, se acordó el informar favorablemente la solicitud para optar a la Mención Internacional del Título de Doctora, por cumplir los requisitos reglamentarios.

Y para que así conste, a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, tribunal defensa y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo el presente en Las Palmas de Gran Canaria, a veinticinco de mayo de dos mil diecisiete.

Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento/Instituto/Facultad_____

Programa de doctorado_____

Título de la Tesis

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_____

Tesis Doctoral presentada por D/Dª_____

Dirigida por el Dr/a. D/Dª. _____

Codirigida por el Dr/a. D/Dª. _____

El/la Director/a,	**El/la Codirector/a**	**El/la Doctorando/a,**
(firma)	**(firma)**	**(firma)**

Las Palmas de Gran Canaria, a _____ de_____ de 20__

A tothom

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Summary

Microorganisms in the ocean conform an extensive microbiome where individuals interact constantly with the particulate matter. However, most of the studies have focused on the free-living microorganisms, and to a lesser extent on the attached microorganisms but have not taken into account the organisms associated to particles of different sizes. The main objective of this thesis is to characterize the diversity of prokaryotes along the particulate matter continuum present in the ocean, as well as to describe its temporal and spatial variability at distinct scales. First of all, we propose a multiple size-fractionation as a sampling method that provides a better comprehension of the prokaryotic diversity than the commonly used sampling methods. Our work shows that each size-fraction contains distinct prokaryotic communities that vary at different spatial and temporal scales. In general, there is an increase of bacterial richness from the smaller to the larger particles, suggesting that larger particles may contribute with new niches. The main exception is the bathypelagic, where richness decreases form the small to the largest size-fractions. In contrast, Archaea presented higher richness in the smaller size-fractions and, although had lower diversity and relative abundance than bacteria, these increased with depth. We moreover classified taxonomic groups depending on whether they have preference for small size-fractions, for larger size-fractions, or do not have a clear preference for any size fraction. This classification is presented as an alternative to the traditional simple separation between free-living bacteria and attached bacteria. Most of the taxonomic groups maintain their preference for certain size fractions in space and time, although some taxonomic groups change their preferences in vertical profiles from the surface to the bathypelagic and along time. We also observed that the bathypelagic is dominated by prokaryotes which are also present in surface waters and that there is a vertical connectivity between prokaryotic communities along the water column through sinking particles. This connectivity causes bathypelagic biogeography to be closely linked to particle colonization in the ocean surface. Overall, this thesis reports on the complexity of prokaryotic communities present in the continuum of sizes and shows the need for disseminating this perspective to define more comprehensively the diversity of ocean prokaryotes

Resum

Els microoganismes oceànics conformen un extens microbioma on els individus interactuen constantment amb la matèria particulada. No obstant això, la gran majoria dels estudis d'ecologia dels microorganismes no tenen en compte aquells associats a la matèria particulada o, si els tenen en compte, no consideren les diferents grandàries que presenten les partícules. L'objectiu principal de la present tesi és la caracterització de la diversitat de procariotes al llarg del continu de mides de les partícules marines presents a l'oceà, així com també descriure la seva variabilitat temporal i espacial a diferents escales. Primer de tot, proposem un fraccionament múltiple de mides de partícules com a mètode de mostreig que proporciona una millor comprensió de la diversitat procariòtica que els mètodes de mostreig més habituals. El nostre treball indica que cada fracció de mida presenta comunitats procariòtiques diferents que a més varien en l'espai, en el temps, i a diferents escales. En general, hi ha un increment de la riquesa de les comunitats bacterianes des de les partícules petites a les més grans, la qual cosa suggereix que les partícules més grans contribueixen nous nínxols ecològics. L'excepció a aquesta tendència s'observa en el batipelàgic, on la riquesa disminueix des de la fracció petita cap a les grans. Per contra, els arqueus presenten en general més riquesa en les fraccions més petites. Comparant bacteris i arqueus, aquests últims presenten menor diversitat i abundàncies relatives, però la seva rellevància augmenta des de la superfície de l'oceà cap al batipelàgic. Hem classificat els grups taxonòmics bacterians segons si tenen preferència per fraccions petites, preferència per fraccions grans, o si no presenten una preferència clara per fraccions petites o grans. Aquesta classificació es presenta com una alternativa a la tradicional separació simple entre bacteris de vida lliure o associats a partícules. La major part de grups taxonòmics mantenen en l›espai i en el temps la preferència per determinats mides de partícula. No obstant això, alguns grups taxonòmics canvien les seves preferències en perfils verticals des de la superfície cap al batipelàgic i també al llarg del temps. Hem vist, a més, que el batipelàgic està dominat per procariotes també existents en aigües superficials, i que hi ha una connectivitat vertical entre les comunitats procariòtiques al llarg de tota la columna d›aigua facilitada per les partícules que sedimenten. Aquesta connectivitat fa que la biogeografia del batipelàgic estigui estretament relacionada amb la colonització de partícules en superfície. En definitiva, aquesta tesi reflecteix la complexitat de les comunitats procariotes presents en el continu de mides de partícules presents a l›oceà i mostra la necessitat de mantenir aquesta perspectiva per descriure així de forma més precisa i completa la diversitat dels procariotes marins.

Resumen

Los microoganismos oceánicos conforman un extenso microbioma donde los individuos interactúan constantemente con la materia particulada. Sin embargo, la gran mayoría de los estudios de microorganismos no tienen en cuenta aquellos asociados a la materia particulada o, si los tienen en cuenta, no consideran los distintos tamaños que presentan las partículas. El objetivo principal de la presente tesis es la caracterización de la diversidad de procariotas a lo largo del continuo de tamaños de las partículas marinas presentes en el océano, así como también describir su variabilidad temporal y espacial a distintas escalas. Primero de todo, proponemos un fraccionamiento múltiple de tamaños de partículas como método de muestreo que proporciona una mejor comprensión de la diversidad procariótica que los métodos de muestreo comunes. Nuestro trabajo indica que cada fracción de tamaño presenta comunidades procarióticas distintas que además varían en el espacio, en el tiempo, y a distintas escalas. En general, existe un incremento de la riqueza de las comunidades bacterianas desde las partículas pequeñas a las más grandes, sugiriendo que las partículas más grandes contribuyen nuevos nichos ecológicos. La excepción a esta tendencia se observa en el batipelágico, donde la riqueza disminuye desde la fracción pequeña hacia las grandes. Por el contrario, las arqueas presentan en general más riqueza en las fracciones más pequeñas. Comparando bacterias y arqueas, estas últimas presentan menor diversidad y abundancias relativas, pero su relevancia incrementa desde la superficie del océano hacia el batipelágico. Hemos clasificado los grupos taxonómicos bacterianos según si tienen preferencia por fracciones pequeñas, preferencia por fracciones grandes, o si no presentan una preferencia clara por fracciones pequeñas o grandes. Esta clasificación se presenta como una alternativa a la tradicional separación simple entre bacterias de vida libre o asociadas a partículas. La mayoría de grupos taxonómicos mantienen en el espacio y en el tiempo la preferencia por determinados tamaños de partícula. Sin embargo, algunos grupos taxonómicos cambian sus preferencias en perfiles verticales desde la superficie hacia el batipelágico y también a lo largo del tiempo. Hemos visto, además, que el batipelágico está dominado por procariotas también existentes en aguas superficiales, y que existe una conectividad vertical entre las comunidades procarióticas a lo largo de toda la columna de agua mediada por las partículas que sedimentan. Dicha conectividad ocasiona que la biogeografía del batipelágico esté estrechamente relacionada con la colonización de partículas en superficie. En definitiva, esta tesis refleja la complejidad de las comunidades procariotas presentes en el continuo de tamaños de partículas presentes en el océano y muestra la necesidad de mantener esta perspectiva para describir así de forma más precisa y completa la diversidad de los procariotas marinos.

General Introduction

General Introduction

Why studying diversity?

We live in the only known planet that supports life, and we are alive thanks to other living beings that share the planet with us. Life diversity directly determines ecosystem functions such as productivity, decomposition rate, nutrient cycling, and resilience. Therefore, biodiversity collectively regulates all processes on the Earth system and, consequently, affects the health of the planet and the well-being of the humans. We are into the sixth mass extinction (Ceballos *et al*. 2015) and there is an urgent call to study everything related to biodiversity to better predict the ecosystems responses to the rapid loss of biodiversity that we are witnessing.

Microbial diversity is of particular interest as microorganisms are recognized as key players in Earth's ecosystems (Bell *et al*. 2005). Besides, multicellular life on Earth would have not been possible without microbes, and life as we know it would not be sustainable (Falkowski *et al*. 2008). Unicellular microbes account for the largest, yet unseen, fraction of Earth's biomass and biodiversity (Whitman *et al*. 1998, Torsvik *et al*. 2002). Thus, a more complete understanding of microbial patterns and processes is essential to disentangle how ecosystems work (Fuhrman 2009, Konopka 2009). Yet, the diversity and ecology of most microbial assemblages remain still poorly understood (Curtis *et al*. 2006, Pedrós-Alió 2006).

Measuring diversity

Biological diversity, or biodiversity, is defined as "*the variability among living organisms from all sources including terrestrial, marine and other aquatic systems and the ecological complexes of which they are part. This includes diversity within species, between species, and of ecosystems/communities*" (Heywood 1995). This description can be simplified to "*the variety and abundance of species in a defined unit of study*" (Magurran 2003). For decades, the discipline of ecology has developed several approaches to analyse diversity from a theoretical and holistic perspective (e.g. Margalef 1963), whereas microbiologists have focused on a reductionist approach (Prosser *et al*. 2007). Recently, thanks to the development of a proxy for (microbial) taxonomic units (Woese 1987, Pace 1997) and the progress of high-throughput sequencing (Goodwin *et al*. 2016), the knowledge developed by the science of Ecology that has been learned from the macroorganisms is being applied to microorganisms (Lennon and Locey 2017).

There are several methods to analyze/describe diversity (reviewed in Magurran 1988, 2004, 2011), and many of them aim at detecting patterns, as patterns imply some sort of repetition, and the existence of repetition implies that some prediction is possible (MacArthur 1965, 1972). Yet, ecosystems vary in space and time, and at distinct scales (Levin 1992), and the scale of observation certainly influences the description of all patterns (Levin 1992). To understand an ecosystem, it is important to take into account the heterogeneity of the habitat, study it at the appropriate scale, and develop models that bridge across scales. As a general rule, when increasing the scale, the variability declines and the predictability increases (Levin 1992). Microorganisms play a role in all the range of scales, from the micro to the macroscale: cells live and interact at the microscale (Azam and Malfatti 2007, Stocker 2015), and the effect of their metabolism influence all the biosphere. Due to the high heterogeneity of the microbial habitats (Pinel-Allou and Ghadouani 2007) and the distinct scales where microorganisms play an important role, the study and description of diversity patterns of microorganisms at an ecologicaly relevant scale is extremely challenging.

Diversity of prokaryotes in the ocean

Oceans cover the 70% of the surface of the Earth and marine marine microbes are responsible for 50% of the oxygen production on Earth (Falkowski *et al*. 1998, Field 1998). Among all microorganisms, prokaryotes dominate the abundance, diversity and metabolic activity of the ocean (Whitman *et al*. 1998, DeLong and Karl 2005, Giovannoni and Stingl 2005, Pomeroy *et al*. 2007). Due to recent advances in technology (as e.g. microscopy, molecular biology, microfluidics and DNA sequencing), contemporary microbial oceanography is truly a sea of opportunity to achieve a comprehensive understanding of marine microorganisms (Karl 2007). In particular, the development of high-throughput sequencing (HTS) has revolutionised the field of microbial oceanography, as it allows to sequence a high number of samples, at a high resolution, and with a relative reasonable price (Logares *et al*. 2012, Goodwin *et al*. 2016) something which can provide a detailed description of the microbial diversity of the oceans. The combination of HTS with intensive sampling provides for the first time a visualization of prokaryotic diversity in large temporal and spatial scales: world-wide circumnavegations, such as the Sorcerer II (Rusch *et al*. 2007), the Malaspina 2010 (http://scientific.expedicionmalaspina.es/) and the TARA Oceans (Bork *et al*. 2015) expeditions have provided samples from all the ocean and described on a global basis the diversity of prokaryotes in surface waters (Sunagawa *et al*. 2015), as well as in the deep ocean (Salazar *et al*. 2015). Moreover, microbial observatories with long data-series (reviewed in Bunse and Pinhassi 2017) are contributing with data for more than 10 years, showing intra-annual and inter-annual patterns of prokaryotic diversity (Fuhrman *et al*. 2006, Gilbert *et al*. 2009, Cram *et al*. 2015). In addition, the increase in sequencing depth provided by HTS allows to explore the rare biosphere (Pedrós-Alió 2007) (i.e., the low abundant prokaryotes). The study of the rare biosphere has unveiled a persistent seed bank throughout the global ocean (Gibbons *et al*. 2013), which provides evidence of the old microbiological tenet: "everything is everywhere" (Baas-Becking 1934). Nonetheless, despite all technological advances, the study of microbial ecology in the ocean does not commonly take into account the particles present in the water column and has mainly focused on those microorganisms that live free in the water column.

Particles in the ocean

Particles in the ocean are composed of various organic and inorganic materials depending largely on the system and on environmental conditions. The composition of the particles can be highly variable and can include living, senescent and dead algae (e.g. diatoms and coccolithophorids), phytoplankton exudates, cysts of thecate dinoflagellates, filamentous cyanobacteria, phytodetritus, diatom frustules, bacteria, protozoans, zooplankton molts and carcasses, fecal pellets, abandoned larvacean houses, pteropod webs, fecal pellets, macrophyte detritus, sand, clay and silt minerals, calcite and other particles scavenged from the surrounding water (see review in Simon *et al*. 2002). Moreover, particles suffer continuous size changes as they can aggregate, forming larger particles, and disaggregate into smaller particles or even dissolved material. The highly heterogeneous composition and high dynamism of the particles make them very difficult to describe and, therefore, there is still a lack of a detailed description of particulate matter composition in the ocean (Zetsche and Ploug 2015).

Particles are important sites for biological processes, e.g., production, decomposition and nutrient recycling in the water column (Alldredge and Silver 1988). Moreover, particles are an interesting niche for prokaryotes because they commonly constitue a local accumulation of nutrients from which cells can benefit, and can be a transport vehicle for prokaryotes through the water column (Pedrós-Alió and Brock 1983). Furthermore, particles with variable sizes, chemical composition and physical properties conform the microspatial architecture that structures the marine microbial environment (Azam *et al*. 1993, Azam 1998, Simon *et al*. 2002, Grossart 2010, Stocker 2012) (**Figure 1**). Therefore, in order to perform a better characterization of prokaryote diversity in the ocean, future descriptions should take into account this high heterogeneity present at the microscale.

Prokaryotic lifestyles: free-living and attached

Depending on their relation with the particulate matter present in the ocean, prokaryotes have been classified as free-living (FL) or attached to particles (ATT) and since free-living and attached prokaryotes are different in genetic, morphological, and physiological

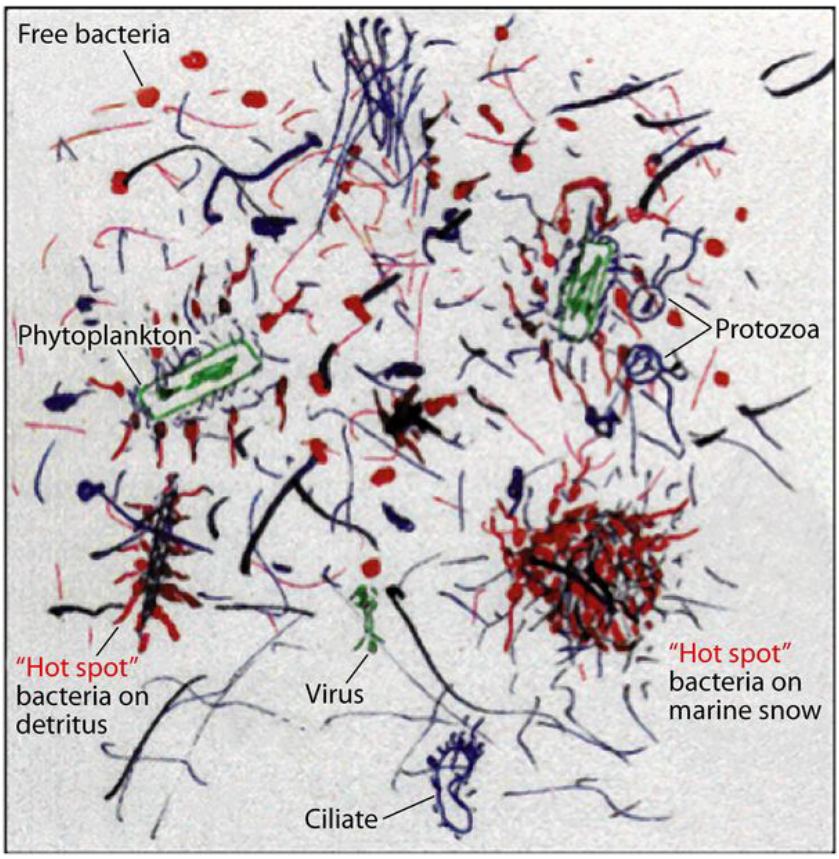

Figure 1. A prokaryotic-eye view of the ocean environment. Seawater contains an organic matter continuum, a gel of tangled polymers with embedded strings, sheets, and bundles of fibrils and particles, including living organisms, as "hotspots". Prokaryots (red) acting on marine snow (black) or algae (green) can control sedimentation and primary productivity; diverse microniches (hotspots) can support high bacterial diversity. The image and the figure caption (adapted) are from (Azam 1998).

aspects these two groups have been considered as two very different "lifestyles": Particle-attached prokaryotes are often larger than free-living prokaryotes (Alldredge *et al*. 1986, Cho 1988, Simon *et al*. 2002), have the ability to chemosensing, have motility and hydrolyze less degradable substrates (Grossart and Simon 1998, Kiørboe and Jackson 2001, Kiørboe *et al*. 2002), and are taxonomically distinct (DeLong *et al*. 1993, Crump *et al*. 1998, Fandino *et al*. 2001, Grossart *et al*. 2005, 2006, Rink *et al*. 2007). The contribution of attached prokaryotes to total prokaryotic abundance varies depending on the environment: generally the attached organisms are <5% of the total, reaching occasionally 10% in pelagic oligotrophic and mesotrophic marine ecosystems and can increase to >60% in

eutrophic environments (Bell and Albright 1981, Bano *et al*. 1997, Crump *et al*. 1998, Garneau *et al*. 2009). Although attached prokaryotes do not always dominate in number, the diversity of attached prokaryotic communities is usually higher than that of free-living communities (Zhang *et al*. 2007, Eloe *et al*. 2011, Fuchsman *et al*. 2011, Crespo *et al*. 2013, Ortega-Retuerta *et al*. 2013, Bižic-Ionescu *et al*. 2014). Moreover, attached prokaryotes living on particles can contribute to increase the diversity and abundance of free-living communities by acting as a "baby-machine" (Jacobsen and Azam 1984), releasing their progeny to the surrounding environment (Azam and Cho 1987, Smith *et al*. 1995, Friedrich *et al*. 1999).

In studies of diversity of aquatic prokaryotes, filtration is commonly used to concentrate the whole community in a 0.2 μm filter, but can also be used to separate the free-living and attached communities. The normal procedure to fractionate both communities is simple: water samples go through two filters. In the first filter, attached communities are retained, and free-living communities are retained in the second filter (normally 0.2 μm). Nonetheless, there is not a consensus on which pore-size is the most appropriate to separate free-living and attached communities. In addition, it has been proposed that the particulate matter is present in the oceans in a continuum of sizes, rather than into the duality of particulate and dissolved (Verdugo *et al*. 2004). Therefore, there is a need to better characterize the diversity of free-living and attached prokaryotes by improving the sampling methods to analyze all prokaryotes into the context of the continuum of the organic matter.

How separation of free-living and attached prokaryotes is commonly done?

We performed a meta-analysis of the published literature where we compiled the existing studies that have analyzed prokaryotic diversity and separated free-living and attached prokaryotic communities using filtration, with special emphasis on the pore-size used to separate both communities. Since the seminal study of DeLong *et al*. (1993) that compared the diversity of free-living microbes with that of those prokaryotes developing in marine snow particles, a range of papers (**Table 1**) have compared the diversity in the free-living communities with that on the particles. **Table 1** includes all types of aquatic planktonic environments, some in very specific sites, but others, such as the GOS, the

Table 1 Papers which present data on particle-attached and free living prokaryotes diversity.

Authors	Date	System	Method
Bidle & Fletcher	1995	Chesapeake Bay estuary	LMW rRNA
Acinas et al.	1997	Mediterranean offshore waters	T-RFLP
Crump et al.	1999	Columbia River estuary	Clones
Acinas et al.	1999	Mediterranean offshore waters	Clones
Hollibaugh et al.	2000	San Francisco Bay	DGGE
Riemann et al.	2001	Lake mesocosms	DGGE
López-García et al.	2001	Antarctic Polar Front	Clones
Moeseneder et al.	2001	Aegean Sea	T-RFLP
Dang et al.	2002	South-Eastern US salt marshes	FISH
LaMontagne & Holden	2003	a human-impacted coastal lagoon	T-RFLP
Selje & Simon	2003	Weser estuary, Germany	FISH, DGGE, clone libraries
Stevens et al.	2005	Wadden Sea is a tidal flat ecosystem	DGGE
Allgaier & Grossart	2006	Mecklenburg Lakes	DGGE, clone libraries
Henriques et al.	2006	Ria de Aveiro estuary	DGGE
Allgaier et al.	2007	Mecklenburg Lakes	Clones
Zhang et al.	2007	Victoria Harbor	DGGE, clone libraries
Ghiglione et al.	2007	NW Mediterranean Sea	CE-SSCP
Kellogg & Deming	2009	Laptev Sea, Arctic	T-RFLP, clone libraries
Garneau et al.	2009	Mackenzie River	DGGE, FISH
Ghiglione et al.	2009	NW Mediterranean Sea	CE-SSCP
Yooseph et al.	2010	GOS	metaG
Eloe et al.	2011	Puerto Rico trench	Clones
Berdjeb et al.	2011	Lake Bourget and Lake Annecy	DGGE
Parveen et al.	2011	Lake Bourget	Clones
Allen et al.	2012	Southern California Bight	metaG
Ortega-Retuerta et al.	2013	Mackenzie River to Beaufort Sea	CE-SSCP
Crespo et al.	2013	NW Mediterranean Sea	454 pyroseq
Smith et al.	2013	Columbia river estuary	metaG
Wilkins et al.	2013	Southern Ocean	454 pyroseq
Ganesh et al.	2014	Eastern Tropical South Pacific OMZ	454 pyroseq, metaG
Mohit et al.	2014	Magdalen Islands coastal lagoon	454 pyroseq
Ortmann & Ortell	2014	Mobile Bay shelf	Ion torrent
Kellogg & Deming	2014	Canadian Arctic Ocean	T-RFLP
D'Ambrosio et al.	2014	coastal North Carolina	Clones
Rieck et al.	2015	Baltic Sea	454 pyroseq
Padilla et al.	2015	OMZ off Manzanillo, Mexico	iTags
Li et al.	2015	western Gulf of Mexico	454 pyroseq
Salazar et al.	2015	bathypelagic tropical oceans	iTags
Bižić-Ionescu et al.	2015	N. Adriatic /Helgoland/German lakes	FISH and 454 pyroseq
Kanukollu et al.	2016	North Sea	Clones, DGGE and pyroseq
López-Pérez et al.	2016	NW Mediterranean Sea	metaG
Tarn et al.	2016	Mariana Trench	454 pyroseq
Milici et al.	2016	Central Atlantic Ocean	iTags
Milici et al.	2016	Central Atlantic Ocean	iTags
Yung et al.	2016	Pivers Island Coastal Observatory	iTags
Mestre et al. (Chapter 1)	2017	NW Mediterranean coastal site	454 tags*
Milici et al.	2017	meso- and bathypelagic S. Ocean	iTags

*Not included in the calculations nor in the graphs

Tara Oceans or the Malaspina studies intended to be more comprehensive (Yooseph *et al*. 2010, Salazar *et al*. 2015, Vargas *et al*. 2015). While the average number of studies per year was 2-3, there has been a renewed interest in looking at the diversity of the two types of communities in recent years (see the increase in the slope for 2013-2016 in **Figure 2**) coincident with the studies collected in this Ph.D. thesis. In total, we identified 47 studies with a total of ca. 900 sampling points (although it is possible that some papers were done in the same station and with the same samples).

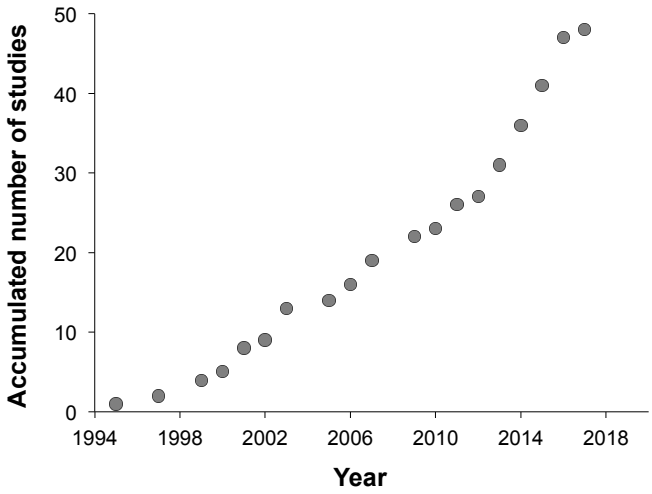

Figure 2. Accumulated number of papers published per year analyzing free-living *vs* attached microorganisms in aquatic systems.

According to **Table 1**, the determination of the diversity of free-living and attached prokaryotes has been mainly done either using clone libraries of the 16S rDNA (11 studies), fingerprinting of this gene (T-RFLP, DGGE or CE-SSCP; 18 studies), FISH (5 studies), 16S rDNA high-throughpout sequencing (either with 454, with Ion Torrent or with Illumina tags, 17 studies), or using metagenomics (4 studies) (**Figure 3**). Some did use the combination of 2 or 3 of these approaches.

While DeLong (1993) and colleagues hand-picked the particles, most of the studies in **Table 1** have used differential filtration, and for most of them only 2 different filters were

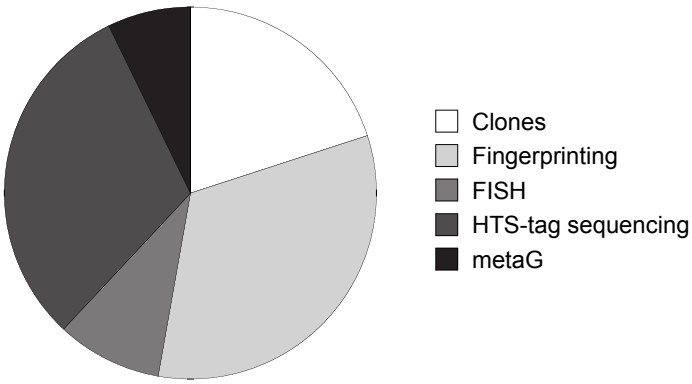

Figure 3. Contribution to the total data set of the distinct diversity analysis methods in studies where separation of free-living and attached microorganisms was performed.

used (**Figure 4**). Before 2012 more than 90% of the studies had used 2 filters and only <10% used 3 filters. None used more than this. It is interesting to realize how this changed after this year and coinciding with the beginning of the sampling that we did for this Ph.D. Thesis: while after 2012, still most studies used 2 filters (more than 70% of them), about 20% of the studies now used 3 filters, and close to 10% of them used 4 filters. Several authors (included us) realized the potential and the need for using more than 2 filters to separate the attached fraction into several different fractions: recent studies, such as the GOS data set or the Tara Oceans sampling have used several filters to characterize prokaryotes or protist diversity (using Illumina 16S or 18S tags) or microbial genes (using metagenomics).

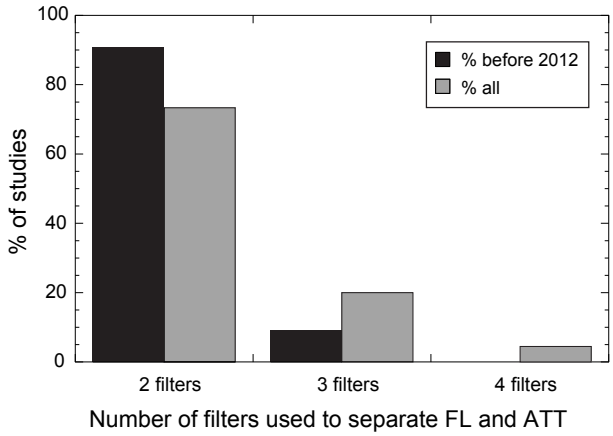

Figure 4. Number of size-fractions utilized in a total of 47 papers published before 2012.

About the filters used, most studies use 0.2 or 0.22 µm filters to characterize the free-living fraction, even though it is well known that some prokaryotes can cross this filter size (see for example Torrella and Morita 1981, Nakai *et al*. 2011, Luef *et al*. 2015). The choice of filter for characterizing the "attached" communities is more diverse, but with a dominance of the 3.0 µm and 5.0 µm filters, followed by the 0.8 µm filter (**Figure 5).**

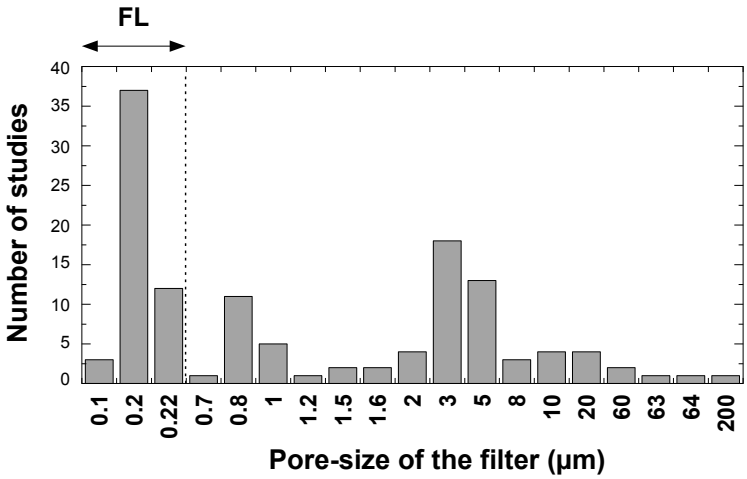

Figure 5. Pore-sizes of the filters utilized and the number of studies where they were used.

Sampling proposal

Taking into account the continuum of sizes of the particulate matter, in this Ph.D. Thesis we postulated to sample with more than one filter as we hypotesize that a multiple size-fractionation will provide a more exhaustive description of the prokaryotic diversity and community structure than the use of only one or two filters to separate free-living and attached lifestyles. Since 2012, not only the number of papers using more than one size-fraction has increased (Smith *et al*. 2013, Wilkins *et al*. 2013, Milici *et al*. 2016) but also has the number of size-fractions used (Bork *et al*. 2015, Vargas *et al*. 2015, Yung *et al*. 2016), supporting that our proposal was well conceived.

To select which filters should be used in the multiple size-fractionation, we first took into account the results of our meta-analysis: the most used pore-sizes where: 0.2, 0.8, 3.0, 5.0,

10 and 20 µm. Considering the Sieburth *et al*. (1978) division of the compartments of the plankton, we included the 20 µm pore-size (frontier between nano- and microplankton), and in addition we decided to prefiltrate all by 200 µm (frontier between micro- and mesoplankton). Therefore, the range of sizes of our samplings comprised from 0.2 to 200 µm, i.e, from the pico- to the microplankton size-fractions.

Concluding, we decided to use a serial fractionation using the pore-size filters: 0.2; 0.8; 3.0; 5.0; 10 and 20 µm placed in series. This defines the fractions: 0.2-0.8; 0.8-3.0; 3.0-5.0; 5.0-10; 10-20 and 20-200 µm (**Figure 6**). In the context of the present thesis, this multiple-size-fractionation filtration system provided samples mainly for diversity analyses, although it may be suitable for other variables such as prokaryotic function or particulate matter biogeochemical studies. We have applied this experimental proposal to samples from the Blanes Bay Microbial Observatory, an oligotrophic coastal site in the NW Mediterranean where we analyze the temporal variability; in a cruise in the NW Mediterranean (the NEMO cruise) where we will study the spatial variability; and in the Malaspina cruise, where we will analyze the spatial variability on a global-ocean scale.

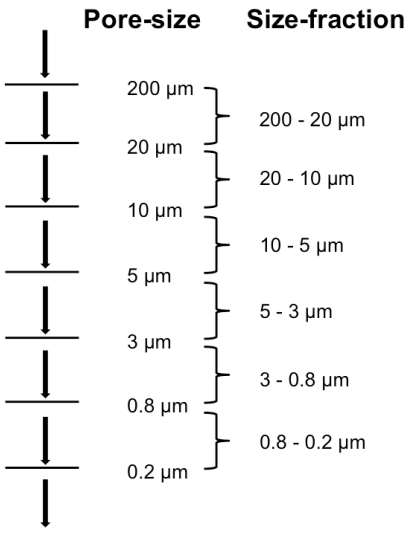

Figure 6. Multiple size-fractionation filtration system proposal. The samples, prefiltered through a 200 µm net mesh, pass sequentially through filters of decreasing pore-sizes. The size of the pores define the size-fractions.

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Aims of the Thesis

Aims of the Thesis

Considering the relevance of prokaryotes in the biogeochemical cycles of the ocean and the need to study them from new perspectives that take into special account the particles to which the prokaryotes interact constantly, the main aim of the present thesis is to characterize the diversity and community composition of the prokaryotics communities along the continuum of particulate matter present into the ocean, as well as to describe its temporal and spatial variability at distinct scales. The specific aims of each chapter are detailed below:

Chapter 1. Patterns of bacterial diversity in the marine planktonic particulate matter continuum

This chapter presents a comprehensive analysis of the bacterial diversity present in distinct size-fractions in a NW Mediterranean coastal site (the Blanes Bay Microbial Observatory). The specific aims of the study were:

- To test whether prokaryotic diversity is related to the size fraction considered (i.e. to the pore-size of the filter used to collect the cells) and, by extension, to the types of particles contained in that size fraction.

- Explore whether the multiple size-fractionation provides a more comprehensive description of the whole community than the use of only one filter to separate free-living and attached communities.

Chapter 2. Seasonality and dynamics of bacterial community structure along the pelagic particulate matter continuum in a temperate oligotrophic coastal site

This chapter presents the dynamism of bacterial community composition in the different size-fractions throught the seasonal cyle at the Blanes Bay Microbial Observatory. The specific objectives were:

- Describe the seasonal variation of bacterial community composition as it depends on the sizes of particles present in the water.

- Test whether the temporal variability in community composition and in individual taxa within each size-fraction presents defined patterns that are repeated annually, and to what extent this is occurring with all or most the bacterial groups.

Chapter 3. Spatial variability of marine bacterial and archaeal communities along the particulate matter continuum

Here we studied the spatial variability of the prokaryotic communities as they structure in different size-fractions. To reach this objective, samples were taken in the Northwestern Mediterranean Sea in a coastal-open ocean transect including vertical profiles. The specific aims of this study were:

- To describe the horizontal (from the coast to the open ocean) and vertical (from the surface to the bathypelagic) variability in diversity and community composition of bacteria and archaea in the different size-fractions.

- To test whether the preference of the dominant prokaryotic taxonomic groups for certain sizes of particles are maintained or vary along the horizontal and vertical gradients.

Chapter 4. Vertical connectivity in the ocean microbiome: Sinking particles as dispersal vectors

Here we explored bacterial composition in distinct size-fractions of samples taken around the global tropical and subtropical ocean (Atlantic, Indic and Pacific) with the following specific aims:

- To explore whether sinking particles of distinct size are a dispersal mechanism for surface dwelling prokaryotes into the deep ocean.

- To test whether the deep ocean prokaryotes are also present in deep ocean samples and in which size fractions do they occur.

- To analyze the connectivity between surface and deep-ocean communities, and examine whether this connectivity determines the prokaryotic biogeography of the deep-ocean.

This thesis is presented as a collection of research articles, each one addressing specific issues. The state of the art for each of those issues as well as specific methodologies are presented within each chapter.

Chapter 1

Patterns of bacterial diversity in the marine planktonic particulate matter continuum

Mireia Mestre, Encarna Borrull, M. Montserrat Sala & Josep M. Gasol

Chapter 1

Patterns of bacterial diversity in the marine planktonic particulate matter continuum

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SUMMARY: 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 6 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.

1.1 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 2 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*. 2010), 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 (e.g: 0.8 µm and 10 µm (Dang and Lovell 2002), 1.0 µm and 60 µm (Kellogg and Deming 2009), 0.8 µm and 3.0 µm (Smith *et al*. 2013 and Wilkins *et al*. 2013), 3.0 µm 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. 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. In order 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 6 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.

1.2 MATERIAL AND METHODS

1.2.1 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 (41º40'N, 002º48'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 & Process Technologies (Trevose, USA) and the rest of the filters from Millipore (Billerica, 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 µm 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 inmediatelly at – 80ºC 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 (i.e. "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**.

1.2.2 DNA extraction, sequencing and sequences processing

The DNA was extracted as described in (Massana *et al*. 1997). Hypervariable V1-V3 16S tags were PCR amplified and 454 GS FLX+ pyrosequenced with primers 28F/519R 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 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 5 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 where 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 dataset. A summary of sequence information can be found in **Supplementary Table 1.**

1.2.3 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 (nMDS) 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 (Dufrene 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 (Bj) (Levins 1968) of each OTU was calculated and OTUs with Bj<3 were considered Specialists, OTUs with Bj∈[3-4] were considered Intermediate and OTUs with Bj>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 more than 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, ANOVAs ($p<0.05$) 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.

1.3 RESULTS

The environmental parameters of the 8 sampled dates presented elevated variability (the CVs were on average 57%) (**Supplementary Table 2**). The nMDS 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 to the communities of the larger size-fractions. Most (94% on average) bacteria were in the >0.2 µm filter while 4.9% were in the 0.8 µm filter, and less than 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 (**Figure 2**). The 20-200 µm size-fraction pre-

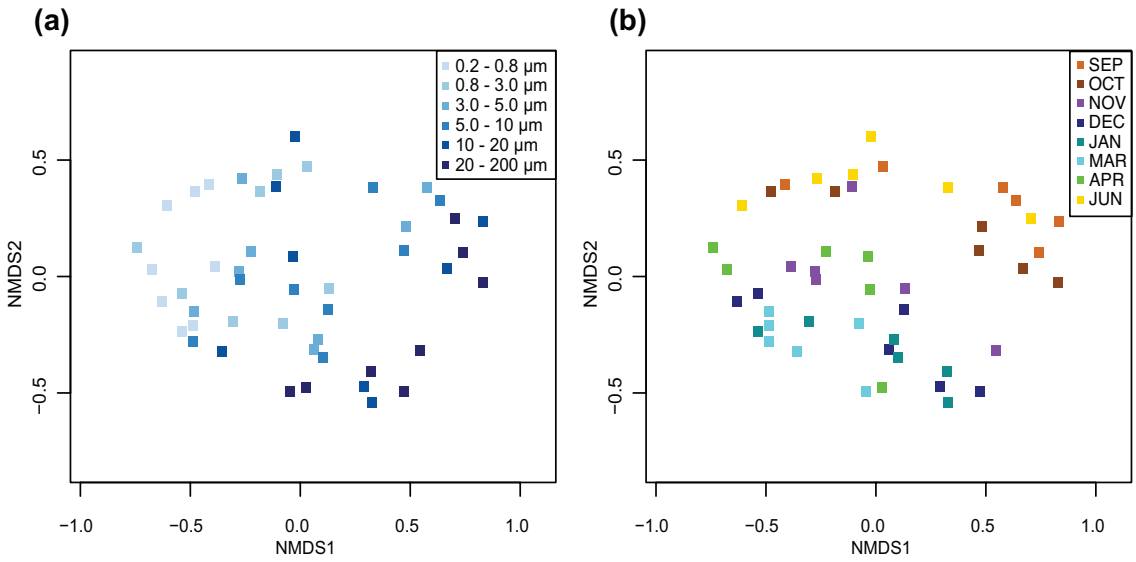

Figure 1. Non-metric multidimensional scaling (nMDS) representing the distance between samples by size-fraction **(a)** and month **(b).** The nMDS was created with a Bray-Curtis distance matrix derived from the OTU table.

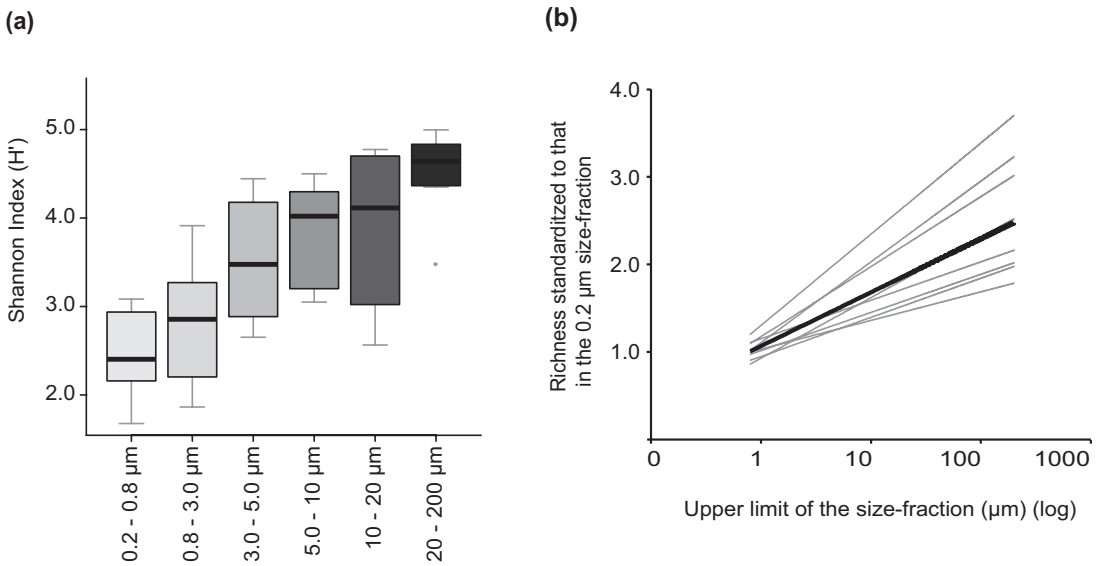

Figure 2. (a) Box-plot of the Shannon diversity Indices for each size-fraction. Upper and lower lines correspond to the 1st and 3rd quartile of the distribution of values. The median values are shown with horizontal black wide lines. Outliers are displayed as dots. **(b)** Richness standardized to that 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.

sented the most diverse community, whereas the 0.2-0.8 µm 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 6 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 (i.e 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 µm) from the rest of fractions, and secondly the 0.8-3.0 µm size fraction from the larger fractions. The larger fractions clustered by pairs: 3.0-5.0 µm with 5.0-10 µm and 10-20 µm with 20-200 µm. 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 (i.e. the smaller fractions) presented less diversity, as can be observed by the steeper slope in this representation.

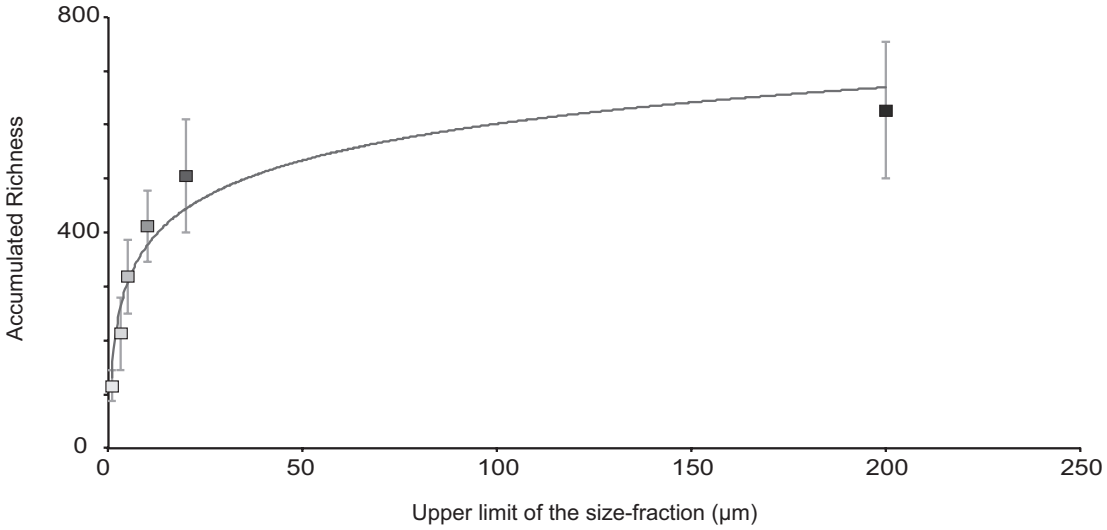

Figure 3. Species accumulation curve representing the number of OTUs ("species") accumulated from the smallest to the largest size-fraction. The curve was constructed with the median values of the 8 months, with its standard deviation. The equation of the curve: $y=97.7\ln(x) + 152$. $R^2=0.95$

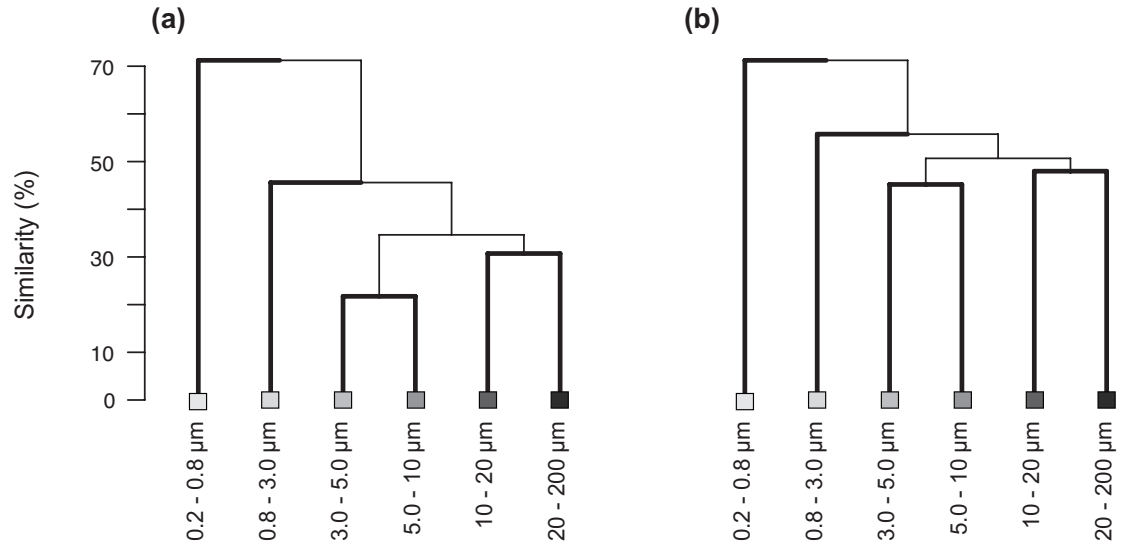

Figure 4. Clustering dendrograms of the 6 size-fractions calculated by average clustering criteria (Unweighted Pair Group Method with Arithmetic Mean, UPGMA). Data used is the average of the distance between two size fractions of the 8 months, with: **(a)** relative abundance data, **(b)** presence-absence (binary) data. Thick lines indicate significant differences (determined by SIMPROF).

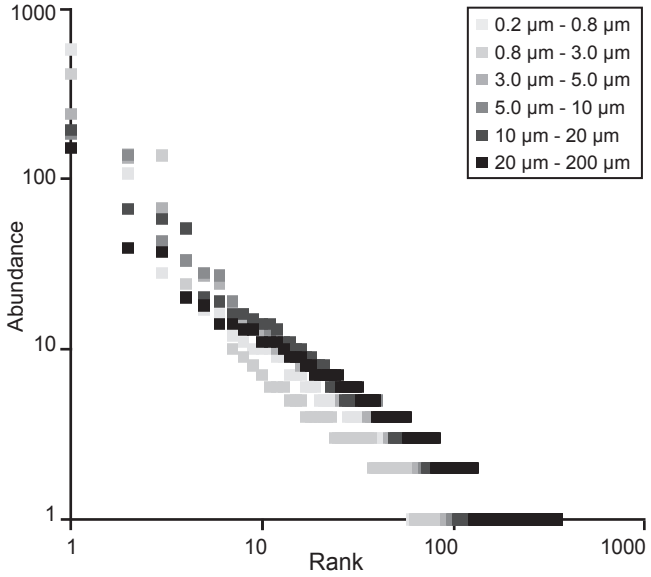

Figure 5. Rank-abundance curve for the 6 size-fractions, represented as a log-log plot. The data represent the average values in each of the 8 months.

On average (±SD), the percentage of unique OTUs in each size fraction ranged from 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 (i.e. ubiquitous, with global co-ocurrence) accounted for 3.3±1.1% of the total OTUs (**Supplementary Table 6**). The percentage of shared OTUs between two size fractions (co-ocurrence 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 20.0-200 µm (24 and 18 respectively).

When separating the values of Niche Breadth (Bj) into 3 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**).

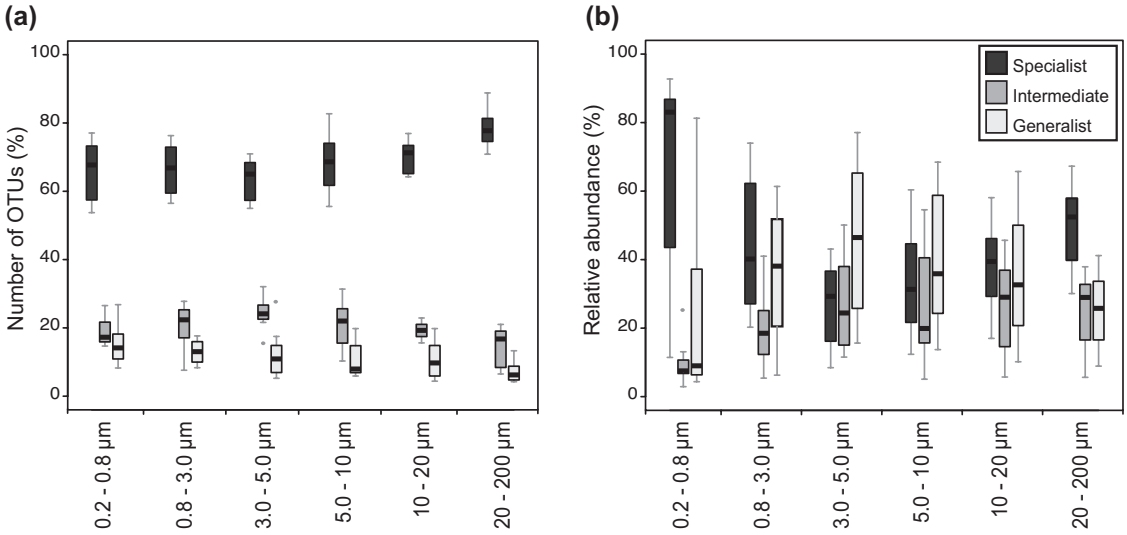

Figure 6. Specialist, Intermediate and Generalist OTUs (see Material and Methods for definition) in each size-fraction, represented as: **(a)** number of OTUs, **(b)** their relative abundances. The boxplots were constructed with the upper and lower lines corresponding to the 1st and 3rd quartile of the distribution. The median values are shown with horizontal black wide lines. Outliers are displayed as dots.

ANOVA tests showed that 16 of the 17 selected (i.e. 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 4 categories could be differentiated. The first category (A) encompassed the taxonomic groups that were enriched in the smallest size-fractions: e.g. 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): e.g. *Synechococcus* sp.. The third category (C) included the taxonomic groups that did not present enrichment when increasing or decreasing the size-fraction: e.g. Deferribacterales, Oceanospirillales, Actinobacteria. The fourth category (D) was composed of taxonomic groups that were enriched in the lager size-fractions: e.g. Rhodobacterales, Cytophagia, Alteromonadales, Verrucomicrobia, Vibrionales, Spingobacteriia, Firmicutes, Planctomycetes, Deltaproteobacteria, Flavobacteria, and Rhizobiales.

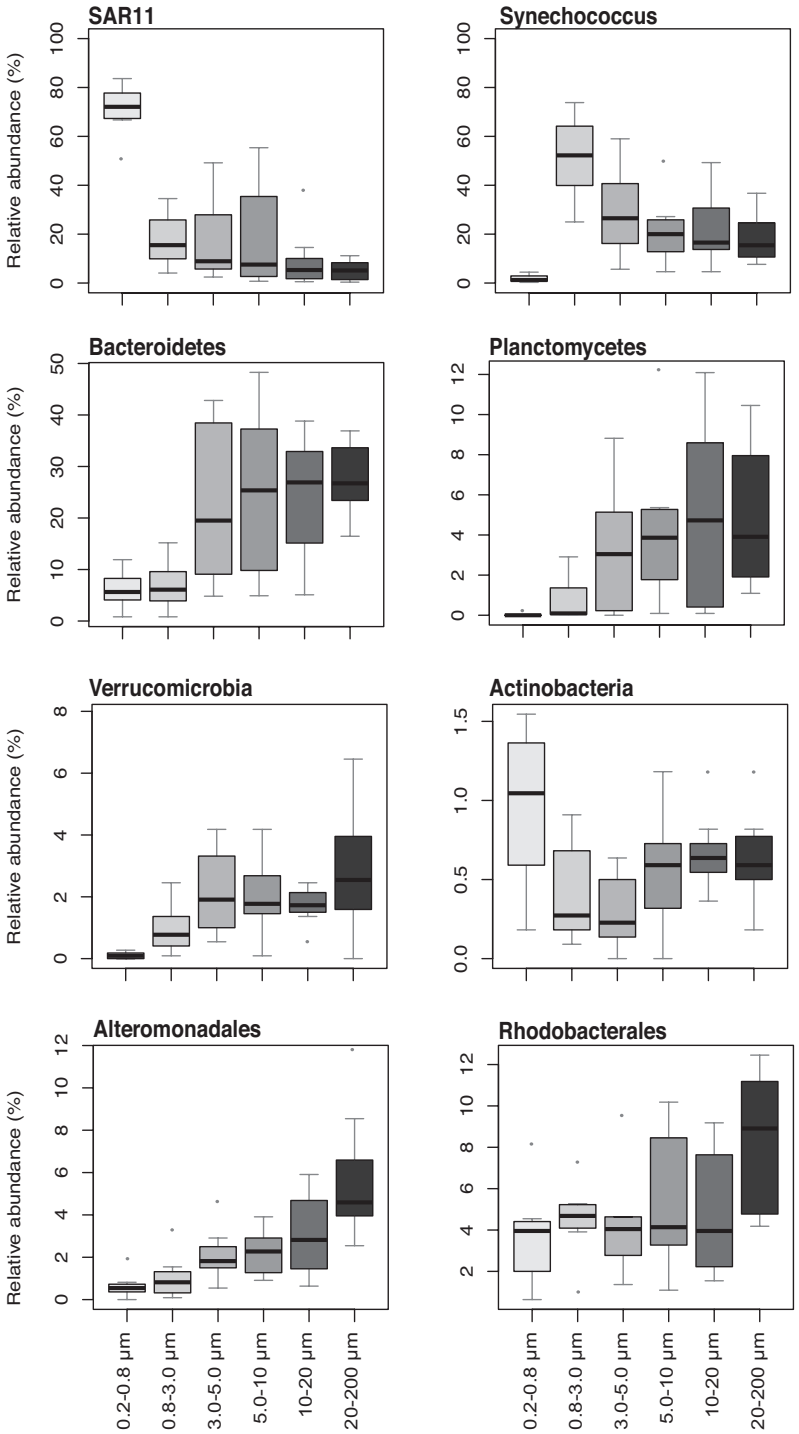

Figure 7. Selected taxonomic groups and their distribution (in relative abundance) among the 6 size-fractions: SAR11, *Synechococcus* sp. Bacteroidetes, Planctomycetes, Verrucomicrobia, Actinobacteria, Alteromonadales, Rhodobacterales. Boxplots were constructed with the upper and lower lines corresponding to the 1st and 3rd quartile of the distribution. The median values are shown with horizontal black wide lines. Outliers are displayed as dots.

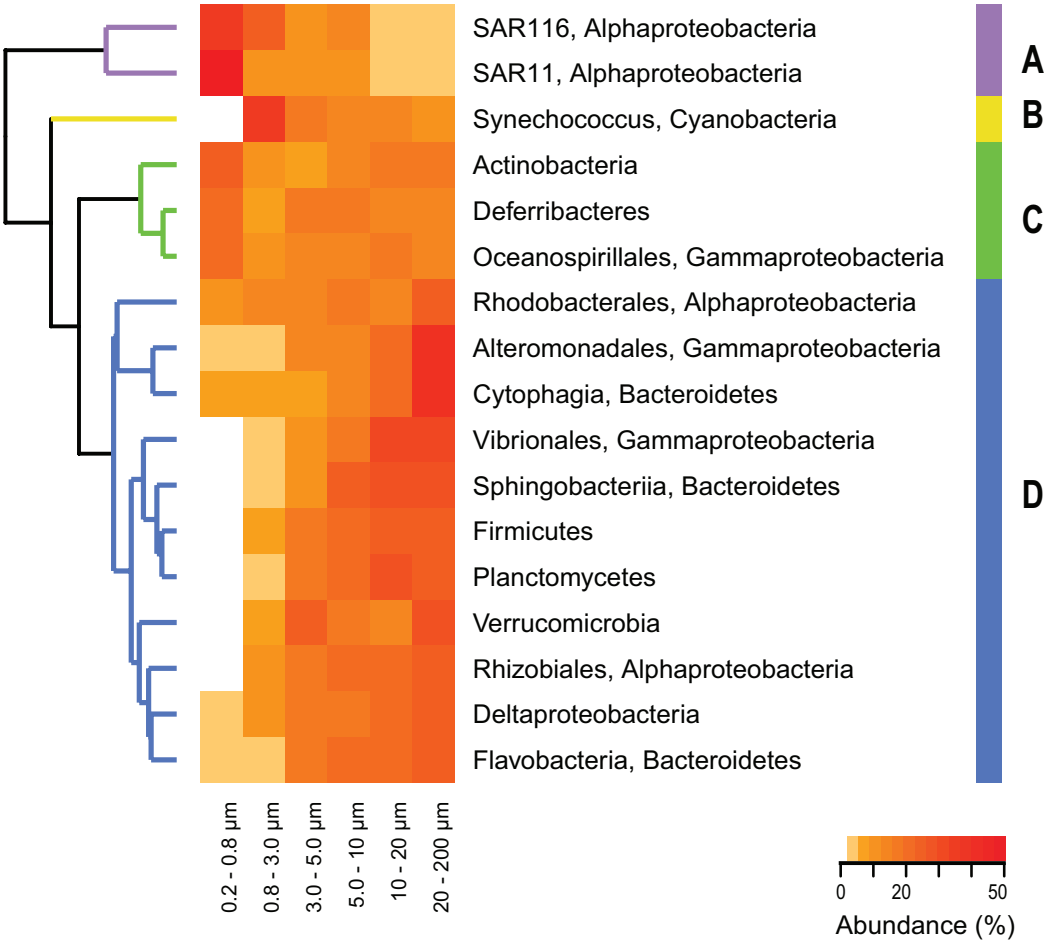

Figure 8. Heatmap representing the relative abundances of individual taxonomic groups among size-fractions. The taxonomic groups were clustered hierarchically by the UPGMA method. A total of 4 categories were differentiated: (A) taxonomic groups enriched in the small size-fractions, (B) taxonomic groups 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, (D) taxonomic groups enriched in increasing size-fractions. The data presented are the average of the 8 months.

1.4 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 (Mestre *et al. in prep.*). 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 dataset of the Blanes Bay Microbial Obsevatory including high variability in terms of environmental parameters and bacterial community composition (**Figure 1, Suppementary Table 3, Supplementary Figure 1, Supplementary Table 2**). 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 6 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 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; Eloe *et al.* 2011; Ortega-Retuerta *et al.* 2013; Bižic-Ionescu *et al.* 2015 Fuchsman *et al.* 2011). 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 (*i.e.* 0.2-0.8 μm) to the largest size fraction (>200 μm, **Figure 2**). Moreover, the larger fractions presented lower levels of dominance compared to 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 (i.e. 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; *Blastopirellula* 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 7**). 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) (Gutierrez-Rodriguez *et al*. 2011), and the 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 (i.e. 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 presumabily be related, at least in part, to the specific bacterial communities attached to phytoplakton 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 vari-

ous 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 (**Figure 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 ca. 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 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 (i.e. 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 Vila-Costa *et al.* study, 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 (i.e. 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 ca. 1 μm (e.g. Morel *et al*. 1993), which is larger than that of most free-living 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 *Synechoccocus* 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. Had *Synechoccocus* been found in the <0.8 μm fraction or abundantly in the >3 μm 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 (Bizic-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*. 2012). In other contrasting environments, such as at 6,000 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 μm, but ca. 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 ca. 20-30% of the communities of sizes >3 μm, 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 play 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 eukaryonts 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 nanoeucaryotes would explain the observed elevated values of Verrucomicobia in the 3.0-5.0 μm size-fraction (ca. 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 free-living 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 µm) to the microplankton sizes (200 µm), 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 multiscale studies focusing on the holistic description of the community (Pinel-Allou 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.

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1.7 SUPPLEMENTARY MATERIAL

1.7.1 Supplementary Methods

To determine prokaryotic abundance on the particles, seawater was fixed with glutaraldehyde (final concentration 1%) and distinct volumes were filtered through black polycarbonate membrane filters (Poretics) of 5 different pore-sizes: 0.2 µm (5 mL), 0.8 µm (20 mL), 3.0 µm (150 mL), 5.0 µm (150 mL), 10.0 µm (150 mL). Before finishing filtering all the volume, the last 5 mL of each sample were maintained for 5 min with 50 µL of DAPI dye (0.5 mg ml^{-1}) in the dark. The filters were placed on microscope slides and with inmersion oil (Type-F, Olympus). DAPI positive cells were enumerated by epifluorescence microscopy (Olympus BX61 epifluorescence microscope).

Temperature and salinity were obtained with a CTD probe (SD2014, SAIV A/S). Chlorophyll *a* was measured according to the procedure of Yentsch and Menzel (1963). Bacterial heterotrophic activity was estimated using the ^{3}H-leucine incorporation method (Kirchman *et al*. 1985). Inorganic nutrients were analyzed using a CFA Bran Luebbe autoanalyser following the methods described by Hansen and Koroleff (1999). Samples for total organic carbon (TOC) determinations were collected in 10 mL precombusted (450 ºC, 24 h) glass ampoules. After acidification with 50 µL 25% H_3PO_4 to pH<2, the ampoules were heat-sealed and stored in the dark at 4ºC until analysis. Analyses were carried out using a Shimadzu TOC-CSV organic carbon analyzer. Particulate organic carbon (POC) was measured by filtering 60 mL (four replicates) on pre-combusted GF/F glass fibre filters. The filters were then frozen in liquid nitrogen and kept at –80ºC until analysis. Prior to analysis, the filters were dried at 60ºC for 24 h and exposed to hydrochloric acid vapours for 48 h to destroy inorganic material. They were then analysed in a Perkin-Elmer 240 C:H:N autoanalyser.

1.7.2 References

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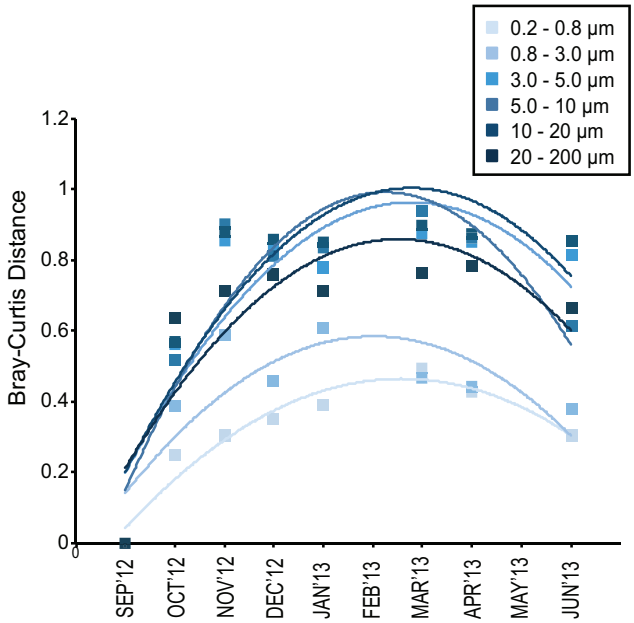
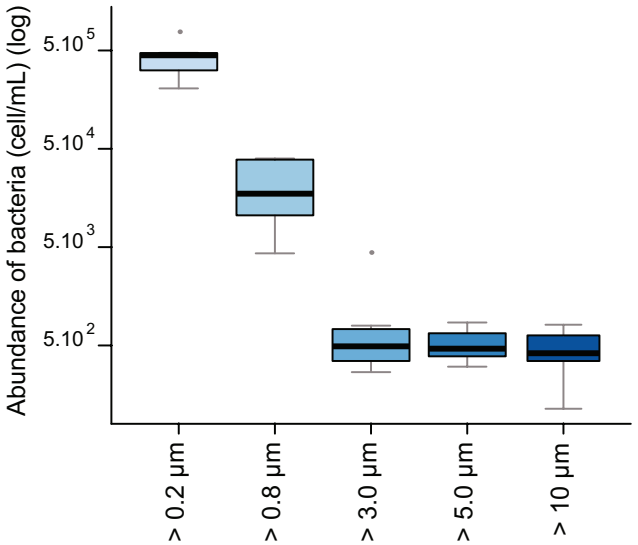
1.7.3 Supplementary Figures



Supplementary Figure 1. Bray-Curtis distances calculated between the same size-fractions of different months. September'12 was taken as a reference for the comparisons.



Supplementary Figure 2. Prokariotic abundance in the filters of the different sizes at each sampling date. The x axes indicate the size of the filter.

1.7.4 Supplementary Tables

Supplementary Table 1. A summary of sequence information and number of identified OTUs.

Sample	Date	Size-fraction	Number of Raw reads	Number of Clean reads	Number of Clean reads (Normalized)	Number of OTUS	Number of OTUS (Normalized)
X114STORM2012SEP01	September	0.2	5443	5442	1000	250	118
X115STORM2012SEP02	September	0.8	6788	5127	1000	392	154
X116STORM2012SEP03	September	3	5309	3892	1000	473	240
X117STORM2012SEP04	September	5	7707	5552	1000	664	266
X118STORM2012SEP05	September	10	3586	3223	1000	546	301
X119STORM2012SEP06	September	20	7299	6408	1000	931	331
X120STORM2012OCT01	October	0.2	5103	5083	1000	332	144
X121STORM2012OCT02	October	0.8	16900	16328	1000	468	110
X122STORM2012OCT03	October	3	19227	12745	1000	1070	258
X123STORM2012OCT04	October	5	6388	4652	1000	621	275
X124STORM2012OCT05	October	10	8774	7094	1000	936	354
X125STORM2012OCT06	October	20	4871	4578	1000	825	366
X126STORM2012NOV01	November	0.2	10448	10326	1000	518	180
X127STORM2012NOV02	November	0.8	7212	5247	1000	727	281
X128STORM2012NOV03	November	3	3961	3524	1000	326	168
X129STORM2012NOV04	November	5	3720	3248	1000	319	162
X130STORM2012NOV05	November	10	5184	4539	1000	347	146
X131STORM2012NOV06	November	20	5862	4415	1000	875	374
X132STORM2012DEC01	December	0.2	3793	3727	1000	193	107
X133STORM2012DEC02	December	0.8	4293	2434	1000	202	113
X134STORM2012DEC03	December	3	7571	2992	1000	433	248
X135STORM2012DEC04	December	5	8081	4943	1000	669	286
X136STORM2012DEC05	December	10	8773	4946	1000	696	306
X137STORM2012DEC06	December	20	8318	4733	1000	769	326
X138STORM2013JAN01	January	0.2	12485	12134	1000	673	178
X139STORM2013JAN02	January	0.8	8075	5073	1000	358	148
X140STORM2013JAN03	January	3	7805	2643	1000	401	242
X141STORM2013JAN04	January	5	4837	1590	1000	332	262
X142STORM2013JAN05	January	10	6850	3047	1000	572	322
X143STORM2013JAN06	January	20	7895	4095	1000	788	335
X150STORM2013MAR01	March	0.2	3661	3126	1000	211	125
X151STORM2013MAR02	March	0.8	8311	4389	1000	482	207
X152STORM2013MAR03	March	3	5705	3653	1000	236	127
X153STORM2013MAR04	March	5	17386	11270	1000	741	192
X154STORM2013MAR05	March	10	13195	7969	1000	412	151
X155STORM2013MAR06	March	20	5443	2067	1000	334	233
X156STORM2013APR01	April	0.2	4308	4296	1000	145	76
X157STORM2013APR02	April	0.8	9030	8581	1000	244	86
X158STORM2013APR03	April	3	9441	6496	1000	400	159
X159STORM2013APR04	April	5	6269	4221	1000	430	201
X160STORM2013APR05	April	10	5522	3709	1000	267	148
X161STORM2013APR06	April	20	5051	3630	1000	317	166
X168STORM2013JUN01	June	0.2	17143	17036	1000	402	117
X169STORM2013JUN02	June	0.8	10499	9559	1000	561	146
X170STORM2013JUN03	June	3	4265	2504	1000	191	123
X171STORM2013JUN04	June	5	2939	2473	1000	336	210
X172STORM2013JUN05	June	10	9784	7212	1000	466	149
X173STORM2013JUN06	June	20	3190	2710	1000	609	319

Supplementary Table 2. Environmental parameters measured in each sampling date and Median, Standard deviation (SD) and Coefficient of variation (CV) of each parameter. Total Number of Bacteria were measured on DAPI counts on 0.2 µm pore-size filters. See Supplementary Methods for details.

Date	Temperature (ºC)	Salinity	Chlorophyll (µg L^{-1})	PO_4 (µM)	NO_3 (µM)	SiO_2 (µM)	Number of Bacteria (DAPI counts) (cell mL^{-1})	Bacterial Production (pM h^{-1})	TOC (µM)	POC (µM)
13-Sep-12	22.46	38.16	0.16	0.027	0.069	1.052	4.11 x10^{05}	364.39		7.91
9-Oct-12	18.31	37.91	0.34	0.026	0.038	0.932	9.14 x10^{05}	139.37		5.5
6-Nov-12	16.66	38.04	0.46	0.221	3.523	2.036	9.37 x10^{05}	51.48	93.94	16.26
11-Dec-12	14.25	38	0.48	0.083	1.36	2.023	7.48 x10^{05}	58.77	66.58	5.43
15-Jan-13	13.27	38.1	0.89	0.124	1.212	1.564	5.08 x10^{05}	8.11	94.99	21.91
12-Mar-13	12.78	38.14	1.08	0.164	3.289	2.475	9.45 x10^{05}	40.93	66.07	8.96
17-Apr-13	14.51	37.87	0.49	0.084	0.646	1.356	1.53 x10^{05}	99.81	73.95	8.66
4-Jun-13	16.92	37.62	0.31	0.103	0.34	1.249	8.72 x10^{05}	41.04	76.43	8.46
Median	16.15	37.98	0.53	0.1	1.31	1.59	8.58 x10^{05}	100.49	78.66	10.39
SD	2.99	0.17	0.29	0.06	1.29	0.51	3.16 x10^{05}	106.6	11.77	5.37
CV	18.49	0.44	54.68	59.41	98.7	32.03	36.85	106.09	14.97	51.7

Supplementary Table 3. Permutational multivariate analysis of variance (PERMANOVA) examining the effects of the factors "month" (seasonality) and "size-fraction" on the bacterial communities on the filters. Key to abbreviations and column headings: D.f, degrees of freedom; MS, mean square; F, F ratio; R^2, coefficient of determination; P, p-value. Probabilities are marked as follows: '***'p<0.001; '**' p<0.01; '*' p<0.05.

Source of variation	D.f	MS	F	R^2	P	Significance
Size-fraction	5	0.74709	6.5717	0.31878	0.0001	***
Month	7	0.57192	5.0308	0.34165	0.0001	***
Residuals	35	0.11368		0.33956		
Total	47					

Supplementary Table 4. True alpha, beta and gamma diversity in each sampling date. Median, Standard deviation (SD) and Coefficient of variation (CV) of each parameter are also presented.

	gamma	beta	alpha
SEP	647	3.3	197.3
OCT	711	3.4	208.2
NOV	637	3.4	187.3
DEC	658	3.2	206.2
JAN	699	3.2	218.5
MAR	535	3.3	163.5
APR	345	3	113.5
JUN	533	3.7	144
Median	595.6	3.3	179.8
SD	113.3	0.2	34.1
CV	19	5.6	19

Supplementary Table 5. Percentage of unique OTUs in each size-fraction and in each sampling date. Size-fractions are referred to by the lowest filter size.

	0.2 µm	0.8 µm	3.0 µm	5.0 µm	10 µm	20 µm
SEP	35.4	33.1	23.9	31.3	32.7	41.2
OCT	51.2	25.5	32.2	27.6	36.4	39.1
NOV	21.3	42.2	19.6	14.9	39.3	54.1
DEC	26.1	27.9	21.1	33.3	32	31.9
JAN	32.9	24.5	19.8	25.8	38.7	41.6
MAR	21.8	43.1	17.1	36.8	21	45
APR	19	12.9	24	41.5	26.7	33.1
JUN	31.8	41.3	26.7	38.4	38.3	54.7
Median	29.9	31.3	23	31.2	33.1	42.6
SD	9.8	9.9	4.5	7.9	6.1	7.9
CV	32.8	31.7	19.5	25.3	18.4	18.6

Supplementary Table 6. Percentage of shared OTUs in all size-fractions (i.e. ubiquitous, with global co-ocurrence) and in each sampling date.

	Number of OTUs presents at the 6 size-fractions	Total number of OTUs	Percentage
SEP	19	647	2.9
OCT	18	711	2.5
NOV	19	637	3
DEC	14	658	2.1
JAN	26	699	3.7
MAR	29	535	5.4
APR	16	345	4.6
JUN	13	533	2.4
Median	19.3	595.6	3.3
SD	5.2	113.3	1.1
CV	27.2	19	32.3

Supplementary Table 8. Indicator OTUs of each size-fraction (INDVAL >0.3, p<0.05).

Size-Fraction	Taxonomy	Number of different OTUs
0.2-0.8 µm	Bacteria;Actinobacteria;Acidimicrobiia;Acidimicrobiales;OCS155 marine group;	2
	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;	1
	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;NS5 marine group;	3
	Bacteria;Proteobacteria;	1
	Bacteria;Proteobacteria;Alphaproteobacteria;	1
	Bacteria;Proteobacteria;Alphaproteobacteria;SAR11 clade;	4
	Bacteria;Proteobacteria;Alphaproteobacteria;SAR11 clade;Surface 1;	7
	Bacteria;Proteobacteria;Alphaproteobacteria;SAR11 clade;Surface 4;	2
	Bacteria;Proteobacteria;Gammaproteobacteria;	1
	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;SAR86 clade;	2
	Total number of Indicator Species:	**24**
0.8-3.0 µm	Bacteria;	2
	Bacteria;Cyanobacteria;	2
	Bacteria;Cyanobacteria;Cyanobacteria;SubsectionI;FamilyI;Synechococcus;	2
	Total number of Indicator Species:	**6**
10-20 µm	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;NS9 marine group;	1
	Total number of Indicator Species:	**1**
20-200 µm	Bacteria;	1
	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;	1
	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;	1
	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Gramella;	1
	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Nonlabens;	1
	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Tenacibaculum;	1
	Bacteria;Cyanobacteria;Cyanobacteria;SubsectionIV;	1
	Bacteria;Fusobacteria;Fusobacteriia;Fusobacteriales;Fusobacteriaceae;Propionigenium;	1
	Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales;Planctomycetaceae;Blastopirellula;	1
	Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales;Planctomycetaceae;Planctomyces;	1
	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;	2
	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Ruegeria;	1
	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Sulfitobacter;	1
	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Alteromonas;	1
	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Pseudoalteromonadaceae;Pseudoalteromonas;	1
	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Alcanivoracaceae;Alcanivorax;	1
	Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae;Vibrio;	1
	Total number of Indicator Species:	**18**

Supplementary Table 9. Percentage of specialist, intermediate and generalist OTUs (see Material and Methods for definition) classified in 3 ranks of relative abundance: Abundant (>1%), Intermediate (1-0.1%), Rare (<0.01-0.1%). Average values of the 8 months and their standard deviations.

		Niche Breadth		
		Specialist	**Intermediate**	**Generalist**
Abundance	**Abundant (>1%)**	4.7±1.2	4.0±1.2	2.7±1.2
	Intermediate (1 to 0.1%)	38.4±1.3	4.7±1.3	0.5±0.3
	Rare (<0.1 %)	45.8±2.8	0	0

Supplementary Table 10. Results of ANOVA test calculated with the relative abundance of each sindividual taxonomic group and among size-fractions. Size-fractions are referred by the lowest filter size. Key abreviations and column headings: F, Fratio; P, p-value. Probabilities are marked as follows: Probabilities are marked as follows: '***'p<0.001; '**' p<0.01; '*' p<0.05. Letters refer to results of post-hoc Tukey tests (p<0.05). Different letters (A, B, C, D) indicate significant differences among treatments. (See next page)

(See caption in previous page)

				Post-hoc Tukey test					
		F	P	0.2 µm	0.8 µm	3.0 µm	5.0 µm	10 µm	20 µm
GROUP A	SAR11, Alphaproteobacteria	28.2426	***	A B	B	B	B	B	
	SAR116, Alphaproteobacteria	3.9604	**	A B	A B	A B	A B	A B	
GROUP B	Synechococcus, Cyanobacteria	20.4772	***	A C	B	B	B	B	
GROUP C	Actinobacteria	3.8047	*	A B	B	A B	A B	A B	
	Deferribacteres	1.6678	n/s	A	A	A	A	A	A
	Oceanospirillales, Gammaproteobacteria	2.7278	*	A B	A B	A B	A B	A B	
GROUP D	Rhodobacterales, Alphaproteobacteria	5.1116	***	B	B	B	B	B	A
	Cytophagia, Bacteroidetes	6.5044	***	B	B	B	B	B	A
	Alteromonadales, Gammaproteobacteria	13.331	***	C	B C	B C	B C	B	A
	Verrucomicrobia	6.9208	*	B	A B	A	A	A	A
	Vibrionales, Gammaproteobacteria	6.8541	***	B	B	B	B	A B	A
	Sphingobacteria, Bacteroidetes	10.9746	***	D	C D	C D	B C	A B	A
	Firmicutes	3.9104	**	B	A B	A B	A	A B	A
	Planctomycetes	4.1519	**	B	A B	A B	A	A B	A
	Deltaproteobacteria, Proteobacteria	5.4162	***	C	B C	A B C	A B	A B	A
	Flavobacteria, Bacteroidetes	8.7272	***	C	B C	A B	A	A	A
	Rhizobiales, Alphaproteobacteria	2.8708	*	B	A	A B	A B	A B	A

Chapter 2

Seasonality and dynamics of bacterial community structure along the pelagic particulate matter continuum in a temperate oligotrophic coastal site

Mireia Mestre, M. Montserrat Sala & Josep M. Gasol

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SUMMARY: The temporal dynamics of ocean prokaryotic community structure in the free-living fraction has been well studied, yet it does not take into account the heterogeneity of habitats present in particles of distinct size that are also colonized by prokaryotes. Using a serial filtration differentiating 6 size fractions (i.e. 6 ranges of particle sizes) spanning from 0.2 to 200 μm, we sampled monthly during two years in a temperate oligotrophic coastal ecosystem (Blanes Bay, NW Mediterranean Sea) to describe the bacterial community structure in each particle-size range, and to test whether the resulting structuring varies seasonally or is stable. While each size of particle had specific bacterial communities, and particle-size was one explanatory factor of global community structure, seasonality was evident in the communities of all size-fractions. The bacterial communities attached to each size-fraction presented gradual changes with time likely related to the variation of day-length and surface-water temperature, with large differences between the warm (ca. May to October) and the cold (ca. November to April) periods. However, the communities in the larger size fractions were also related to variables such as water turbidity. In addition, the communities in smaller size-fractions changed less throughout the year than the communities in the larger particles. Total bacterial diversity increased through the warm season to reach a maximum at the limit between seasons in the Fall.

Differentiation of the communities also increased through the warm season. Warm and cold seasons were dominated by distinct taxa, and while some taxonomic groups (such as *Synechococcus* or *Rhodobacterales*) maintained the preference for small or large size factions during most of the year, others (such as SAR11 or *Planctomycetes*) changed their distribution into different fractions in different months. Our data indicate that the diversity of the various groups in the different size fractions should be integrated to obtain a more comprehensive view of the dynamics of planktonic bacterial communities.

2.1 INTRODUCTION

Ecological communities are dynamic over time (Magurran and Henderson 2010) and microbial communities vary over different timescales (e.g. Fuhrman *et al*. 2006, Kara and Shade 2009, Jones *et al*. 2012, Gilbert *et al*. 2012, Hatosy *et al*. 2013). In surface marine waters, a dynamic seasonal succession of free-living bacterioplankton communities, with repeatable patterns between years, has been well described (reviewed in Bunse and Pinhassi 2017). In temperate regions, shifts in free-living bacterial community composition follow changes in temperature and chlorophyll *a,* as bacterial growth tends to be limited by nutrients (Pinhassi and Hagström 2000, Pinhassi *et al*. 2006, Gilbert *et al*. 2009, Andersson *et al*. 2010). In summer, water stratification is accompanied by communities dominated by Cyanobacteria, *Roseobacter*, SAR86 and SAR11 (Schauer *et al*. 2003, Alonso-Sáez *et al*. 2007, Lindh *et al*. 2015). In winter and spring, a mixed water column facilitates phytoplankton blooms and bacterial communities are dominated by *Flavobacteria*, *Roseobacter* and some *Gammaproteobacteria* (Pinhassi and Hagström 2000, Teeling *et al*. 2012, Buchan *et al*. 2014, Taylor *et al*. 2014, Lindh *et al*. 2015). However, while the majority of these studies have described the temporal changes of free-living bacteria, less is known on the seasonal variability of the bacterial communities attached to particles.

Free-living and attached bacteria are taxonomically distinct (e.g. DeLong *et al*. 1993, Crump *et al*. 1998, Grossart *et al*. 2005, Rink *et al*. 2007, Ortega-Retuerta *et al*. 2013) and represent two radically distinct lifestyle strategies: Free-living microorganisms tend to be adapted to low substrate concentrations (Satinsky *et al*. 2014), to have small genomes (Smith *et al*. 2013) and to exhibit higher motility (Mitchell *et al*. 1995, Fenchel 2001, Grossart *et al*. 2001). In contrast, particle-attached bacteria are often larger than free-living bacteria (Alldredge *et al*. 1986, Cho 1988, Simon *et al*. 2002), form dense communities of cells (Simon *et al*. 2002), have higher production (Kirchman and Mitchell 1982) or respiration rates (Grossart *et al*. 2007), extracellular enzyme activities (Karner and Herndl 1992, Smith *et al*. 1992), and have the ability to hydrolyze more recalcitrant substrates (Grossart and Simon 1998, Kiørboe and Jackson 2001, Kiørboe *et al*. 2002). Being both lifestyles such different, we could expect that the taxonomic differences are

maintained seasonally. However, only few studies have explored the seasonal dynamics of free-living and attached bacterial community composition: one in the Weser estuary (Selje and Simon 2003) using FISH and DNA fingerprinting and another in a coastal lagoon using high throughouput sequencing of the 16S rDNA gene (Mohit *et al*. 2014). Both found marked seasonal trends in the free-living and attached communities along the year. Yet, both studies used only one filter to separate free-living and attached lifestyles, thus neglecting the possible differences in community composition and seasonal changes in communities associated to particles of distinct size. Recent studies have shown that the compositon of the bacterial community attached varies among diferent size fractions (Mestre *et al*. 2017, Mestre *et al*. submitted, Yung *et al.* 2016) and a multiple size-fractionation with 4 filters performed over 1 year in the mouth of a eutrophic estuary (Pivers Island Coastal Observatory) observed that the communities were different in the distinct sizes of particles (Yung *et al*. 2016) and the ecological drivers of community structure in each fraction were distinct.

Here, we aimed at characterizing the bacterial communities in particles of distinct size (ranging from 0.2 to 200 μm) in a temperate oligotrophic site in the NW Mediterranean Sea, along a 2 years study. We focus on the seasonal succession dynamics of bacteria to test whether the variability over time in diversity, community composition and in individual taxa varies depending on the size-fraction (i.e. on the size of the particles to which bacteria are associated).

2.2 MATERIALS AND METHODS

2.2.1 Study area, sampling and basic parameters

A total of 25 samples were taken between June 2011 and June 2013 at the Blanes Bay Microbial Observatory (BBMO, www.icm.csic.es/bio/projects/icmicrobis/bbmo/) an oligotrophic coastal station (20 m depth) placed at 0.5 miles offshore (41º40'N, 002º48'E) in the NW Mediterranean Sea, which has been regularly sampled for microbial ecology studies during the last decades (e.g. Gasol *et al*. 2012, 2016). Surface water (0.5 m depth) was taken monthly and pre-filtered through a 200-µm mesh net and transported to the laboratory in darkness. For bacterial diversity analysis, a total of 10 L were filtered using a peristaltic pump at very low speed and pressure, and sequentially through 10, 5, 3, 0.8 and 0.2 µm pore-size polycarbonate filters (Millipore. Billerica, USA). A 20 µm pore-size polycarbonate filter (GE Water & Process Technologies. Trevose, USA) was added the second year of the temporal series, whereas the first year incorporated a mesh net of 20 µm. All filters were stored immediately at – 80ºC 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 along the manuscript by the lowest size (i.e. the "0.8 fraction" indicates from 0.8 to 3 µm). In parallel, a set of environmental parameters (day length, temperature, salinity, secchi disk depth, chlorophyll *a*, inorganic nutrients, total organic carbon (TOC), particulate organic carbon (POC), bacterial production and bacterial abundance) were measured. Methods for determination of these environmental parameters were described previously in Mestre *et al*. (2017).

2.2.2 DNA extraction, sequencing and sequence processing

The DNA was extracted as described in Massana *et al*. (1997). Hypervariable V1-V3 16S tags were PCR amplified and 454 GS FLX+ pyrosequenced with primers 28F/519R in the hypervariant V1-V3 region of the ribosome gene by Research and Testing Laboratory (Lubbock, TX, USA; www.researchandtesting.com). 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. The

reads were clustered into 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 5 sequences in total) were removed. The samples were randomly subsampled to the number of reads present in the sample with the lowest amount of reads (1000).

2.2.3 Characterization of the microbial community

Statistical analyses and graphs were done in R (www.r-project.org) and using the packages *vegan* (Oksanen *et al*. 2017) and *simba* (Jurasinski and Retzer 2015). To determine the environmental variables that best explained the distribution of communities the function *bioenv* (R package *vegan*) was performed with the environmental matrix, which included the variables day length, temperature, salinity, secchi disk depth, chlorophyll *a*, nutrients, POC, TOC, bacterial production and bacterial abundance. A subsequent Mantel test was performed to determine the correlation between the diversity matrix and environmental matrix and its significance. To visualize the environmental variables that most influenced the community composition of each sample, a distance-based redundancy analysis (dbRDA) was performed with function *capscale*. The Bray-Curtis distance was used as an estimator of taxonomic dissimilarity between samples and communities were clustered by non-metric multidimensional scaling (nMDS) analysis. Statistical differences between the principal factors (size-fraction, month, year and season) were explored with a permutational multivariate analysis of variance (PERMANOVA) test (*adonis* function, R *vegan* package). The analyses of dbRDA and nMDS are different even if both are multivariate and in both the samples are represented as points when visualized in plots, but each one has distinct objectives and the graphical representation has distinct interpretation: First, the data used in both representations is not the same: whereas the nMDS requires only an OTU table, the dbRDA is a constrained analysis and requires both an environmental (explanatory variables) and an OTU table (response variables). The nMDS aims to represent the similarity between samples: in the plot, samples that are more similar to one another are placed closer together. The dbRDA attempts to represent the impact of the

explanatory variables on the response data. The plot shows the influence of an explanatory variable on a sample (projection of samples onto a vector, the closer is the sample to a vector, the more influenced is by the variable represented by the vector) and the angles approximate the correlation between the variables they represent (smaller angles, higher correlation between variables). Therefore, the distance between samples in the dbRDA is not informative.

To elucidate the variability of community composition along time in a particular size-fraction, Bray-Curtis distances were calculated between a given size-fraction in January 2013 and the same size-fraction at other time-points. January 2013 was selected as reference because it was the month with higher diversity and included all the 6 size-fractions. The diversity of each size-fraction was calculated using the total number of OTUs (richness) and to define the diversity in the context of the 6 size-fractions and along time, the True Alpha (the average richness among the 6 size-fractions), the True Gamma (the total richness of the 6 size-fractions) and the True Beta diversity (the taxonomic differentiation between the 6 size-fractions) were calculated for each month with the R package *simba* following (Tuomisto 2010). A harmonic analysis of the annual component of the variables day length, surface-water temperature, average alpha-, beta- and gamma- diversity was performed using the damped least-squares (DLS) method. Data was adjusted to the following trigonometric equation:

$$Y = b1 + b2 \cdot \cos\left(\frac{2\pi}{365} \cdot t + b3\right)$$

Where Y is the variable analyzed, b1 is the annual mean, b2 the amplitude, b3 the diphase (Seasonal maxima), 365 the period, and t the ordinal date, ranging from 1 to 365.

In order to visualize the taxonomic composition in each size-fraction and month, the most abundant taxonomic groups, i.e. those that represented >1% of the total abundance in at least one size-fraction, (a total of 17) were selected. The remaining taxonomic groups were considered “rare” and pooled together as “other bacteria”. To describe the temporal variability of each taxonomic group along time, the average relative abundances of each taxonomic group in each size-fraction and at each month were represented.

To evaluate whether the various taxonomic groups selected maintained (or varied) their contributions to bacterial community structure in the different size fractions, we calculated an averaged distribution using the data of all months, and the deviations (the absolute difference between a given value and the average) that occurred every month from that "seasonal average" were then calculated. The sum of all absolute deviations (in each size fraction) for each group and month provided a single value that could then be averaged across all months for a given group, or across all groups for a given month. This relative value, which we named HDI for Homogeous Distribution Index has no units and is a relative measure, being low when all distributions are similar, high when they are very distinct and equal to 1 if all situations were identical.

2.3 RESULTS

2.3.1 Seasonality of the bacterial communities

The size-fractionation sampling scheme was used at the BBMO, an oligotrophic coastal ecosystem located in an open bay with seasonal changes typical of a temperate site, characterized by a strong seasonal forcing, with warm summers and colder winters. This seasonal variance was well represented in this 2-year study (**Supplementary Figure 1**). Day length and surface water temperature presented a harmonic variation along time with a maximum in June (average 15.2 h) and a minimum in December (average 9.2 h) in day length, and a maximum in August (24.1ºC) and a minimum in February-March (12.5ºC) in surface water temperature. These values were not distinct from previous determinations using more than 10 years of sampling at the BBMO (i.e. Gasol *et al*. 2016).

Surface water temperature (r=-0.92) and day length (r=-0.82) were the variables that best predicted the temporal changes in community composition of the overall dataset (**Supplementary Figure 2**, **Supplementary Table 1**). The dbRDA performed with each size-fraction separately showed that each size-fraction communities were always determined by surface-water temperature and day length but also by a particular combination of environmental factors (**Supplementary Figure 3**). As an example, we observed that turbidity (i.e. Secchi depth) played an important role in the largest size-fractions (i.e. 5.0-10 µm, 10-20 µm and 20-200 µm), whereas PO_4 concentration was relevant in smaller size-fractions (i.e. 0.2-0.8 µm and 0.8-3.0 µm) (**Supplementary Table 2**).

Overall, prokaryotic communities were structured by size-fraction and season (**Figure 1**). As in the dbRDA, the nMDS separated samples along the first two axes in two major clusters. Taking both clusters into account, we defined two periods: the warm season (from May to October) and the cold season (from November to April). Prokaryotic communities presented statistically significant differences due to the factors "size-fraction" (communities in large fractions were different from small size-fractions, $PERMANOVA_{bySize\text{-}fraction}$ R^2=0.279, p<0.001), and "month" (communities differed from one month to the other, $PERMANOVA_{byMonth}$ R^2=0.275, p<0.001). The communities were also different according

to the season (communities from cold periods were different from those of warmer periods, $PERMANOVA_{bySeason}$ R^2=0.095, p<0.001).

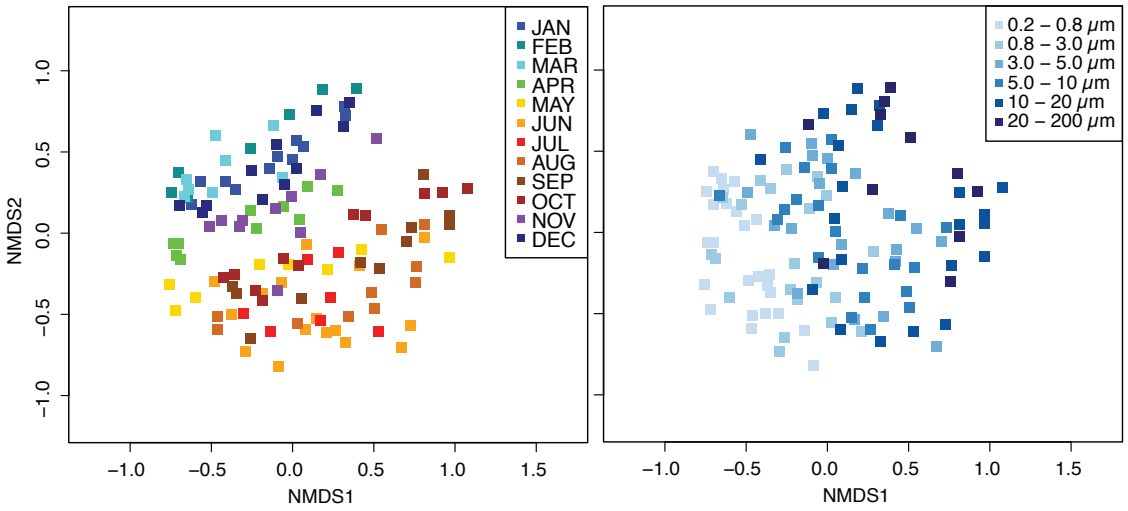

Figure 1. nMDS ordinations representing spatially the Bray-Curtis distance between bacterial communities. Distances were calculated from the rarefied OTU table. Samples are color-coded depending on month (left panel) and size-fraction (right panel).

The variation of community composition throughout the year was gradual and this was true for all size-fractions (**Figure 2a**) yet the magnitude of this variation was distinct in each size-fraction, being the smallest size-fractions the ones with less variation of community composition along the year (**Figure 2b**). In general, the magnitude of this variability increased towards the larger size-fractions yet the 10-20 µm was the one with higher seasonal variability as the variability decreased in the largest size-fraction (20-200 µm).

2.3.2 Diversity changes along time

When analyzing community composition at the species (OTUs) level, we observed a large variability in richness (number of OTUs) between size-fractions and along time (**Supplementary Figure 4**). Average richness increased with increasing size-fraction as found previously (Mestre *et al*. 2017 and **Supplementary Figure 5**). In addition, we observed

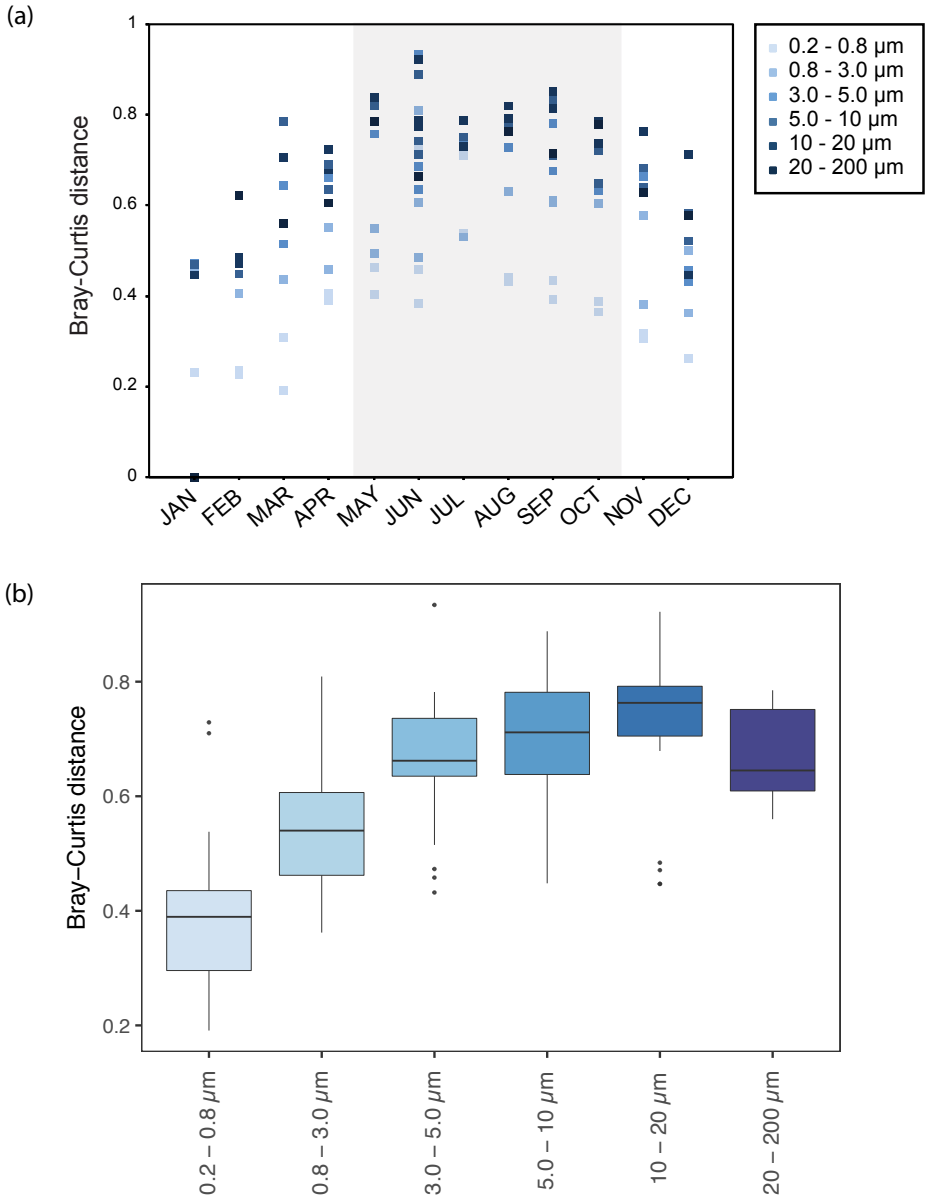

Figure 2. Bray-Curtis distances calculated between the same size-fractions of different months. January of 2013 was taken as a reference for the comparisons. (**a)** Representation of Bray-Curtis values along time, from January to December. Gray background: warm season. White background: cold season. (**b)** Box-plot of the average of Bray-Curtis distance calculated between the same size-fractions of distinct months. The upper and lower lines correspond to the 1st and 3rd quartile of the distribution of values. The median values are shown with horizontal black wide lines.

that the total number of species and the average number of species in each month increased during the warm periods to reach a maximum that lasted through most of the winter (**Figure 3 a,c**). Furthermore, this increase in the number of species was accompanied by an increase in global community differentiation among the size-fractions of a given month (i.e. beta-diversity) (**Figure 3 b**).

We modeled the seasonality of some variables using a harmonic regression of the annual component (that with a period of 365 days). The number of species and the community differentiation among size-fractions presented a harmonic distribution similar to that of day length and temperature (**Supplementary Figure 6, Supplementary Table 3**), yet with a lag between the variables, which ordered as follows: increase of day length (maximal in June), increase of temperature (max in July/August), increase of differentiation of particles (i.e. beta diversity) and increase in alpha diversity (maximum in October). This sequence of events allows us to put forward the hypothesis that each variable is influenced and promoted by the previous one.

2.3.3 Temporal variability of the preference for a given size class of the dominant taxonomic groups

The taxonomic composition of the bacterial communities was highly variable among size-fractions and all along the two years (**Supplementary Figure 7**). Some differences were also observed when comparing the taxonomy of the warmer months (May through October pooled together) with that of the colder period (November through April) (**Supplementary Figure 8**). We selected those bacterial groups that at least in one sample reached 1% of the community and they were grouped into 4 categories, following the classification of (Mestre *et al*. 2017): (a) taxonomic groups enriched in the small size-fractions (for example SAR11); (b) taxonomic groups enriched in the smallest size-fractions, but depleted or absent in the smallest one (0.2–0.8 μm, such as *Synechococcus*); (c) taxonomic groups that did not present enrichment in relation with the size-fraction (e.g. *Deltaproteobacteria*); and (d) taxonomic groups enriched in the larger size-fractions (e.g. *Flavobacteria*). The representation of relative abundances of each taxonomic group along time (**Supplementary Figure 9**) revealed that, while some bacterial groups maintained their

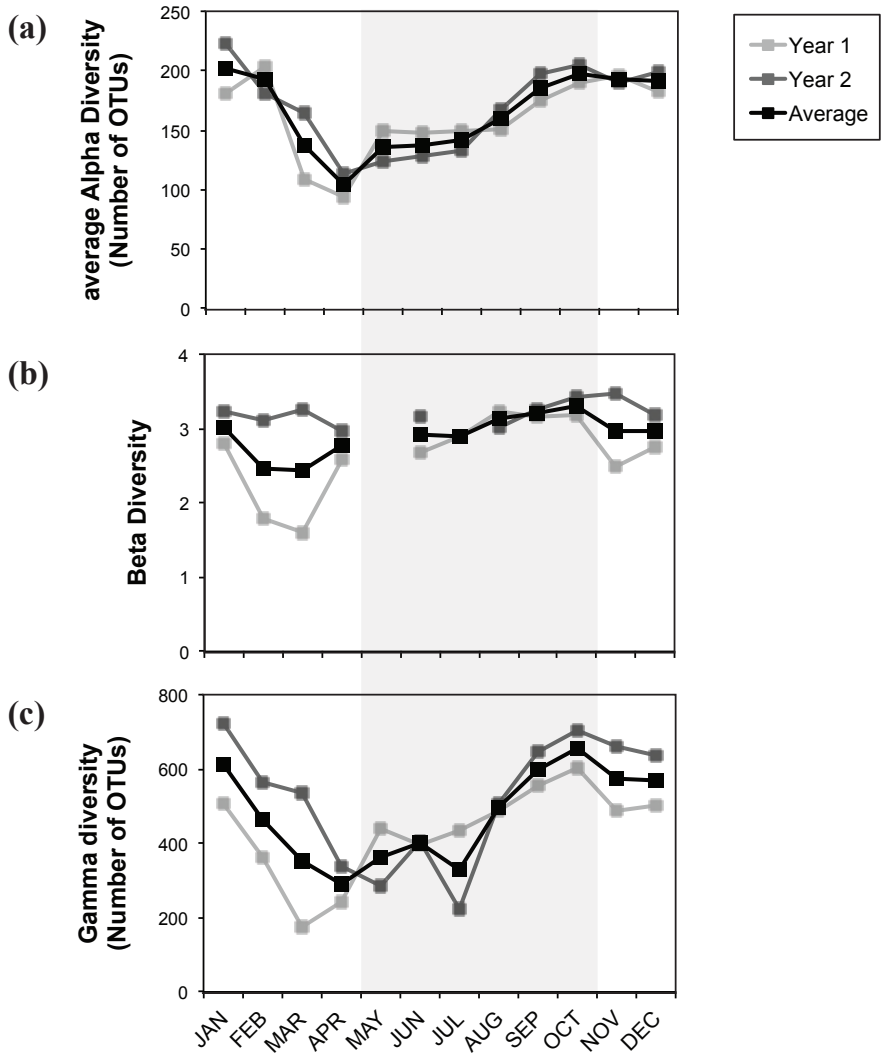

Figure 3. Average alpha-**(a)**, beta-**(b)** and gamma-**(c)** diversity at each month. Values of each year and its average were represented separately. Gray background: warm season. White background: cold season.

structure across size classes, others did not. We devised a way of quantifying the degree of conservation of the average contribution of the various groups to the whole community, what we named the Homogeneous Distribution Index, or HDI (**Table 1**). Calculated for the various groups considered, it varied from a value of ca. 2 for those groups that changed little their structure, such as *Flavobacteria*, *Synechococcus*, *Rhodobacterales*, *Oceanospirillales* or *Verrucomicrobia*; to a value of ca. 6 for those groups that presented very different structures into the size classes at different times of the year, such as SAR11,

Table 1. Homogeneous Distribution Index (HDI) values for each taxonomic group and in each month. Annual averages are also included. See Material and Methods for details related to the calculation of HDI.

		Months											
Taxonomy	**Average**	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
Actinobacteria	**3.2**	5.27	2.35	1.76	7.17	3.07	2.6	3.24	2.61	1.8	3.39	3.23	1.4
Cytophagia (Bacteroidetes)	**4.8**	7.23	1.72	4.03	5.77	3.83	4.75	7.02	8.85	3.03	2.51	5.15	4.04
Flavobacteria (Bacteroidetes)	**1.8**	1.05	1.4	1.71	4.37	1.43	1.62	3.06	2.09	0.93	0.7	2.65	1
Sphingobacteriia (Bacteroidetes)	**3.9**	2.18	2.24	4.46	9.59	2.89	3.38	4.96	7.24	2.55	1.37	3.38	2.72
Synechococcus (Cyanobacteria)	**2.2**	2.1	3.43	2.26	1.79	3.73	1.35	2.22	1.8	2.78	1.64	2.32	1.42
Deferribacteres	**5.3**	6.27	1.63	4.78	3.75	9.13	8.37	8.46	5.21	4.47	3.44	4.79	2.69
Firmicutes	**3.3**	1.28	1.71	5.11	5.93	2.14	5.02	4.68	3.92	2.24	1.65	3.5	1.9
Planctomycetes	**6.2**	3.67	3.37	7.17	1.84	5.66	17.97	17.44	2.76	4.02	4.18	3.93	2.16
SAR11 clade *(Alphaproteobacteria)*	**6.8**	5.56	7.17	9.41	1.34	7.15	6.2	3.13	9.34	16.31	5.08	7.55	3.9
SAR116 clade *(Alphaproteobacteria)*	**6.7**	12.38	4.35	9.59	7.69	7.23	6.07	4.83	4.17	7.8	6.15	5.47	5.12
Rhizobiales (Alphaproteobacteria)	**2.6**	1.11	3.09	4.05	2.52	2.53	2.94	3.55	1.8	2.01	3.99	2.01	1.37
Rhodobacterales (Alphaproteobacteria)	**2.6**	6.18	2.01	1.15	3.37	1.81	3.49	3.15	1	2.61	2.25	2.84	1.89
Alteromonadales (Gammaproteobacteria)	**3.1**	2.57	2.39	3.19	2.62	3.12	3.46	3.45	3.92	1.92	2	5.63	3.23
Oceanospirillales (Gammaproteobacteria)	**2.5**	2.01	1.03	2.03	3	8.74	1.14	2.34	3.54	1.14	1.79	1.52	1.3
Vibrionales (Gammaproteobacteria)	**5.4**	6.75	8.6	2.09	4.21	4.57	3.38	13.47	6.5	3.68	2.03	5.21	4.78
Deltaproteobacteria	**4**	1.6	4.9	1.77	2.03	5.61	4.21	8.92	2.46	2.86	3.1	6.47	4.49
Verrucomicrobia	**2.3**	1.99	1.96	3.85	4.54	1.82	1.37	2.79	1.02	1.73	1.02	4.17	1.04
All groups together	3.9	3.96	3.04	3.89	4.14	4.23	4.48	5.54	3.87	3.55	2.71	4.28	2.54

Planctomycetes (**Figure 4**) or SAR116. We could also calculate an average HDI for each month of the seasonal cycle, just by averaging the HDIs of all the groups considered. This "whole community HDI" was higher in the last months of the spring and into summer (April through July) and again in November. It was lower in October and December. November was the month where, in both years, a lower Secchi disk water transparency and lower salinity were recorded, concomitant to high chlorophyll *a* concentrations caused mainly by diatoms (details not shown).

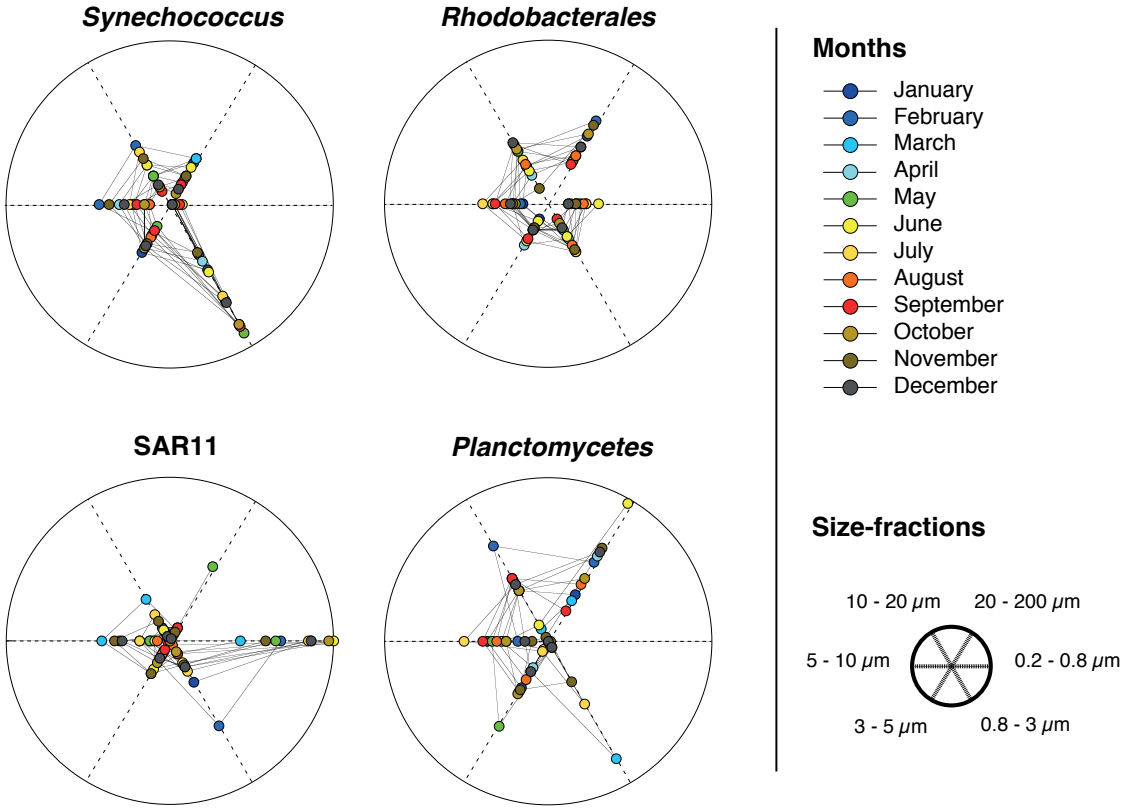

Figure 4. Examples of taxonomic groups with low (*Synechococcus* and *Rhodobacterales*) and high (SAR11, *Planctomycetes*) Homogeneous Distribution Index (HDI) values: *Synechococcus* and *Rhodobacterales* are relatively stable throughout the year whereas SAR11 and *Planctomycetes* vary significantly through the year (See Material and Methods and Table 1 for details). The spider chart represents the relative abundances (average values of both years) of each taxonomic group, in each month and in each size-fraction.

2.4 DISCUSSION

While we have a relatively good knowledge of the seasonality of marine bacterial communities (reviewed in Bunse and Pinhassi 2017) including the broad patterns at the Blanes Bay Microbial Observatory (BBMO) (Schauer *et al*. 2003, Pinhassi *et al*. 2006, Alonso-Sáez *et al*. 2007, Galand *et al*. 2010), we know little of the seasonal variability in the composition of the bacterial communities attached to particles. As far as we know, there are only three studies describing the seasonality of attached bacteria in aquatic systems: one in a temperate coastal lagoon during one summer (Mohit *et al*. 2014), another in a North Sea estuary during less than a year (Selje and Simon 2003) and a recent one studying one year in the mouth of an eutrophic Atlantic estuary (Yung *et al*. 2016). These studies have shown contrasting results: in some cases the communities attached to the particles varied a lot seasonally, while in others varied less than the free-living communities, but the temporal extend of these studies was rather limited. Our multiple size-fractionation along 2-years identified day length and surface water temperature as the environmental variables that mainly determined the changes in community composition through the particle sizes and along the year (**Supplementary Figure 2 and Supplementary Table 1**). Temperature and day length have been extensively described as key drivers determining the variability in community composition elsewhere (e.g.: Gilbert *et al*. 2012, Chow *et al*. 2013, Sunagawa *et al*. 2015, Yung *et al*. 2016, Ward *et al*. 2017). Yet, these studies only took into account the composition of the free-living bacteria and it is interesting to remark that variables other than these two appear to play a role in determining the structure of bacterial communities in distinct size-fractions (**Supplementary Figure 3 and Supplementary Table 2**). Furthermore, we observed that the size of the particles played a more relevant role than surface water temperature in shaping bacterial communities (the size-fractions explained 27% of the vatiation in community composition, whereas day length and surface water temperature only 8% and 5% of the variability, respectively). The importance of particle size over any other environmental factor was also observed before (Yung *et al*. 2016). This study and our results highlight the importance of taking into account the spatial context of the microbial habitat to better describe the global ecology of the microorganisms in aquatic environments.

The intra-annual variability of community composition in temperate plankton sites has been described as having two distinct dynamics: either a non-continuous and rapid transition between warm and cold months (Ward *et al*. 2017) or gradual changes throughout the year (Schauer *et al*. 2003). Here we observed that both, the free-living but also the communities associated to various size-fractions exhibited gradual changes in community composition along the year (**Figure 2a**). In addition, we also observed that the variability of community composition along the year seemed to be higher in some size-fractions than in others (**Figure 2b**). The fraction with less variability in community composition was the smallest (i.e. 0.2-0.8 μm), suggesting that the free-living communities might have a more homogeneous niche along the year than the attached counterparts. The highest variability in community composition in larger size-fractions is probably linked to the annual variability of particle composition, that are likely to be chemically and ecologically more variable than the dissolved phase. We observed that, generally, the larger the size-fraction the more variable the community was along the year, with the exception of the largest size fraction (i.e. 20-200 μm), which had less variability than their closest counterparts (i.e. particles of 3.0-5.0, 5.0-10 and 10-20 μm). Microzooplankton, such as nauplii and/or copepodites, dominate the zooplankton community in BBMO and are present in the 53-200 μm and 20-200 μm size-fractions throughout the year (Calbet *et al*. 2001, Almeda *et al*. 2011). Since some bacteria are known to be associated to microzooplankton (e.g. Tang *et al*. 2014), the fraction 20-200 μm may harbour a more stable bacterial composition throughout the year than the lower size-fractions.

2.4.1 Dynamics of bacterial community composition in the various size fractions

The richness of the bacterial communities in each size-fraction was highly variable throughout the year (**Supplementary Figure 4**). Yet, a clear pattern could be observed: larger size-fractions harbored higher diversity than their smaller counterparts (**Supplementary Figure 5**). The increase in diversity with the size-fraction was observed before in another temperate site (Yung *et al*. 2016), and in these same samples (Mestre *et al*. 2017). The Yung *et al*. (2016) study found that the attached was more diverse than the free-living size fraction and that a large proportion of the attached bacterial taxa were never detected in a 3-yr weekly time series of the free-living community in the same site (Ward *et al*. 2017).

Moreover, particles contribute highly to the total diversity of an aquatic system, as the accumulated diversity in size-fractions from 0.8 µm to 200 µm is ca. 6 times higher than observed in the 0.2-0.8 size-fraction) (Mestre *et al*. 2017). Therefore, this relevant contribution of attached communities to the total site diversity reinforces the role of attached communities in terms of ecosystem diversity.

Another interesting pattern that we observed is that the total bacterial diversity increased during the warm periods (from May to October), had a maximum at the end of the warm period, and decreased in the cold periods (from November to April) (**Figure 3 a,c**). In the same sampling point, it had been reported that bacterial diversity in the free-living fraction is higher in winter than in summer (Alonso-Sáez *et al*. 2007). These results were corroborated in particular for the aerobic anoxygenic phototrophic bacteria (AAPs) (Ferrera *et al*. 2013) and the Archaea (Galand *et al*. 2010). Moreover, higher diversity during winter has also been observed in other temperate sites in the free-living fraction (García *et al*. 2015, Rieck *et al*. 2015). Such apparent discrepancies with our results might have been produced by the distinct criteria used to define the seasons.

We have shown that bacterial community composition in a given size-fraction varies over time, but also the community composition in a given month varies among size-fractions: we calculated the degree of differentiation of community composition among the distinct size-fractions of a time-point (beta-diversity) and we observed that it varies along time (**Figure 3b**), being the size-fractions more similar among them at the end of the cold period, and being more distinct among them at the end of the warm period. The differentiation of community composition among size-fractions must be likely linked to the differential composition of the particles as each size-fraction comprises different types of particles and therefore distinct niches. This again supports the importance of taking particles into account in regular diversity sampling, as they contribute to better understand the niche structuring of prokaryotes and the changes in total diversity.

Interestingly, when we fit a harmonic model to the variability of richness with time, we observed that the model presented a harmonic pattern similar to that of day length and temperature (**Supplementary Figure 6**). We, moreover, observed a time lag between

the maximum peaks of day length, temperature, diversity and community differentiation that might suggest that day length directly affects seawater temperature, and temperature mediates changes in diversity and in the differentiation between the particles. A similar previous analysis of the organic matter pools at the BBMO concluded that dissolved organic carbon (DOC) accumulated in late summer, reaching the annual maximum by early September, half a month later than water temperature (Romera-Castillo *et al*. 2013) and that this accumulation was paralelled by a progressive shift of the organic carbon pool to a more refractory material, in a process probably promoted by phosphorous limitation. We could put forward the hypothesis that this progressively complex set of organic molecules facilitates a larger diversity of the bacteria growing on particles.

2.4.2 Some bacterial taxonomic groups seasonally maintained the preference for particular size fractions, while others did not

The dominant bacterial groups throughout the year at the Blanes Bay Microbial Observatory are Alphaproteobacteria, *Synechococcus* and Bacteroidetes, as has previously been observed with distinct techniques as fingerprinting, clone libraries, 454 pyrosequencing, and FISH (Schauer *et al*. 2003, Alonso-Sáez *et al*. 2007, Pommier *et al*. 2010). Seasonality of given taxa in the free-living fraction of temperate sites has been previously described (reviewed in Bunse and Pinhassi 2017), but we here describe that taxa attached to particles presented also seasonality (**Figure 2, Supplementary Figure 3**). We can classify taxa by their preference for small or large size-fractions (as in Mestre *et al*. 2017), but here we were particularly interested in describing whether the taxa that presented a certain preference for a given particle size-range maintained constant this preference throughout the seasonal cycle or, in contrast, varied this preference in some extent. As an example, we observed that SAR11 was enriched in the smallest size-fraction for most of the year. SAR11 was described mostly as free-living bacteria (Giovannoni 2012) but there are also ecotypes adapted to particles (Allen *et al*. 2012). Here we observed that SAR11 attached to particles appeared mainly during the cold season, and especially in February-March, coinciding with the late-winter phytoplankton bloom typical of the NW Mediterranean Sea (Duarte *et al*. 1999, Gasol *et al*. 2016) suggesting that the SAR11-attached populations are related with particulate matter derived from phytoplankton. In the NW Mediter-

ranean Sea, not only the abundance, but also the diversity of SAR11 is higher in the cold months than in the warm months (Salter *et al*. 2014). Our results suggest that this high diversification occurs likely in the particulate size-fraction. This fact opens a new vision of the ecology of SAR11, which has been mainly described as a free-living bacteria.

Synechococcus sp. was present in relatively high abundances all over the year and generally more abundant in the 0.8-3.0 μm size-fraction, specially in the warm period. *Synechococcus* is an example of a taxonomic group that maintained relatively constant their structure along size classes through the seasonal cycle (i.e. low HDI index, **Table 1, Figure 4**), and SAR11 an example of a taxonomic group that changed their structure along the seasonal cycle (i.e. high HDI index, **Table 1, Figure 4**). Other groups that maintained their structure were the *Rhodobacterales*, *Flavobacteria* or the *Verrumicrobia* and other groups that changed their structure were the SAR116, *Planctomycetales*, or *Vibrionales*. *Flavobacteria* and *Rhodobacterales* appeared preferently in warm months and in large size-fractions. *Flavobacteria* and *Roseobacter* (within the Rhodobacterales) are known to dominate communities after spring blooms (Buchan *et al*. 2014, Needham and Fuhrman 2016). Here we observed that both groups were relevant (5-20%) along the year with highly variable peaks in abundance representing rapid changes of presence among months and among size-fractions. It is possible that these groups respond fast not only to seasonal phytoplankton blooms, but also to specific compounds within particles that may appear sporadically throughout the year.

Interestingly, our approach allowed to estimate the average degree of change of the structure of bacteria into groups for each part of the seasonal cycle. Spring and the transition into summer was the part of the year where the structure was less maintained, together with November. In both studied years November presented low salinity and very low values of Secchi disk depth (i.e. less transparency) and high chlorophyll *a* concentrations, something that could explain the changes observed in the distribution of the bacterial groups in the different size fractions.

2.5 CONCLUSIONS

A multiple size-fractionation scheme to describe bacterial diversity in particles of 6 distinct sizes along the year indicates that both free-living and attached microbial communities present gradual changes over time, likely related with the variation of day-length and surface-water temperature. We observed the largest values of total diversity occurring at the end of the warmer part of the year, accompanied with a higher differentiation of the communities among particles. Regarding dominant taxonomic groups, while some taxonomic groups maintained their preference for small or large size-fractions all over the year, others did not. Our results show that the temporal seasonality of taxonomic groups, community composition and the diversity of bacteria associated to particles presents robust and clear seasonal patterns throughout the year. Furthermore, the study of bacteria in the various size-fractions generates a more comprehensive vision of bacterial dynamics over time and reinforces the importance of taking particles into account to better understand the aquatic microbial habitat.

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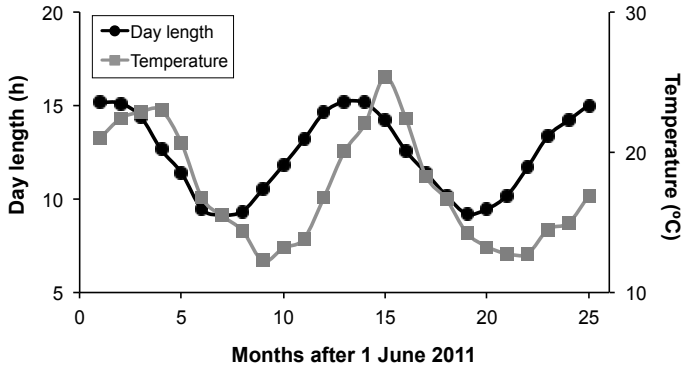
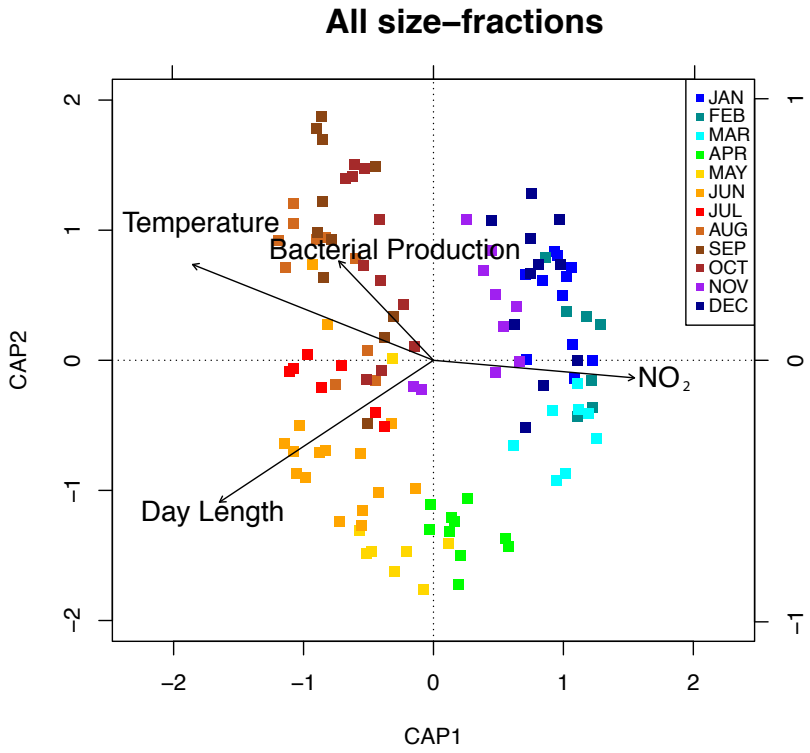
2.8 SUPPLEMENTARY MATERIAL

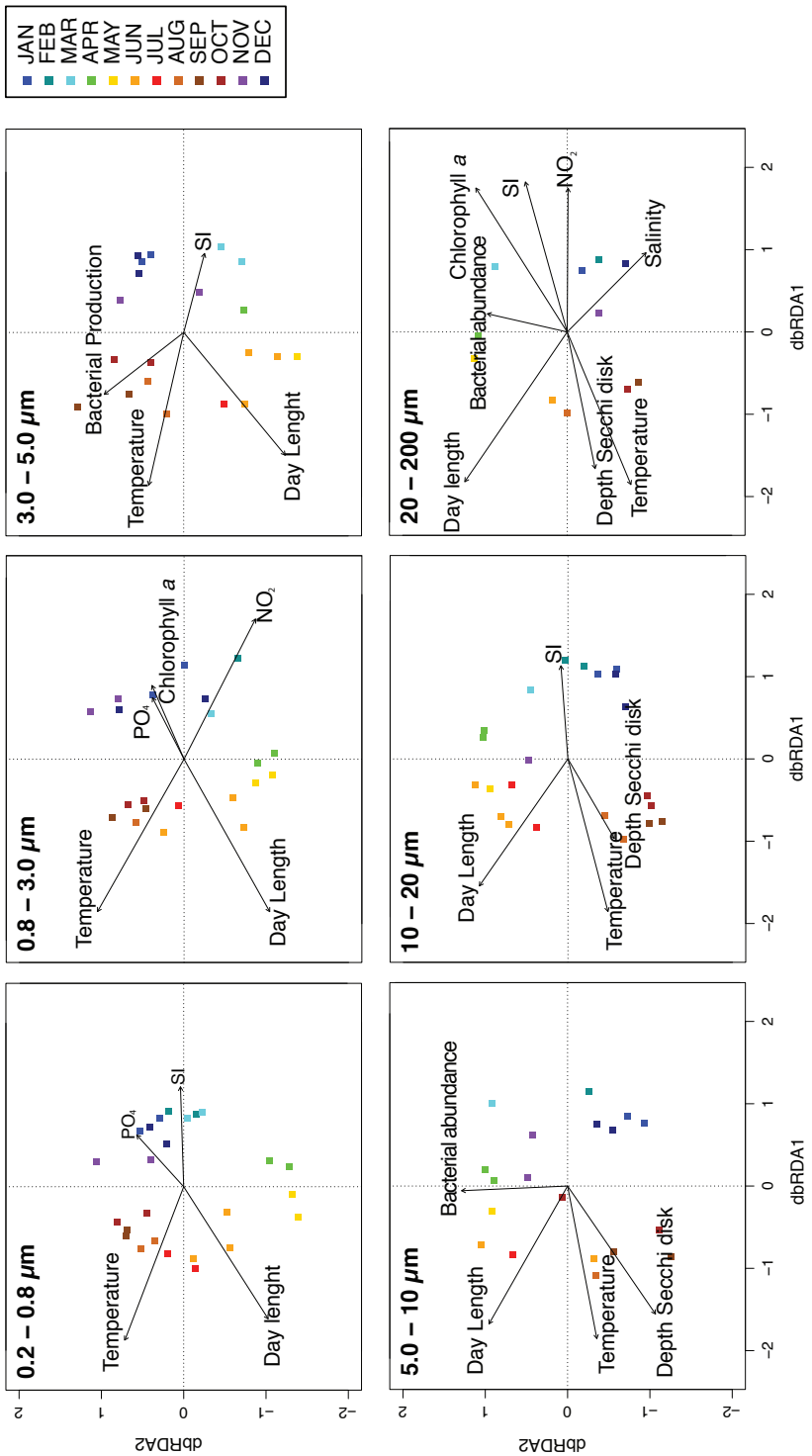
2.8.1 Supplementary Figures



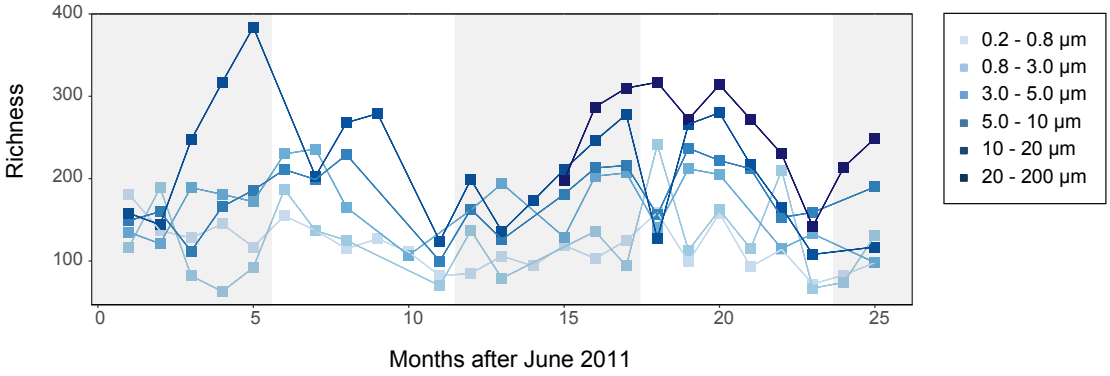
Supplementary Figure 1. Day length and surface-water temperature variability in Blanes Bay Microbial Observatory, from June 2011 to June 2013. Samples were taken monthly.



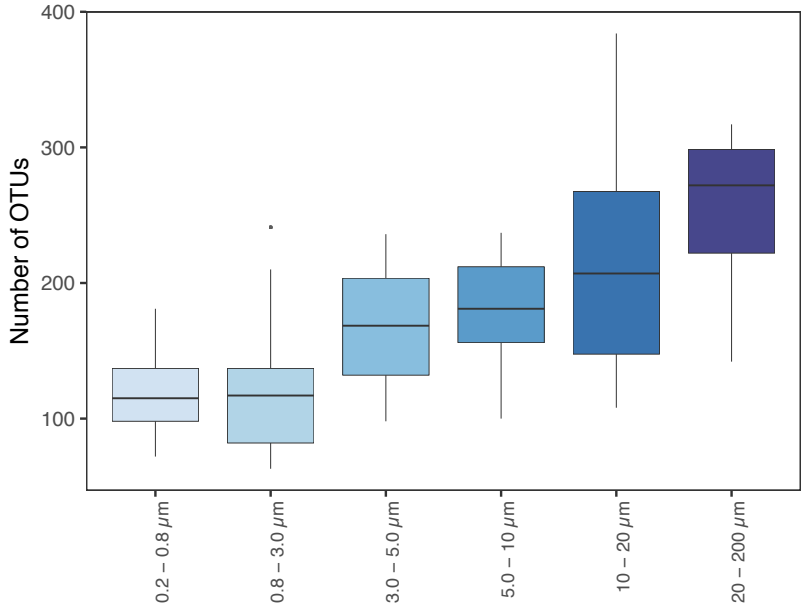
Supplementary Figure 2. Distance-based redundancy analysis (dbRDA) representing all samples (dots) and the environmental parameters that correlated with the distribution of communities (arrows) (See Material and Methods for details).



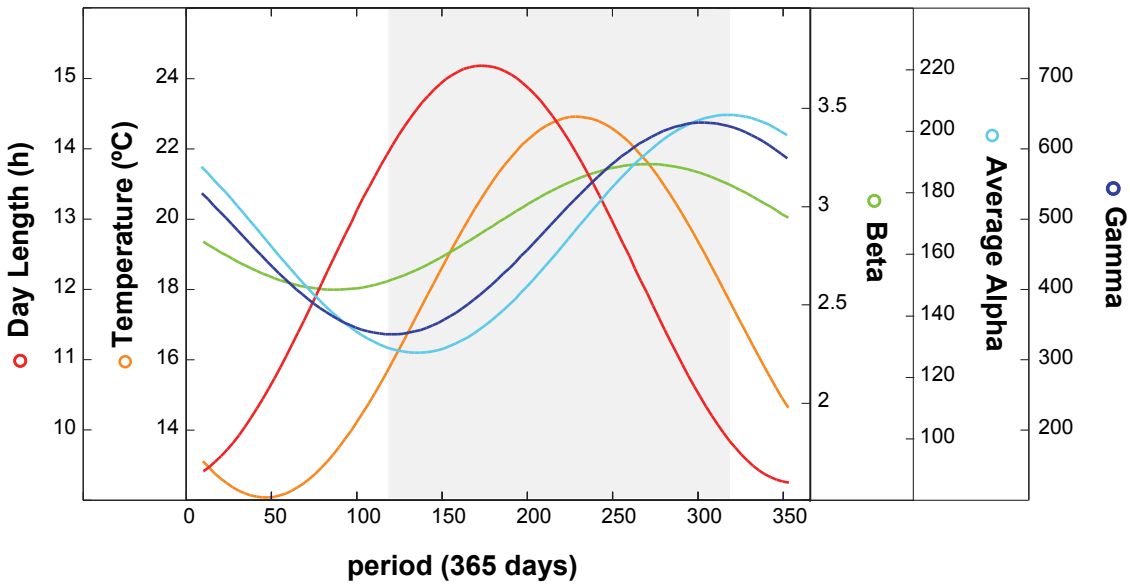
Supplementary Figure 3. Distance-based redundancy analysis (dbRDA) representing samples of a given size-fraction (dots) and the environmental parameters that best explain the distribution of communities (arrows). Samples of each size-fraction are represented in different panels.



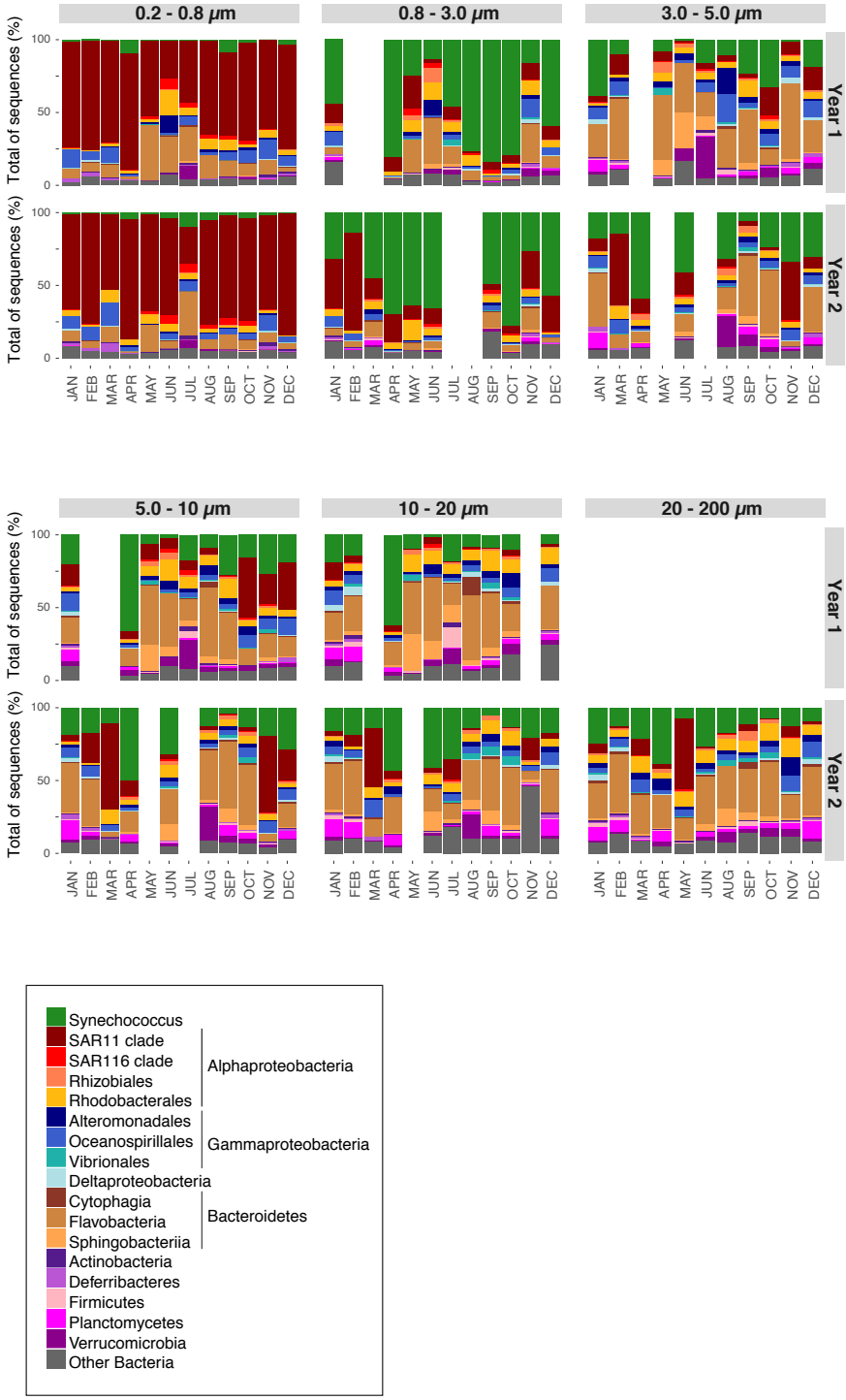
Supplementary Figure 4. Richness (number of OTUs) in each size-fraction and in each month from June 2011 to June 2013. Gray background: warm seasons. White background: cold seasons.



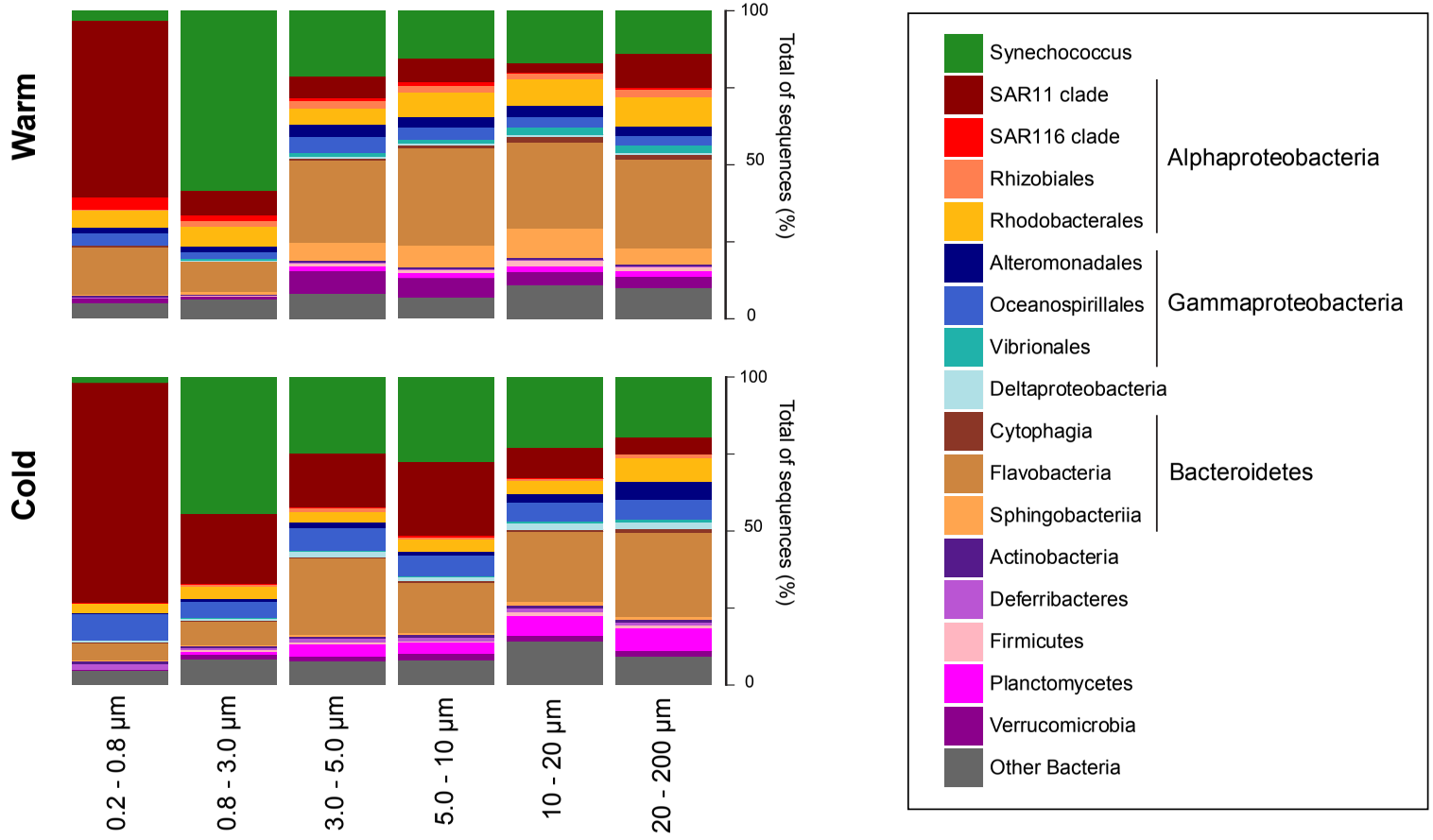
Supplementary Figure 5. Box-plot of the Richness (number of OTUs) of each size-fraction. Upper and lower lines correspond to the 1st and 3rd quartile of the distribution of values. The median values are shown with horizontal black wide lines. Outliers are displayed as dots.



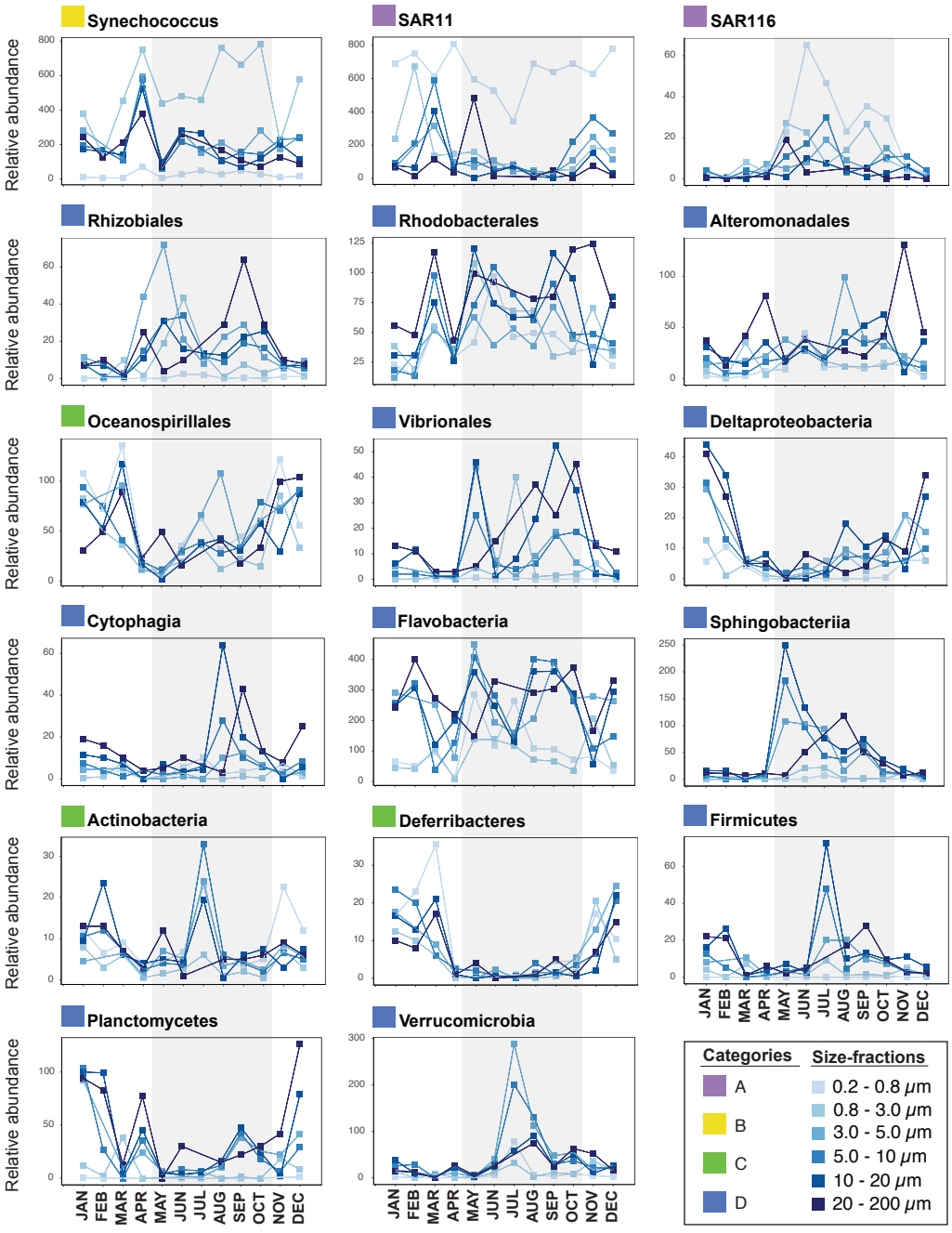
Supplementary Figure 6. Modeled values of day length, surface-water temperature, average alpha-, beta- and gamma- diversity in each month as defined with an harmonic regression. To better compare values, data were standardized. Gray background: warm season. White background: cold season.



Supplementary Figure 7. Taxonomic composition in each month and in each size-fraction. Abundances are expressed as percentage of the total number of sequences in each size fraction (for more details see Material and Methods).



Supplementary Figure 8. Average taxonomic composition of cold (November to April) and warm (May to October) seasons, and in each size-fraction. Abundances are expressed as percentage of the total number of sequences in each size fraction (for more details see Material and Methods).



Supplementary Figure 9. Relative abundances (average values of both years) of the most abundant taxonomic groups in each size-fraction and from January to December. Taxonomic groups were grouped into 4 categories, following the classification of Mestre *et al*. (2017): 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. Gray: warm season. White: cold season.

2.8.2 Supplementary Tables

Supplementary Table 1. Correlations between each environmental variable and dbRDA coordinate axis.

	dbRDA1	dbRDA2	dbRDA3	dbRDA4
Day Length	-0.82	-0.54	-0.03	-0.18
Temperature	-0.92	0.37	0.12	-0.05
NO_2	0.77	-0.07	-0.07	-0.63
Bacterial production	-0.36	0.38	-0.83	0.18

Supplementary Table 2. Correlations between each environmental variable and the dbRDA axes, for each size-fraction separately.

Size-fraction	Variable	dbRDA1	dbRDA2	dbRDA3	dbRDA4	dbRDA5	dbRDA6	dbRDA7	dbRDA8
0.2 - 0.8 µm	**Day length**	-0.81	-0.52	-0.19	0.21				
	Temperature	-0.93	0.36	0.03	0.04				
	PO_4	0.32	0.29	-0.9	0.09				
	SI	0.61	0.02	-0.37	0.7				
0.8 - 3.0 µm	**Day length**	-0.84	-0.47	-0.24	0.09	-0.07			
	Temperature	-0.84	0.48	0.02	0.23	-0.11			
	Chlorophyll *a*	0.41	0.18	-0.47	0.02	0.76			
	PO_4	0.34	0.17	-0.75	0.03	-0.55			
	NO_2	0.77	-0.39	0.08	0.45	-0.19			
3.0 - 5.0 µm	**Day length**	-0.77	-0.63	-0.09	0.08				
	Temperature	-0.95	0.22	-0.08	-0.2				
	SI	0.49	-0.13	-0.85	0.13				
	Bacterial Production	-0.39	0.5	0.05	0.78				
5.0 - 10 µm	**Day length**	-0.8	0.46	-0.2	0.34				
	Temperature	-0.88	-0.17	0.43	-0.09				
	Secchi depth	-0.74	-0.51	-0.38	0.22				
	Bacterial abundance	-0.03	0.61	-0.17	-0.77				
10 - 20 µm	**Day Length**	-0.8	0.56	0.18	0.09				
	Temperature	-0.97	-0.25	-0.06	0.01				
	Secchi depth	-0.5	-0.29	0.81	-0.06				
	SI	0.59	0.04	-0.23	0.77				
20 - 200 µm	**Day length**	-0.79	0.54	0.09	-0.16	0.09	-0.01	0.05	-0.22
	Temperature	-0.8	-0.33	-0.22	0.07	0.42	-0.02	-0.11	-0.04
	Secchi depth	-0.72	-0.15	0.51	0.03	0.07	-0.37	-0.23	-0.04
	Salinity	0.42	-0.41	-0.22	-0.03	0.72	0.11	-0.09	0.25
	Chlorophyll *a*	0.76	0.48	0.34	0.18	0.08	0.08	0.04	0.19
	NO_2	0.76	0	0.26	-0.27	0.02	-0.43	0.31	-0.03
	SI	0.79	0.22	-0.09	0.29	-0.06	0.4	0.02	-0.27
	Bacterial abundance	0.1	0.42	-0.66	-0.07	-0.53	-0.08	-0.29	0.04

Supplementary table 3. Fitting parameters (±standard error) of the harmonic analyses of the studied variables: annual mean (b1), amplitude (b2) and diphase (b3). Coefficients b1 and b2 are expressed in the units of each variable and b3 is expressed in days of the year. R^2, determination coefficient; n, number of data of each time series; p-level, significance of the linear fitting for the measured versus the modelled data.

Variable	b1 (annual mean)	b2 (amplitude)	b3 (seasonal max)	R^2	N	P-level
Temperature	17.5 ± 0.23	10.83 ± 0.66	222.3 ± 0.06	0.923	25	P< 0.001
Day length	12.2 ± 0.02	8.79 ± 0.04	169.8 ± 0.01	0.998	25	P< 0.001
Alpha	166.42 ± 4.06	77.30 ± 11.36	299.25 ± 0.15	0.682	25	P< 0.001
Beta	2.88 ± 0.10	0.63 ± 0.30	249.82 ± 0.45	0.166	25	P= 0.043
Gamma	487.00 ± 21.51	300.10 ± 61.50	280.66 ± 0.20	0.524	25	P< 0.001

Chapter 3

Spatial variability of marine bacterial and archaeal communities along the particulate matter continuum

Mireia Mestre, Isabel Ferrera, Encarna Borrull, Eva Ortega-Retuerta, Susan Mbedi, Hans-Peter Grossart, Josep M. Gasol & M. Montserrat Sala

Chapter 3

Spatial variability of marine bacterial and archaeal communities along the particulate matter continuum

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SUMMARY: Biotic and abiotic particles shape the microspatial architecture that defines the microbial aquatic habitat, and particles are highly variable in size and quality along oceanic horizontal and vertical gradients. We analyzed the prokaryotic (bacterial and archaeal) diversity and community composition present in 6 distinct particle size classes ranging from the pico- to the microscale (0.2 to 200 μm). Further, we studied their variations along oceanographic horizontal (from the coast to open oceanic waters) and vertical (from the ocean surface into the meso- and bathypelagic ocean) gradients. In general, prokaryotic community composition was more variable in depth than by the transition from the coast to the open ocean. Comparing the 6 size-fractions, distinct prokaryotic communities were detected in each size-fraction, and whereas bacteria were more diverse in the larger size-fractions, archaea were more diverse in the smaller size-fractions. Comparison of prokaryotic community composition among particle size-fractions showed that most, but not all, taxonomic groups present a depth-conserved preference for certain size-fractions. Environmental filtering, or the presence of diverse ecotypes with distinct size-fraction preferences, may explain why depth conservation of particle attachment preference is absent in some taxa.

3.1 INTRODUCTION

The variability in the community composition of the prokaryotes present along the particulate matter size continuum, that stretches from nearly dissolved to large visible particles (Verdugo *et al*. 2004), has received little attention from aquatic microbial ecologists (Grossart 2010). However, the patterns of prokaryotic diversity are known to vary greatly with the size of the particle (Mestre *et al*. 2017). In addition, prokaryotic diversity changes with the composition of the particles (Simon *et al*. 2002; Rieck *et al.* 2015). Yet, particle composition is highly variable along the water column, with freshly-formed particles at the surface to more degraded ones in the deep ocean (Herndl and Reinthaler 2013). Moreover, particle types vary from coastal to open waters, as coastal particles are more influenced by river inputs, land runoff, or anthropogenic activities (Simon *et al*. 2002; Rieck *et al*. 2015) than offshore particles, which are more dependent on biotic processes. Particles with variable sizes, chemical composition and physical properties conform the microspatial architecture that structures the microbial environment (Azam *et al*. 1993; Simon *et al*. 2002; Grossart 2010). There are numerous examples of spatially structured microbial habitats: the phycosphere (Bell and Mitchell 1972), zooplankton and fecal pellets (Tang *et al*. 2011), particles such as transparent exopolymeric particles (Delong *et al*. 1993) or marine snow (Simon *et al*. 2002), nutrient plumes (Smith *et al*. 1992; Kiørboe and Jackson 2001) and oxygen gradients outside and inside particles (Alldredge and Cohen 1987; Ploug *et al*. 1999).

Analyzing how bacterial communities respond to this heterogeneity at the microscale is challenging, but recent technologies, such as atomic force microscopy (Malfatti and Azam 2009), nanoSIMS (Wagner 2009) or microfluidics (Seymour *et al*. 2008; Stocker 2012) have allowed visualisation and quantification of the microbial distribution and structure at the microscale. However, these techniques are time-consuming and have limitations to provide an overview on how prokaryotic community composition varies in an oceanographic context. In contrast, the systematic size-fractionation of water samples collected across horizontal and vertical gradients represents a quicker and cheaper strategy to provide insight into prokaryotic community composition along the particle matter continuum in contrasting oceanic sites (Mestre *et al*. 2017). Hence, it can contribute to a better un-

derstanding of the microscale framework, offering a reasonable alternative between these new but time consuming and expensive technologies, and traditional oceanographic procedures that largely neglect the microscale distribution of aquatic microbes.

Considering the importance of the microspatial architecture of the ocean, we aim at characterizing the bacterial and archaeal communities recovered in various pico- to microsize fraction (i.e. ranging from 0.2 to 200 μm) and describing their variability along horizontal (from the coast to the open ocean across the continental slope) and vertical (from surface waters down into the bathypelagic ocean, including deep chlorophyll maxima and bottom nepheloid layers) gradients in the NW Mediterranean Sea. Therefore, we used high-throughput sequencing to evaluate the diversity and community composition of bacteria and archaea, and the potential preference of specific taxonomic groups for certain size classes, and whether these preferences change across the gradients studied.

3.2 MATERIALS AND METHODS

3.2.1 Sampling and basic parameters

Samples were collected in a transect from coastal to offshore waters in the NW Mediterranean sea during the NEMO cruise aboard the R/V García del Cid, in May 2012. Sampling started near the coastal Blanes Bay Microbial Observatory (Gasol *et al*. 2012) and continued through the continental slope, from the continental shelf (41º 27'N, 2º 42'E) and until the ocean-basin floor (2,315 m depth, 41º 13'N, 2º 49'E). Samples were collected in 4 vertical profiles from surface to near benthic waters with Niskin bottles. The depth layers were defined as: SFC (surface, 5 m), DCM (deep chlorophyll maximum, 50-60 m), MESO (the upper layer of mesopelagic waters, around 200 m), and BNL (samples at 10 m above the sea bottom and associated to the benthic nepheloid layer, ranging from 600 to 2,300 m). Additionally, in Station 8, three extra depths were analysed to obtain a more in-detail view of the vertical changes across the water column: TOP-DCM (ca. 20 m above the DCM), BOTTOM-DCM (15 m below the DCM), and BATHY (bathypelagic waters, 1,600 m) (for details, see **Table 1**). The samples were first screened through a 200 µm mesh and a total of 10 L were sequentially filtered through 20, 10, 5, 3, 0.8 and 0.2 µm pore-size filters (47 mm polycarbonate filters: all from Nuclepore except the 20 µm pore-size, GE Water & Process Technologies), using a peristaltic pump at very low speed and pressure. The filters were stored at -80ºC until further processing. 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 to simplify nomenclature are named by the lowest filter pore size throughout the manuscript (i.e. "0.2 fraction" indicates the fraction from 0.2 to 0.8 µm).

3.2.2 DNA extraction, sequencing and sequence processing

A DNA extraction protocol was optimized to enhance the quantity and quality of the DNA recovered. Filters were cut into small pieces and subjected to a bead-beating step with 510 µL lysis buffer (50 mM Tris-HCl, 40 mM EDTA 0.5 M, 0.75 mM sucrose) and 0.1 mm and 0.7 mm zirconium beads). Then, they were incubated with 15 µL of lysozyme (0.04 mg mL^{-1}) at 37º for 30 min. Afterwards, 60 µL of sodium dodecyl sulphate (SDS) (10%)

and 30 µL of proteinase K (0.008 mg μL^{-1}) were added, and the samples were incubated at 55ºC for 12 h. The lysate (liquid phase) was recovered. Filters were washed with 500 µL of Tris-EDTA buffer (TE), and the liquid was pooled with the lysate. To precipitate the DNA, 40 µL of glycogen (5 mg mL^{-1}) and 110 µL sodium acetate (NaAc) 3 M were added. The final volume was separated into two subsamples and precipitated overnight with 1.35 mL of ethanol (70%) at -80ºC. The samples were centrifuged at 20,000 g (4ºC, 30 min) and the supernatant was removed. The DNA was dried with a vacuum evaporator and finally resuspended in 30 µL Tris-HCl 10 mM.

The V4 region of the 16S rDNA gene of bacteria and archaea was PCR amplified with the bacteria/archaeal primer pair 515F/806R (Caporaso *et al.* 2012). PCR products were purified individually and ligated to barcoded Illumina-Adaptors using the Ovation® Rapid DR Mulitplex System 1-96 library preparation kit (NuGen Technologies/ USA). The pooled, barcoded samples were sequenced with the Illumina MiSeq platform using the v2 500 cycles sequencing kit (paired-end reads 2 x 250 bp) at the Berlin Center for Genomics in Biodiversity Research. Only one sample resulted in extremely low read numbers and thus was discarded for further analyses. Computing analyses were run at the MARBITS bioinformatics platform of the Institut de Ciències del Mar (ICM). The amplicons were processed with UPARSE (Edgar 2013). Briefly, the reads were merged with PEAR (Zhang *et al.* 2014), and those with >100 nucleotides were selected. Quality check, dereplication, OTU clustering (97%), and chimera filtering (with SILVA v.119 as reference database) were processed with USEARCH (Edgar 2010). Further details can be found in the following Github site (github.com/ramalok/amplicon_processing). Taxonomic assignment was done using SILVA Incremental Aligner (SINA v1.2.11). The OTUs affiliated with eukaryotes, chloroplasts and mitochondria were removed and the samples were randomly subsampled to the lowest number of reads present in the samples (10,000 reads).

3.2.3 Data analysis

Statistical analyses and graphs were performed in R (www.r-project.org). The samples were categorized according to size-fraction, station and depth. A Permutational Multivariate Analysis of Variance (PERMANOVA) (Adonis test, R package vegan) was performed

to discern statistically significant differences in community structure explained by the different factors: size-fraction, station, depth and their interactions. The diversity in a given size-fraction (alpha diversity), the average diversity in the 6 size-fractions (average alpha diversity), the diversity within the 6 size-fractions (gamma diversity) and the differentiation between the 6 size-fractions (beta diversity) was calculated according to Tuomisto (2010) with R package *Simba*. The diversity of bacteria and archaea in each size-fraction was calculated using the total number of OTUs (richness). The OTUs were grouped by taxonomy at Phylum, Class and Order levels and the higher-rank taxonomic groups that represented more than 1% of the total abundance in at least one size-fraction and/or in one depth were selected for further analyses. The other taxa were grouped as "Other bacteria". With these criteria, a total of 42 taxonomic groups were considered for in detail analyses. An Analysis of Variance (ANOVA) was conducted (with previous arcsine square-root transformation of the relative abundance of each taxa) to test whether the distribution (i.e. the relative abundance, enrichment or percentage contribution to the community) of each taxonomic group along the size-fractions was conserved at the various stations and depths.

3.3 RESULTS

3.3.1 Environmental setting

All sampled stations (**Figure 1, Table 1**) were stratified, with temperatures ranging from ~17ºC at the surface (SFC) to ~13ºC at the sea bottom. At each station, we observed a well-developed deep chlorophyll maximum (DCM), typical for the Mediterranean Sea during this time of the year, with a peak of chlorophyll *a* (Chl *a*, 0.41-1.73 µg L^{-1}) located around 50-60 m. Chlorophyll *a* decreased significantly until the mesopelagic (MESO), located at 200 m. We also detected a benthic nepheloid layer (BNL) at all stations, characterized by an increase in turbidity (indicative of higher particle abundances) near the bottom (0.1-0.14 NTU). These particularities lead us to collect the samples in these 4 main layers: SFC, DCM, MESO and BNL, yet we also sampled 3 extra-layers at Station 8: above and below the DCM and in bathypelagic waters, labelled as TOP-DCM, BOTTOM-DCM and BATHY (see Material and Methods for details), respectively.

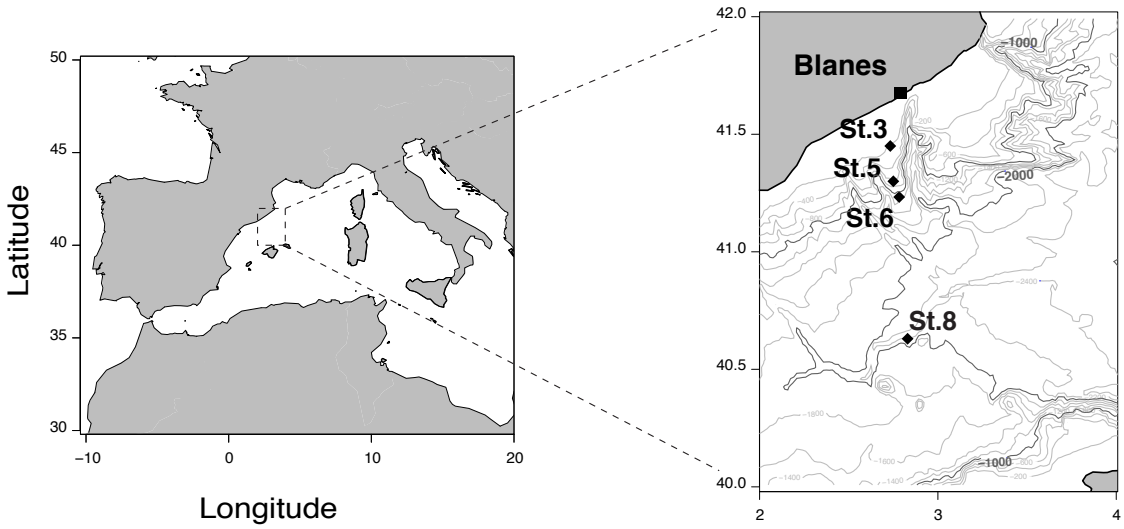

Figure 1. Location of the study area in the NW Mediterranean and bathymetric map showing the stations sampled.

Table 1. Detailed information about the stations sampled during the NEMO cruise, where samples for prokaryotic diversity were obtained.

Station	Sampling Date (dd/mm/yy)	Lat /Lon	Bottom depth (m)	Layer	Sampling depth (m)	Temperature (°C)	Salinity (PSU)	Chl *a* (µg L-1)	Turb (NTU)
3	13/05/2012	41° 27'/ 2° 43'	195	SFC	5	16.47	38.12	0.09	0.07
5	14/05/2012	41° 18' / 2° 45'	620	SFC	5	17.68	38.15	0.20	0.14
				DCM	51	13.99	38.27	0.41	0.09
				MESO	200	13.26	38.50	0.03	0.05
				BNL	610	13.12	38.50	N.D	0.10
6	15/05/2012	41° 13'/ 2° 46'	1039	SFC	5	17.71	38.17	0.25	0.14
				DCM	55	13.51	38.26	1.73	0.13
				MESO	200	13.11	38.49	0.01	0.04
				BNL	1029	13.09	38.49	N.D	0.14
8	17/05/2012	40° 37'/ 2° 49'	2315	SFC	5	17.59	38.21	0.10	0.09
				TOP-DCM	39	14.16	38.19	0.35	0.12
				DCM	60	13.44	38.21	0.89	0.10
				BOTTOM-DCM	76	13.27	38.22	0.29	0.06
				MESO	202	13.02	38.29	0.02	0.09
				BATHY	1600	13.15	38.48	N.D	0.09
				BNL	2305	13.24	38.48	N.D	0.11

Headers: Lat/Long (latitude and longitude), chlorophyll *a* (Chl a), turbidity (Turb). Layers: SFC (surface), DCM (deep chlorophyll maximum), MESO (the upper layer of mesopelagic waters), BNL (benthic nepheloid layer: layer 10 m above the sea bottom with high turbidity), TOP-DCM (above the DCM), BOTTOM-DCM (below the DCM), and BATHY (bathypelagic waters). Units: PSU (Practical Salinity Units), NTU (Nephelomeric Turbidity Units). N.D: no date

3.3.2 Spatial variability of prokaryotic communities

We described the prokaryotic (archaea and bacteria) community composition at all depths in the same 6 filter-size classes. Our choice of the filters was based on the most common filters used to separate the various types of particles found in the literature. The size-fractions were equivalent to particle-sizes ranging from 0.2 to 0.8 µm, 0.8 to 3.0 µm, 3.0 to 5.0 µm, 5.0 to 10 µm, 10 to 20 µm and 20 to 200 µm. Thereby, the size-fraction of 0.2 to 0.8 µm was considered as free-living prokaryotes, and the others as attached prokaryotes. A total of 5,979,503 sequences were obtained by Illumina sequencing, and grouped into 2,851 OTUs (at the 97% cut-off). After processing the sequences and normalizing the OTU table to the sample with the lowest read number (10,000), the average

number of OTUs per sample was 560±253. A PERMANOVA test was used to describe which factors mainly explained the differences in community structure. The variability of the prokaryotic communities related to particle size (i.e. size-fractions) was represented by the factor "size-fraction", the coastal to oceanic variability of the communities by the factor "station", and their depth variability by the factor "depth". This analysis revealed statistically significant differences in prokaryotic community structure due to all three factors (P<0.001) (**Supplementary Table 1**), yet the variability due to "size-fraction" (15%) and "station" (8%) were much lower than that explained by "depth" (40%). The higher relevance of the factor "depth" compared to "size-fraction" and "station" was encountered even if we considered a smaller surface-depth gradient, i.e. only the SFC and DCM depths. Thus, given the importance of the vertical gradient on the variability in prokaryotic community composition (even at small distances), we focused on analysing in more detail the vertical gradient rather than the horizontal one, which seems to be less relevant. Taking into account the most abundant taxonomic groups and their contribution to community structure in each sampling point (**Figure 2**), we observed that its relative abundances were more variable in depth than between stations. Overall, the small size-fractions of the SFC depths were dominated by members of the SAR11 clade and the large size-fractions by Flavobacteria. In the DCM, Cyanobacteria accounted for a high sequence proportion in the 0.8-3.0 μm size-fraction and larger, but accounted for a low proportion in the 0.2-0.8 μm size-fraction. In MESO, the contribution of Planctomycetes was higher in larger size-fractions and contrarily; archaea contributed more in the smallest size-fractions. In the BNL, there was an increase in the abundance of Deltaproteobacteria and Vibrionales in all size-fractions as compared to other depths. In contrast, the contributions of these taxonomic groups to each size-fraction were generaly maintained between stations.

3.3.3 Variability of diversity with depth

The species accumulation curve (or species discovery curve, i.e. accumulation of species number with increasing particle sizes) showed a logarithmic form in each of the 4 depth layers (SFC, DCM, MESO and BNL) (**Supplementary Figure 1**). The total values of accumulated richness were larger at aphotic depths with the BNL reaching a maximum of ~1700 OTUs vs. ~700 OTUs in the SFC. SFC and DCM had similar values of accumu-

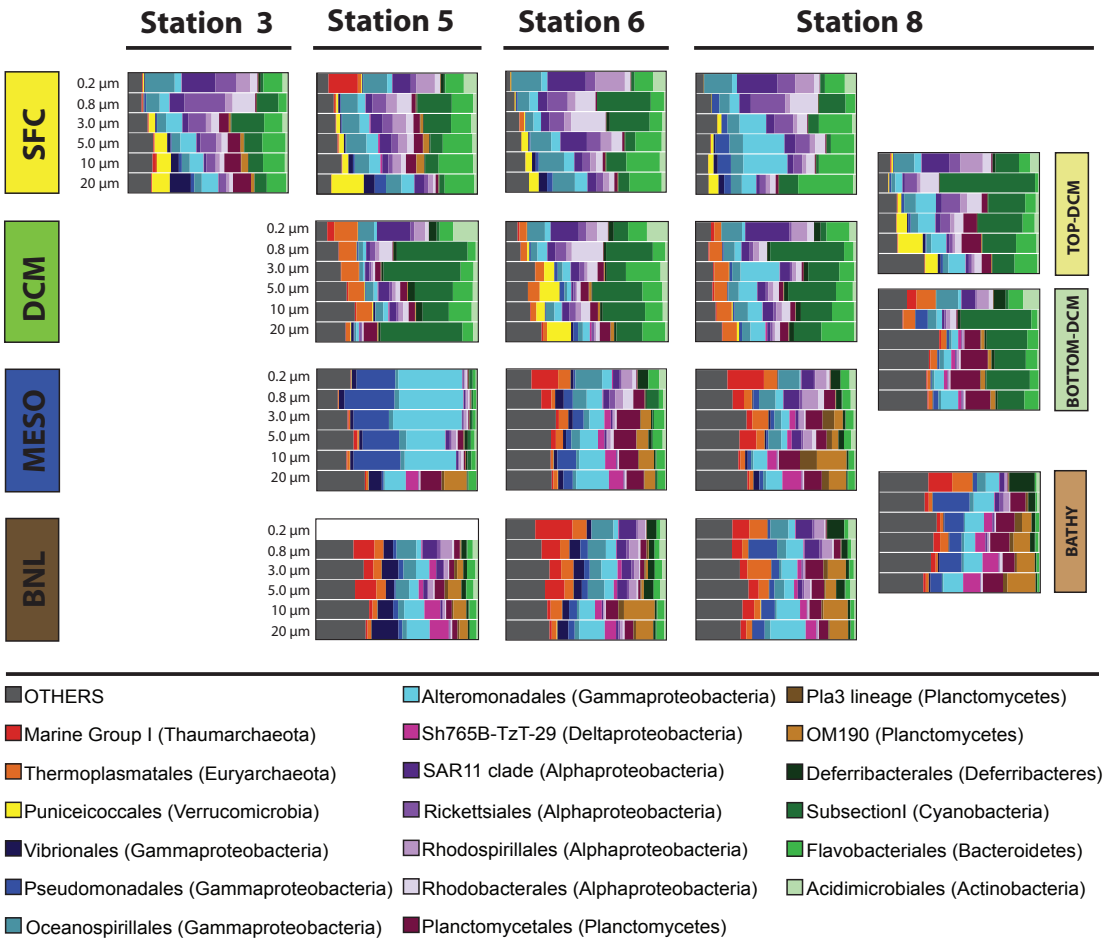

Figure 2. Taxonomic composition of Archaea and Bacteria at each station, depth and size-fraction. In order to simplify the figure, only taxonomic groups with >10% abundance in at least one sample are represented. The remaining sequences were pooled as "Others". SFC: surface; DCM: Deep Chlorophyll Maximum; MESO: Mesopelagic; BNL: Benthic Nepheloid Layer. BATHY: Bathypelagic.

lated richness along all studied particle size-fractions and stations. The average richness of all six size-fractions (average alpha diversity) and the total richness of all size-fractions combined (gamma diversity) both increased with depth. Conversely, the rate of microbial community differentiation among size-fractions (i.e. beta diversity, with smaller values indicating a higher similarity) decreased with depth (**Figure 3**). At Station 8, where we sampled 3 additional depths, we detected a substantial increase in average alpha-, beta- and gamma diversity and in the richness of each size-fraction in the TOP- and BOTTOM-DCM layers as compared to SFC and DCM layers. In the dark ocean, the BATHY layer

showed higher values of average alpha-, beta-, gamma diversity and richness in each size fraction than in the BNL (**Figure 4**), yet this was analyzed in only one station.

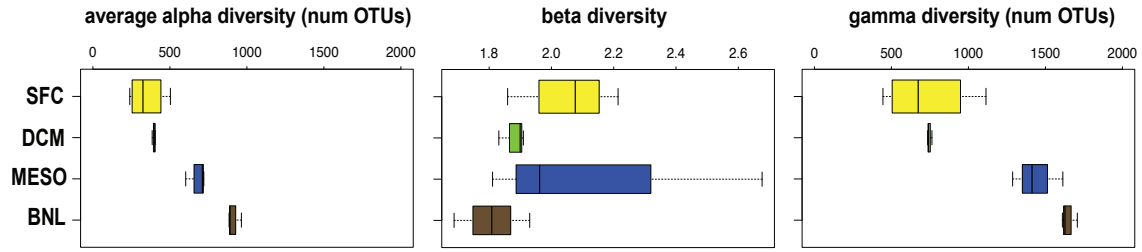

Figure 3. Distribution of average alpha, beta and gamma diversity at each depth and for all stations. The boxplots are constructed with the upper and lower lines corresponding to the 1st and 3rd quartile of the distribution of values. The median values are shown with horizontal black lines.

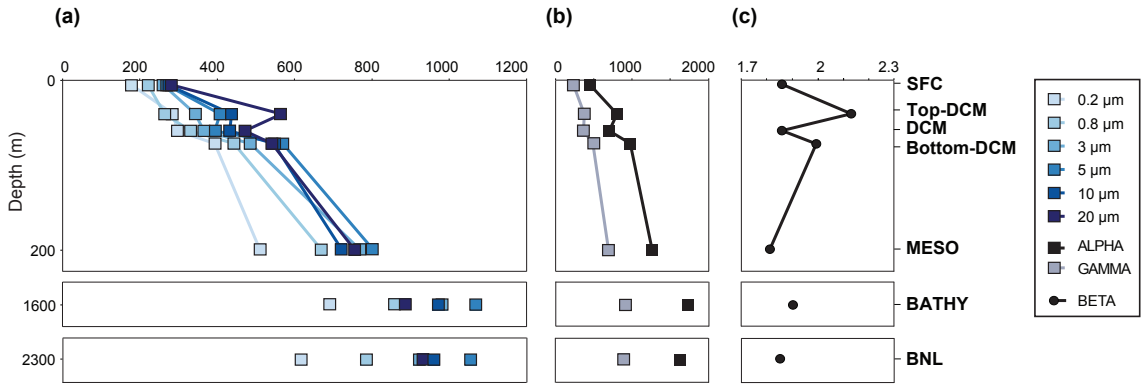

Figure 4. Vertical profiles of **(a)** richness in each size-fraction, **(b)** average alpha- and gamma diversity, and **(c)** beta diversity at Station 8.

3.3.4 Bacterial *vs*. archaeal vertical variability

Bacteria and archaea presented distinct patterns of relative abundances and richness in the different size-fractions and depths. Overall, the relative abundance of archaea was low (on average 8% of the total sequences) as compared to bacteria (on average 92% of the total sequences). The proportion of archaea increased from the surface to depth (average SFC = 1.5% ± 4.0; average BNL = 13.5% ± 7.2), and was higher in the smaller size-fractions, particularly in the free-living fraction (**Supplementary Figure 2**). Bacterial richness

(number of OTUs) was much higher than archaeal richness (average ratio richness bacteria/archaea = 26.5% ± 18.9), but the ratio decreased from the surface to deeper waters (from 43.6% ± 25.8 in the SFC layer to 14.2% ± 3.2 in the BNL layer). Bacterial richness increased with increasing particle size at all depths (**Figure 5**, left). This generalized and persistent pattern includes singularities at each depth: the net increase of richness from the smallest to the largest size-fraction had its minimum in the SFC and its maximum in the BNL layer. Moreover, this increase was gradual in the SFC, DCM, and MESO layers, but not in the BNL, where we observed a marked increase in diversity from 0.2 to 3.0 µm. Archaea presented richness patterns in the particle size fractions different from those of

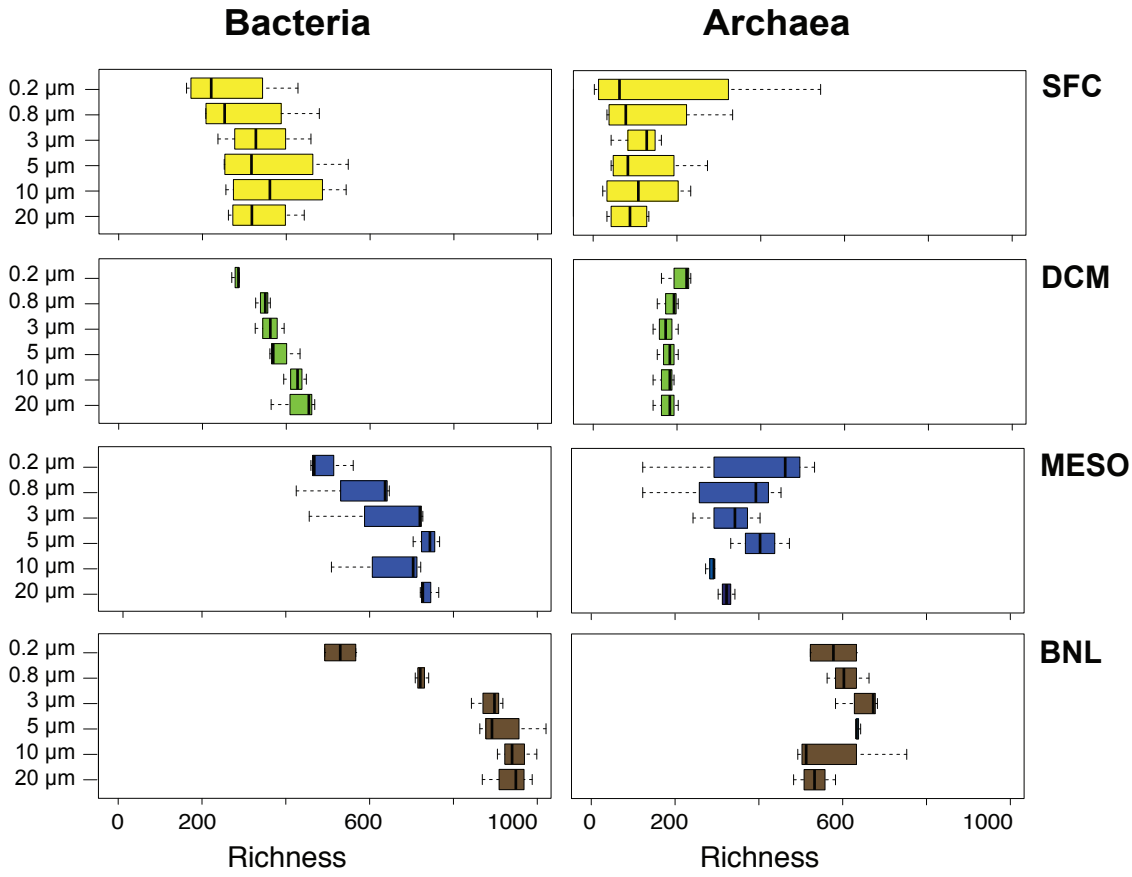

Figure 5. Richness of Bacteria (left panels) and Archaea (right panels) of each particle size-fraction at each depth and for all stations. Boxplots are constructed with the upper and lower lines corresponding to the 1st and 3rd quartile of the distribution of values. The median values are shown with horizontal black lines.

bacteria, characterized mainly by a higher richness in the smallest size-fractions (**Figure 5**, right). This pattern was not uniform throughout the water column (e.g. archaea showed an increase of richness in the intermediate size-fractions, such as the 5.0 µm fraction, in the MESO layer, and in the 3.0 µm fraction in the BNL layer).

3.3.5 Vertical variability of most abundant taxonomic groups

In the overall dataset, 42 taxonomic groups represented at least 1% of the sequences in one size-fraction or sample (see Material and Methods and **Table 2**) and thus were selected for further analysis. The taxonomic groups were classified depending on whether the enrichment (i.e. increase in relative abundance) in the different size-fractions was maintained (ME) or varied (VE) with depth. We further sub-classified ME groups, taking into account whether the enrichment was in the small (MES) or in the large size-fractions (MEL) respectively, whereas in the type labelled MEN the enrichment was not apparent in any size-fraction (see details in **Supplementary Figure 3**). This classification was done based on our ANOVA outputs, where we tested the effects of the factors "station", "depth", and "size-fraction" on the relative abundance of each individual taxonomic group. Overall, we observed that, while the relative abundances of each taxonomic group changed greatly with depth, most taxa (30 out of the 42 taxonomic groups studied) yielded patterns of enrichment in the large or small size-fractions conserved with depth (i.e. type ME) (**Table 2**). From these groups, 8 showed an enrichment in the small size-fractions conserved with depth (i.e. MES, e.g: Thaumarchaeota and Euryarchaeota), 13 groups showed an enrichment in the large size-fractions conserved with depth (i.e. MEL, e.g: Planctomycetes), and 9 groups did not show any enrichment in the large nor in the small size-fractions at any depth (i.e, MEN, e.g: Acidobacteria). Conversely, 12 groups showed enrichment in variable size-fractions in different depths (VE, e.g: Cyanobacteria, certain members of Proteobacteria, such as SAR11, Rhodobacterales or the Deltaproteobacteria Sh765B, and Acidimicrobiales, among others).

Table 2. Classification of the selected taxonomic groups depending on whether the enrichment in the size-fractions was maintained (ME) or varied (VE) with depth. The taxonomic groups were selected for being those which presented >1% of abundance in at least one sample.

Classification		Taxa
Type	**Subtype**	
ME (Maintained enrichment among depths)	**MES** (Enriched in small size-fractions)	Arctic97B-4 marine group (Verrucomicrobia)
		Deferribacteres
		Marine Group I (Thaumarchaeota)
		Oceanospirillales (Gammaproteobacteria)
		Rickettsiales (Alphaproteobacteria)
		Salinisphaerales (Gammaproteobacteria)
		SAR202 (Chloroflexi)
		Thermoplasmatales (Euryarchaeota)
	MEL (Enriched in large size-fractions)	Bdellovibrionales (Deltaproteobacteria)
		Corynebacteriales (Actinobacteria)
		Desulfuromonadales (Deltaproteobacteria)
		Flavobacteriales (Bacteroidetes)
		Phycisphaerales (Planctomycetes)
		Planctomycetales (Planctomycetes)
		Pla3 (Planctomycetes)
		OM190 (Planctomycetes)
		Puniceicoccales (Verrucomicrobia)
		Rhizobiales (Alphaproteobacteria)
		Sphingobacteriales (Bacteroidetes)
		Thiotrichales (Gammaproteobacteria)
		Verrucomicrobiales (Verrucomicrobia)
	MEN (Enriched neither in larger nor in small size-fractions)	Acidobacteria
		Alteromonadales (Gammaproteobacteria)
		Bacillales (Firmicutes)
		Burkholderiales (Betaproteobacteria)
		Cytophagia (Bacteroidetes)
		KI89A (Gammaproteobacteria)
		Pseudomonadales (Gammaproteobacteria)
		Sphingomonadales (Alphaproteobacteria)
		Vibrionales (Gammaproteobacteria)
VE (Variable enrichment among depths)		Acidimicrobiales (Actinobacteria)
		E01 (Gammaproteobacteria)
		GR WP33 30 (Deltaproteobacteria)
		Incertae Sedis (Deltaproteobacteria)
		Myxococcales (Deltaproteobacteria)
		Rhodobacterales (Alphaproteobacteria)
		Rhodospirillales (Alphaproteobacteria)
		SAR11 (Alphaproteobacteria)
		SAR324 (Deltaproteobacteria)
		Sh765B (Deltaproteobacteria)
		Subsection I (Cyanobacteria)
		Xanthomonadales (Gammaproteobacteria)

3.4 DISCUSSION

Several studies have shown differences in the diversity and taxonomy of free-living vs. attached marine microbial communities (e.g. Bižic-Ionescu *et al*. 2014; Rieck *et al*. 2015, and references therein). However, most of these works differentiated only between two particle size classes to distinguish between free-living and attached bacteria. Consequently, these studies ignored the fact that the particulate matter fraction encompasses a size continuum in the ocean (e.g. Verdugo *et al*. 2004). Therefore, in this study, we separated the particle size continuum into 6 distinct particle size-fractions in order to analyse in great detail the prokaryotic community structure at scales ranging from 0.2 to 200 μm. Moreover, we described how these patterns vary along horizontal and vertical gradients.

3.4.1 Depth is a stronger driver of prokaryotic community structure than station or size-fraction

Microbial community composition is known to be more variable along depth gradients than in surface transects from the coast to offshore (e.g. Baltar *et al*. 2007; Pommier *et al*. 2010) or in surface transects in the open ocean (Hewson *et al*. 2006). Nonetheless, these studies did not size-fractionate their samples. Other studies which have used size-fractionation to separate free-living and attached lifestyles have revealed that prokaryotic community structure differs more with particle-size than in space, including surface transects and depth gradients (Acinas *et al*. 1997; Moeseneder *et al*. 2001; Ghiglione *et al*. 2009; Crespo *et al*. 2013). Yet, other studies described that the similarity or difference between free-living and attached communities strongly depends on the sampling site (Ortega-Retuerta *et al*. 2013). The studies conducted by Acinas *et al*. (1997), Ghiglione *et al*. (2009) and Crespo *et al*. (2013) were performed close to our study area, but they used only one (yet different) filter pore size to separate between free-living and attached prokaryotic fractions (2.0 μm, 0.8 μm and 3.0 μm, respectively), and involved only shallow depths. In a previous study (Mestre *et al*. 2017), we showed that the use of various particle size-fractions reveals a more comprehensive view of microbial community composition along the particle size continuum. Consequently, depth gradients analyzing only 2 size-fractions integrate across various particle sizes and provide a restricted visualization of the prokary-

otic community composition at the microscale at each depth. In our more comprehensive effort, we considered 6 particle size-fractions in a horizontal gradient of ca. 100 km, and a vertical gradient up to 2,300 m depth. In our case, the variability in prokaryotic community composition due to depth was much larger than between size-fraction and stations. Given the importance of the vertical gradient on the variability in prokaryotic community composition, we focused on further analysis on the depth gradient.

3.4.2 Communities along the particulate size continuum become more similar with depth

The sinking particles that reach the deep ocean are younger, and their carbon content is more labile than the deep dissolved organic matter pool, which is considered to mainly contain recalcitrant organic matter (Druffel and Williams 1990). This has been related to the differentiation of free-living and attached prokaryotic communities of the dark ocean, where free-living communities would be more adapted to the recalcitrant dissolved organic matter pool, whereas attached communities would be more adapted to growing under nutrient-rich conditions (reviewed in Herndl and Reinthaler 2013). But, are the differences between free-living and attached prokaryotic communities in the dark ocean indeed more pronounced than in the sunlit ocean? The decrease in beta-diversity with depth indicates that in deeper water layers the prokaryotic communities of each particle size-fraction becomes more similar. Even though strong differences between free-living and attached prokaryotic communities have been recently described in the dark ocean (Salazar *et al*. 2015), we observed that these differences were smaller than in the sunlit ocean. This observation could point to higher particle heterogeneity in surface waters as compared to the deep ocean and the benthic nepheloid layer. It has been recently shown that particle composition varies between smaller and larger particles (sizes 11-64 μm and > 64 μm, Durkin *et al*. 2015) whereas, on the other hand, an exchange exists between differently sized particles throughout the water column (e.g.: Bacon *et al*. 1985). In the deep ocean there are less particle sources and it is likely that the exchange (particle fragmentation and re-aggregation) is higher. Yet, this could explain why the prokaryotic communities of distinct size-fractions including the free-living fraction are more similar in the deep.

3.4.3 High variability in alpha-, beta- and gamma diversity in the vicinity of the DCM

Considering the whole water column (the 4 main depth layers defined here), we observed a general trend of increasing average alpha- and gamma diversity, and decreasing in beta diversity with depth. Nonetheless, when analysing the vertical profile at a higher resolution (tens of meters) in surface waters, we observed pronounced changes in average alpha, beta- and gamma diversity values in and around the DCM (**Figure 4**). We also observed changes in bacterial community composition, as, e.g. a decrease of Verrucomicrobia and an increase in the contributions of archaea and Planctomycetes from the top to the bottom of the DCM. A well-developed DCM is a prominent feature of the temperate ocean including the Mediterranean Sea during extended periods of the year (Berman *et al*. 1984; Estrada *et al*. 1993). Previous studies in the same area showed that the levels of bacterial and phytoplankton biomass as well as production differed from the top to the bottom of the DCM (Pedrós-Alió *et al*. 1999), and that there was a distinct distribution of heterotrophic and photosynthetic ciliates (Dolan and Marrasé 1995) in the vicinity of the DCM. In the Atlantic Ocean, the community structure of pico- (Cabello *et al*. 2016) and larger phytoplankton (Latasa *et al*. 2016) also presented a distinct spatial distribution in the DCM, and several studies have shown changes in prokaryotic community composition with phytoplankton composition (reviewed in Amin *et al*. 2012). Although there are plenty of studies describing prokaryotic community structure at the DCM, there are, to the best of our knowledge, no studies describing the variability of prokaryotic community composition above and below the DCM. Despite being restricted to one station, our results represent the first attempt to describe changes in community structure at a higher spatial (depth) resolution and suggest that complex processes and interactions along the different particle sizes occur in this prominent oceanographic feature, with presumably strong implications for particle fluxes.

3.4.4 Bacteria and archaea exhibit opposite patterns of richness along the particulate size continuum and with depth

Consensus on the generality of trends in prokaryotic richness along vertical gradients has not yet been reached. For bacteria, some studies described an increase of richness with depth (Pommier *et al*. 2010; Kembel *et al*. 2011; Walsh *et al*. 2015) while others observed a decrease (Brown *et al*. 2009; Agogué *et al*. 2012, Bryant *et al*. 2016). Moreover, diversity analyses in which distinct size-fractions are considered reported contradictory conclusions about which size-fraction is more diverse: some studies reported the free-living size-fraction to be more diverse (Acinas *et al*. 1999, Moeseneder *et al*. 2001, Ghiglione *et al*. 2009), whereas others the attached size-fraction (Crespo *et al*. 2013, Ganesh *et al*. 2014). Further, there are divergent conclusions about how the ratio of free-living *vs* attached bacterial richness varies with depth: some studies reported a general increase in depth (Acinas *et al*. 1997; Ghiglione *et al*. 2009), others a decrease (Ganesh *et al*. 2014), and others first showed an increase with depth and then a decrease at the last sampling depth (Moeseneder *et al*. 2001 -at 1000 m-; Crespo *et al*. 2013 –at 500 m-). In the present study, bacterial diversity increased with size-fraction and with depth (up to 2,300 m). An increase in bacterial diversity with size-fraction has been recently described in a NW Mediterranean surface coastal station (Mestre *et al*. 2017) demonstrating the relevance of implementing particle size fractionation for prokaryotic biodiversity assessment to fully appreciate its magnitude. This study was based on a single location and thus did not include the spatial dimension as a possible driver of bacterial diversity. Here, we demonstrate that the increase in bacterial diversity with size-fractions is widespread in all sampled stations in the NW Mediterranean, covering transects from the coast to 100 km offshore and from the surface to 2,300 m depth.

In contrast to bacteria, there are only very few studies describing archaeal richness along a depth gradient, and those existing have revealed contrasting results: some show an increase in richness with depth (De Corte *et al*. 2009) and others a decrease (Brown *et al*. 2009; Bryant *et al.* 2016). To our knowledge, this is the first study that analyzed Archaea in distinct particle size-fractions along a depth gradient (up to 2,300 m). Archaeal diversity increased with depth and, in contrast to bacteria, presented a generally higher

diversity in the smallest size-fractions. In fact, certain archaeal taxa have recently been associated to particles (Galand *et al*. 2008; Martin-Cuadrado *et al*. 2014; Orsi *et al*. 2015). Moreover, we observed that archaeal richness increased in the intermediate size-fractions in both mesopelagic and benthic nepheloid layers coinciding with an increase in their relative abundance. This suggests that the attachment of archaea to particles can be related to certain taxa and environmental changes that are related to depth.

3.4.5 Most prokaryotic groups maintain their preferences for certain size-fractions through depth

In a recent study in which the multiple size-fractionation approach was also applied, it was obvious that certain taxonomic groups were enriched in the smaller size fraction and others in the larger size-fractions, whereas others did not show any enrichment neither in the larger nor smaller size-fractions (Mestre *et al*. 2017). Our present study highlights that the observed preference for a given particle size-fraction is a rather depth-independent and conserved trait for most prokaryotic taxonomic groups. This includes the two major groups of archaea, i.e. Thaumarchaeota and Euryarchaeota. Previous studies have reported that certain archaea can show either a preference for the small size fractions (Smith *et al*. 2013, Salazar *et al*. 2015) or a more general association to particles (Galand *et al*. 2008; Martin-Cuadrado *et al.* 2014; Orsi *et al.* 2015). Our results, despite showing enrichment on the small particle size-fractions, demonstrate that archaea are indeed present in all size-fractions. Contrarily, Planctomycetes represent a group which is highly enriched in the larger particle size-fractions independent of depth (e.g. Crespo *et al*. 2013; Ganesh *et al*. 2014). Planctomycetes are known to be associated to distinct substrates (e.g. Delong *et al*. 1993; Fuerst *et al*. 1997; Crump *et al*. 1999; Bengtsson and Øvreås 2010; Lage and Bondoso 2011) where they likely contribute to biopolymer degradation (Woebken *et al*. 2007), which may explain their clear preference for larger particle size-fractions. On the other hand, groups like the Acidobacteria showed neither a preference for small nor large particles. Despite being highly abundant in soils, Acidobacteria represent a rather small fraction of microbial communities in aquatic systems (Crump *et al*. 2009; Eloe *et al*. 2011; Vila-Costa *et al*. 2012; Zhu *et al*. 2013). Members of this group, however, have an extensive metabolic versatility (Quaiser *et al*. 2003), which may allow them to occupy differ-

ent niches, and this would explain their presence in all determined particle size-fractions. However, we cannot rule out that their globally low abundances may have masked a potential size-fraction preference.

3.4.6 Environmental filtering and/or distinct ecotypes may explain the lack of conserved patterns of enrichment on distinct particle-size fractions with depth

Even though most taxonomic groups are enriched in either the small or large size-fractions, there were some exceptions. Changes of enrichment of certain groups along the size continuum with depth could be due to environmental filtering along surface-deep ecological gradients. An obvious example is the enrichment of autotrophic unicellular Cyanobacteria on smaller particle size-fractions in photic waters. However, below the photic zone, they were equally present in all size-fractions and occurred in very low relative abundances in the dark ocean. Cyanobacteria are photoautotrophs and thus their presence in aphotic waters is most likely related to their transport via fast-sinking particles, a mechanism recently described to be present worldwide (Agustí *et al*. 2015). A similar example is represented by the Alphaproteobacteria group Rhod obacterales, whose relative abundances decreased strongly with depth and were very low at the BNL. This pattern could stem from their photoheterotrophic lifestyle and close associations to phytoplankton (Rosenberg *et al*. 2006; Buchan *et al*. 2014). Conversely, Deltaproteobacteria of the Order Sh765B were nearly absent in surface waters and had their maximal relative abundance in deep waters where they were clearly enriched in the large particle size-fractions. Despite their unknown ecology, it has been suggested that this group is possibly linked to the anaerobic oxidation of methane (Siegert *et al*. 2011) in the absence of molecular oxygen. It is likely that large particles represent anaerobic microniches (Michotey and Bonin 1997) at specific depths where oxygen concentration is low, e.g. in the deeper waters. An alternative explanation would be that they colonize the anoxic bottom sediment and are resuspended on large particles into the deeper water layer.

Another plausible explanation for the lack of depth-conserved patterns of enrichment is the presence of depth-related ecotypes with distinct preferences for specific particle size-fractions. As an example, the abundant SAR11 showed a distinct distribution of different

clades: the "SAR11 clade Surface 1" was present mainly in surface waters and in the small particle size-fractions, whereas the "SAR11 clade Family Deep 1" was mainly enriched in the 5.0-10 μm size-fraction in the MESO and BNL layers. Clades of SAR11 restricted only to free-living fractions or attached to particles have been described before (Bižic-Ionescu *et al*. 2014). Yet, all this is in agreement with the occurrence of distinct ecotypes and an extensively described microdiversity within the SAR11 clades (García-Martínez and Rodríguez-Valera 2000; Brown and Fuhrman 2005). Additionally, we observed a distinct distribution of the Acidimicrobiales (Actinobacteria) with depth: whereas the family "OCS155 marine group" predominated in the sunlit layers and was enriched in small size-fractions, the family "Sva0996 marine group" predominated in the dark layers and was enriched in the 5.0-10 μm particle size-fraction. Therefore, even though members of the Acidimicrobiales have been described as free-living microbes prevalent at the DCM (Mizuno *et al*. 2015), some families are enriched in aphotic waters and related to larger particle size-fractions.

3.4.7 Concluding remarks

We have studied, for the first time, how structure and composition of prokaryotic communities associated to particles in the size range from 0.2 to 200 μm vary in space. Changes in prokaryotic community composition were more pronounced from the surface to deeper water layers than from coastal to offshore waters. Both bacteria and archaea showed an increase in alpha diversity from surface to deep waters, but differed in their structure along size-fractions: whereas bacterial richness generally increased with particle size, archaeal richness decreased. This may suggest divergent strategies of each kingdom regarding their attachment to particulate matter. When using a higher phylogenetic resolution, we could discriminate two types of microbes: those that are enriched on certain size-fractions throughout the whole water column, and those that showed a variable enrichment on the different particle size-fractions with depth. Most taxonomic groups, however, maintained their enrichment in specific particle size-fractions. In summary, this study contributes to our understanding on how the architecture of prokaryotic communities along the particulate size continuum varies over spatial and vertical gradients in the ocean.

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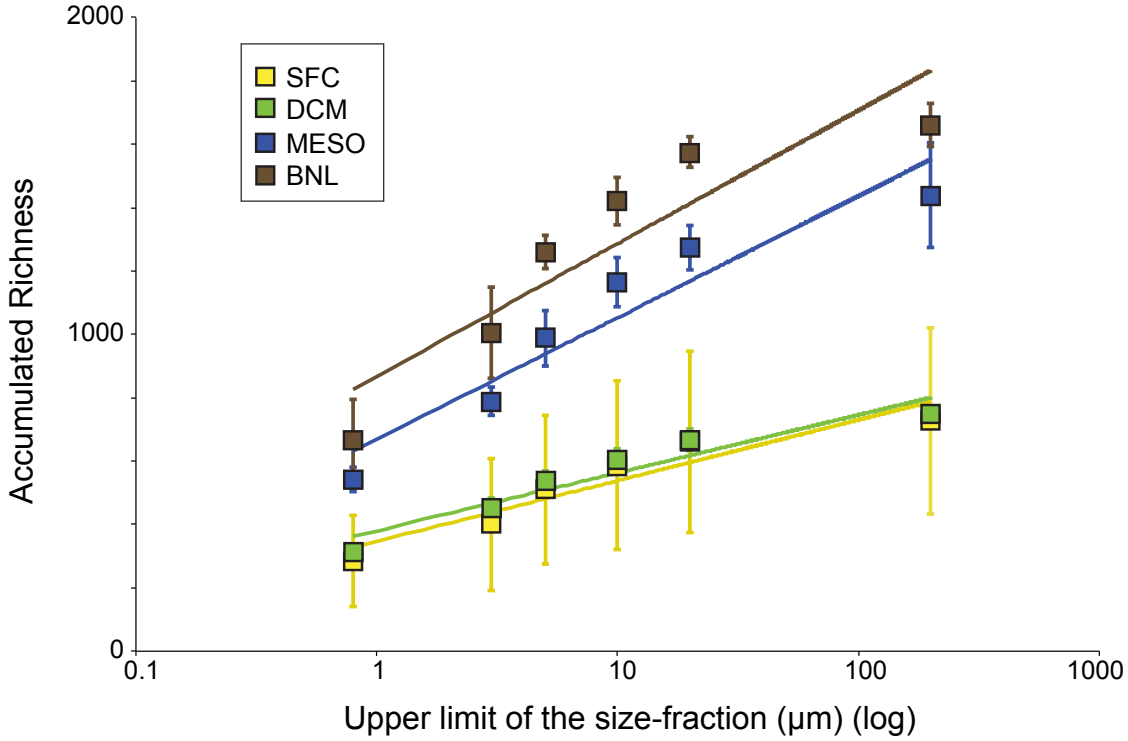
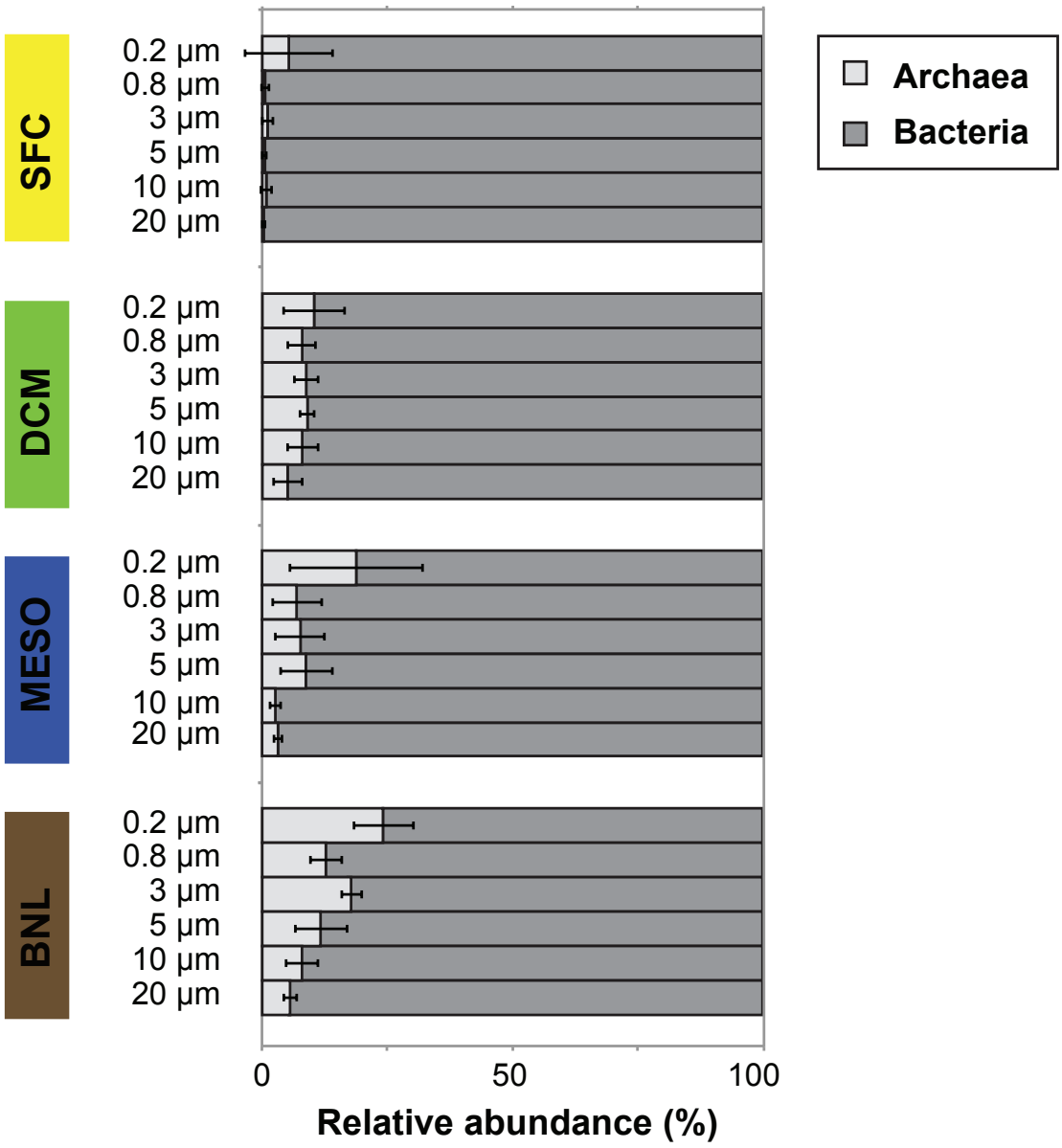
3.7 SUPPLEMENTARY MATERIAL

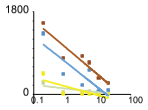
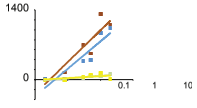
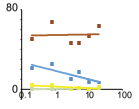
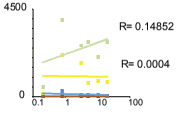
3.7.1 Supplementary Figures



Supplementary Figure 1. Species accumulation curves representing, for each depth, the number of OTUs ("species") accumulated from the smallest to the largest size-fraction. The curve fits the median values calculated among stations with their standard deviation for each depth.



Supplementary Figure 2. Relative abundance of Archaea and Bacteria in each particle size-fraction and depth layer. The data shown are the average values calculated among the distinct stations, with their standard deviation.

Type		Significant diferences			Interpretation	Example	
		Depth	Size-fraction	Interaction Depth:size-fraction			
ME (Maintained enrichment among depths)	**MES** (enriched in small size-fractions)	Yes	Yes				Marine Group I (Thaumarchaeota)
	MEL (enriched in large size-fractions)	Yes	Yes				OM190 (Planctomycetes)
	MAN (not enriched in larger nor in small size-fractions)	Yes					Acidobacteria
VE (Variable enrichment among depths)		Yes	Yes	Yes	…		SubsectionI (Cyanobacteria)

Supplementary Figure 3. Examples of each pattern of distribution discussed in the text and its interpretation. Significant differences correspond to the results of the ANOVA test between depths, size-fraction and its interactions.

3.7.2 Supplementary Tables

Supplementary Table 1. Permutational multivariate analysis of variance (PERMANOVA) examining the effects of the factors "station" (distance coast-ocean), "depth" (surface-deep variation) and "size-fraction" on the composition of the prokaryotic communities (Archaea and Bacteria). Key to abbreviations and column headings: D.f, degrees of freedom; MS, mean square; F, F ratio; R^2, coefficient of determination; P, p-value.

Source of variation	D.f	MS	F	R^2	P
Station	3	0.568	4.919	0.085	0.0001
Depth	3	2.657	22.993	0.395	0.0001
Size-fraction	5	0.594	5.146	0.147	0.0001
Residuals	65	0.115		0.372	
Total	76			1.000	

Chapter 4

Vertical connectivity in the ocean microbiome: Sinking particles as dispersal vectors

Mireia Mestre, Clara Ruiz-González, Ramiro Logares, Josep M. Gasol & M. Montserrat Sala

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Vertical connectivity in the ocean microbiome: Sinking particles as dispersal vectors

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SUMMARY: One of the dominant processes exporting carbon into the deep ocean is the sinking of organic particles formed in the photic layer, which are rapidly colonized by microbes. Even though this is a well-recognized process, the role of particles as vectors transferring prokaryotic taxa from the surface to the deep oceanic realms has not yet been addressed. Here, we explored the vertical connectivity of the ocean microbiome by using a serial filtration system to separate marine prokaryotic communities into five different size-fractions, characterizing them by sequencing of the 16S rRNA gene, and examining their compositional variability from the surface down to 4,000 m across eight globally distributed oceanic stations sampled during the Malaspina-2010 expedition. Our results show that the most abundant prokaryotes in the deep-ocean are also present in surface waters, and that "endemic" taxa of the deep-ocean account for a very small fraction of total sequences. Vertical connectivity seemed to occur through the largest particles, because: (1) particle-attached prokaryotic communities were more similar throughout the water column than free-living prokaryotes and (2) particle-attached prokaryotes from surface where found in both free-living and attached deep-sea communities. Finally, we found that the particle colonization processes occurring in surface waters can determine the composition of the communities inhabiting particles in the deep ocean, since the biogeographic patterns of surface particle-attached communities were reflected on those of their deep-ocean counterparts. Overall, we argue that particles may function as a vector that inoculates viable surface microbes into the deep-sea realm determining, to a considerable extent, their biogeography.

4.1 INTRODUCTION

A main goal of microbial ecology is to unveil the spatiotemporal distribution patterns of microbial taxa and comprehend the factors that determine them. Previous studies have suggested that variation in prokaryotic community composition is primarily due to changes in the relative abundance of cosmopolitan taxa (Gibbons *et al*. 2013). For example, dominant prokaryotes within specific depths tend to be ubiquitous (Salazar *et al*. 2015, Sunawaga *et al*. 2015; Lindh *et al*. 2016), which points to a high dispersal potential of microbes and thus a significant connectivity of oceanic communities at the horizontal scale. In contrast, the vertical connectivity between communities from surface to bathypelagic waters remains poorly understood, as spatial surveys focusing in the vertical dimension often describe the communities found at each depth but without assessing their potential connectivity throughout the water column (e.g. Baltar *et al*. 2007, Pommier *et al*. 2010, Ganesh *et al*. 2014, Walsh *et al*. 2015).

A few recent studies have shown that communities from surface and deep waters may be connected through water mass circulation (Wilkins *et al*. 2013, Tamburini *et al*. 2013, Luna *et al*. 2016), while others have suggested that migrating organisms or particle export may link communities throughout the water column (Zinger *et al*. 2015; Cram *et al* 2015). In particular, the sinking of particles, which is known to be widespread throughout the global ocean (Ducklow *et al*. 2001), could represent a major dispersal pathway of taxa to the deep ocean. Nevertheless, since most of these studies have focused on microbes freely suspended in the water column, we do not know whether sinking particle-attached microbial communities comprise a source of diversity to deeper waters.

Particles formed in surface waters are rapidly colonized by prokaryotes, and particle-attached communities are often more metabolically active (Karner and Herndl 1992, Grossart, Hietanen, *et al*. 2003, Grossart *et al*. 2007) and phylogenetically diverse (e.g: Eloe *et al*. 2010, Crespo *et al*. 2013, Ortega-Retuerta *et al*. 2013, Bižic-Ionescu *et al*. 2014, Ganesh *et al*. 2014) than suspended (or free-living) assemblages. As particles sink, they carry with them the attached prokaryotes, thus potentially acting as vectors that transport surface prokaryotes to deeper waters. To date, most studies on marine particle sinking

have focused on the biogeochemistry of carbon (e.g.: carbon export (Alldredge and Silver 1988, Guidi *et al*. 2009, Bochdansky *et al*. 2016) and carbon remineralization (Martin *et al*. 1987, Kwon *et al*. 2009)), or on the role of sinking particles as food sources for deep-sea prokaryotes (reviewed in Arístegui *et al*. 2009, Herndl and Reinthaler 2013). And thus, even though it is clear that the vertical transport of particles has a major role in carbon sequestration and in the maintenance of deep sea metabolism, whether sinking particles constitute a global dispersion vector of viable prokaryotes to the deep ocean remains unexplored.

Particle-attached prokaryotic communities change compositionally with depth (Acinas *et al*. 1999, Moeseneder *et al*. 2001, Ghiglione *et al*. 2009, Crespo *et al*. 2013, Ganesh *et al*. 2014, Thiele *et al*. 2015, Milici *et al*. 2017), yet it is not clear whether these changes are due to an ecological succession of taxa in the degrading particle or due to a continuous colonization of particles during sinking. Assemblages attached to sinking particles could influence the structure of deeper prokaryotic communities in two ways: 1) by bringing dormant or slow growing surface taxa (Jones and Lennon 2010, Lennon and Jones 2011) that thrive when reaching a certain depth or when the nature of the particle changes, or 2) by transporting surface bacteria that slowly die during transport because they cannot survive in deep waters. In cases where the transported surface prokaryotes thrive after reaching deeper depths, particle sinking would represent a continuous source of immigrants for the deep ocean. Given that in surface waters the origin and composition of particles vary spatially (Longhurst 1998, Buesseler and Boyd 2009, Guidi *et al*. 2009, Bach *et al*. 2016), and that deep sea particle-attached communities often present a much clearer biogeography than their free-living counterparts (Salazar *et al*. 2015), it is possible that diversity patterns in the deep ocean are conditioned by the dynamics of surface prokaryotic colonization of particles of varying origins and composition. In other words, variable species sorting or different colonization dynamics occurring in geographically separated surface particles could be reflected in the biogeography of deep-sea particle-attached prokaryotes. Although the comparison of particle-attached communities between surface and bathypelagic waters is essential for understanding the relevance of such dispersal processes, it has never been done at a local nor at a global scale.

Large particles sink fast and small sink slow or remain buoyant (Smayda 1970, Richardson and Jackson 2007, Buesseler and Boyd 2009), and therefore communities associated to larger particles should have greater chances to reach the deepest layers. Given these different rates of sinking, we could expect communities inhabiting larger particles to be vertically more similar between themselves than those associated to smaller particles. To date, most literature on particle-attached prokaryotes has been restricted to the dichotomic exploration of free-living versus attached populations (e.g. Acinas *et al*. 1999, Hollibaugh *et al*. 2000, Moeseneder *et al*. 2001, Ghiglione *et al*. 2007, Eloe *et al*. 2011, Crespo *et al*. 2013, Ortega-Retuerta *et al*. 2013, Bižic-Ionescu *et al*. 2014, Ganesh *et al*. 2014, Salazar *et al*. 2015), without taking into consideration the continuum of particle sizes that might be colonized by largely different microbial populations (Mestre *et al*. 2017). In this context, here we explore whether particles represent a dispersal vector for prokaryotes when they sink into the deep ocean, contributing to the vertical connectivity of the marine microbiome and whether particle size has an effect on vertical dispersal and particle community composition. In order to do so, we investigated the composition of suspended prokaryotic communities as well as that of those attached to particles of different sizes (ranging from 0.2 μm to 200 μm) in 8 stations across the global tropical and subtropical ocean. In particular, we explored the vertical changes of community structure in different size fractions from surface (3 m) to bathypelagic waters (4000 m). We expect, that, if sinking particles are a dispersal vector of viable microbes to the deep sea, most suspended and particle attached deep sea prokaryotes should also be present in surface waters, and suspended prokaryotic communities should be more isolated between depths than particle-attached assemblages, which should be more similar vertically. We also test the hypothesis that communities attached to the largest particles show the strongest vertical similarity due to their assumed faster sinking rates, and that deep ocean biogeographic patterns should resemble to some extent those of the surface particle-attached communities.

4.2 MATERIALS AND METHODS

4.2.1 Study area and sampling

We selected a total of 8 stations of those sampled during the Malaspina 2010 expedition (http://scientific.expedicionmalaspina.es/), between December 2010 and July 2011. The selected stations were distributed across the global tropical and subtropical ocean (Latitudes between 30º N and 40º S), 3 in the Atlantic Ocean, 2 in the Indian Ocean and 3 in the Pacific Ocean. At each station, 4 depths were sampled corresponding to the surface (SFC, 3 m), the deep chlorophyll maximum (DCM, 48-150 m), the mesopelagic (MESO, 250-670 m) and the bathypelagic waters (BATHY, 3105-4000 m). Water was sampled with niskin bottles attached to a conductivity–temperature–depth (CTD) profiler and was prefiltered through a 200 μm net mesh. Vertical profiles of salinity, potential temperature and dissolved oxygen were recorded continuously with the CTD sensors installed in the rosette sampler. Nutrients (nitrate, phosphate and silica were determined as explained in Catalá *et al*. (2016). Bacterial abundance, and bacterial size were determined by flow cytometry as described in Gasol and Moran (2016). Bacterial heterotrophic production was estimated using the ^{3}H-leucine incorporation method (Kirchman *et al*. 1985) as explained in Moran *et al*. (2017).

Prokaryotic biomass from different size fractions was collected by sequentially filtering 10 L through 20, 5.0, 3.0, 0.8 and 0.2 μm pore-size filters (all 47 mm polycarbonate filters, Nuclepore), using a peristaltic pump at very low speed and pressure, resulting in size fractions that consider suspended prokaryotes (0.2-0.8 μm), as well as various particle-attached ones (0.8-3.0; 3.0-5.0; 5.0-20 and 20-200 μm). The filters were flash-frozen in liquid N_2 and stored at –80ºC until DNA extraction. In order to simplify the nomenclature, hereafter we will refer to the different size fractions by the smallest size filter (e.g. the "0.8 fraction" refers to prokaryotes retained between 0.8 to 3 μm pore-sized filters). Moreover, we assume that, whereas the 0.2 μm fraction will harbor mostly free-living prokaryotic communities, the rest of the fractions will comprise prokaryotes associated to distinct kinds of particles (living or not living organisms -e.g. protists-, organic or not organic) of different sizes.

4.2.2 DNA extraction, sequencing and sequence processing

The DNA was extracted with a phenol-chloroform protocol (as described in Massana *et al*. 1997). The hypervariable V4-V5 region of the 16S rRNA gene was PCR amplified with primers 515F-926R (Parada *et al*. 2015) and sequenced in an Illumina MiSeq platform using 2x250 bp paired-end approach at the Research and Testing Laboratory facility (Lubbock, TX, USA; http://www.researchandtesting.com/). Computing analyses were run at the MARBITS bioinformatics platform of the Institut de Ciències del Mar (ICM). The amplicons were processed through a protocol (detailed in Logares 2017) based on UPARSE (Edgar 2013). Briefly, reads were assembled with PEAR (Zhang *et al*. 2014), and those with >100 nucleotides were selected. Quality check, dereplication, OTU clustering (97%), and reference-based chimera filtering (using SILVA v.119) were processed with USEARCH (Edgar 2010). Taxonomy assignment of the representative sequences was done using the SILVA Incremental Aligner v.123 with SINA v.1.2.11. Non-prokaryotic OTUs (Eukaryotes, Chloroplast, Mitochondria), as well as singletons, were removed. In order to allow comparisons between samples, the OTU table was randomly subsampled to the number of reads present in the sample with the lowest amount of reads (which was n=5,598).

4.2.3 Data analyses

Statistical analyses and plots were done in R (www.r-project.org) using the *vegan* (Oksanen *et al*. 2017), *simba* (Jurasinski and Retzer 2015), *spaa* (Zhang 2016), *betapart* (Baselga and Orme 2012) and *BiodiversityR* (Kindt 2017) packages. The OTU richness of each size-fraction and at each depth was calculated using the rarefied OTU table. The Bray-Curtis metric was used as an estimator of community dissimilarity. Communities were clustered using non-metric multidimensional scaling (nMDS) analyses based on Bray-Curtis distances. Statistical differences between categories such as size-fraction, station and depth were explored with permutational multivariate analyses of variance (PERMANOVA) tests (*adonis* function, R *vegan* package). In order to elucidate the connectivity between communities of a given size-fraction throughout the water column, a set of parameters was calculated considering each station separately: Vertical beta-diversity

was calculated using the *trudi* function from R package *simba*. Vertical OTU turnover (i.e. species replacement) and nestedness (i.e. species loss) were estimated using the *beta.multi* function in the R *betapart* package, and based on the Sorensen index. The vertical niche breath of each OTU was calculated using the *niche.width* function in the R *spaa* package using the Levins (Levins 1968) index. Niche breadth was defined as the number of distinct depths where an OTU appeared (i.e: OTUs with niche breadth values of 4 were present across the four depths, while OTUs with niche breadth values of 1 were present in only one depth). Correlations between communities from different fractions and depths were calculated using Mantel tests based on Bray-Curtis distances.

In order to differentiate between the OTUs prevalent in smaller or larger size fractions, we defined the Particle-Association Niche Index (PAN-Index). The PAN-Index indicates in which size-fraction is an OTU more abundant and was calculated using the abundance-weighted mean of each OTU among the 5 size-fractions. This PAN-Index defines the size-fraction preference of every OTU in the continuum of sizes and is a modification of the PAN-Index presented in Salazar *et al*. (2015) where only 2 size-fractions (free-living *vs*. attached) were considered. Values of PAN-Index are comprised from 1 to 5 and each number reflects the size-preference of a given OTU as follows: 1 = preference for 0.2-0.8 μm; 2 = preference for 0.8-3.0 μm; 3 = preference for 3.0-5.0 μm; 4 = preference for 5.0-20 μm; 5 = preference for 20-200 μm.

We defined "seed" OTUs as those OTUs present in the surface transported by particles that find a more suitable environment when reach deeper depths, i.e. that increased their abundances towards deeper waters. To detect the "seed" OTUs, we selected all surface OTUs with PAN-Index >3 (i.e. that had preference for particles larger than 3.0 μm. We calculated the euclidean distance of their relative abundances between all pairs of samples, and selected those OTUs with the largest changes in relative abundances (mean distance >10 following Ruiz-González *et al*. 2015). From those we selected OTUs that increased in their relative abundances towards deeper layers (i.e. showing mean relative abundances in surface and/or DCM lower than in meso- and/or bathypelagic). These OTUs were named 'seed' OTUs, because they could potentially represent taxa seeding deeper communities.

4.3 RESULTS

The sampled stations were located in tropical and subtropical latitudes, and spanned a broad longitudinal gradient across the Pacific, Atlantic and Indian oceans (**Supplementary Figure 1a**). The averaged values of the measured environmental variables per layer are shown in **Supplementary Figure 1b**: variables such as temperature, salinity and turbidity decreased with depth, concentrations of nutrients such as phosphate, nitrate and silicate increased pronouncedly towards bathypelagic waters, and fluorescence showed a maximum at the DCM. The number of free-living prokaryotes and heterotrophic prokaryotic production decreased with depth, but the mean cell size of prokaryotes inhabiting deep waters was larger than that of surface bacteria (**Figure 1**).

4.3.1 Taxonomic composition of the prokaryotic assemblages

We recovered 3,947,217 sequences that clustered into 4,534 OTUs at 97% similarity threshold. After subsampling the OTU table to 5,598 reads per sample, 3,632 OTUs were retained. Richness (number of OTUs) ranged between a minimum of 77, a maximum of 576 and an average of 251 OTUs per sample. Richness was highly variable among size-fractions and depths, but in general, richness increased towards larger size-fractions in SFC and DCM, and decreased with the size-fraction in BATHY, whereas richness in MESO depths increased toward intermediate size-fractions (**Supplementary Figure 2**). The composition of the prokaryotic communities differed between stations, depths and size-fractions (**Supplementary Figure 3**). Overall, prokaryotic communities were mainly structured by depth (meso- and bathypelagic communities clustered separately from surface and DCM communities, $PERMANOVA_{byDepth}$ $R^2=0.11$, $p<0.001$), by size-fraction (communities in the large size fractions were different from those free-living and those in the smallest size fractions, $PERMANOVA_{byFraction}$ $R^2=0.12$, $p<0.001$) but also depending on the geographic location ($PERMANOVA_{byStation}$ $R^2=0.23$, $p<0.001$) (**Supplementary Figure 4, Supplementary Table 1**). Interestingly, we observed that compositional differences among depths (vertical) were higher for the smallest size-fraction ($R^2=0.49$) than for the largest size-fractions ($R^2=0.10$, **Supplementary Table 2**). In contrast, the differences between stations (horizontal) were higher in the largest size-fractions ($R^2=0.55$) than in the smallest size-fraction ($R^2=0.15$).

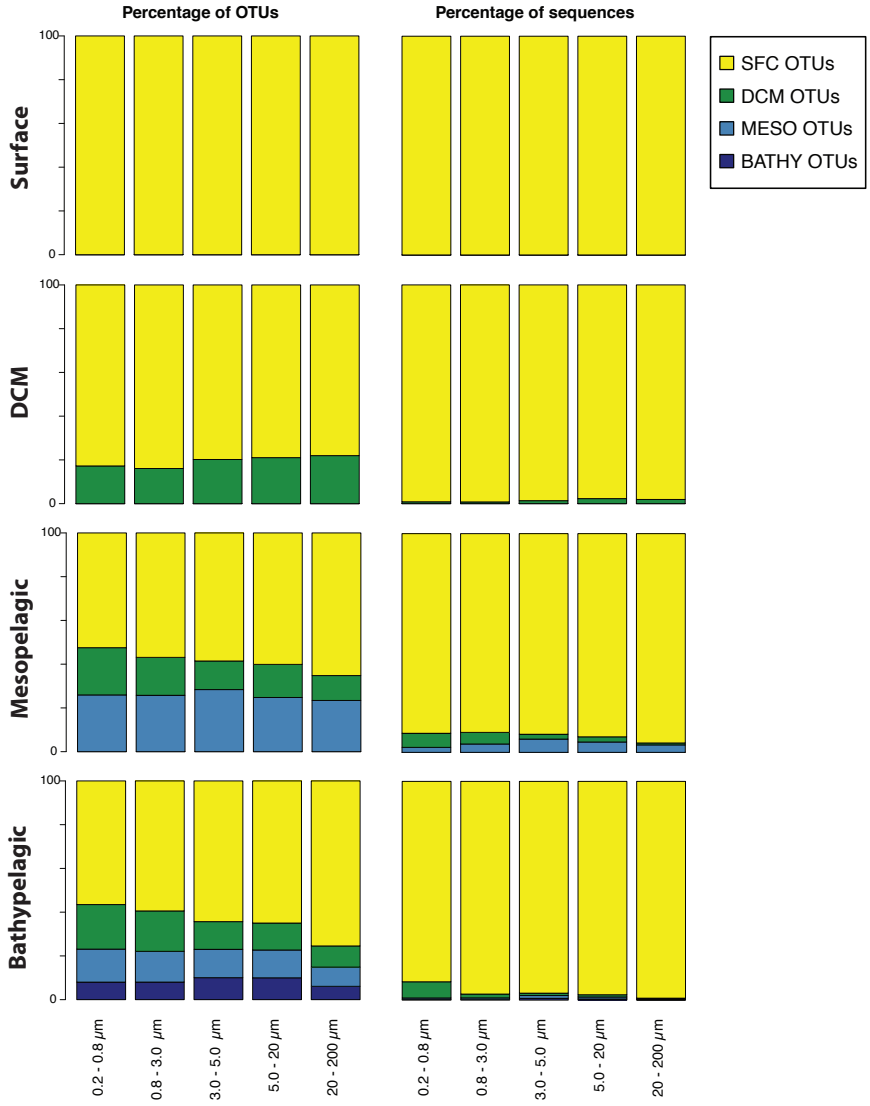

Figure 1. Contribution, in percentage of OTUs and percentage of sequences, of those OTUs categorized as 'SFC', 'DCM', 'MESO' and 'BATHY', in each depth and size fraction and considering all stations together. The category of each OTU was defined as the depth where they were first detected, assuming a directionality from surface to bathypelagic waters, and considering all stations together (see Results for details).

In order to differentiate between OTUs more dominant in smaller or larger size fractions, we defined a particle-association niche index (PAN-Index, see details in the Material and Methods section). OTUs were assigned PAN-Index values from 1 to 5 according to which of the 5 size fractions they were more prevalent (e.g. OTUs with PAN-Index=1 were more

prevalent in the 0.2-0.8 μm size-fraction, whereas OTUs with PAN-Index=5 were more prevalent in the 20-200 μm size-fraction). Intermediate values, as e.g. PAN-Index=2.3 indicate that this OTU appears preferentially in the 0.8–3.0 μm size-fraction in most sampling points, but in some it appears more abundant in size-fractions larger than 0.8 μm. The distribution of PAN-Index values showed two modes around values 3 and 3.5 (**Supplementary Figure 5**) and we used this distribution to differentiate two groups of OTUs, those enriched in small size-fractions (ES, PAN-Index <3) and those enriched in large size-fractions (EL, PAN-Index ≥ 3). Interestingly, we found that the preference for one lifestyle or the other seemed phylogenetically conserved to some extent, since whereas some orders such as SAR11, SAR324 or *Rhodospirillales* showed a preference for a free-living style, others like *Rhizobiales, Pseudomonadales, Cythophagales* or *Flavobacteriales* were preferentially enriched in large size-fractions (PAN indices ≥ 3, **Supplementary Figure 6**). Accordingly, large changes in taxonomic composition were observed between size fractions (**Supplementary Figure 3**), although in general, the three size-fractions larger than 3 μm were more similar among each other than they were to the smallest size fractions, at least at the Phyla level.

4.3.2 Vertical connectivity between oceanic prokaryotic communities

In order to assess the vertical connectivity between prokaryotic communities, we explored whether OTUs present at one depth could be detected in the other depths. To do so, all OTUs were categorized into 4 groups defined by the depth where they were first detected assuming directionality from surface to bathypelagic waters, and considering all stations together. For example, if an OTU was detected in any of the surface samples, it was categorized as 'SFC' OTU; instead, if an OTU was not detected in any surface sample but appeared in DCM waters, it was categorized as 'DCM', and so on. Finally, an OTU was categorized as 'BATHY' if it was only detected in bathypelagic waters, but not in any of the previous depths (surface, DCM or mesopelagic). For this categorization the non-rarefied OTU table was used, so that we could detect the largest number of OTUs per sample. This analysis showed that even though there were new OTUs appearing when moving from surface to bathypelagic waters (**Figure 1**), in terms of numbers of sequences, communities from all depths and size fractions were largely dominated by OTUs first detected

in surface waters ('SFC' OTUs). When this categorization of OTUs was done considering each station separately, we observed a similar pattern, but in some stations we detected a larger contribution of OTUs to deep layers that were not present in surface waters, particularly in the free-living fraction (**Supplementary Figure 7**). This indicates that some of the 'DCM', 'MESO' or 'BATHY' OTUs in some stations were not present at the surface of those particular stations, but were present in other surface stations. In any case, in all stations bathypelagic communities were still numerically dominated by SFC OTUs, including the suspended fraction, pointing to high vertical connectivity of the microbial communities in the open ocean.

Interestingly, we observed that suspended prokaryotic communities were vertically more different between each other than the communities from the largest particles. For example, we found the highest OTU turnover between assemblages from the 0.2-0.8 μm fraction (**Figure 2**), indicating a higher replacement of OTUs within the suspended communities across depths compared to communities attached to the largest particles. Conversely, moderately higher nestedness and niche breath values were found among communities from the largest size fraction when compared to smaller ones, suggesting that communities attached to larger particles are somewhat more connected throughout the water column than those free-living or attached to small particles (**Figure 2**). In order to further explore the latter, we divided the 'SFC' OTUs (i.e. OTUs detected in any of the surface stations, see above) into those enriched in small size-fractions (PAN-Index <3) and those enriched in large size-fractions (PAN-Index ≥ 3), and compared their distribution along the water column in both small (<3.0 μm) and large (>3.0 μm) size-fractions (**Figure 3**). Interestingly, whereas the relative abundance of SFC OTUs enriched in small fractions decreased with depth (from an abundance of 75% in surface to 25% in bathypelagic waters in small size-fractions (<3.0 μm) and from an abundance of 25% in surface to <12% in bathypelagic waters in large size-fractions (>3.0 μm), **Figure 3**, left), 'SFC' OTUs enriched in large fractions increased their abundance towards deeper layers, contributing to more than the 75% of community sequences in small size-fractions (<3.0 μm) and almost 100% of community sequences in large size fractions (>3.0 μm), in deep waters (**Figure 3**, right). This suggests that suspended prokaryotes and those associated to the smallest particles from surface waters have a relatively limited connectivity with deep-sea communities. Conversely, a very large percentage of deep-sea communities of all size-fractions

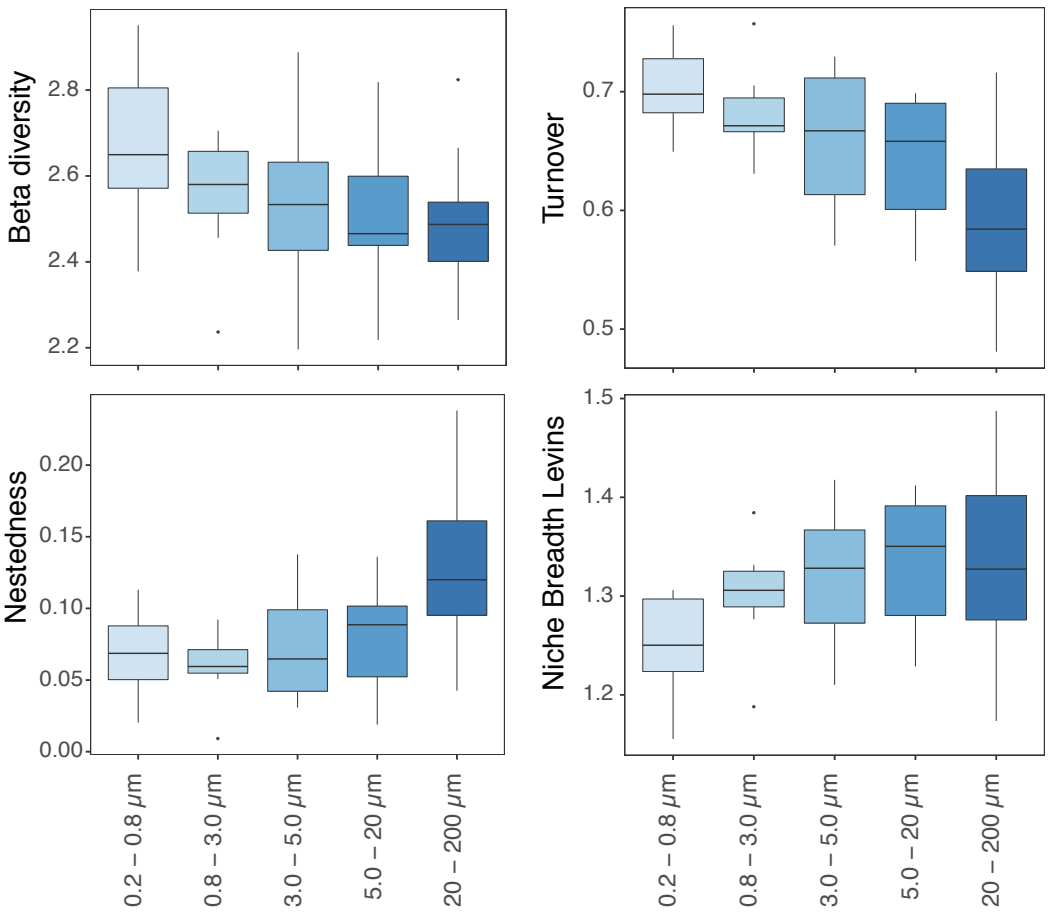

Figure 2. Vertical variation in OTU composition (beta-diversity), spatial OTU turnover (i.e. species replacement) and nestedness (species loss), and habitat specialization (niche breadth, calculated based on the number of depths where an OTU was found) across the different size fractions. Values were calculated separately for each station and size-fraction and were pooled together for each box-plot. See Material and Methods for details.

are surface OTUs attached to particles, pointing to the importance of the vertical transport of cells driven by the sinking of the largest particles.

Given the higher vertical transport of microbes in larger particles, we would expect that spatial differences (i.e. differences between stations) among communities from the largest particles are maintained vertically, whereas suspended communities should be more isolated vertically and thus more different across depths. We tested this hypothesis by comparing, for each size fraction, community dissimilarities between surface communi-

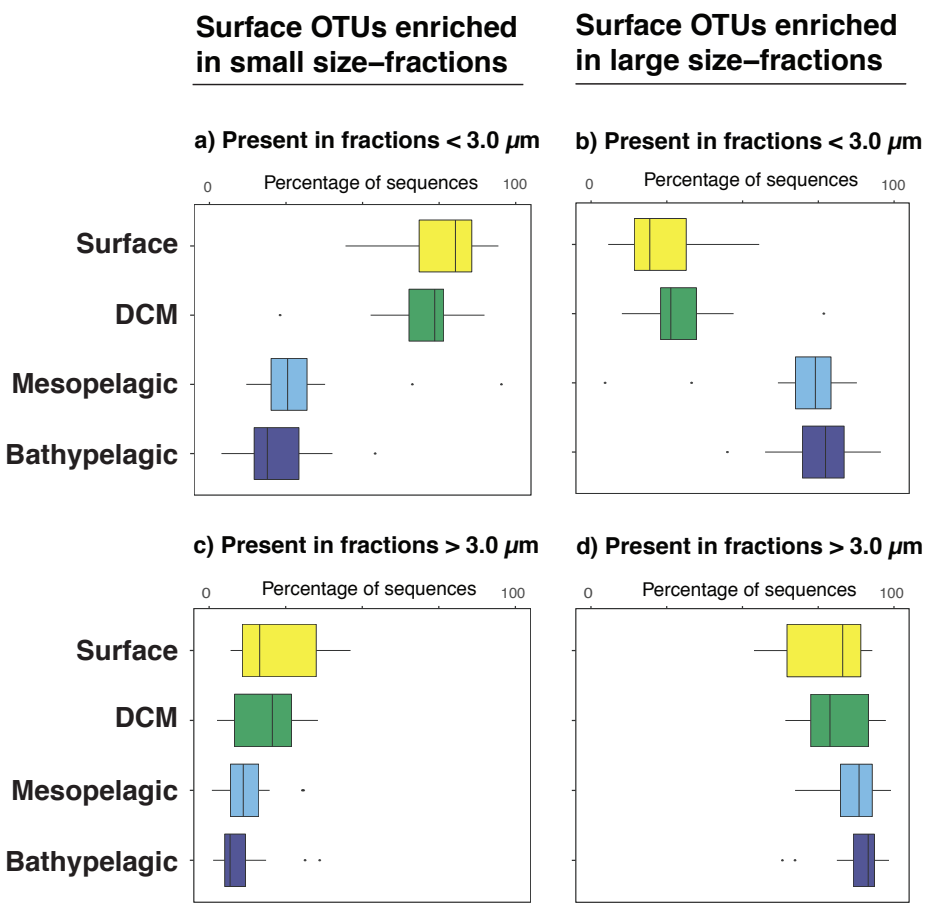

Figure 3. Vertical variation of the contribution (in percentage of sequences) of surface OTUs enriched in small size-fractions (PAN-Index<3) (left) and surface OTUs enriched in large size-fractions (PAN-Index≥3) (right) to communities present in fractions < 3.0 µm (a,b) and fractions > 3.0 µm (c,d) and at each depth. See Material and Methods for further details.

ties and i) meso- or ii) bathypelagic communities using Mantel tests (**Figure 4**). We found that the dissimilarities between suspended communities from the surface and meso- or bathypelagic waters were not significantly correlated. Yet, dissimilarities between particle-attached communities from the surface and the deep waters presented a higher significant correlation (**Figure 4**), suggesting that the compositional differences between deep sea particle-attached communities were caused, at least partially, by the biogeographic patterns of surface particle-attached assemblages.

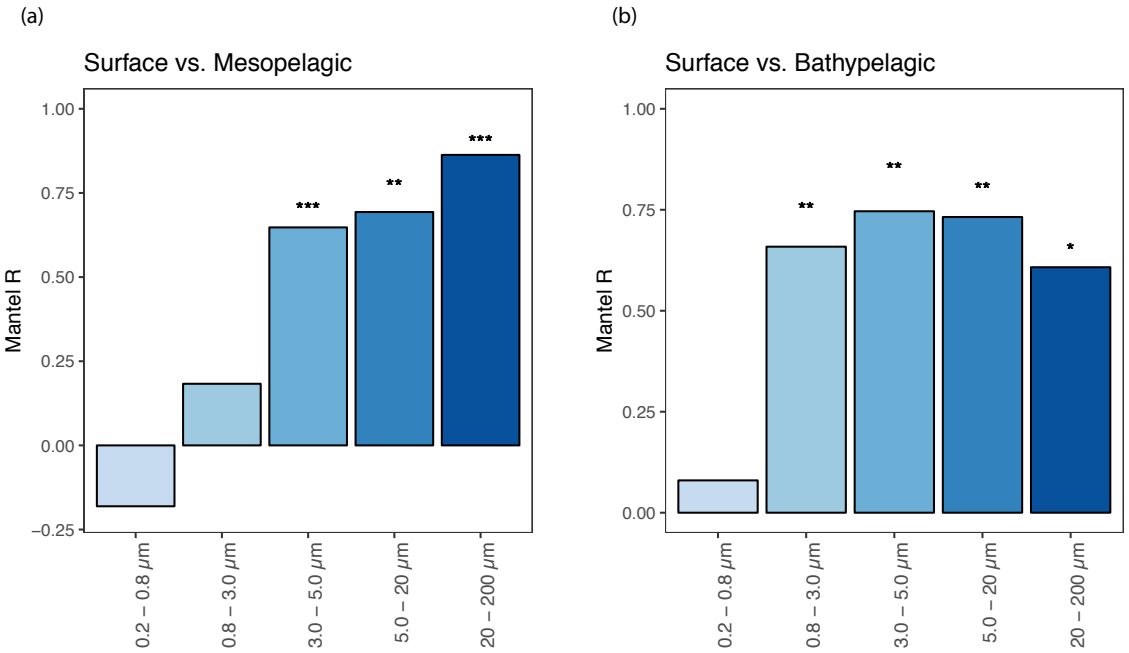

Figure 4. R coefficients of the Mantel correlations between the taxonomic dissimilarity matrices from surface and mesopelagic communities (a) and surface and bathypelagic communities (**b)** for each of the five size fractions. Higher R values mean that the compositional differences between communities at a given depth were highly correlated (and thus were similar) to differences between communities from a different depth. Significance of the correlations is stated as follows: '***' p<0.001; '**' p<0.01; '*' p<0.0

The previous results indicate that the biogeography of surface particle-attached prokaryotes is transferred to deeper waters via particle sinking. However, the fact that surface and deep communities were compositionally different (**Supplementary Figures 3, 4**) suggests that this vertical dispersal of particle-attached microbes must be accompanied by changes in their abundances during sinking which, however, maintain the community differentiation observed in the surface. To further test this idea, we detected and analyzed, for each station, the surface OTUs prevalent in the larger size-fractions that increased in relative abundance with depth, hereafter 'seed' OTUs (see details in Methods). We also compared whether these OTUs were similar or different between stations. We identified 69 seed OTUs in total, that showed clear increases in relative abundance towards deeper waters, comprising up to 60% (average ~40%) of abundance in bathypelagic particle-attached communities (**Figure 5**). Interestingly, different genera dominated seed sequences at different stations (e.g.: *Oceanospirillales* in Station 20, *Sphingomonadales* in Station

56, *Corynebacteriales* in Station 94) (**Figure 5**), and the nMDS of the seed subcommunities showed eight clusters, corresponding to the eight stations (**Supplementary Figure 8**). This would indicate that the biogeography of the surface particle-attached seed prokaryotes partially determines the biogeography of both particle-attached and free-living deep ocean communities.

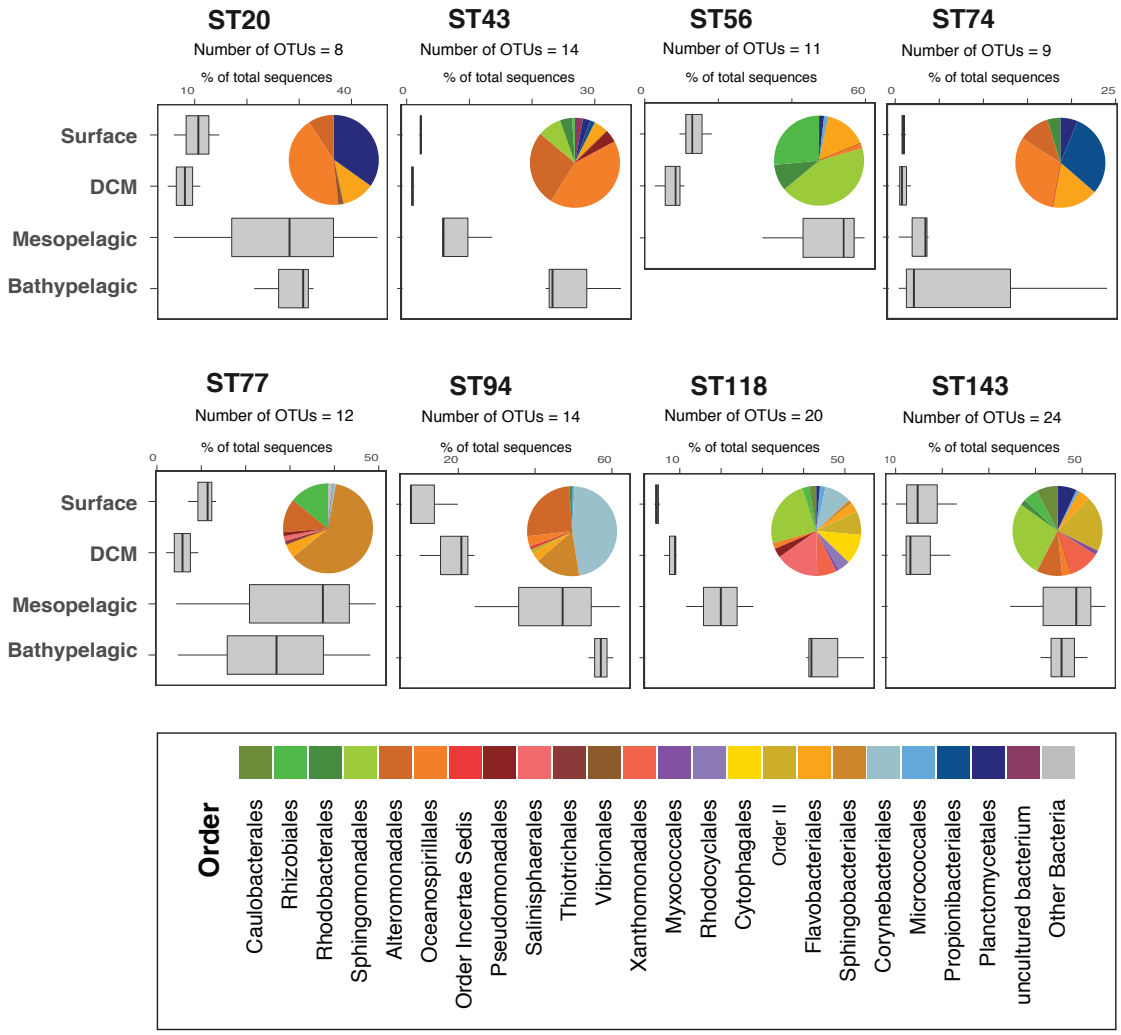

Figure 5. Dynamics of 'seed' OTUs per station and across depths. Seed OTUs are those surface OTUs enriched in the larger size-fractions that increased in relative abundance with depth. Data represent the contribution of the OTUs categorized as 'seeds' to the total sequences of communities associated to the largest size fractions (>3.0 µm, see Results for details). Pie charts indicate the taxonomic composition at the Order level (in % of sequences) of the seed OTUs at each station.

4.4 DISCUSSION

One of the dominant processes exporting biological material into the deep ocean is the sinking of particles formed in the photic layer (Ducklow *et al*. 2001), which are rapidly colonized by bacterial communities (Kiørboe *et al*. 2002, Grossart *et al*. 2003). Even though this is a well-recognized process, the role and generality of particles as vehicles transferring prokaryotic taxa from the surface to the deep oceanic realms has not been addressed until now. Here, we explored the vertical connectivity in the ocean microbiome by using a serial filtration system to separate marine prokaryotic communities into five different size-fractions and exploring their compositional variability from the surface down to 4,000 m across eight globally distributed open ocean stations. We show that most abundant prokaryotes in the deep-ocean are also present in surface waters in all size-fractions, pointing to vertical connectivity for most of the members of the community. In particular, since we observed that most abundant prokaryotes living in suspension in the deep ocean were also present in surface waters, suspended or in particles, we can argue that particles may function as a vector that inoculates, to a certain extent, deep-sea suspended communities. Besides, we observed other less abundant taxa that seem indigenous of the deep ocean. We also found that particle colonization processes occurring in surface waters can determine to a considerable extent the structure of the communities inhabiting particles in the deep ocean, as well as their biogeography; these patterns were stronger with increasing particle size. Finally, we observed that particle-attached local prokaryotic assemblages were vertically linked by particle sinking.

Communities from the meso- and bathypelagic realms clustered separately from those inhabiting surface and DCM as has been previously observed and is well known (e.g. Baltar *et al*. 2007, Brown *et al*. 2009, Pommier *et al*. 2010, Agogué *et al*. 2011, Kembel *et al*. 2011, Ganesh *et al*. 2014, Walsh *et al*. 2015, Milici *et al*. 2017), yet we add that this occurs in all size fractions (**Supplementary Figures 3, 4**). As most abundant deep-sea taxa were also present in surface waters, both concepts necessarily imply important changes in species relative abundances with depth, rather than in species turnover. Our results agree also with previous studies indicating that prokaryotic communities differ largely between suspended and attached fractions both in epipelagic (Thiele *et al*. 2015, Milici *et al*. 2016)

and also in bathypelagic (Eloe *et al*. 2011, Salazar *et al*. 2015) waters. Vertical differences in free-living and attached prokaryotic community composition were also observed in the NW Mediterranean in profiles from the surface to 2,300 m depth, and in particles ranging from 0.2 to 200 μm (Mestre *et al*. *submitted*).

4.4.1 Substantial vertical connectivity between oceanic microbial assemblages

The finding that all communities, including those inhabiting the bathypelagic, were numerically dominated by OTUs that could be detected in the surface (**Figure 1, Supplementary Figure 7**) supports a strong vertical connection between surface and bathypelagic communities at the global scale. Only a few recent studies have assessed this vertical connectivity, indicating that the advection and the convection of water masses can shape the structure of surface and deep microbial communities by promoting their transport and increasing chances for colonization (Wilkins *et al*. 2013, Luna *et al*. 2016, Severin *et al*. 2016). These studies, however, have focused only on the free-living assemblages. Studies exploring changes in prokaryotic assemblages during particle sinking have also been restricted to shallower depths and to our knowledge ours is the first attempt to assess the extent to which bathypelagic communities associated to different size-fractions are also present in surface waters.

Our results supporting a strong vertical connection within local communities lead us to hypothesize that there is dispersion from surface to deeper waters driven by sinking particles. We expected this transport to be faster in larger particles, which are likely to sink more rapidly than small ones. Accordingly, our results indicate (**Figure 2**) that the connectivity along the vertical column is higher for communities associated to the largest particles than for those attached to the smaller ones. For example, the community composition of the suspended communities and those associated to the smallest particles differed more between depths (larger beta-diversity) than those in the largest size-fractions (**Figure 2, Supplementary Table 2**). Also, the highest OTUs replacement (turnover) across depths was found between communities from the smallest size-fractions, and the highest vertical nestedness in larger particles. Accordingly, OTUs associated to the largest particles were present across a higher number of depths (i.e. had broader niche breadths) than OTUs as-

sociated to smaller particles (**Figure 2**). Altogether, this suggests that the vertical changes in prokaryotic assemblages attached to the largest particles are due to changes in the relative abundances of taxa present through several depths, whereas prokaryotic communities from the smallest size fractions present more restricted depth-distributions. In addition, we observed that most meso- and bathypelagic community sequences were associated to large particle-attached OTUs already present in the surface, whereas OTUs enriched in the smallest fractions in the surface comprised less that 20% of meso- and bathypelagic sequences (**Figure 3**), and this pattern was comparable across the 8 stations. The fact that also the deep free-living communities (in size-fractions <3.0 μm) were composed of mostly surface-attached prokaryotes (in size-fractions >3.0 μm) suggests that large particles are indeed vectors transporting viable prokaryotes that can thrive in the free-living fraction. Thus, the transport of prokaryotes from surface to deep waters would be mostly via large particles, which would then be a source of potential immigrants (or inoculum) to the suspended community living in the deep ocean.

4.4.2 Transfer of biogeographic patterns from surface to the deep sea

Despite a general perception of an homogeneous dark ocean, genomic approaches have unveiled the enormous and dynamic genetic variability of the deep sea microbial communities (reviewed in Arístegui *et al*. 2009). Indeed, a recent global survey of prokaryotic communities in the bathypelagic realm showed that they differed between basins and that this biogeographic signal was stronger for the particle-attached members (Salazar *et al*. 2015), which agrees with our observation that, at all depths, communities from the largest particles presented much clearer differences between stations than the free-living communities (**Supplementary Table 2**). But, more interestingly, we found that the compositional differences between surface stations tended to be correlated with spatial differences in the deep-sea layers for particle attached communities, while no such a pattern was observed when correlating suspended surface communities vs. their deep-layers particle-attached counterparts (**Figure 4**). Altogether, this points to a transmission of surface biogeographic patterns to the deep-sea in particle associated communities, and suggests that the biogeography of deep ocean microbial communities may be partially determined by the attached bacteria coming from the surface. Salazar *et al*. (2015) suggested that submarine moun-

tains that divide the deep ocean into basins might act as 'ecological barriers' for prokaryotic communities, thus favoring their differentiation. Our results indicate, in contrast, that bathypelagic communities may be influenced more by communities arriving in sinking particles than by geographic (basin) isolation. Particles have a highly heterogeneous organic and inorganic composition that, even if altered during sinking, is mainly defined by the environmental conditions of the surface waters (Buesseler and Boyd 2009, Guidi *et al*. 2009, Bach *et al*. 2016). Such composition would determine colonization dynamics and the initial microbial community (reviewed in Simon *et al*. 2002).

Given the considerable variability in community composition populating large particles with depth (**Supplementary Figures 3, 4**), it looks like that the influence of surface particles on deep waters should be through the transport of attached-taxa that would change their abundances during sinking. Also, if the initial colonization of surface particles plays a role, then the pool of particle-dispersed taxa that has the potential to increase their abundances when arriving to deeper layers should differ across stations. We tested this idea by identifying the pool of surface particle-attached bacteria that increased their abundances as we moved towards deeper waters ('seed' taxa, see Methods). We could detect 'seed' OTUs in all stations (**Figure 5**), but interestingly, they belonged to different taxonomic groups in the different stations. This may indicate the importance of the initial surface inoculum in determining deep ocean microbial communities in all size-fractions, which is further supported by the clear station-specific taxonomic signature of the 'seed' OTUs (**Supplementary Figure 8**). This scenario would imply that sinking particles transport diverse communities, yet some of these taxa (maybe dormant or slowly growing) have the potential to grow and dominate deep-ocean communities when the surrounding environmental conditions or the nature of the particle changes.

Focusing on the free-living prokaryotes, Wilkins *et al*. (2013) suggested that advection of seawater masses can shape microbial community structure by increasing opportunities for colonization. This has also been observed in other ecosystems, such as the river-to-lake freshwater continuum, where the transport of rare bacteria from a terrestrial source environment was shown to be crucial for determining the structure of the receiving aquatic communities (Ruiz-González *et al*. 2015). Given the limited water mixing between dif-

ferent ocean layers, sinking particles may play a crucial role in determining and shaping the vertical connectivity of oceanic microbial communities by allowing the continuous dispersal of viable organisms into the dark ocean. This is of particular importance in the deep ocean, where particles and their attached bacteria likely play major biogeochemical roles (Bochdansky *et al*. 2016): The bathypelagic is constituted mostly by slow sinking or buoyant particles, younger than the dissolved counterpart (Herndl and Reinthaler 2013), which are resource-rich habitats for microbes (Bochdansky *et al.* 2016). Prokaryotes in the deep ocean are more adapted to the attached lifestyle than the surface ones (DeLong *et al*. 2006, Martín-Cuadrado *et al*. 2007, Lauro and Bartlett 2008, Lauro *et al*. 2009) and microbial activity might be concentrated on particles (DeLong *et al*. 2006, Arístegui *et al*. 2009, Baltar *et al*. 2009).

4.5 CONCLUSION

In summary, we found that global ocean prokaryotic communities exhibit strong vertical connectivity through the entire water column, and that such connection occurs via particle sinking. The latter also highlights the role of particles as microbial vectors that bring viable surface taxa into the deep ocean. In addition, our results indicate that all local communities seem dominated at all depths by OTUs that are already present in surface waters, and that the biogeography of the bathypelagic realm is influenced, to some extent, by particle-colonization events occurring in surface waters. The contribution of both particles and their attached communities to the bathypelagic realm seems therefore crucial: First, particles are a well demonstrated source of carbon and nutrients essential for the development of heterotrophic life in the deep ocean (Arístegui *et al*. 2009, Herndl and Reinthaler 2013). In addition, our results indicate that sinking particles comprise a source of viable diversity to deeper ocean layers. Overall, our work contributes to increase our understanding of the role of sinking particles and their attached prokaryotes in the assembly and structuring of deep ocean communities as well as in the connectivity of communities across the water column.

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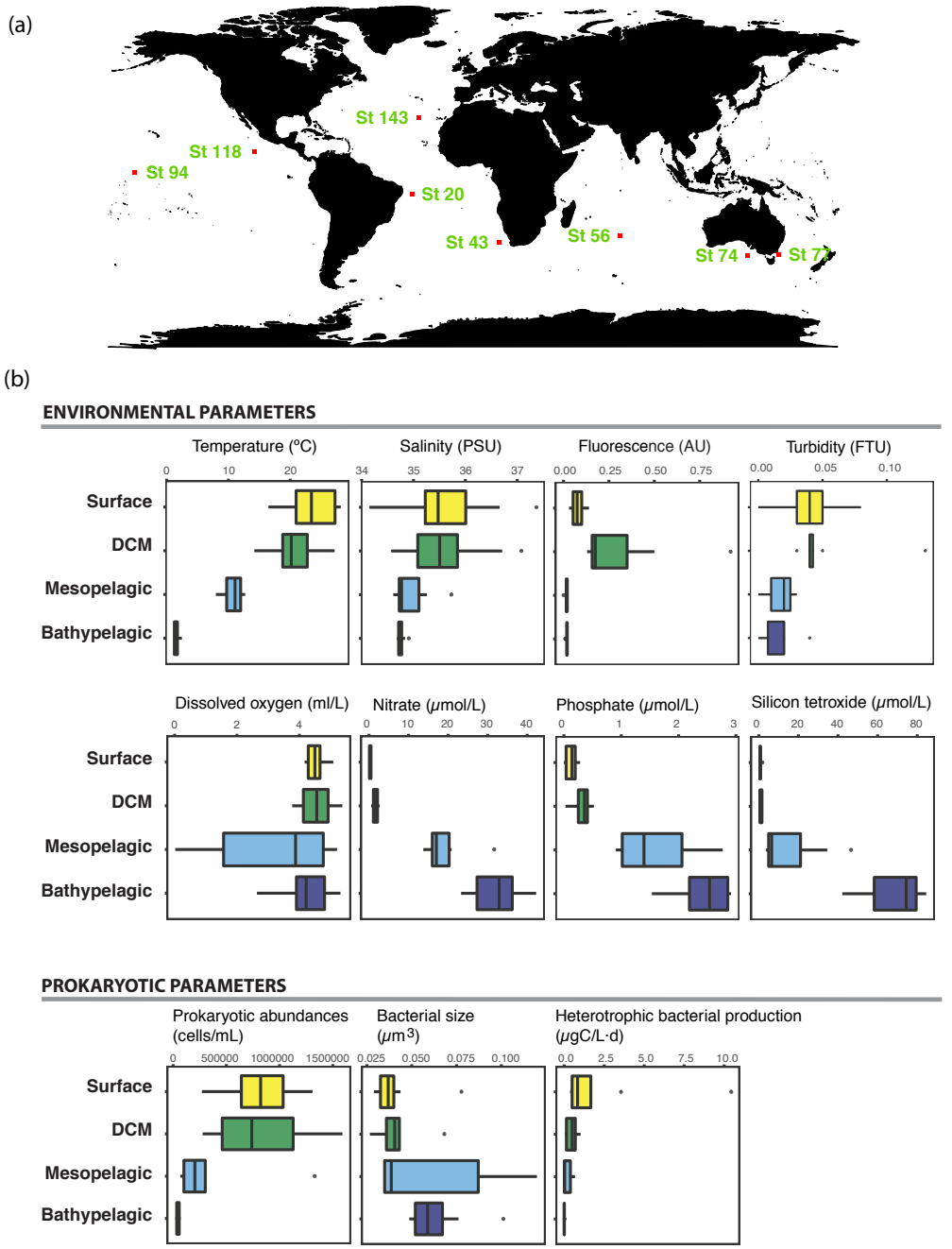
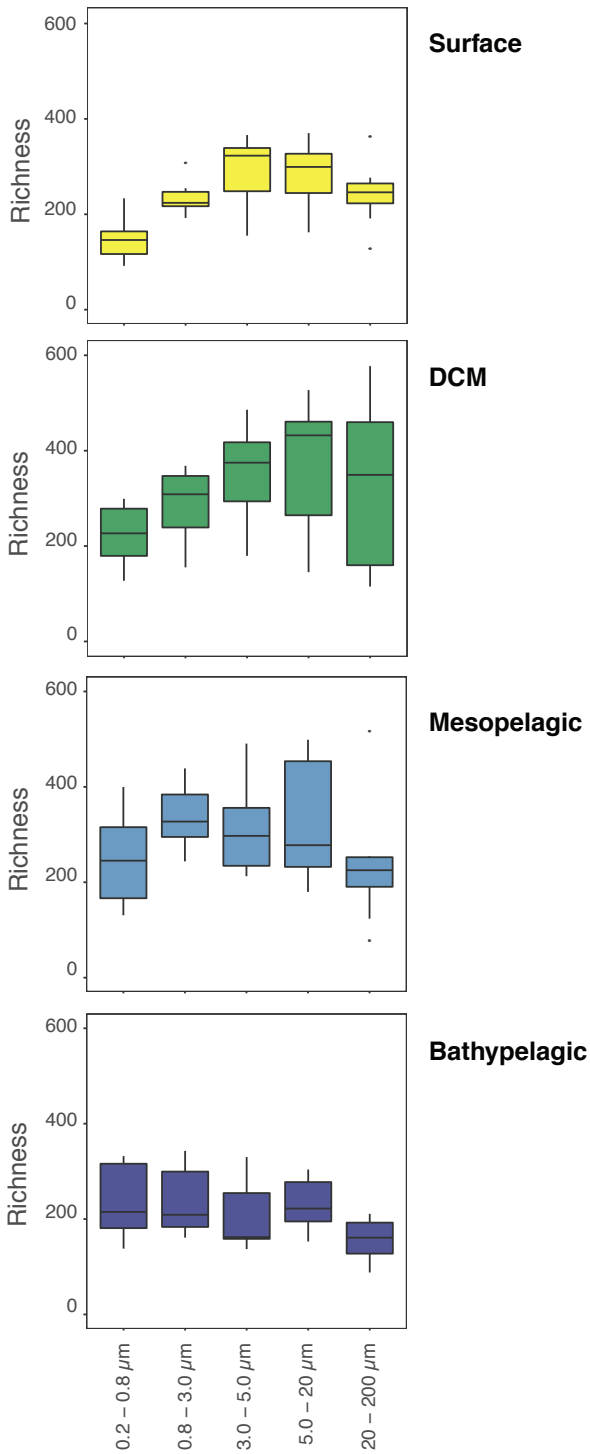
4.8 SUPPLEMENTARY MATERIAL

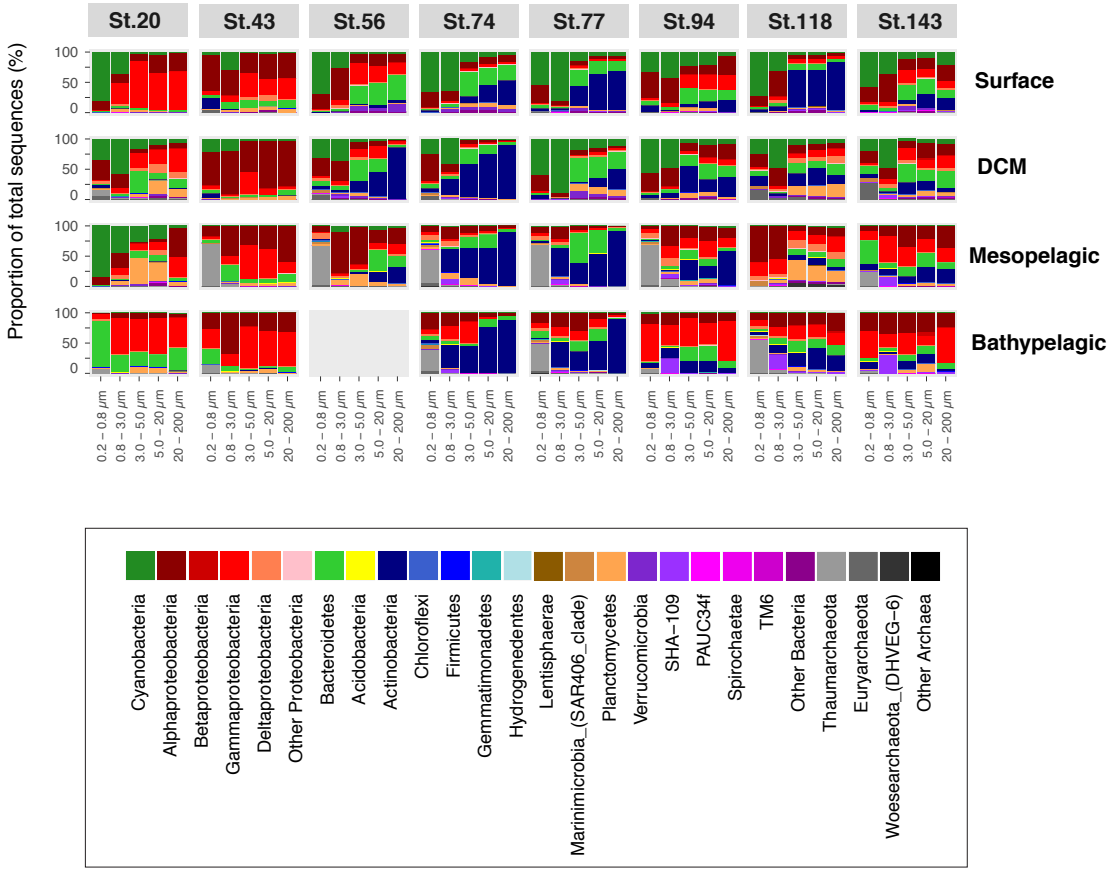
4.8.1 Supplementary Figures



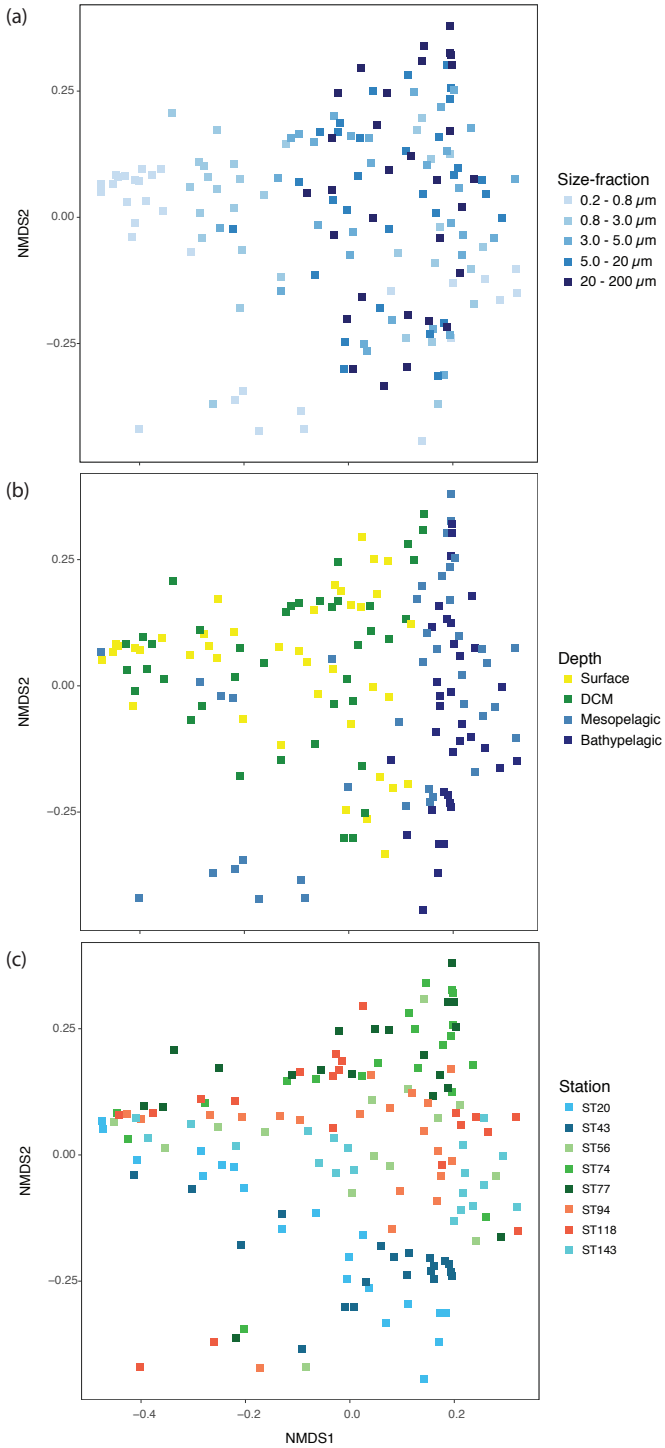
Supplementary Figure 1. Oceanographic context. **(a)** World map showing the sampled Malaspina cruise stations. **(b)** An overview of environmental and prokaryotic data at each depth. Distribution of values in the studied stations.



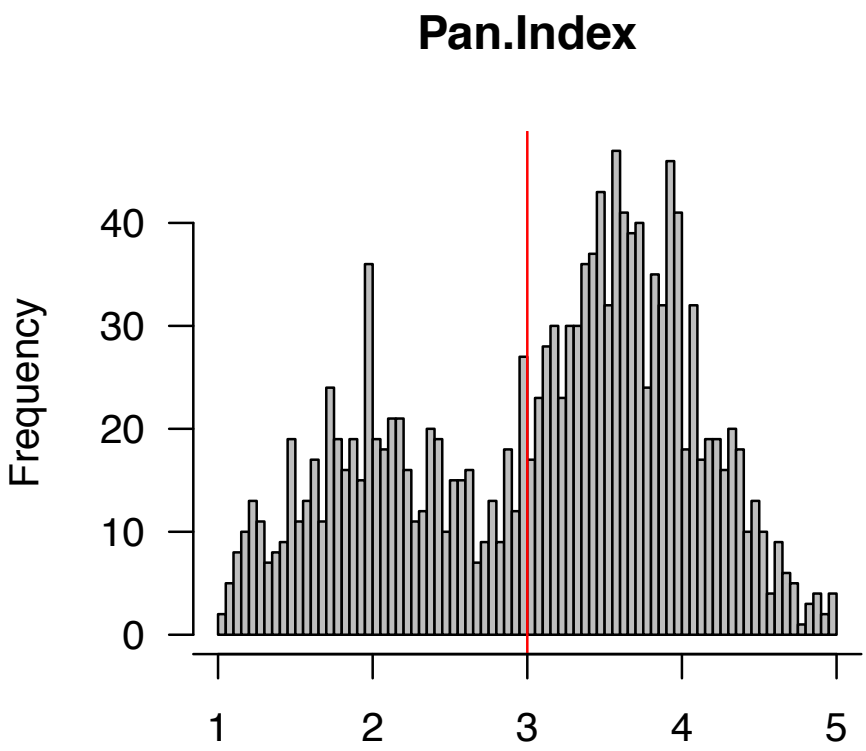
Supplementary Figure 2. OTU richness (number of OTUs) at each size-fraction and depth. Boxplots comprise data from the 8 stations.



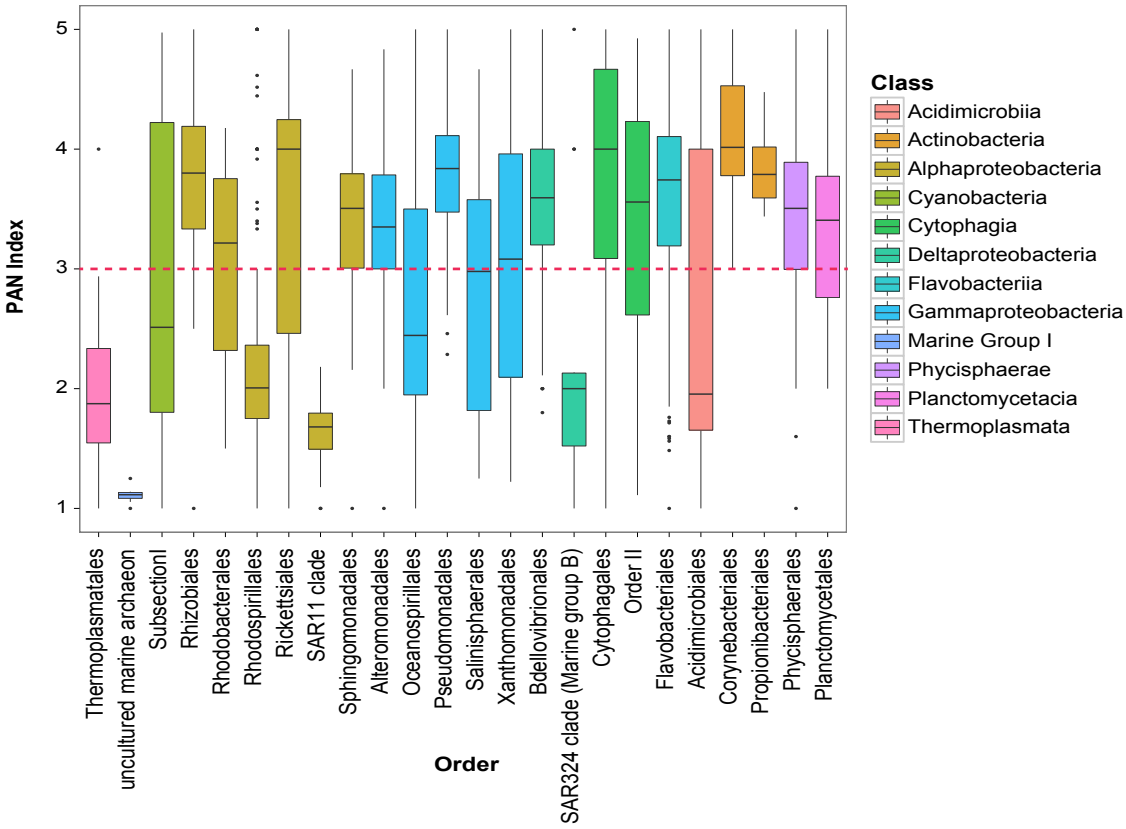
Supplementary Figure 3. Taxonomic composition, at the phylum level, at each station, depth and size-fraction. The phylum proteobacteria is separated into the different classes. Values represent the fraction of the total sequences.



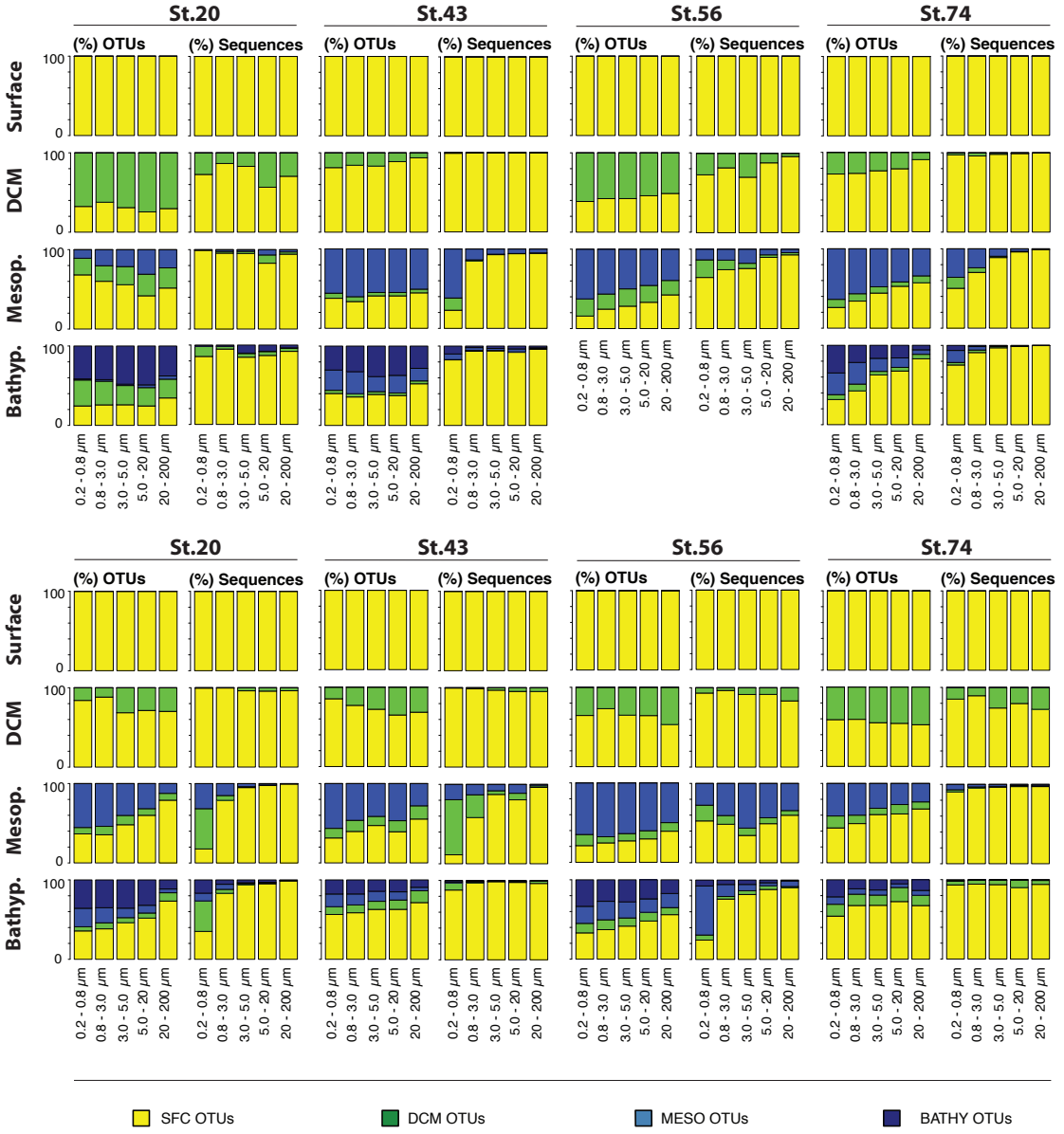
Supplementary Figure 4. nMDS ordinations representing spatially the Bray-Curtis distances between prokaryotic communities. Distances were calculated from the rarefied OTU table. Samples are color-coded depending on size-fraction (**a**), depth (**b**), and sampling station (**c**).



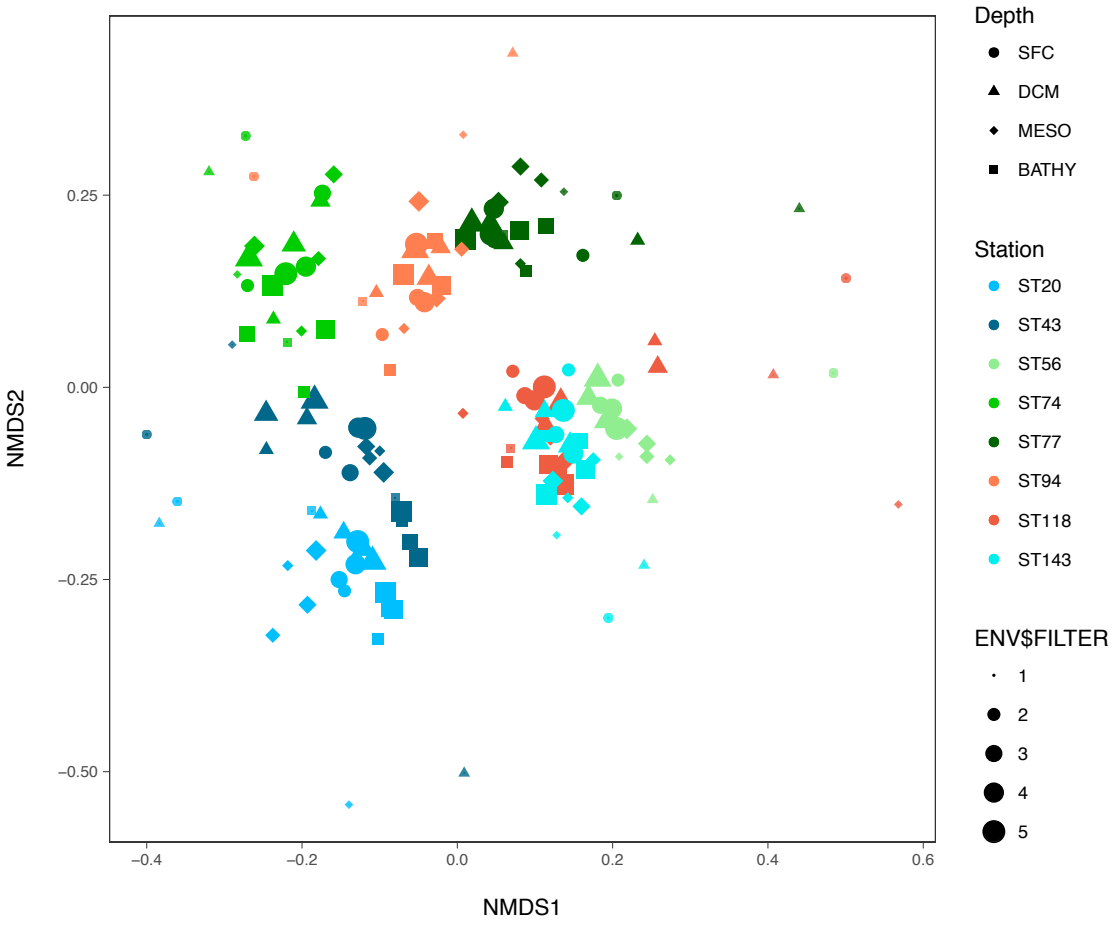
Supplementary Figure 5. Histogram of the distribution of the Particle Association Niche (PAN)-Index values of all the OTUs observed with more than 10 reads (N=3616). The PAN-Index takes values from 1 to 5, relative to the 5 size-fractions. OTUs were assigned values from 1 to 5 depending on the size-fraction where they were enriched (see Methods for details). The red line indicates what we took as the middle point, PAN-Index=3, which we used to distinguish two main groups of OTUs: those mainly enriched in small size-fractions (PAN-Index<3), and those mainly enriched in large size-fractions (PAN-Index≥3).



Supplementary Figure 6. Particle-association niche index (PAN-Index) values of the 24 most abundant Orders. The red dashed line corresponds to a PAN-Index of 3, and separates those Orders that are more prevalent in large size-fractions from those that are enriched in the small size-fractions (see Suppl. Figure 5). Colors indicate the different taxonomic Classes to which the Orders studied belong.



Supplementary Figure 7. Contribution, in percentage of OTUs and percentage of sequences, of those OTUs categorized as 'SFC' (surface, yellow), 'DCM' (DCM, green), 'MESO' (mesopelagic, blue) and 'BATHY' (bathypelagic, dark blue) in each depth and size fraction and for each station separately. The category of each OTU was defined as the depth where they were first detected, assuming a directionality from surface to bathypelagic waters (see Results for details), and was estimated for each station separately.



Supplementary Figure 8. nMDS ordinations representing the Bray-Curtis distance between prokaryotic communities considering only the OTUs identified as 'seeds' at each station.

4.8.2 Supplementary Tables

Supplementary Table 1. Permutational multivariate analysis of variance (PERMANOVA) examining the effects of the factors station, depth and size-fraction on the prokaryotic communities. Key to abbreviations and column headings: D.f, degrees of freedom; SS, sum of squares; MS, mean square; F, F ratio; R^2, coefficient of determination; P, p-value. Significance was stated as follows: '***' p<0.001; '**' p<0.01; '*' p<0.05.

	Df	SS	MS	F	R^2	P	
Size-fraction	4	6.80	1.70	7.90	0.12	<0.001	***
Station	7	13.10	1.88	8.73	0.23	<0.001	***
Depth	3	6.32	2.11	9.78	0.11	<0.001	***
Residuals	140	30.13	0.22		0.53		
Total	154	56.40			1.00		

Supplementary Table 2. Summary of the coefficient of determination (R^2) from the Permutational multivariate analysis of variance (PERMANOVA) examining the effects of the factors station and depth into each of the five size-fractions.

	by Station		by Depth	
	R^2	signif	R^2	signif
0.2-0.8 µm	0.16	0.96	0.50	<0.001***
0.8-3.0 µm	0.36	0.004 **	0.33	<0.001***
3.0-5.0 µm	0.48	1e-04 ***	0.14	<0.05*
5.0-20 µm	0.55	1e-04 ***	0.11	0.28
20-200 µm	0.56	1e-04 ***	0.10	0.39

General discussion and Future perspectives

General discussion and Future perspectives

The complexity in composition and structure of organic matter, along with variable supply regimes, is probably one of the major factors that help to maintain a high diversity of prokaryote communities in the oceans. Organic matter-bacteria interactions also exert a large influence on the major properties and patterns of ecosystems, including primary production, food web organization, and biogeochemical fluxes.

Nagata (2008). Organic matter-bacterial interactions in seawater

In: Kirchman, D.L. (ed). Microbial Ecology of the Oceans, 2nd Ed. Wiley

This discussion aims to examine and summarize the main findings of this thesis by highlighting our contribution to a better understanding of the structure of the prokaryotic communities in the pelagic habitat and their relation with the dissolved and particulate matter. We moreover try to connect our findings to what is known about carbon fluxes and biogeochemical cycles, and provide hints that can be useful for biogeochemists and modellers. In addition, we try to open future questions that still need to be answered and we discuss about the main problems that we encountered during the development of the work here reported and how we solved them. In addition, we discuss how new ideas and new advances in technology can help us better understand the role of prokaryotes in the ocean.

1 Dividing the plankton into size-fractions

Research in marine biology taking into account the distinct sizes of plankton has been performed for more than a century. Schütt (1892) was likely the first to introduce size categories to plankton studies and used the terms "micro-", "meso-" and "macro-". Almost a century later, Sieburth *et al*. (1978) completed the range of sizes, which comprised from the "femto-" (0.02 μm) to the "mega-" (200 cm), linking the sizes of plankton with those of the nekton (**Figure 1**).

Body size is the most important trait characterizing pelagic organisms (Andersen *et al*. 2015) and still now the Sieburth *et al*. (1978) compartments are the most common way of classifying plankton by size. Now, almost 40 years after the Sieburth *et al*. (1978) classifi-

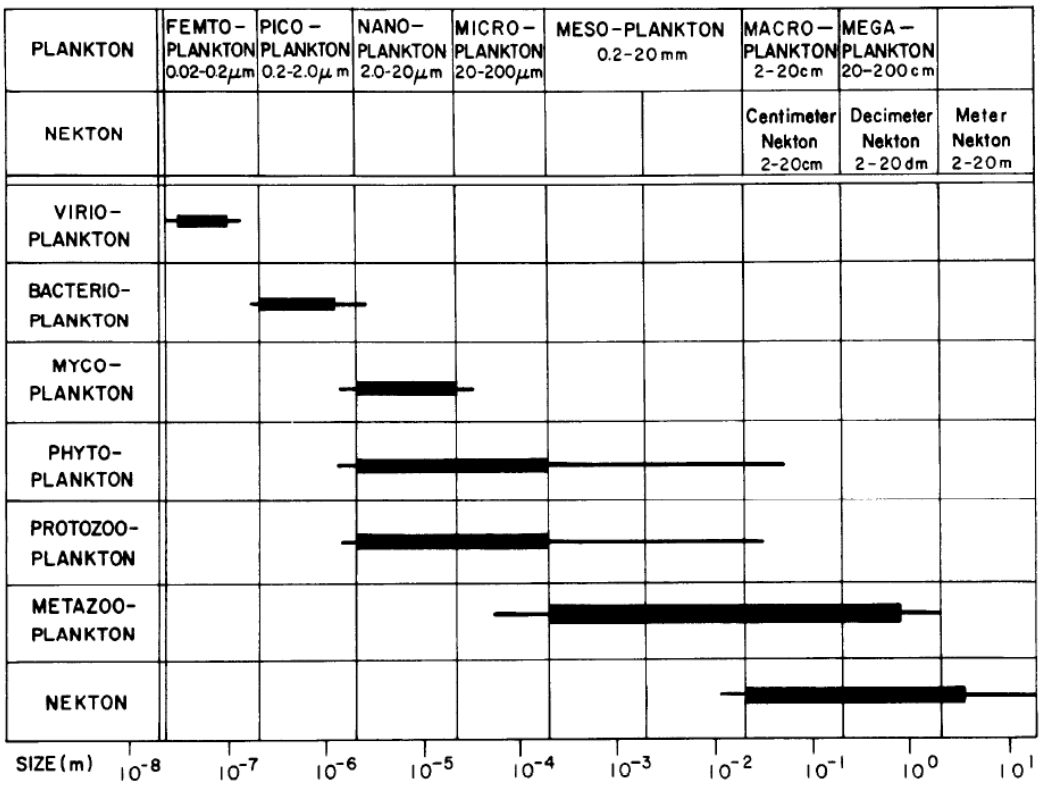


Figure 1. Distribution of different compartments of plankton in a spectrum of size fractions, with a comparison to the size range of nekton (Figure from Sieburth *et al*. 1978).

cation, knowledge about the unicellular organisms present in the plankton has increased a lot and it is likely that this Sieburth *et al*. (1978) classification has to be modified partially. For example: mycoplankton and protozooplankton could be placed into the "unicellular eukaryotes" or "protists", the "pico-eukaryotes" should also be included, and the Bacterioplankton should be called Prokaryoplankton to also include Archaea.

The classification by size is also useful for sampling optimization, as the organisms of each compartment also have a characteristic concentration (abundance per volume) which demands a particular sampling methodology: as an example, nano- and picoplankton is normally sampled with a niskin bottle, whereas zooplankton is traditionally sampled with a plankton net (Karsenti *et al*. 2011). However, the needs for development of new sampling strategies to better access the whole spectrum of bacterial lifestyles, as well as to examine the ecology of prokaryotes at the scale at which they experience the environment, have recently been voiced (Azam and Malfatti 2007, Grossart 2010).

In this thesis, we used a multi-fractionation sampling scheme that allowed us to better characterize the archaea and bacteria present in distinct size-compartments (from 0.2 to 200 μm), where the content of the compartments can be plankton, organic detritus or particles of distinct size. With this sampling procedure we show that prokaryoplankton appear associated to structures of distinct sizes, and we also describe that whereas there are OTUs present in all size-ranges, there are others present only in a given size-fraction, and we also observed that prokaryotes present in distinct size-fractions can have distinct temporal and spatial dynamics. Therefore, the study of planktonic prokaryotes should not be restricted to the size-compartment correspondent to its cell size (0.2-2.0 μm), and shoud be extended to all size-ranges of structures that are present in the pelagic habitat. There are some studies that have taken into account the prokaryotes attached to particles (most of them cited through the chapters of the thesis). Yet, most studies analyzing archaea and bacteria in the oceans are mostly focused on the free-living ones, i.e. only those prokaryotes present in the compartment correspondent to their cell size.

Finally, during this thesis we have seen that consideration of the size-compartment where prokaryotes are associated is important not only to better describe the communities and

their variability over time and space, but also as to consider them as parts of the communities dispersal mechanisms: in **Chapter 4** we describe how large particles contribute to the dispersal of surface communities towards the bathypelagic realm. Since it would be interesting to analyze what occurs with the prokaryotes attached to certain plankton compartments, as e.g. zooplankton, an interesting question arises: Is nekton also a dispersal vector for prokaryotes?

2 Spatial and temporal scale framework

Environmental heterogeneity is fundamental for the structure and dynamics of ecosystems (Levin 1992). Natural ecosystems are heterogeneous at scales ranging from microhabitats to landscapes and in the water column, planktonic organisms have a strongly heterogeneous distribution (Giller *et al*. 1994, Pinel-Allou 1995). As ecological systems are hierarchically organized (O'Neill *et al*. 1986), defining and integrating distinct scales of study is the best strategy to describe and understand this high heterogeneity. Pinel-Allou described the distinct categories of spatial and temporal scales in marine systems where microorganisms play a role (**Figure 2**). The scale can also give us a hint about what types of processes dominate: when the scale increases, the system is more dominated by abiotic processes, and when it decreases, the system is more dominated by biotic processes (Hewson *et al*. 2006). Moreover, as a general rule, when increasing the scale, the variability of the system declines and the predictability increases (Levin 1992).

The spaltial and temporal framework of the studies performed during this thesis are represented in **Figure 2**. We have analyzed microbes present in particles ranging from 0.2 μm to 200 μm. Thus, if we take the definition strictly, we have analyzed the pico-, nano- and micro-scale. Yet, there are no publications using the term microscale to refer to filtered particles but the term "microenvironment" has already been used in a multiple size-fractionation approach similar to the one we used (Yung *et al*. 2016). We also observed that in the range of sizes from 0.2 μm to 200 μm the species-accumulation curve of the microorganisms tended to saturation (**Chapter 1** and **Chapter 2**). And as we have discussed in **Chapter 1**, this information is relevant as it indicates that the whole communities present from the pico to the micro-structures are well characterized.

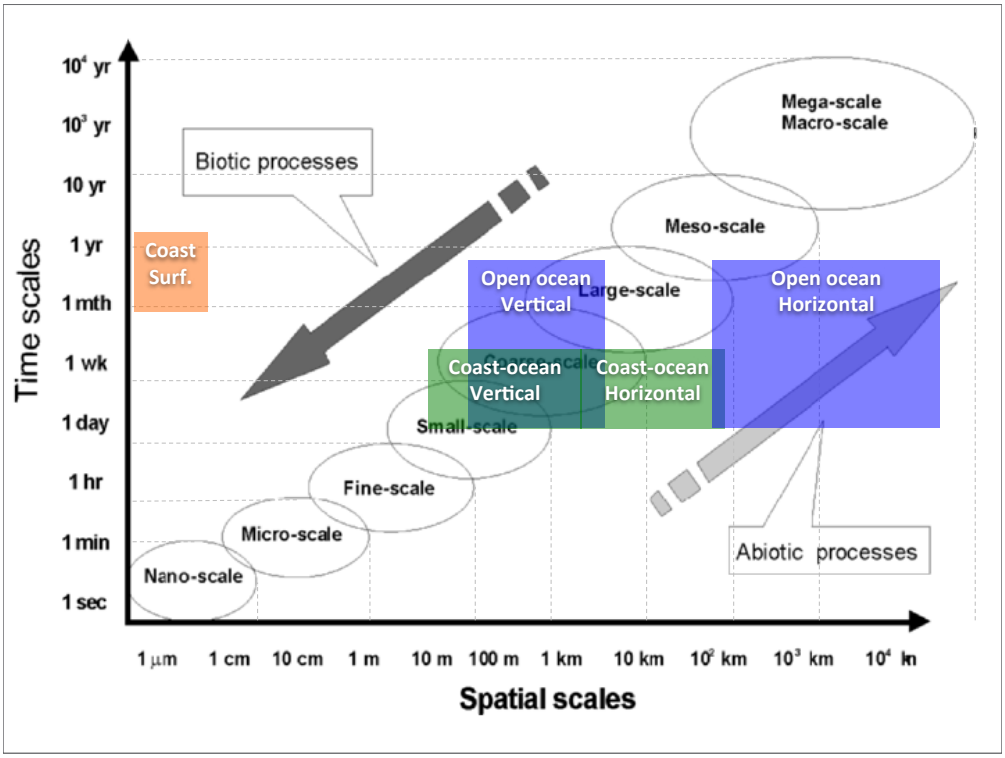


Figure 2. Categories of spatial and temporal scales in marine systems (adapted from Pinel-Allou and Ghadouani 2007). The rectangles represents the scales studied during the present thesis: In **Chapter 1** and **Chapter 2** we sampled monthly in a temperate site for over 2 years, i.e. the temporal variability present at the large- and meso-scale. In **Chapter 3** we analyzed a coast-ocean transect of 100 km in an oligotrophic temperate region, and thus we analyzed an horizontal gradient at the coarse- and large-scale. In open-ocean stations situated in the tropic and subtropic we sampled horizontally at the meso-, and macro-scale (**Chapter 4**). Moreover, we also studied the vertical variability from the surface to 2300 m (**Chapter 3**) and from surface to 4000 m (**Chapter 4**) thus we sampled at small- (when samples were taken at distances of few meters) and coarse- and large-scale vertically. **Chapter 1** and **Chapter 2** in orange; **Chapter 3** in green; **Chapter 4** in blue.

Microbial communities are known to vary across the ocean surface, differing between coastal and open ocean regions, with latitude and from the surface to the bathypelagic, but normally these studies analyze only the free-living size-fraction. In this thesis we provided a novel view focusing on the microbial communities present in particles of distinct sizes. We observed that prokaryotic communities are variable among fractions, and with space and time. As an example of this variability, here we synthesize the results obtained about richness, the simpler of the measured parameters: bacterial richness increased with the size-fraction, and this occurred each month and during 2 years, in a temperate site.

The same pattern was observed in an horizontal transect (ca 100 km) from the coast to the open ocean and in a vertical profile from the surface to 2300 m. Yet, archaea, on the contrary had richness decreasing from small to large size-fractions. Interestingly, these robust patterns were much less so in the open-ocean (tropical and subtropical latitude), where we observed that in the surface, the deep chlorophyll maximum, and the mesopelagic, the intermediate size-fractions were more diverse, even more interesting: richness decreased with size-fraction in the bathypelagic.

Ecological scaling laws can be used for prediction. Locey and Lennon (2016) used a global-scale compilation of microbial data and showed a unified scaling law that was used to predict that in Earth there likely are 1 trillion (10^{12}) microbial species. Yet, the data that they compiled from the oceans were mainly from the free-living fraction. During this thesis, we observed in the species-accumulation curve that, if diversities on particles are taken into account, we could retrieve ca. 5 times more species in the same volume of water. Therefore, the predictions of Locey and Lennon (2016) are perhaps under-estimated by 5 times, and maybe the microbial diversity on Earth is not 1 trillion but 5 trillions. Yet, our calculations are not precise as they have not been performed in the detail required for this complex analysis, and a new estimation, including the diversity on particles, should be calculated. Still, we can argue that the values of (Locey and Lennon 2016) are underestimates.

This thesis is one of the first in which a multiple size-fractionation has been systematically performed and, as far as we know, the first in which 6 size fractions have been used. Therefore it is the first systematic attempt to analyze prokaryotic communities in particles of distinct size and their variability in space and time at distinct scales. Yet, more effort should be done at describing the variability in further scales, especially the temporal. A study that must be done in the future is to see how much change the prokaryotic community in the distinct size-fractions on a daily basis. To compare the communities in distinct size-fractions from day to night is interesting as these changes might be strongly related to the vertical migration of zooplankton at night and the production of biomass by phytoplankton during the day. We should also analyze the temporal variability in the particles not only in surface waters, but also in a vertical gradient. As an example, an interesting

question to solve could be: how this seasonality affects the prokatyotic communities present in the distinct size-fractions in the bathypelagic realm?

Concluding, we have described novel and interesting patterns concerning how bacterial and archaeal communities structure along the particulare matter plankton continuum. Yet, we have not tested the mechanisms that cause these patterms and an important future challenge would be to elucidate the mechanisms underlying the observed patterns. Knowledge of the quality, quantity and dynamics of particles will be essential to define these mechanisms.

3 Links between diversity, particulate matter and carbon fluxes

The sinking of particulate matter is a major component of the "biological pump" that contributes to the sequestration of carbon in deep waters (Longhurst 1998). Globally, the export of carbon via sinking of particles below the euphotic zone is about 10 Gt C/ year, accounting for 20 percent of primary production in the ocean (Tréguer *et al*. 2003). Macroscopic aggregates (>500 μm) are considered an important vehicle for the vertical transport of organic matter from the surface to deep waters (Simon *et al*. 2002). However, particles between 50 μm to several millimeters contribute most to the mass flux, as smaller particles do not sink sufficiently fast and larger particles are too rare to play a major role (Guidi *et al*. 2008, McDonnell and Buesseler 2010). Microbial processes modulate the chemical and physical structures of particulate matter, affecting the rate at which particulate matter is solubilized and mineralized, and therefore greatly affecting the vertical flux of organic carbon from the surface to the ocean interior (Simon *et al*. 2002). Thus, clarifying the interactions between particulate matter and prokaryotes is key to better understanding particulate fluxes and thus the function of the biological pump.

But, is it possible to link prokaryotic community structure and particulate matter fluxes in the oceans? We can link communities to biogeochemical processes if certain taxonomic groups exploit certain compounds, and thus play a specific role in the regulation and turnover of organic and inorganic matter in marine systems. While there are hints to this, the phylogenetic specificity to certain compounds is not totally evident: in one hand,

distinct taxa can have the same genes for degradation, adquired via lateral gene transfer (as e.g. suggested for chitinase in Cottrell *et al*. 2000) and individuals of the same taxa can have differential gene expression, thus exploiting different types of organic matter (Baty *et al*. 2000). On the other hand, it has been shown that certain groups of prokaryotes use preferentially specific components of the DOC pool (e.g. Cottrell and Kirchman 2000, Malmstrom *et al*. 2004, Elifantz *et al*. 2005, Teira *et al*. 2006). Sarmento and Gasol (2012) analyzed the preferences of prokaryotic phylogenetic groups for organic matter derived from various phytoplankton species, and described high specificity in the use of specific algal compounds by some bacterial lineages, but weak interactions also occurred and were relevant as well. Overall, we can conclude that prokaryotic communities can be related, to some extent, to the dissolved and particulated compounds present in the water column, and this is the main reason why exploring bacterial community composition can contribute to a better modeling of ecosystem processes. As our data describe prokaryotic taxonomic compositions in distinct size-fractions, we can contribute to better define the role of prokaryotes on particles of distinct size, and therefore to better define their influence in biogeochemical fluxes. Yet, the specificity of certain taxa to certain compounds and how this influences the biochemical cycles of aquatic ecosystems needs still further investigation.

4 The seed-bank cycle

Particles constitue a local accumulation of nutrients from which cells can benefit, but can also be a transport vehicle for prokaryotes through the water column (Pedrós-Alió and Brock 1983). In the present thesis we have seen that prokaryotes observed in the surface ocean reach the bathypelagic thanks to the transport via sinking particles. As we know from biogeochemical studies, part of the carbon coming from surface is sequestered permanently in the deep ocean. However, do prokaryotes from surface waters have the same destiny? Are they sequestred permanently in the deep ocean? This does not make sense from an evolutionary point of view, especially for those prokaryotes that find in the deep ocean a more suitable environment (i.e. those prokaryotes that are adapted to bathypelagic conditions). Since these prokaryotic organisms are also observed in the surface ocean, one would expect a connection between the deep-ocean back and the surface waters. There-

fore, deep-ocean prokaryotes may have similar opportunities to be back to the surface as the temporarily sequestered carbon has, via upwelling of deep water.

Dormancy is a strategy used by prokaryotic taxa that consits in entering a reversible state of low metabolic activity when faced with unfavourable environmental conditions. Furthermore, dormant microorganisms can be considered a seed bank, with individuals capable of reactivating ("resuscitating") following environmental change. This dormancy can last decades, centuries and even thousands or millions of years (Lennon and Jones 2011).

Trying to link our observations with the carbon cycle and the dormancy strategy, we propose what we name "a seed-bank cycle" (**Figure 3**) that consists of four major steps: (1) dormant microorganisms from surface waters attach to particles (they attach actively after resuscitating, or by physical adherence) and then sink with the particles. (2) Some of the attached bacteria start to grow when they find more suitable environment in deeper depths. (3) After centuries or millennia these communities might return back to the surface in upwellings. (4) When they arrive to the ocean surface they are dispersed by the air or by water circulation as dormant seeds. Eventually, these taxa will find a particle and will sink, starting again the seed-bank cycle. Note that substances such as TEP (transparent exopolysaccharide particles) might contribute to capture the prokaryotes, even if dormant, and help them sink.

Our theory can be supported by some pieces of evidence: There are many studies evidencing the colonization of particles by prokaryotes in aquatic environments (reviewed in Simon *et al*. 2002) and particularly in sea water (e.g. Kogure 1982, Vaqué *et al*. 1989) and therefore supporting Step 1. In **Chapter 4** we describe that prokaryotes from surface sink with particles and dominate the community in deep waters, and this would support Step 2. Nagata *et al*. (2010) noticed that many of the microbial groups that are suited to deep ocean condition are also dominant members of terrestrial soil environments, suggesting that there might be a link between these taxa and the particles. Microbial studies relating water masses with prokaryotic community composition are scarce, so there are no direct evidences for the third step. Yet, Wilkins *et al*. (2013) described that water advection can shape microbial community structure by increasing the opportunities for colonization of

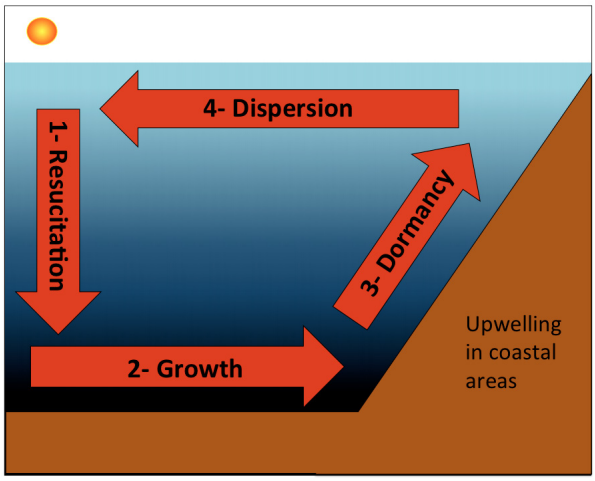


Figure 3. Schematic proposal of the "Seed-bank cycle". The cycle consists of four major steps: (1) prokaryotes attach to particles in the surface and sink with the particles, (2) they grow and reproduce at depth, (3) they return to surface by upwelling, likely in a dormant stage and (4) they disperse as microbial seeds by air or by water-masses circulation.

some taxa and suggested that, for a better understanding of the effects of advection on prokaryotic communities, specific taxonomic groups must form dormant spores. Finally, Step 4 can be supported by numerous studies analyzing the dispersion of microbes in the ocean using different approaches from a more descriptive to a more theoretical point of view, and also including air-sea fluxes and atmosferic processes (e.g. Mayol *et al*. 2014, Müller *et al*. 2014, Jönsson and Watson 2016, Whittaker and Rynearson 2017).

In this thesis we have described the first and second steps of this seed-bank cycle. To complete the cycle and demonstrate the third and fourth steps, we should use an oceanographic expedition in an upwelling zone. During that expedition, the oceanography of the region should be well studied and samples should be taken in three main areas: in deep waters masses just before upwelling, all along the water mass that upwells, and in surface water masses affected by the upwelling. Moreover, a langragian study following the newly formed surface water mass would be useful. Once the samples analyzed, if we would detect that prokaryotes present in deep waters become less abundant and dormant at the surface when water rises, this would support Step 3 above. By analyzing not only rDNA tags but also rRNA tags, we could check which taxonomic groups become active

and which ones become inactive. We also hypotetize that during the dispersion in surface waters (Step 4), the taxonomic groups that arrived dormant to surface would be diluted over time. So we expect that dormant prokaryotes will be less abundant when we move away from the upwelling zone. If we plot the rank-abundance curve of each surface station, we would see that dormant prokaryotic taxa would be in the right part (tail) of the rank abundance curve (i.e. would be rare organisms), and would move further to the right part (would become more rare) in the stations most distant from the upwelling zone, and therefore contribute to the seed-bank reservoir.

5 Technical challenges

5.1 Sampling

Sampling is the first step when analysing microbial diversity in aquatic environments Most of the studies have sampled the prokaryotes in the free-living fraction (FL) while only a small percentage have sampled the prokaryotes attached to particulate matter (ATT). There is a lack of consensus on the filter that should be used to separate free-living and attached prokaryotes among studies, something that has been discussed along this work, specially in the **Introduction** and **Chapter 1**. Our results show that each filter retains distinct communities (Mestre *et al*. 2017). Furthermore, we show that, instead of using one filter to divide the communities into FL and ATT, our proposal of a multiple size-fractionation provides a better description of prokaryotic communities along the continuum of particulate matter and, consequently, provides a more detailed picture of the composition and role of prokaryotes in the ocean.

We have been asked several times about which filter we recommend to separate the ATT from the FL communities. Despite we defend the advantages of a multiple size-fractionation, we have adapted our knowledge to the needs of aquatic microbial ecologists. Therefore, we consider that the election of filters must be between pore-size 0.8 μm (where we might find FL cells in the ATT size-fraction), and 3.0 μm (where we might find ATT bacteria in the FL size-fraction). More specifically, we recommend to use the pore-size of 3.0 μm as in the plankton there are many prokaryotic cells with sizes larger than 0.8 μm

(e.g. the abundant *Synechococcus*). We think this is the option with less inaccuracies.

The traditional differentiation between FL and ATT communities is similar to the operational differentiation between particulate and dissolved organic matter and one issue that must be addessed is the connection of FL and ATT with the dissolved and particulate matter. Since bacterial growth relies strongly on the organic and inorganic matter present in the water column, it would be desirable to compare FL vs ATT prokaryotes and dissolved vs particulate organic matter in the same study. However, to the best of our knowledge, studies combining both approaches do not exist, and similarly to the lack of consensus on the optimal filter to separate FL and ATT communities, there is no agreement on the pore-size to separate between dissolved and particulate organic matter (e.g. dissolved defined from 0.2 to 0.45 µm (Simon *et al*. 2002), from 0.45 to 1.7 µm (Samo *et al*. 2008), <0.22 µm or <0.7 µm (depending on the filter used) (Jiao *et al*. 2010)). Therefore, we propose that FL vs ATT prokaryotes and dissolved vs particulate organic matter should be separated by the same pore size (see **Figure 4**) to allow a better association of free-living prokaryotes and dissolved organic matter, and attached prokaryotes with particulate carbon, something that would allow a better insight into the relationship of prokaryotes and organic and inorganic matter present in the two distinct fractions.

5.2 DNA extraction

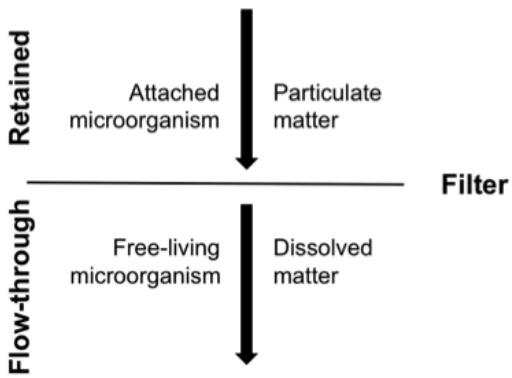


Figure 4. A convergent vision of FL *vs* ATT and Dissolved *vs* Particulate matter analysis.

Apart from the diversity in sampling and filtration procedures, DNA extraction protocols vary widely among labs. Yet, the extraction of DNA produces biases as it affects the recovery of distinct taxonomic groups (e.g. Ferrera *et al*. 2010). In our lab in the Institut de Ciències del Mar (ICM), the most common protocol is the one described in Massana *et al*. (1997). This protocol is generally used with samples of the BBMO (Blanes Bay Microbial Observatory) data series, where 10 L of water are filtered through 2 filters (3.0 μm and 0.2 μm). The samples analyzed in this thesis consist of the same volume of water, but the DNA was divided among 6 filters. Therefore, the concentration of DNA in each filter is lower. As the concentration of DNA recovered in some of the filters was very low, the PCR amplification was difficult and sometimes even impossible, specially in the largest size-fractions, where the number of bacteria (and therefore the DNA yield) was the lowest. This technical problem was an important issue during this thesis: if the PCR amplification could not be accomplished, the description of the community composition (and therefore the experimental part of this thesis), could not be performed. Since solving this critical step was crucial, we developed a new extraction protocol to recover more DNA yield from each filter. Our protocol was based on (Boström *et al*. 2004) and included some steps of the ICM protocol, and the protocol used in the IGB (Leibniz-Institut für Gewässerökologie und Binnenfischerei) (e.g. Rieck *et al*. 2015), and we also included a phenol-chloroform step by precipitation with glycogen. This protocol can be found in the Materials and Methods section of **Chapter 3**. Luckily, the Research and Testing Laboratory (http://www.researchandtesting.com/), to where samples where sent for sequencing, eventually offered a new service: to perform the PCR step. Moreover, they made an effort to improving their PCR amplification protocol, and whereas in **Chapter 1** and **Chapter 2** they could not amplify all samples, in **Chapter 4** all samples could be amplified and sequenced successfully, despite having relatively low DNA concentrations when extracted with the Massana *et al*. (1997) protocol.

5.3 Sequencing

The rapid technological advances in DNA sequencing technologies have strongly changed in the last decades and this has provided a leap in knowledge of microbial communities. The present thesis has been carried out during this “revolution” and this has forced us to

quickly adapt to changing methodologies.

At the very beginning of the thesis, the DGGE technique (Denaturing Gradient Gel Electrophoresis) and clone libraries were the most common methodologies used to describe microbial communities in microbial ecology labs. Yet, DGGE was basically a fingerprinting technique and the taxonomic assignment was a complicated step as only the most abundant organisms could be described (Sánchez *et al*. 2007). Moreover, only a maximum of 20-30 samples could be compared in one gel. ARISA (Automated Ribosomal Intergenic Spacer Analysis) appeared as an interesting fingerprinting alternative to DGGE, as was more rapid and sensitive (Fisher and Triplett 1999). Due to its advantages, ARISA was then successfully implemented in our lab and we planned to use this methodology to analyse all samples in this thesis. The 454 pyrosequencing was at that time a quite new tool, that provided a more detailed information on the taxonomic composition of the community, but it was really expensive and required bioinformatic tools and expertise that still did not exist in our institution. Soon, 454 tag sequencing became cheaper and the bioinformatics platform at the ICM was implemented. Therefore, ARISA became obsolete. Shortly after, the Illumina sequencing technology appeared, offering a cheaper alternative to 454 pyrosequencing. In less than 2 years, the 454 pyrosequencing company broke and now Illumina is the most common sequencing procedure. Still, these rapid changes continue as we go to the third generation sequencing platforms (Glenn 2011).

Furthermore, something that has sometimes been forgotten during this sequencing revolution is that the metabarcoding is PCR-based and that requires specific primers, and this means that it is PCR-biased as PCR can preferentially amplify some sequences than others (Reysenbach *et al*. 1992, Suzuki and Giovannoni 1996, Wintzingerode *et al*. 1997, Polz and Cavanaugh 1998, Suzuki *et al*. 1998). Moreover, the election of primers may lead to variable results. The primers of choice for PCR amplification have also changed in few years. In our case, we started using the bacterial primers 28 F/519 R in **Chapter 1** and **Chapter 2**. Later on, the Earth Microbiome Project (http://www.earthmicrobiome.org/) recommended the bacteria/archaeal primer pair 515F/806R to analyze any environmental sample and we used it in **Chapter 3**. Yet, more recently, Parada *et al*. (2015) found that the primer pair 515F/806R greatly underestimated (e.g. SAR11) or overestimated

(e.g. *Gammaproteobacteria*) common marine taxa and they proposed that the primers 515F-926R are the most suitable for marine samples, and thus, in **Chapter 4** we decided to use these primers. As a consequence, the use of different primers during this thesis may explain some of the differences in the relative abundances of particular groups. For example, compared to the pair 515F/806R, the 515F-926R pair yields higher proportion of *Cyanobacteria* and lower abundances of *Proteobacteria* in surface oceanic samples (Ruiz-González, personal communication).

As a final comment, miTags (16S rDNA fragments derived from Illumina-sequenced environmental metagenomes) is nowadays the apparently less biased option available to analyze taxonomic diversity and community composition as does not need the PCR step (Logares *et al*. 2013). Comparisons between 515F-926R amplicons and miTags showed that although the proportions may change, the patterns (i.e. the variability across stations) remain the same (Ruiz-González, personal communication).

Overall, the development of sequencing technologies has contributed to a better understanding of prokaryotic communities. Yet, despite its advantages, and during the transition from old to new methods, the need exists for an important effort and time allocation in adaptation and optimization. We consider that this should be specially taken into account in future studies. Finally, despite sequencing methodologies are advancing fast and there is no doubt that these can help us improve the description of ocean microbes, we should be aware that there is still an urgent need to improve the previous steps: the molecular lab procedures (extraction, PCR amplification) and the sampling. Relevant advances in microbial ecology can occur only if all steps are taken into account.

5.4 Further techniques

The study of prokaryotes in the context of the particulate and dissolved matter can also take advantage of other techniques which have been stablished recently. For example, we could collect individual particles and afterwards analyze SAGs (Single Amplified Genomes), which should provide information about the whole genome of each individual cell, and therefore generate a further insight of, e.g. the metabolic routes that occours in

one specific particle. With single-cell NanoSIMS (nanoscale secondary ion mass spectrometry) isotope analysis it is possible to quantify the metabolism of free-living and attached bacteria (Arandia-Gorostidi *et al*. 2016). And with metagenomics of each size-fraction we would be able to know which genes are present, and therefore the potential metabolic routes present, and thus the functional role of the prokaryotes (e.g. Ganesh *et al*. 2014). Besides, with metatranscriptomics of each size-fraction we would know which prokaryotes are active and which metabolic routes are active, and thus provide information about processes, (e.g degradation routes, and from there to infer rates of remineralization), which would help to better describe biogeochemical cycles.

The methods for "Big Data" analysis are also advancing fast and now we can apply co-occurrence networks and detect positive and negative interactions between species and define the interdependencies within each size-fraction (e.g. Milici *et al*. 2016) or among fractions of distinct sizes. Interactions among grazers, viruses, primary producers and symbionts can also be hypothesized from network analysis (Lima-Mendez *et al*. 2015) and networks also allows to relate specific plankton communities with carbon export as well as to predict its variability (Guidi *et al*. 2015).

Yet, to understand the environmental context of the prokaryotic community, we should analyze not only the common environmental parameters (as e.g. temperature, nutrients chlorophyll), but also the composition of the particulate and of the dissolved matter. Mass spectrometry technologies are advancing as fast as sequencing technologies do, and marine chemists are already applying them to oceanographic samples to elucidate the molecular composition of dissolved organic matter. An integrated perspective of microbial ecologists and chemical oceanographers is needed to create interesting connections to better understand the biogeochemical cycles in the ocean (Moran *et al*. 2016).

6 Closing remarks

Much has been learned about microbes in the last decades but much is still unknown. It is already recognized that prokaryotes in the ocean conform an extense microbiome where individuals do not operate as stand-alone cells in a watery soup but are better described

if the microscale structuring of marine organic and inorganic matter is taken into account (Moran 2015). Nowadays, thanks to technologic improvements, we can describe prokaryotes in detail so that they cannot be considered any more a "black box" for oceanographers nor modelers. Here, we opened the box and described prokaryotic communities taking on account the context where prokaryotes inhabit by describing their composition along the continuum of sizes of particulate matter and this approach provides a novel vision of prokaryotic communities as they appear in the pelagic habitat that can also be extrapolated to other aquatic systems.

Microbiology has been approaching macroecology since the advent of environmental sequencing. Contrary to what the name implies, macroecology is not the ecological study of macroscopic organisms. Rather, macroecology is the study of ecological relationships through patterns in abundance, distribution, and diversity with the goal of providing general explanations for robust and predictively powerful patterns that, together, can lead to unified ecological theories (Lennon and Locey 2017). During this thesis we tried (to some extent) to use macroecological inspiration as we practiced microbial ecology. Yet, the macroecology focus is still new in microbial ecology and sometimes our goals collides with the more traditional views. It is likely that in the following years the macroecologic view of microbes becomes common.

Finally we would like to add a short thought: Nowadays we live in an strange period for politics, society and culture, and this also affects science. Science is now measured with quantity and not with quality. And there is no time left to develop a deep understanding of a theme and the tendency for most people is to just follow fashionable topics. Therefore, it is in our hands to decide whether we let ourselves be carried by the current, or if we try to make a difference. My recommendation is to think about where we came from, where we are, and where we go. For this, it is important to do an effort and revise old studies (it is incredible how many ideas written in old books we echo now as new ones). It is also interesting to learn and understand the distinct points of views about the same topic that have different disciplines, and identify the main gaps. And then use the new advantages that the emerging technologies provide. This "easy" recipe can make a difference and help us reach interesting advances in science. And I am sure we will.

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General Conclusions

General Conclusions

1) Beyond common aquatic microbial ecology procedures, a multiple size-fractionation protocol allows to describe the diversity of microorganisms in the context of the particulate matter continuum and provides a more complete description of how prokaryotic communities and individual taxonomic groups are structured in the pelagic habitat.

2) When we apply this protocol to samples from both the coastal ocean and the open sea, and both from surface and deep waters, we observe that each size-fraction contains different prokaryotic communities that show variation over distinct temporal and spatial scales.

3) Each size-fraction presents unique OTUs, whereas there are OTUs that are present in all size-fractions.

4) As a general rule, we observe an increase in bacterial richness from the smallest to the largest size-fractions, suggesting that increasingly larger particles contribute with new prokaryotic niches. The main exception occurs in the open ocean bathypelagic, where bacterial richness decreases from the smallest to the largest size-fractions.

5) In contrast,archaea was generally more relevant in the smallest size-fractions. Archaea represented always a small percentage of total prokaryotic diversity, but this percentage increased with depth.

6) Three main categories of taxonomic groups can be differentiated: taxonomic groups

with preference for small size-fractions (such as *Synechococcus* and SAR11), taxonomic groups with preference for large size-fractions (such as *Cytophagia* and *Vibrionales*), and taxonomic groups without a clear preference for larger nor for smaller size-fractions (such as *Actinobacteria* and *Deferribacteres*). This classification is an alternative to the traditional dichotomy between free-living and attached bacteria.

7) While some taxonomic groups (such as *Synechococcus* or *Rhodobacterales*) maintained the preference for small or large size factions during most of the year, others (such as SAR11 or *Planctomycetes*) changed their distribution into different size-fractions in different seasons. Moreover, most (but not all) taxonomic groups have a depth-conserved preference for small or large size-fractions.

8) The bathypelagic is dominated by prokaryotes that are also present in surface waters: The pelagic prokaryotic communities are vertically connected via sinking particles, and particle colonization processes occurring in surface waters determine in part the biogeography of the bathypelagic.

Summary in Spanish / Resumen en Castellano

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Los microorganismos marinos son los responsables del 50% de la producción de oxígeno en el planeta Tierra (Falkowski et al. 1998, Field 1998) y los procariotas son los organismos que dominan en abundancia, diversidad y actividad metabólica en el océano (DeLong & Karl 2005, Giovannoni & Stingl 2005, Pomeroy et al. 2007). Además, los microoganismos del océano conforman un extenso microbioma donde los individuos son mejor descritos si se tiene en cuenta la materia particulada presente en el agua (Moran 2015). Sin embargo, la gran mayoría de los estudios no tienen en cuenta los procariotas asociados a partículas.

Dependiendo de su relación con la materia particulada presente en el oceáno, los procariotas han sido clasificados como de vida libre (FL) o ligados a partículas (ATT), los cuales representan comunidades distintas y tienen distintos rasgos morfológicos, genéticos y fisiológicos. Comúnmente, los métodos oceanográficos para muestrear los procariotas FL y ATT consisten en una filtración diferencial, donde el filtro con mayor tamaño de poro retiene las comunidades ATT, mientras que las FL pasan a través del poro y son recogidas en un segundo filtro. Sin embargo, la materia particulada marina está presente en un continuo de tamaños, en lugar de estar repartidas en la dualidad de particulado y disuelto (Verdugo et al. 2004). Por ello, existe la necesidad de una mejora de los métodos de muestreo de los procariotas FL y ATT para así caracterizar mejor los procariotas en el contexto del continuo de

tamaños de las partículas marinas.

En la **Introducción**, hemos realizado un meta-análisis donde hemos recopilado la literatura existente que trata de separar los procariotas FL de los ATT, con énfasis en los tamaños de poro utilizados para separar ambas comunidades. El objetivo final del meta-análisis fue proponer un nuevo protocolo de filtración, que proporcionase una mejor comprensión de la diversidad procariótica que los métodos de muestreo comunes.

Así pues, proponemos un fraccionamiento múltiple de tamaños de partículas, que consiste en una filtración secuencial donde se colocan en cadena 6 filtros distintos con poros que abarcan desde las 0.2 μm a las 200 μm. En el contexto de la presente tesis, los filtros son utilizados principalmente para el análisis de la diversidad procariótica, aunque pueden ser útiles también para otras variables como podría ser la función procariótica o el análisis del material particulado.

Considerando la importancia de los procariotas en el océano y la necesidad de estudiarlos desde nuevas perspectivas que tengan especialmente en cuenta las partículas con las que interactúan constantemente, el objetivo principal de la presente tesis es la caracterización de la diversidad de procariotas a lo largo del continuo de tamaños de las partículas marinas presentes en el océano, así como también describir su variabilidad temporal y espacial a distintas escalas.

En el **primer capítulo** ("*Patterns of bacterial diversity in the marine planktonic particulate matter continuum*"; Patrones de diversidad bacteriana en el continuo de la materia particulada marina) se analiza por primera vez las distintas comunidades presentes en partículas de distinto tamaño, con los siguientes objetivos específicos:

- Testar la hipótesis que la composición bacteriana difiere entre las distintas frac-

ciones de tamaño.

- Explorar si el fraccionamiento múltiple de tamaños de partículas proporciona una descripción más completa de la totalidad de la comunidad, que el uso de un sólo filtro para separar comunidades de vida libre y asociadas a partículas.

En este primer capítulo se observó que cada fracción de tamaño presenta comunidades bacterianas distintas, con un rango de 23-42% de OTUs (unidades taxonómicas operacionales) únicos en cada fracción, apoyando la idea de que cada fracción contiene distintos tipos de partículas. Se observó un aumento de riqueza de taxones desde la fracción pequeña hacia las más grandes, sugiriendo que las partículas cada vez más grandes aportan nuevos nichos ecológicos. Nuestros resultados muestran que el fraccionamiento de tamaños múltiple proporciona una descripción más exhaustiva de la diversidad bacteriana y de la estructura de comunidades que el uso de un solo filtro. Además, y en base a nuestros resultados, proponemos una alternativa a la dicotomía habitual de los estilos de vida FL o ATT, en el cual diferenciamos los grupos taxonómicos con preferencia por las fracciones pequeñas, los grupos que no tienen preferencia por fracciones pequeñas o grandes, y aquellos grupos que aparecen preferentemente en fracciones grandes.

En el **segundo capítulo**, ("*Seasonality and dynamics of bacterial community structure along the pelagic particulate matter continuum in a temperate oligotrophic coastal site*"; Estacionalidad y dinámicas de la estructura de comunidades bacteriana a lo largo del continuo de partículas marinas en una zona oligotrófica y temperada) se estudió la variabilidad temporal de las comunidades asociadas a partículas de distinto tamaño. Se cogieron muestras mensualmente y durante dos años en el Observatorio Microbiano de la Bahía de Blanes. Los objetivos principales fueron:

- Describir la sucesión estacional y dinámica de las comunidades de bacterias para

comprobar la hipótesis que la variabilidad temporal, tanto en la composición de comunidades como en taxones individuales, depende del tamaño de la partícula.

- Comprobar la hipótesis que la variabilidad temporal en composición de comunidades y en taxones individuales tiene patrones definidos, y que dichos patrones son repetitivos anualmente.

Los resultados mostraron que tanto las bacterias FL como las ATT presentaron cambios graduales a lo largo del tiempo relacionados con la variación de la temperatura del agua. Teniendo en cuenta las variables ambientales y la distribución de la composición de la comunidad a lo largo del tiempo, definimos dos estaciones principales: la cálida (desde Mayo a Octubre) y la fría (desde Noviembre a Abril). En el período cálido, observamos un incremento gradual de diversidad y diferenciación de partículas, mientras que en el período cálido ocurrió lo contrario. A pesar de que las estaciones cálida y fría estaban dominadas por distintos grupos taxonómicos, algunos taxones mantuvieron su preferencia por fracciones pequeñas o grandes durante todo el año. En conjunto, en este capítulo demostramos que si se tienen en cuenta las partículas de distinto tamaño, obtenemos una visión mucho más amplia de las dinámicas estacionales de las comunidades bacterianas así como de los grupos taxonómicos individuales.

En el **tercer capítulo**, ("*Spatial variability of marine bacterial and archaeal communities along the particulate matter continuum*"; Variabilidad espacial de comunidades de bacterias y arqueas a lo largo del continuo de partículas marinas), se estudió la variabilidad espacial de las comunidades asociadas a partículas de distinto tamaño. Para ello, se muestreó en el Mar Mediterráneo Noroccidental en un transecto costa-océano abierto de 100 km y hasta profundidades de 2300 m, con los siguientes objetivos específicos:

- Describir la variabilidad horizontal (desde la costa hacia el océano abierto, a lo largo de la plataforma continental y talud) y vertical (desde aguas superficiales hacia el batipelágico, incluyendo el máximo de clorofila y las capas nefeloides profundas).

- Evaluar la diversidad y la composición de comunidades de bacteria y arquea, las preferencias de los grupos taxonómicos por tamaños específicos, y si estas preferencias cambian o se mantienen a lo largo de los gradientes horizontales y verticales.

En este capítulo se observó que, en general, la composición de comunidades fue más variable en profundidad que en la transición de cosa hacia el océano abierto. Comparando las 6 fracciones de tamaño, se detectaron distintas comunidades en cada fracción, y mientras que las bacterias fueron más diversas en las fracciones más grandes, las arqueas fueron más diversas en las fracciones más pequeñas. La comparación de la composición de las comunidades entre las distintas fracciones de tamaño mostraron que la mayoría de los grupos taxonómicos (pero no todos), eran conservativos en profundidad en su preferencia por ciertas fracciones. El filtrado ambiental o la presencia de diferentes ecotipos con distintas preferencias por tamaños de fracciones, podría explicar por qué ciertos taxones no tienen una preferencia por ciertos tamaños de partículas mantenida en profundidad.

En el **cuarto capítulo** y último capítulo, ("*Vertical connectivity in the ocean microbiome: Sinking particles as dispersal vectors*"; Conectividad vertical en el microbioma oceánico: partículas en hundimiento como vectores de dispersión), se analizaron muestras del océano global (océanos Atlántico, Índico, y Pacífico), en ocho estaciones situadas en el trópico y subtrópico, con los siguientes objetivos específicos:

- Explorar si las partículas en hundimiento de distinto tamaño son un mecanismo

de dispersión de procariotas en el océano.

-Analizar la conectividad entre las comunidades de la superficie y las comunidades profundas, y ver si esta conectividad influye en la diversidad microbiana de las comunidades del océano profundo.

En este capítulo se observó que los procariotas más abundantes del océano profundo estaban presentes en las aguas superficiales, y que los taxones "endémicos" del océano profundo representan sólo una fracción muy pequeña del total de las secuencias. La conectividad vertical tenía lugar a través de las partículas más grandes, ya que los procariotas asociados a partículas eran más similares a través de la columna de agua que los procariotas de vida libre, y los procariotas asociados a partículas de la superficie fueron encontrados tanto en las fracciones pequeñas como en las grandes. Finalmente, encontramos que el proceso de colonización de partículas que ocurre en aguas superficiales puede determinar la composición de las comunidades que habitan en el océano profundo, ya que los patrones biogeográficos de superficie se ven reflejados en las comunidades profundas. En general, argumentamos que las partículas funcionan como un vector que inocula microorganismos superficiales viables en el océano profundo, determinando en gran medida su biogeografía.

A raíz de este último capítulo proponemos el ciclo del banco de semillas (seed-bank cycle), que consiste en 4 pasos principales: (1) microoganismos latentes de aguas superficiales se adhieren a partículas y entonces se hunden con ellas; (2) algunas de las bacterias adheridas a partículas crecen ya que encuentran un ambiente propicio en las aguas profundas; (3) tras siglos o milenios estas comunidades vuelven a la superficie por afloramientos y durante este viaje de vuelta a la superficie, retornan a su latencia; (4) cuando allegan a la superficie del océano, están latentes y se dispersan como esporas por aire o por la circulación de masas de agua. Eventualmente, estos taxones encontrarán una partícula, se asociaran activa o pasivamente a ella y se hundirán, empezando de nuevo el ciclo del banco de semillas.

Como conclusión, en la presente tesis hemos descrito las comunidades procariotas a distintos niveles: especie (OTUs), poblaciones (grupos taxonómicos) y comunidades. Además, hemos tenido especialmente en cuenta el contexto donde los procariotas habitan y los hemos descrito en el continuo de tamaños de la materia particulada. Esta aproximación ha hecho posible proporcionar una visión novedosa y más completa de las comunidades procarióticas en el hábitat pelágico que además se puede extrapolar a otros sistemas acuáticos y puede ser de interés para estudios de ciclos biogeoquímicos.

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