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Community analysis of high- and low-nucleic acid-containing bacteria in NW Mediterranean coastal waters using 16S rDNA pyrosequencing

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Summary

The ecological significance of the marine bacterial populations distinguishable by flow cytometry on the basis of the fluorescence (FL) of their nucleic acid (NA) content and proxies of cell size (such as side scatter, SSC) remains largely unknown. Some studies have suggested that cells with high NA (HNA) content and high SSC (HS) represent the active members of the community, while the low NA (LNA) cells are inactive members of the same phylogenetic groups. But group-specific activity measurements and phylogenetic assignment after cell sorting have suggested this is not be the case, particularly in open-ocean communities. To test the extent to which the different NA subgroups are similar, and consequently the extent to which they likely have similar ecological and biogeochemical roles in the environment, we analysed the phylogenetic composition of three populations after cell sorting [high NA-high SC (HNA-HS), high NA-low SC (HNA-LS), low NA (LNA)] by 454 pyrosequencing in two contrasting periods of the year in NW Mediterranean coastal waters (BBMO, Blanes Bay Microbial Observatory) where these three populations have recurrent seasonal patterns. Statistical analyses showed that summer and winter samples were significantly different and, importantly, the sorted populations within a sample were composed of different taxa. The majority of taxa were associated with one NA fraction only, and the degree of overlap (i.e. OTUs present simultaneously in 2 fractions) between HNA and LNA and between summer and winter communities was very small. *Rhodobacterales*, SAR116 and *Bacteroidetes* contributed primarily to the HNA fraction, whereas other groups such as SAR11 and SAR86 contributed largely to the LNA fractions. *Gammaproteobacteria* other than SAR86 showed less preference for one particular NA fraction. An increase in diversity was observed from the LNA to the HNA-HS fraction for both sample dates. Our results suggest that, in Blanes Bay, flow cytometric signatures of natural communities track their phylogenetic composition.

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Introduction

Each introduction of a new technique in the field of microbial ecology results in novel insights into the structure and function of microbial communities. Flow cytometry was introduced early on in microbial ecology (Bailey *et al*., 1977; Robertson and Button, 1989) but did not gain widespread use until the appearance of desktop cytometers that use blue wavelength emission and nucleic acid stains (Li *et al*., 1995; del Giorgio *et al*., 1996; Marie *et al*., 1997). One of the first observations reported from the application of this technology to aquatic bacteria was the presence of at least two subgroups which had different nucleic-acid staining properties, then called Type I and II, and later HighDNA and LowDNA, or HNA and LNA bacteria (Li *et al*., 1995; Jellett *et al*., 1996; Gasol *et al*., 1999). Image analysis of epifluorescence micrographs conducted at maximal resolution had also identified these two groups of aquatic microorganisms (Sieracki and Viles, 1992). Later work showed that almost all marine and freshwater samples harboured these groups, independent of the specific staining protocol (e.g. Bouvier *et al*., 2007; Wang *et al*., 2009), representing a characteristic feature of planktonic bacteria.

Based on the relative nucleic acid content of these different populations and different relationships with chlorophyll (CHL), Li and colleagues (1995) suggested that the HNA cells correspond to the 'active' cells of the community and the LNA cells to the 'inactive', and their ratio

represented an 'active cell' index (Jellett *et al.*, 1996). Further work showed that the HNA cells were those growing in dilution experiments and preferentially depleted by predators (Gasol *et al.*, 1999). Many other studies have in some way or another associated the HNA cells with the most active members of the bacterial community (e.g. Morán *et al.*, 2007). However, the relative abundance of HNA and LNA was found to be a be poor predictor of ecosystem properties (e.g. Morán and Calvo-Díaz, 2009).

One definitive test of the relative activity of the two subgroups of bacteria involves the sorting of a bacterial community following incorporation of universal organic tracers (i.e. leucine or thymidine). Lebaron and colleagues (2001; 2002) and Servais and colleagues (2003) found that most HNA bacteria were highly active in incorporating the substrates, although other studies showed that the LNA fraction could be active as well, and in some cases equally or even more active than the HNA fraction if scaled to cell biovolume (Zubkov *et al.*, 2001a; 2006; Mary *et al.*, 2006; Wang *et al.*, 2009). Interestingly, open ocean oligotrophic communities appeared to have a higher proportion of total heterotrophic bacterial activity attributable to LNA cells than coastal or continental shelf communities (Servais *et al.*, 2003; Longnecker *et al.*, 2005; 2006).

The degree of activity of each population is in itself interesting, but also raises the question of whether the two populations are composed of identical phylotypes that segregate into the HNA fraction when active and into the LNA fraction when inactive. This idea was based on the observation that the DNA content of the HNA population was approximately four- to sixfold higher than that of the LNA fraction (Li *et al.*, 1995; Jellett *et al.*, 1996) and it was a reasonable possibility that the LNA fraction was composed of individual cells with one genome copy, while the HNA was composed of cells in the process of replication and containing two, four or eight copies of the genome. Bouvier and colleagues (2007) suggested four possible explanations for the universal occurrence of HNA and LNA populations: that bacteria begin in the HNA fraction and move to the LNA upon death or inactivity (i.e. there is similar community structure in both fractions); that bacteria from the LNA fraction move temporarily into the HNA fraction when dividing (similar community structure); that HNA and LNA fractions contain distinct taxa that never appear in the other (different community structure, maybe with completely different organisms in each population); and that bacteria move constantly between fractions such that at any one time, some taxa are present in only one fraction but others are in both (different community structure, but with overlapping communities).

To test for taxonomic differences in the two bacterial subgroups, sorted populations have been the subject of molecular analyses, including clone libraries (Zubkov *et al.*, 2001b; Fuchs *et al.*, 2005; Trigui *et al.*, 2011), fingerprinting techniques (SSCP or DGGE, Bernard *et al.*, 2000a; Fuchs *et al.*, 2000; Servais *et al.*, 2003; Longnecker *et al.*, 2005) and FISH (Zubkov *et al.*, 2001a,b; 2002a; 2007; Fuchs *et al.*, 2005; Mary *et al.*, 2006; Schattenhofer *et al.*, 2011). Some of these studies concluded that the HNA and LNA populations were not very different (Servais *et al.*, 2003; Longnecker *et al.*, 2005), whereas others indicated completely different taxonomic composition (Bernard *et al.*, 2000b; Zubkov *et al.*, 2001a). Interestingly, FISH-based studies along a north-south transect in oceanic Atlantic waters consistently showed active LNA populations consisting of the widespread but slow-growing SAR11 group (Mary *et al.*, 2006), challenging the model that LNA cells are inactive versions of the HNA cells. A specific test of the four scenarios of Bouvier and colleagues (2007), again based on FISH analysis of the sorted populations, likewise identified completely different HNA and LNA communities in North Atlantic water samples (Schattenhofer *et al.*, 2011).

The studies concluding that the composition of the HNA and LNA populations is different were conducted primarily in open ocean waters (most in the central Atlantic), while a number of coastal studies report similar composition of these groups (e.g. Servais *et al.*, 2003). The use of FISH approaches may influence these different outcomes, since it involves lumping of species-level phylotypes into larger taxonomic groups and potentially obscures finer-scale taxonomic differences between the HNA and LNA fractions. Clone library construction after cell sorting could potentially resolve this situation, but the studies done to date produced relatively low-coverage libraries (~ 40 sequences per sample in Trigui *et al.*, 2011; ~ 50 sequences in Zubkov *et al.*, 2001b; ~ 130 sequences in Fuchs *et al.*, 2005) and this limited the ability to robustly test for differential OTU composition. The contradictory results found in the literature might thus result from real ecosystem-level differences (i.e. coastal communities are different from open ocean in their HNA versus LNA community structure) or may simply reflect incomplete description of the subgroups.

New sequencing technologies that produce larger sequence libraries can now be used to more deeply describe microbial community structure (Sogin *et al.*, 2006), making it possible to test HNA versus LNA hypotheses at high taxonomic resolution. Since the discrepancies in the literature were more common in coastal communities than in open ocean ones, we conducted flow cytometric sorting of winter and summer coastal bacterioplankton communities into several NA content fractions, and used pyrotagging technology (454 sequencing of amplified 16S rRNA genes) to compare taxonomic composition of the different fractions in the two seasonal samples.

Results

Seasonality of flow cytometric signatures

The Blanes Bay Microbial Observatory is an oligotrophic coastal site with a significant seasonal signal over the annual cycle that drives microbial activities (e.g. Alonso-Sáez *et al*., 2007; 2008; Vila-Costa *et al*., 2007). The cytograms obtained using light scatter versus NA fluorescence usually reflect this year-round variability with a dynamic presence of three distinguishable populations: HNA-HS, HNA-LS and LNA (Fig. 1). The winter season is characterized by relatively high chlorophyll concentrations (10-year average: $0.98 \pm 0.06\ \mu g\ l^{-1}$) and non-limiting concentrations of dissolved inorganic nitrogen and phosphorus, whereas summer stratification is characterized by lower CHL ($0.32 \pm 0.02\ \mu g\ l^{-1}$) and depleted inorganic nutrients. While LNA bacteria are a relatively constant component of the bacterial community (average seasonal values of 42–47%, Fig. S1), HNA-HS and HNA-LS switch in relative abundance between winter and summer (Fig. S1). For this study, we chose two contrasting periods of the year, August and March. In August 2008, LNA represented 48% of the cells (8-year average for summer: 47%), HNA-LS were 23% (21%) and HNA-HS 33% (32%). In March 2009, LNA represented 45% of the cells (8-year average for winter: 42%), HNA-LS were 30% (average, 29%) and HNA-HS were 25% (average: 28%). Thus, the summer sample had more LNA and HNA-HS cells and fewer HNA-LS cells than the winter sample, representative of the seasonally different communities appearing at Blanes Bay.

Flow cytometric sorting

Negative controls run with PBS solution as sheath fluid showed no obvious cells but some noise; subsequent PCR reactions gave no amplicons. Positive controls consisting of *Synechococcus* cells present in the natural samples were sorted based on their inherent pigment fluorescence (phycoerythrin, FL3 signature, see Fig. 1); subsequent PCR amplification resulted in $86.3 \pm 3.7\%$ (CV = 4.3%) of the sequences classified as *Synechoccoccus*.

Replicates for all samples represented true experimental replicates since they were sorted from different initial samples collected in parallel. In general, assignment of OTUs from the sorted populations into major taxonomic groups were within a 10% deviation from the equality line for replicate samples (Fig. S2, CV = 10.7 ± 2.42). The summer replicates were more variable than the winter ones, and the summer LNA samples in particular had some groups that differed by more than 10% contribution to community structure between replicates.

Diversity analysis

An average of 1918 ($\pm$ 1338 SD) sequences were obtained per sample, representing 393 ($\pm$ 172) OTUs for HNA-HS, 240 ($\pm$ 80) OTUs for HNA-LS and 220 ($\pm$ 115) OTUs for the LNA fractions. Not all rarefaction curves reached an asymptote, indicating insufficient sequencing to capture the full diversity of the communities (Fig. S3). The Shannon diversity index and richness consistently increased from the LNA to the HNA-HS fraction in both months (Fig. 2). For each fraction, the winter community tended to be richer than the summer community. Rank abundance curves showed a dominance of few phylotypes in all fractions but with a longer tail of less frequent OTUs in the HNA-HS fraction (Fig. S4). We constructed distance matrices with Bray-Curtis distances from winter and summer abundance data and found no correlations, indicating significantly different communities in summer and winter ($r_M = 0.08$, $P = 0.428$, Mantel test). To identify the main discriminating factor correlating with bacterial community structure (i.e. DNA content, season, sorted gate), we performed permutational MANOVA analyses. The results indicated that the primary factor was the period of sampling (season) ($R^2 = 0.27$, $P < 0.001$) and the assignment to a sorted gate ($R^2 = 0.27$, $P < 0.01$) with lesser contributions from DNA content (high or low) ($R^2 = 0.18$, $P < 0.01$). Accordingly, an NMDS analysis clearly distinguished summer from winter populations and grouped replicates for each NA fraction together (Fig. S5).

Taxonomy of sorted populations

Overall taxonomic characterization of the bacterial community was conducted at the phylum level, and only *Proteobacteria* were classified at the class or order level (the latter just for selected environmentally relevant groups such as SAR11 and SAR86). Both winter and summer bacterial communities were dominated by *Alphaproteobacteria* and *Gammaproteobacteria*, followed next by *Bacteroidetes* (Fig. 3), a pattern commonly observed in marine systems and particularly in the BBMO (Alonso-Sáez *et al*., 2007). Contributions of *Alphaproteobacteria* other than *Rhodobacterales* and SAR11 were relatively similar in summer and winter whereas a higher contribution of *Rhodobacterales* and SAR11 was observed in all three NA populations in winter (Fig. 3, Fig. S6). *Gammaproteobacteria* contribution to the sorted populations differed between the seasonal samples (SAR86 plus other *Gammaproteobacteria*; Fig. 3 and Fig. S6). *Rhodobacterales* accounted for the highest proportion of sequences in the HNA-HS fraction, whereas SAR86 and SAR11 were highest in HNA-LS and LNA fractions respectively. SAR86 members were always substantially more abundant in the HNA-LS and LNA fractions and a

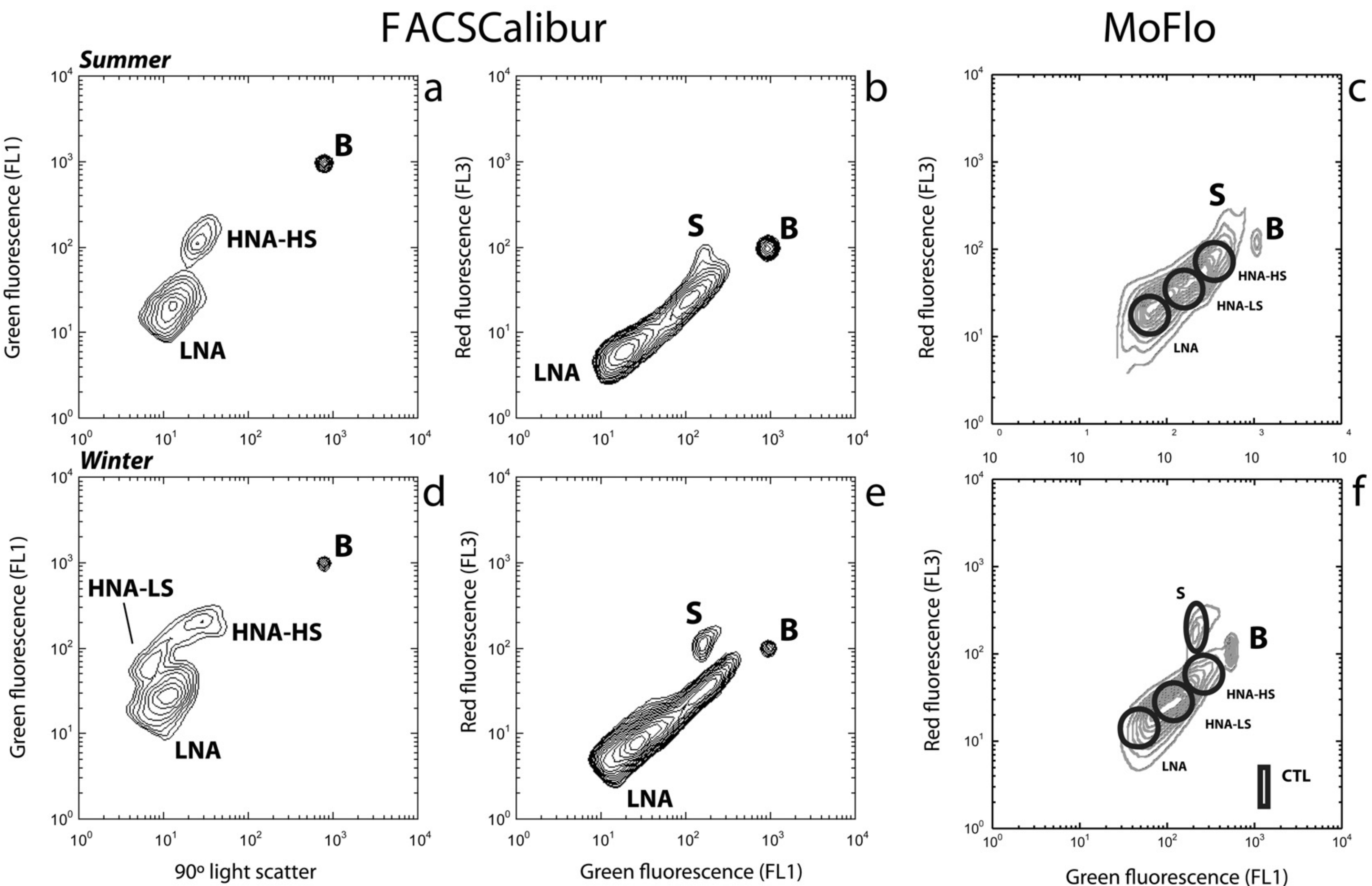

Fig. 1. Flow cytometry plots of typical seawater samples in summer (August 2008, a–c) and winter (March 2009, d–f) at the Blanes Bay Microbial Observatory. Plots on the left (a, b, d, e) were obtained with the FACSCalibur; plots on the right (c, f) were obtained with the MoFlo flow sorter. The sorting gates (marked in c and f) were used to collect cells with high nucleic acid content and high light scatter (HNA-HS), high nucleic acid content and low light scatter (HNA-LS) and low nucleic acid content (LNA). A *Synechococcus* gate (S) was established as a positive control and a CTL gate as a negative control (winter sample). Fluorescent bead standards are indicated (B).

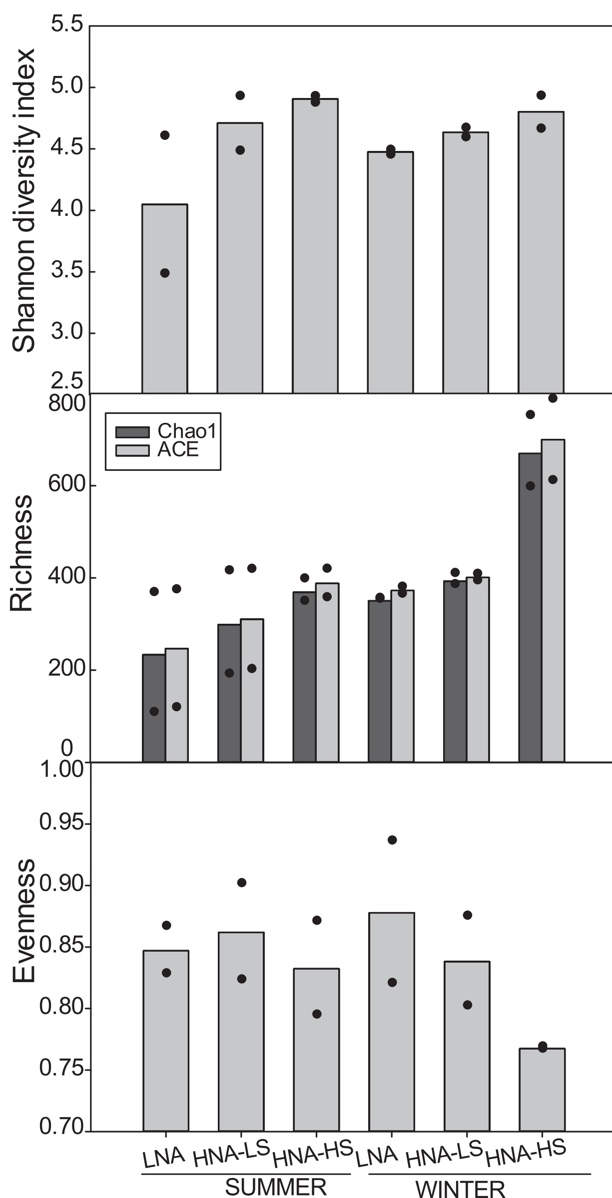

Fig. 2. Shannon H′ diversity index (upper panel), Chao1 and ACE richness indexes (middle panel) and evenness (lower panel) for HNA-HS, HNA-LS and LNA populations in summer and winter samples. Dots represent the standard deviation of the values.

minor contribution to the HNA-HS fraction, in contrast to *Rhodobacterales* and SAR116 which were always more abundant in the HNA-HS community (Fig. 4). Similarly, *Bacteroidetes* were always found to be more abundant in high NA fractions. SAR11 was the main contributor to the LNA fraction in winter, whereas in summer, an unidentified group of *Gammaproteobacteria* mostly accounted for this fraction.

We also calculated the relative enrichment of each fraction for major taxonomic groups by computing the ratio between the proportions of sequences in the HNA versus those in the LNA fraction for both samples (winter and summer). We combined the two HNA populations (HNA-HS plus HNA-LS) to reduce the number of pairwise ratios (Fig. 4). Three categories of bacterial groups emerged: (i) those that were more important in the HNA fractions (in both summer and winter), such as *Rhodobacterales*, SAR116, *Bacteroidetes*, and the 'other' *Alphaproteobacteria*, (ii) those that were always more important in the LNA fraction, such as SAR11, SAR86, *Betaproteobacteria*, *Firmicutes* and *Fibrobacter/Acidobacteria*, and (iii) those that were more important in the HNA in one season but in LNA in the other, such as *Actinobacteria* and 'other' *Gammaproteobacteria*. The most consistent preference between NA-content fractions was for *Rhodobacterales* (up to 33 times more important in the HNA fraction) and SAR11 (up to 10 times more important in the LNA fraction).

Patterns of dominant and rare OTUs within seasons

For a more detailed taxonomic characterization, we investigated the extent to which specific OTUs (defined as 95% similarity clusters) overlapped across sorted populations. There was little overlap of dominant OTUs between the HNA and LNA community (Fig. S7, Table 1) even though our criteria for defining an OTU was conservative and could potentially group sequences with up to 5% sequence divergence. Only 3 abundant OTUs (those that make up at least 1% of the community) overlapped in the summer out of 20 total abundant OTUs in the HNA community and 24 in the LNA community, while only 1 abundant OTU overlapped in the winter out of the 10 and 22 abundant OTUs in the HNA and LNA communities respectively (Table 1). Considering all abundance categories, the majority of OTUs did not overlap across co-occurring communities, with 70% of HNA OTUs and 67% of LNA OTUs undetected in the other community in the summer, and 73% and 49% in the winter (Table 1). Our data therefore suggest that the exchange of OTUs between fractions is limited, typically does not involve the most abundant OTUs, and is inconsistent with the idea that the LNA pool acts as a 'seed bank' of inactive bacteria for the HNA fraction.

Patterns of dominant and rare OTUs between seasons

There was also low overlap of OTUs within a population but between summer and winter. For dominant HNA OTUs, none overlapped between the summer and winter samples out of 10 abundant OTUs in the summer community and 20 in the winter community, while for dominant LNA OTUs, only 4 abundant OTUs overlapped out of 21 abundant OTUs in the summer community and 26 in the

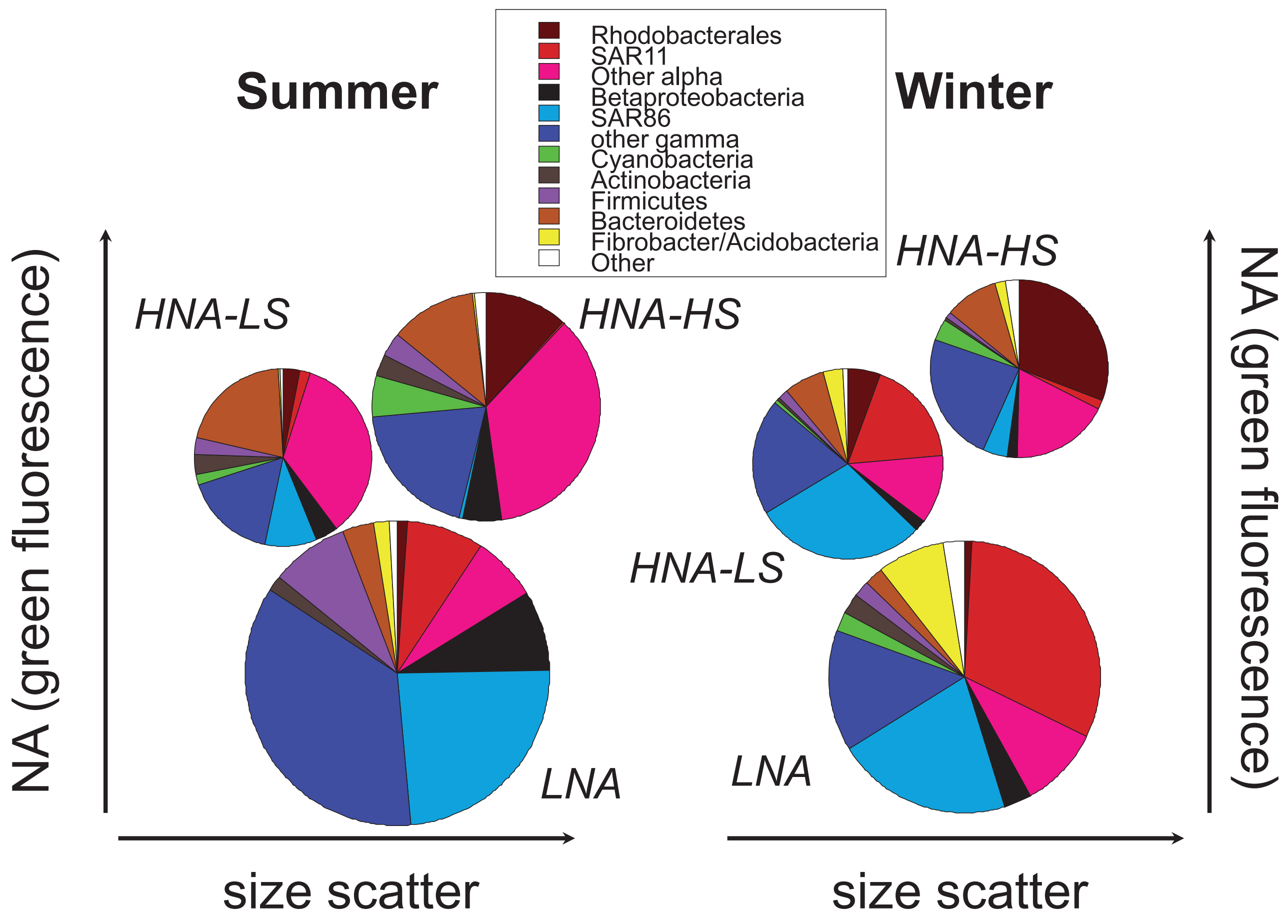

Fig. 3. Contribution of each phylogenetic group to the total sequences from the sorted communities. The communities (= spheres) are drawn roughly proportional to their abundance, and placed on a Side Scatter versus Nucleic Acid fluorescence plot similar to those in Fig. 1.

winter (Table 2). Thus dominant OTUs were typically found in just one population and one season. Considering all abundance categories, 72% of summer OTUs and 47% of winter HNA were undetected in the other season, and 58% of summer LNA OTUs and 52% of winter LNA OTUs were undetected in the other season (Fig. S7).

Discussion

Flow cytometric cell sorting followed by high-throughput sequencing of 16S rRNA genes was used to distinguish and characterize three bacterioplankton communities by DNA content and size in summer and winter coastal seawater. Duplicate independent sorting and sequencing carried out for each community showed < 10% variation in cell assignment to the three NA categories for almost all samples (Fig. S2), and a control gate sorted on *Synechococcus* pigment signature and size (Fig. 1) showed that > 85% of the PCR amplicons were from the intended taxonomic group. Over 1200 OTUs were identified from 12 samples, with an average of 285 OTUs per sample. This taxonomic analysis is more comprehensive than previous studies based on either 16S rRNA clone library construction or fingerprinting of sorted bacterioplankton fractions.

Bacterial populations that can be distinguished by size and nucleic acid content have been found consistently using flow cytometry, and have raised the question of whether they are determined by phylogeny, by physiological status of the cells, or by ecological roles of the cells. In line with several previous studies (Fuchs *et al.*, 2000; 2005; Zubkov *et al.*, 2001a,b; 2002a; 2007; Mary *et al.*, 2006; Schattenhofer *et al.*, 2011), our results show that bacterial taxa differ between three cytometric categories (HNA-HS, HNA-LS and LNA fractions), although some studies have shown similar composition (based on HNA and LNA fractions; Servais *et al.*, 2003; Longnecker *et al.*, 2005). Our results are also more in line with studies of open ocean environments in that previous studies of coastal sites typically showed poorer discrimination across cytometric communities (Servais *et al.*, 2003).

Several major taxonomic groups contributed to only one cytometric fraction regardless of the month of sampling (Fig. 4). Notably, *Rhodobacterales* (largely

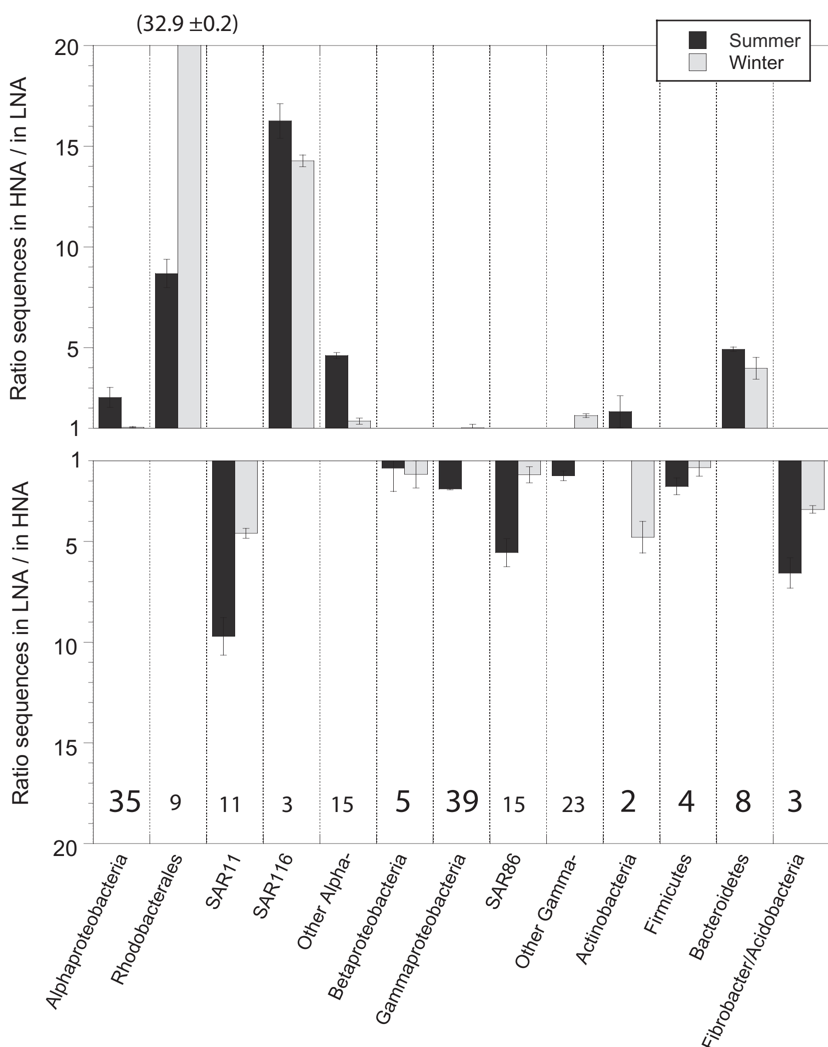

Fig. 4. Relative enrichment of each fraction for each major bacterial group calculated by computing the ratio between the proportions of sequences in the HNA versus those in the LNA fraction. The HNA fraction includes both the HNA-HS and the HNA-LS cells. The graph shows the HNA/LNA ratio for those groups more abundant in the HNA fraction and the LNA/HNA ratio for those more abundant in the LNA fraction. Note that the winter *Rhodobacterales* ratio is off scale. The numbers at the bottom of the plot indicate the average % of sequences for each group. Numbers for groups at the phylum or class level are larger and include those at the subclass level. Specific ratios of the different subgroups within the *Alphaproteobacteria* and *Gammaproteobacteria* are included after the general pattern observed at order level. Other Alpha: alphaproteobacterial cells other than *Rhodobacterales*, SAR11 and SAR116. Other Gamma: gammaproteobacterial cells other than SAR86.

Roseobacter) contributed primarily to the HNA-HS community, as had previously been observed (Zubkov *et al*., 2001b; 2002b; Fuchs *et al*., 2005; Schattenhofer *et al*., 2011). This taxon can be common in algal blooms and appears to actively consume low-molecular-weight organic compounds (González *et al*., 2000; Alonso and Pernthaler, 2006; Alonso-Sáez and Gasol, 2007; Vila-Costa *et al*., 2007). SAR116 showed the same

Table 1. Comparison of OTU abundance in flow cytometry fractions.

		Category in LNA				
		Abundant	Frequent	Rare	Undetected	Total
(A) Summer						
Category in HNA	Abundant	3	4	2	11	20
	Frequent	13	42	31	209	295
	Rare	0	6	7	40	53
	Undetected	8	94	121		
	Total	24	146	161		
(B) Winter						
Category in HNA	Abundant	1	3	2	4	10
	Frequent	16	46	25	193	280
	Rare	3	49	45	331	428
	Undetected	2	100	80		
	Total	22	198	152		

HNA-LS and HNA-HS are combined for this analysis. Abundant = > 1% of the community; frequent = 0.05–1%; and rare = < 0.05%.

Table 2. Comparison of OTU abundance for the same flow cytometry population occurring in different months.

		Category in Summer				
		Abundant	Frequent	Rare	Undetected	Total
(A) HNA						
Category in Winter	Abundant	0	13	1	6	20
	Frequent	7	60	89	139	295
	Rare	0	15	18	19	52
	Undetected	3	194	317		
	Total	10	282	425		
(B) LNA						
Category in Winter	Abundant	4	12	2	8	26
	Frequent	9	38	24	69	140
	Rare	7	33	24	91	155
	Undetected	1	111	99		
	Total	21	194	149		

HNA-LS and HNA-HS are combined for this analysis. Abundant = > 1% of the community; frequent = 0.05–1%; and rare = < 0.05%.

occurrence pattern as *Rhodobacterales*, and the only representative of the SAR116 clade with a genome sequence thus far appears to be a metabolic generalist (Oh *et al*., 2010). *Bacteroidetes* also contributed primarily to the HNA fractions, and members of this taxon are considered efficient consumers of the high-molecular-weight compounds that may be abundant in late stages of phytoplankton blooms (Cottrell and Kirchman, 2000; Newton *et al*., 2011).

The SAR11 taxon contributed primarily to the LNA fraction, particularly in winter, as had been observed previously at oceanic sites (Fuchs *et al*., 2005; Longnecker *et al*., 2005; Mary *et al*., 2006; Schattenhofer *et al*., 2011). This group is abundant and ubiquitous in the surface waters of the ocean (Morris *et al*., 2002) and contains small and efficient genomes (averaging 1.6 ± 0.4 Mbp, Giovannoni *et al*., 2005; Kottmann *et al*., 2010). The gammaproteobacterial SAR86 group also contributed primarily to the LNA fraction, as was also observed by Zubkov and colleagues (2001a; 2002a) in the Celtic Sea and the northern North Sea, although Schattenhofer and colleagues (2011) found SAR86 cells in HNA fractions in North Atlantic waters. The absence of sequenced SAR86 genomes limits speculation about their lifestyle, but co-occurrences of SAR86 with SAR11, *Prochlorococcus* and *Actinobacteria* (HGC I type) suggest they may also be adapted to oligotrophic conditions. Gammaproteobacterial OTUs belonging to the order *Enterobacteriales* were also important in LNA fractions, primarily in the summer sample (Fig. 3, Table S1) but we have limited knowledge about their ecological lifestyles.

Two HNA cell fractions that can be distinguished based on their SSC (called HNA-LS and HNA-HS here) have been seen previously (Zubkov *et al*., 2001a,b; 2002b; 2006; Fuchs *et al*., 2005; Mary *et al*., 2006). In this study, we found evidence that a seasonal difference exists in the contribution of these different NA-containing groups to the total bacterioplankton community. In Blanes Bay, the summer HNA community was largely HNA-HS cells (dominated by *Rhodobacterales*) while the winter the HNA community was mainly composed by HNA-LS cells (dominated by *Bacteroidetes*) (Fig. S1). In addition, it appeared that LNA cells made up a larger component of the community in summer than in winter, a pattern that had been observed in other sites (e.g. Cantabrian Sea, Calvo-Díaz and Morán, 2006; Morán *et al*., 2007; Morán and Calvo-Díaz, 2009). Overall, these results indicate a strong phylogenetic signal in the composition of the different fractions.

To evaluate the ecological coherence of the sorted communities, we calculated the percentage of OTUs that were found primarily in one NA fraction. Over 97% (summer) and 93% (winter) of the *Rhodobacterales* OTUs contributed more to the HNA fraction than the LNA fraction. For SAR11, 70% (summer) and 89% (winter) of the OTUs contributed more to the LNA fraction than to HNA. Less consistency was observed among *Gammaproteobacteria* OTUs (excluding SAR86), with over 73% of the OTUs in winter showing a higher contribution to the HNA fraction but 65.6% of the OTUs in summer showing a higher contribution to the LNA fraction (Fig. 3 and Fig. S6). This high ecological diversity within the *Gammaproteobacteria* may warrant finer phylogenetic analysis since, for example, about half the *Gammaproteobacteria* orders had a higher contribution to the LNA fraction regardless the month of sampling. An analysis of the marine genomic database indicates a median genome size of 5 Mbp for marine *Gammaproteobacteria* (average 4.6 Mbp, $n = 43$), which falls in the larger end of the range for marine genomes and may suggest a culture bias towards fast-growing and ecologically adaptable members of this group (Massana *et al*., 2001). Alternative cultivation strategies (Rappé *et al*., 2002) or single-genome amplification (e.g. Swan *et al*., 2011) may provide

better insights into the *Gammaproteobacteria* more typical of the LNA fractions.

The four alternative hypotheses of Bouvier and colleagues (2007) to explain the cytometric signatures of natural communities can be tested with our data. We observed relatively little overlap between NA fractions within and between months (Table 1, Table 2), with few OTUs abundant under more than one condition. This is at odds with two of the Bouvier and colleagues (2007) scenarios: (i) that LNA communities consist of inactive groups that move to HNA when they become active, and (ii) that HNA communities consist of active groups that move into the LNA fraction after death or inactivation. The rare bacterial biosphere has been considered a 'seed-bank' of taxa that could become abundant when conditions turn favourable (Pedrós-Alió, 2007). However, if the LNA fraction functioned as a seed bank for the HNA community, it should exhibit high OTU diversity harbouring a variety of potentially active phylotypes, but this was not observed (Fig. 2, Fig. S3). Furthermore, the seed bank hypothesis predicts that some OTUs present in the LNA fraction should also be found in the HNA, but this was not often observed. Rather, our data better support the Bouvier and colleagues (2007) scenarios 3 or 4. Scenario 3 (that flow cytometric communities contain distinctly different taxa) is consistent with most SAR11, SAR86, SAR116 and *Rhodobacterales* OTUs having fidelity to a specific fraction. Scenario 4 (that there is a constant flux of cells between flow cytometric communities) is consistent with the observation that some *Gammaproteobacteria* OTUs and more minor groups (*Actinobacteria* and *Betaproteobacteria*) are equally likely to appear in both, the HNA and LNA fractions.

The highest OTU diversity and richness was found in the HNA-HS fraction, and the lowest in the LNA fraction (Fig. 2, Fig. S3). Several studies have found that the percentage of HNA cells correlate positively to bacterial production and activity (Lebaron *et al.*, 2001; 2002; Zubkov *et al.*, 2001a; 2006; Mary *et al.*, 2006; Talarmin *et al.*, 2011), but the relationship between diversity and productivity in aquatic microbial communities is not yet well established (Smith, 2007). Pommier and colleagues (2010) suggested that a positive correlation between productivity and diversity in a transect in NW Mediterranean waters was due to the presence of more ecological niches in coastal waters as compared with open ocean ones. Philippot and colleagues (2010) associated large genomes with bacterial groups able to adapt to changing environments, using their repertoire of transport and regulation genes. Our data are consistent with the idea that the HNA fraction is composed of these versatile fast-growing bacteria (Schattenhofer *et al.*, 2011) whose larger genomes allow them to occupy more ecological niches.

Conclusions

Substantially different bacterial communities were found in the flow cytometrically defined populations of NW Mediterranean coastal waters. The HNA fractions were composed primarily of versatile bacteria predicted to have large and flexible genomes, such as members of the *Rhodobacterales* and *Bacteroidetes* groups, whereas LNA fractions were composed primarily of cells thought to have less adaptability and smaller genomes, such as SAR11. Most OTUs appeared in one fraction and one season only. The *Gammaproteobacteria* group (other than SAR86) was the exception to this rule, having OTUs with inconsistent contributions to the different NA fractions across the two seasonal samples. We conclude that most bacterial taxa are predictably present in a single cytometric population, although some (at least as defined by 95% 16S rRNA sequence similarity) might sort into different populations according to their physiological state.

Experimental procedures

Samples

Surface seawater samples were collected 1 km offshore in Blanes Bay, NW Mediterranean (the Blanes Bay Microbial Observatory, 41°40′N, 2°48′E), filtered through a 200 µm mesh net and kept in the dark in 25 l polycarbonate carboys at *in situ* temperature until processed in the laboratory (usually ~ 2 h after sampling). The average values of abundances of flow-cytometrically derived populations from samplings from 2003 to 2010 are shown in Fig. S1. Samplings were carried out at least once a month, from a depth of 0.5 m at 10 AM. The samples used for cell sorting were from 17 March 2009 (winter sample; winter covered December to early March) and from 5 August 2008 (summer sample; summer covered June to late September). In all replicates, subsamples (1.6 ml) were preserved with 1% paraformaldehyde + 0.01% glutaraldehyde (final concentrations) at room temperature for 10 min in the dark and then flash-frozen in liquid nitrogen. The samples were stored at −80°C until flow-cytometry analyses. CHL was measured by fluorometry in 90% acetone extracts (extraction overnight at 4°C) from 150 ml of samples filtered through GF/F filters.

Flow cytometry

Flow-cytometric analyses were routinely performed with a Becton-Dickinson FACSCalibur machine with a blue-light (488 nm) laser operating at 15 mW and a bandpass filter at 530/30 nm (green fluorescence) and a longpass filter at 650 nm (red fluorescence). Staining with SYBRGreen I (1:100 final dilution of the commercial stock, or 10× final concentration of the commercial stock; Molecular Probes) was carried out for 5–10 min in the dark at room temperature, and the sample was run at low speed (*c.* 15 µl min^{-1}) with MilliQ water as sheath fluid. Cells were detected by SSC-

green fluorescence (FL1) as previously described (Gasol and del Giorgio, 2000). Based on their size and fluorescence characteristics, we discriminated three populations: LNA, HNA-LS and HNA-HS (Fig. 1). Other authors have found the same populations, and labelled them similarly (e.g. Zubkov *et al*., 2001a; 2002a; 2003; 2004).

Cell sorting

Cell sorting was done with a MoFlo flow cytometer (DakoCytomation, Glostrup, Denmark. Laser at 488 nm, 100 mW, bandpass filters at 529/28 and 625/26 nm), which has lower discriminating power for SSC than the FACSCalibur but electrostatically sorts rapidly into a very small volume amenable to subsequent molecular analysis. The cleaning protocol of the instrument included 2 h of constant flow of 10% bleach solution through the system followed by a 2 h run of sterile filtered DI water and 4 h of sterilized phosphate-buffered saline (PBS) solution (Puraflo; Dakocytomation, Fort Collins, CO). Sterilized PBS served as the sheath fluid. Samples were thawed and stained with SYBRGreen I in the dark for 5 min at room temperature and then mixed with an internal bead standard (1.00 μm YG-Polysciences Fluoresbrite) prior to analysis. The three bacterial populations were discriminated in the MoFlo based on their FL1 and FL3 signals (red fluorescence, Fig. 1) in the absence of a strong SSC signal (see Fig. 1). Since the same samples observed in the FACSCalibur also showed three populations in the FL1–FL3 scatterplot (Fig. 1), we were confident that the populations as ordered by increasing fluorescence in the MoFlo were the same as those identified as LNA, HNA-LS and HNA-HS in the SSC-FL1 plot with the FACSCalibur (see Fig. 1).

The MoFlo sorter was set in 'purify 1–2 drop' sort mode and a flow sorting rate of 150–200 events s^{-1}. A *Synechoccoccus* population evident only in the winter sample was sorted as a positive control and to detect sample cross-contamination. The populations were simultaneously sorted into several sterilized 0.5 ml polypropylene tubes containing 1 ml of sterilized PBS until a minimum of 50 000 events for each FC population was obtained.

DNA preparation and sequencing

We followed the protocol used by Mou and colleagues (2005) with minor modifications. Briefly, sorted cells from each population were filtered onto 0.2-μm-pore-size polycarbonate filter (Poretics Products) and washed two times with sterile PBS and two times with 0.2 μm filtered deionized water. The edges of the filter were trimmed off to avoid PCR inhibition. The filter was air dried and transferred to a 0.5 ml tube. Ten microlitres of Lyse-N-Go PCR reagent (Pierce, Rockford, IL) was added to the tube to lyse the bacterial cells on the filter according to the manufacturer's protocol. A second lysis step was conducted by adding 82 μl of 0.2 μm filtered deionized water to the same tube and subjecting the tube to three freeze–thaw cycles of 10 min at −80°C and 65°C. The resulting lysate served as the DNA template for PCR amplification.

Partial bacterial 16S ribosomal RNA genes including the hypervariable region V6 were PCR-amplified using a mix of five forward primers (967F) and four reverse primers (1046R) (Sogin *et al*., 2006), with unique tags on the primers to discriminate samples. The reaction was carried out with four Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, NJ) with a final concentration of forward and reverse primers mixtures of 0.4 μM. PCR conditions were as follows: 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, primer extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PBS sheath fluid used for sorting, and a sort from a 'noise' gate (a gate placed on a cytogram region of high SSC and low FL1 where no bacteria should appear; Fig. 1) were used as negative controls. PCR amplification was confirmed by electrophoresis on ethidium bromide-stained 1.5% agarose gels. The PCR products were cleaned using the QIAquick PCR Purification kit (Qiagen) and a second cleaning step was performed using the Agencourt AMPure XP kit (Agencourt Bioscience, Beckman Coulter). The PCR products were quantified using PicoGreen dsDNA quantification kit (Molecular Probes, Eugene, OR) and sequenced using a Roche GS FLX sequencer. The partial run yielded a total of 14.7 Mbp from 233 007 reads representing an average of 1918 sequences per sample (63 bp average length). All 454 sequences can be accessed from the CAMERA database under project id CAM_P_0000908.

Data analysis

Reads were eliminated if they did not perfectly match the PCR primer at the beginning of a read, were shorter than 50 bp after the proximal primer, or contained more than one N. The remaining sequences were then clustered at a 95% identity threshold (*c*. 3 bp average difference) using CD-HIT (Li and Godzik, 2006), and singletons were removed (from sequences pooled across all samples) in order to prevent inflation of the diversity estimates (32% of total clusters) (Kunin *et al*., 2010). As observed in other studies (e.g. Quince *et al*., 2011), we checked that the differences between using DeNoiser or our own data purging protocol were very minor (details not shown). Some singletons (7.8%) remained present in this partial run since they were not singletons in the total run. Taxonomic affiliation of the 95% clusters (Operational Taxonomic Units, OTUs) was determined by taxonomic assignment of the reference sequence (the longest sequence of the cluster, Li and Godzik, 2006) using two different criteria: a minimum 80% sequence similarity and 80% overlap of the reference sequence to a 16S rDNA database (RDP) and a minimum of 90% sequence identity and an overlap of 80% of the reference sequence in a pairwise Smith-Waterman alignment to members of an in-house database containing marine 16S rRNA *Bacteria* and *Archaea* gene sequences retrieved from the GOS database (Biers *et al*., 2009); the reference database is available at http://www.simo.marsci.uga.edu/public_db/. Unmatched sequences were manually inspected and assigned to a group based on the local Smith-Waterman alignment. Alpha-diversity was estimated by the abundance-based indices Chao1, ACE and the Shannon and evenness indices. Shannon index was defined as H = −sum p_i log(b) p_i, where p_i is the proportional abundance of OTU i and b is the base of the logarithm. Beta-diversity analyses were based on the Bray-Curtis distance measure

for community similarity and represented in a non-metric multidimensional scaling (NMDS) after normalizing to the total number of sequences within a sample to account for differences in read counts between the libraries. A permutational MANOVA (McArdle and Anderson, 2001) was used to elucidate the factors significantly correlated with community composition (i.e. season, DNA content, gate). We considered that an OTU was abundant if it accounted for > 1% of total reads, frequent if it accounted 0.05–1%, and rare it accounted for less than 0.05%. All statistical analyses were carried out in R (http://www.r-project.org/) with the Vegan package version 1.17 (Oksanen *et al.*, 2010). Marine genome sizes were downloaded from http://www.megx.net (Kottmann *et al.*, 2010).

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References

Alonso, C., and Pernthaler, J. (2006) Concentration-dependent patterns of leucine incorporation by coastal picoplankton. *Appl Environ Microbiol* **72:** 2141–2147.

Alonso-Sáez, L., and Gasol, J.M. (2007) Seasonal variation in the contribution of different bacterial groups to the uptake of LMW-DOM compounds in the NW Mediterranean coastal waters. *Appl Environ Microbiol* **73:** 3528–3535.

Alonso-Sáez, L., Balagué, V., Sà, E.L., Sánchez, O., González, J.M., Pinhassi, J., *et al*. (2007) Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiol Ecol* **60:** 98–112.

Alonso-Sáez, L., Vázquez-Domínguez, E., Cardelús, C., Pinhassi, J., Sala, M.M., Lekunberri, I., *et al*. (2008) Factors controlling the year-round variability in carbon flux through bacteria in a coastal marine system. *Ecosystems* **11:** 397–409.

Bailey, J., Fazel-Makjlessi, J., McQuitty, D., Lee, Y., Allred, J., and Oro, J. (1977) Characterization of bacterial growth by means of flow microfluorometry. *Science* **198:** 1175–1176.

Bernard, L., Schäfer, H., Joux, F., Courties, C., Muyzer, G., and Lebaron, P. (2000a) Genetic diversity of total, active and culturable marine bacteria in coastal seawater. *Aquat Microb Ecol* **23:** 1–11.

Bernard, L., Courties, C., Servais, P., Troussellier, M., Petit, M., and Lebaron, P. (2000b) Relationships among bacterial cell size, productivity and genetic diversity in aquatic environments using cell sorting and flow cytometry. *Microb Ecol* **40:** 148–158.

Biers, E.J., Sun, S., and Howard, E.C. (2009) Prokaryotic genomes and diversity in surface ocean waters: interrogating the global ocean sampling metagenome. *Appl Environ Microbiol* **75:** 2221–2229.

Bouvier, T., Del Giorgio, P.A., and Gasol, J.M. (2007) A comparative study of the cytometric characteristics of high and low nucleic-acid bacterioplankton cells from different aquatic ecosystems. *Environ Microbiol* **9:** 2050–2066.

Calvo-Díaz, A., and Morán, X.A.G. (2006) Seasonal dynamics of picoplankton in shelf waters of the southern Bay of Biscay. *Aquat Microb Ecol* **42:** 159.

Cottrell, M.T., and Kirchman, D.L. (2000) Natural assemblages of marine proteobacteria and members of the Cytophaga–Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66:** 1692–1697.

Fuchs, B.M., Zubkov, M.V., Sahm, K., Burkill, P.H., and Amann, R. (2000) Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. *Environ Microbiol* **2:** 191–201.

Fuchs, B.M., Woebken, D., Zubkov, M.V., Burkill, P., and Amann, R. (2005) Molecular identification of picoplankton populations in contrasting waters of the Arabian Sea. *Aquat Microb Ecol* **39:** 145–157.

Gasol, J.M., and Del Giorgio, P.A. (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci Mar* **64:** 197–224.

Gasol, J.M., Zweifel, U.L., Peters, F., Fuhrman, J.A., and Hagstrom, A. (1999) Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Appl Environ Microbiol* **65:** 4475–4483.

del Giorgio, P.A., Bird, D.F., Prairie, Y.T., and Planas, D. (1996) Flow cytometric determination of bacterial abundance in lake plankton with the green nucleid acid stain SYTO 13. *Limnol Oceanogr* **41:** 783–789.

Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D., *et al*. (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309:** 1242–1245.

González, J.M., Simó, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedrós-Alió, C., and Moran, M.A. (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing north Atlantic bloom. *Appl Environ Microbiol* **66:** 4237–4246.

Jellett, J.F., Li, W.K.W., Dickie, P.M., Boraie, A., and Kepkay, P.E. (1996) Metabolic activity of bacterioplankton communities assessed by flow cytometry and single carbon substrate utilization. *Mar Ecol Prog Ser* **136:** 213–225.

Kottmann, R., Kostadinov, I., Duhaime, M.B., Buttigieg, P.L., Yilmaz, P., Hankeln, W., *et al*. (2010) Megx.net: integrated database resource for marine ecological genomics. *Nucleic Acids Res* **38:** D391–D395.

Kunin, V., Engelbrektson, A., Ochman, H., and Hugenholtz, P. (2010) Wrinkles in the rare biosphere: pyrosequencing errors lead to artificial inflation of diversity estimates. *Environ Microbiol* **12:** 118–123.

Lebaron, P., Servais, P., Agogue, H., Courties, C., and Joux, F. (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Appl Environ Microbiol* **67:** 1775–1782.

Lebaron, P., Servais, P., Baudoux, A.C., Bourrain, M., Courties, C., and Parthuisot, N. (2002) Variations of bacterial-specific activity with cell size and nucleic acid content assessed by flow cytometry. *Aquat Microb Ecol* **28:** 131–140.

Li, W., and Godzik, A. (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22:** 1658–1659.

Li, W., Jellet, J.F., and Dickie, P.M. (1995) DNA distributions in planktonic bacteria stained with TOT0 or TO-PRO. *Limnol Oceanogr* **40:** 1485–1495.

Longnecker, K., Sherr, B.F., and Sherr, E.B. (2005) Activity and phylogenetic diversity of bacterial cells with high and low nucleic acid content and electron transport system activity in an upwelling ecosystem. *Appl Environ Microbiol* **71:** 7737–7749.

Longnecker, K., Sherr, B.F., and Sherr, E.B. (2006) Variation in cell-specific rates of leucine and thymidine incorporation by marine bacteria with high and with low nucleic acid content off the Oregon coast. *Aquat Microb Ecol* **43:** 113.

McArdle, B.H., and Anderson, M.J. (2001) Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82:** 290–297.

Marie, D., Partensky, F., Jacquet, S., and Vaulot, D. (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl Environ Microbiol* **63:** 186–193.

Mary, I., Heywood, J.L., Fuchs, B.M., Amann, R., Tarran, G.A., Burkill, P.H., and Zubkov, M.V. (2006) SAR11 dominance among metabolically active low nucleic acid bacterioplankton in surface waters along an Atlantic meridional transect. *Aquat Microb Ecol* **45:** 107–113.

Massana, R., Pedrós-Alió, C., Casamayor, E.O., and Gasol, J.M. (2001) Changes in marine bacterioplankton phylogenetic composition during incubations designed to measure biogeochemically significant parameters. *Limnol Oceanogr* **46:** 1181–1188.

Morán, X.A., Bode, A., Suarez, L.A., and Nogueira, E. (2007) Assessing the relevance of nucleic acid content as an indicator of marine bacterial activity. *Aquat Microb Ecol* **46:** 141–152.

Morán, X.A.G., and Calvo-Díaz, A. (2009) Single-cell vs. bulk activity properties of coastal bacterioplankton over an annual cycle in a temperate ecosystem. *FEMS Microbiol Ecol* **67:** 43–56.

Morris, R.M., Rappé, M.S., Connon, S.A., Vergin, K.L., Slebold, W.A., Carlson, C.A., and Giovanonni, S.J. (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420:** 806–810.

Mou, X., Moran, M.A., Stepanauskas, R., González, J.M., and Hodson, R.E. (2005) Flow-cytometric cell sorting and subsequent molecular analyses for culture-independent identification of bacterioplankton involved in dimethylsulfoniopropionate transformations. *Appl Environ Microbiol* **71:** 1405–1416.

Newton, R.J., Jones, S.E., Eiler, A., McMahon, K.D., and Bertilsson, S. (2011) A guide to the natural history of freshwater lake bacteria. *Microbiol Mol Biol Rev* **75:** 14–49.

Oh, H.-M., Kwon, K.K., Kang, I., Kang, S.G., Lee, J.-H., Kim, S.-J., and Cho, J.-C. (2010) Complete genome sequence of '*Candidatus* Puniceispirillum marinum' IMCC1322, a representative of the SAR116 clade in the *Alphaproteobacteria*. *J Bacteriol* **192:** 3240–3241.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., O'Hara, R.B., Simpson, G.L., *et al*. (2010) vegan: community ecology package. R package version 1.17-2 [WWW document]. URL http://CRANR-projectorg/package=vegan.

Pedrós-Alió, C. (2007) Dipping into the rare biosphere. *Science* **315:** 192–193.

Philippot, L., Andersson, S.G.E., Battin, T.J., Prosser, J.I., Schimel, J.P., Whitman, W.B., and Hallin, S. (2010) The ecological coherence of high bacterial taxonomic ranks. *Nat Rev Microbiol* **8:** 523–529.

Pommier, T., Neal, P., Gasol, J., Coll, M., Acinas, S., and Pedrós-Alió, C. (2010) Spatial patterns of bacterial richness and evenness in the NW Mediterranean Sea explored by pyrosequencing of the 16S rRNA. *Aquat Microb Ecol* **61:** 221–233.

Quince, C., Lanzen, A., Davenport, R., and Turnbaugh, P. (2011) Removing noise from pyrosequenced amplicons. *BMC Bioinformatics* **12:** 38.

Rappé, M.S., Connon, S.A., Vergin, K.L., and Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418:** 630–633.

Robertson, B.R., and Button, D.K. (1989) Characterizing aquatic bacteria according to population, cell size, and apparent DNA content by flow cytometry. *Cytometry* **10:** 70–76.

Schattenhofer, M., Wulf, J., Kostadinov, I., Glöckner, F.O., Zubkov, M.V., and Fuchs, B.M. (2011) Phylogenetic characterisation of picoplanktonic populations with high and low nucleic acid content in the North Atlantic Ocean. *Syst Appl Microbiol* **34:** 470–475.

Servais, P., Casamayor, E.O., Courties, C., Catala, P., Parthuisot, N., and Lebaron, P. (2003) Activity and diversity of bacterial cells with high and low nucleic acid content. *Aquat Microb Ecol* **33:** 41–51.

Sieracki, M.E., and Viles, C.L. (1992) Distributions and fluorochrome-staining properties of submicrometer particles and bacteria in the North Atlantic. *Deep Sea Res A* **39:** 1919–1929.

Smith, V.H. (2007) Microbial diversity-productivity relationships in aquatic ecosystems. *FEMS Microbiol Ecol* **62:** 181–186.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., *et al*. (2006) Microbial diversity in the deep sea and the underexplored 'rare biosphere'. *Proc Natl Acad Sci USA* **103:** 12115–12120.

Swan, B.K., Martinez-Garcia, M., Preston, C.M., Sczyrba, A., Woyke, T., Lamy, D., *et al*. (2011) Potential for chemolithoautotrophy among ubiquitous bacteria lineages in the dark ocean. *Science* **333:** 1296–1300.

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Talarmin, A., Wambeke, F.V., Catala, P., Courties, C., and Lebaron, P. (2011) Flow cytometric assessment of specific leucine incorporation in the open Mediterranean. *Biogeosciences* **8:** 253–265.

Trigui, H., Masmoudi, S., Brochier-Armanet, C., Barani, A., Grégori, G., Denis, M., *et al.* (2011) Characterization of heterotrophic prokaryote subgroups in the Sfax coastal solar salterns by combining flow cytometry cell sorting and phylogenetic analysis. *Extremophiles* **15:** 347–358.

Vila-Costa, M., Pinhassi, J., Alonso, C., Pernthaler, J., and Simó, R. (2007) An annual cycle of dimethylsulfoniopropionate-sulfur and leucine assimilating bacterioplankton in the coastal NW Mediterranean. *Environ Microbiol* **9:** 2451–2463.

Wang, Y., Hammes, F., Boon, N., Chami, M., and Egli, T. (2009) Isolation and characterization of low nucleic acid (LNA)-content bacteria. *ISME J* **3:** 889–902.

Zubkov, M.V., Fuchs, B.M., Burkill, P.H., and Amann, R. (2001a) Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. *Appl Environ Microbiol* **67:** 5210–5218.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2001b) Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea. *Environ Microbiol* **3:** 304–311.

Zubkov, M.V., Fuchs, B.M., Tarran, G.A., Burkill, P.H., and Amann, R. (2002a) Mesoscale distribution of dominant bacterioplankton groups in the northern North Sea in early summer. *Aquat Microb Ecol* **29:** 135–144.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2002b) Rapid turnover of dissolved DMS and DMSP by defined bacterioplankton communities in the stratified euphotic zone of the North Sea. *Deep Sea Res Part II Top Stud Oceanogr* **49:** 3017–3038.

Zubkov, M.V., Fuchs, B.M., Tarran, G.A., Burkill, P.H., and Amann, R. (2003) High rate of uptake of organic nitrogen compounds by *Prochlorococcus* cyanobacteria as a key to their dominance in oligotrophic oceanic waters. *Appl Environ Microbiol* **69:** 1299–1304.

Zubkov, M.V., Allen, J.I., and Fuchs, B.M. (2004) Coexistence of dominant groups in marine bacterioplankton community: a combination of experimental and modelling approaches. *J Mar Biol Assoc UK* **84:** 519–529.

Zubkov, M.V., Tarran, G.A., and Burkill, P.H. (2006) Bacterioplankton of low and high DNA content in the suboxic waters of the Arabian Sea and the Gulf of Oman: abundance and amino acid uptake. *Aquat Microb Ecol* **43:** 23–32.

Zubkov, M.V., Mary, I., Woodward, E.M., Warwick, P.E., Fuchs, B.M., Scanlan, D.J., and Burkill, P.H. (2007) Microbial control of phosphate in the nutrient-depleted North Atlantic subtropical gyre. *Environ Microbiol* **9:** 2079–2089.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Eight-year average values of the contribution of HNA-HS, HNA-LS and LNA to total prokaryote abundance at Blanes Bay Microbial Observatory (J.M. Gasol, L. Alonso-Sáez, R. Simó and M. Vila-Costa, in preparation).
Fig. S2. Contribution to bacterial community composition of different bacterial groups (classified at phylum level or below) for each sorted fraction (symbols) in two replicate samples. The continuous line is the 1:1, and the dashed lines indicate 10% deviation from 1:1.
Fig. S3. Rarefraction curves for HNA-HS, HNA-LS and LNA fractions.
Fig. S4. Rank abundance curves for HNA-HS, HNA-LS and LNA fractions combining summer and winter samples.
Fig. S5. Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distance matrices comparing bacterial community composition of HNA-HS, HNA-LS and LNA fractions in summer and winter samples.
Fig. S6. Phylogenetic composition of HNA-HS, HNA-LS and LNA fractions. Abbreviations of taxonomic groups are as follows: cyano, *Cyanobacteria*; prochl, *Prochlorococcus*; synech, *Synechococcus*; euk, eukaryotes.
Fig. S7. Venn diagram of abundant OTUs (> 1%) in the HNA (HNA-LS plus HNA-HS) and LNA populations in summer and winter. The position and size of the spheres approximate the position and size of the populations in flow cytometry plots (as in Fig. 1).
Table S1. Number of OTUs in order-level grouping within the *Gammaproteobacteria* present in the LNA and HNA fractions in summer and winter. Per cent contribution to the indicated NA fraction is given in parentheses.

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Contails 1 Table (S1) and 7 figures (SF1-SF7)

Table S1. Number of OTUs in order-level grouping within the *Gammaproteobacteria* present in the LNA and HNA fractions in summer and winter. Percent contribution to the indicated NA fraction is given in parentheses.

Order	Summer-LNA	Summer-HNA	Winter-LNA	Winter-HNA
Alteromonadales	5 (11.4)	2 (25.0)	1 (3.1)	2 (7.1)
Chromatiales	1 (2.3)	0 (0)	1 (3.1)	1 (3.6)
Enterobacteriales	11 (25.0)	2 (25.0)	4 (12.5)	5 (17.9)
Oceanospirillales	7 (15.9)	2 (25.0)	7 (21.9)	10 (35.7)
Pseudomonadales	1 (2.3)	1 (12.5)	4 (12.5)	2 (7.1)
Thiotrichales	1 (2.3)	0 (0)	1 (3.1)	0 (0)
Vibrionales	4 (9.1)	0 (0)	1 (3.1)	0 (0)
Unidentif. Gamma	8 (18.2)	1 (12.5)	13 (40.6)	8 (28.6)

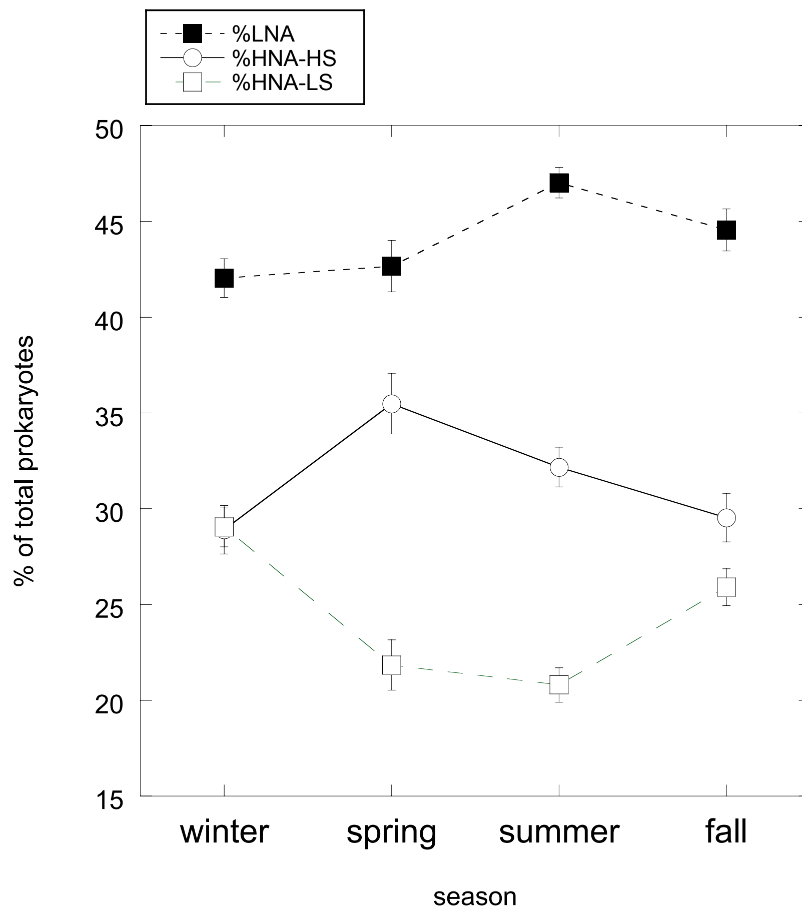

Fig. S1. Eight-year average values of the contribution of HNA-HS, HNA-LS and LNA to total prokaryote abundance at Blanes Bay Microbial Observatory (J.M. Gasol, L. Alonso-Sáez, R. Simó and M. Vila-Costa, in preparation).

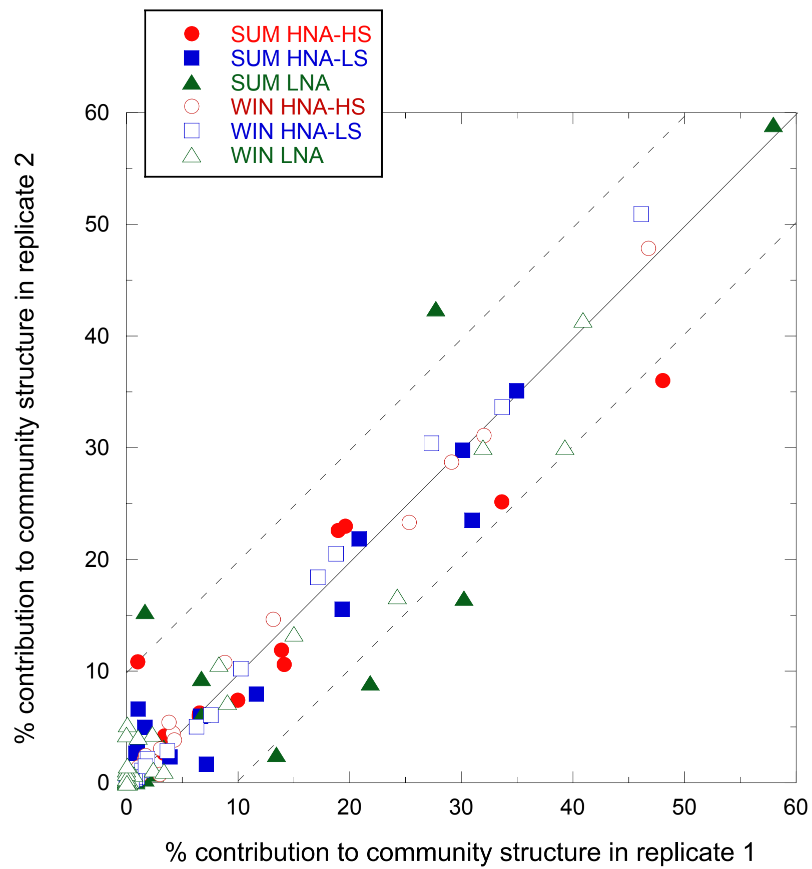

Fig. S2. Contribution to bacterial community composition of different bacterial groups (classified at phylum level or below) for each sorted fraction (symbols) in two replicate samples. The continuous line is the 1:1, and the dashed lines indicate 10% deviation from 1:1.

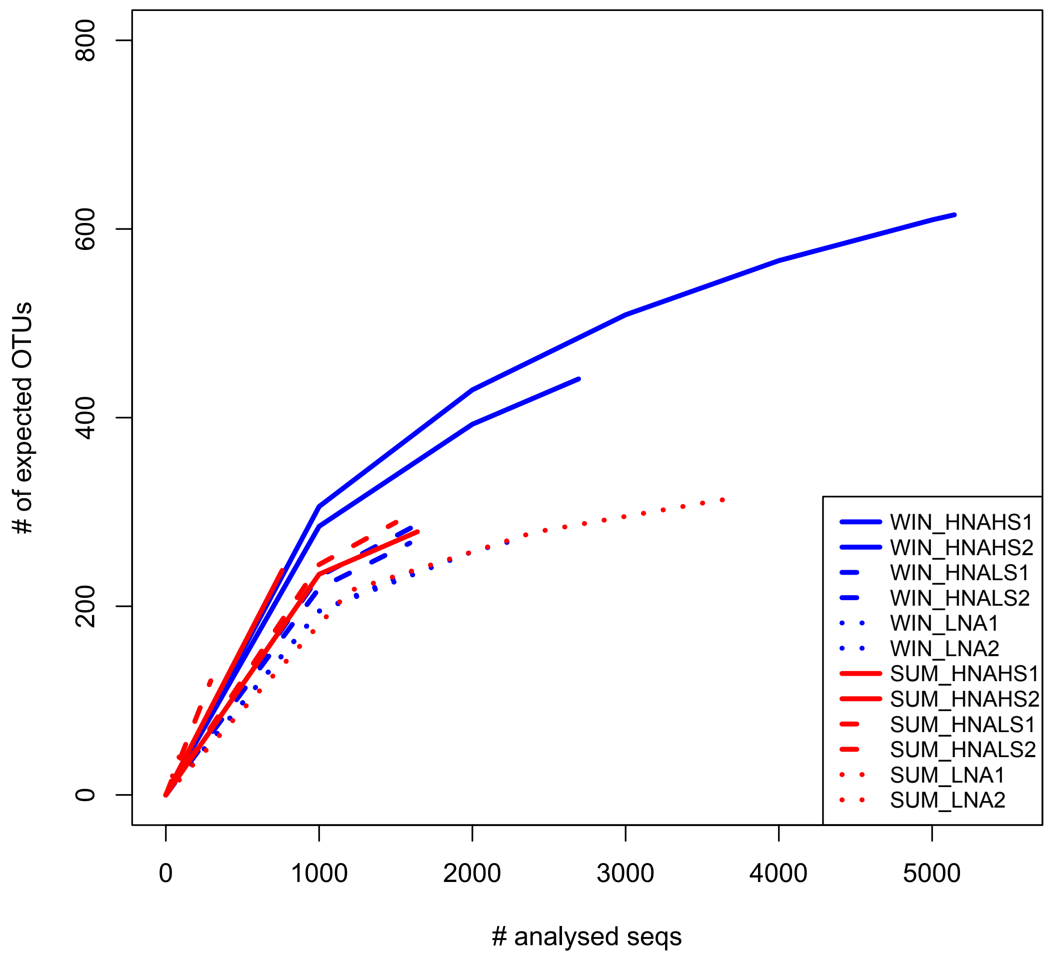

Fig. S3. Rarefraction curves for HNA-HS, HNA-LS and LNA fractions.

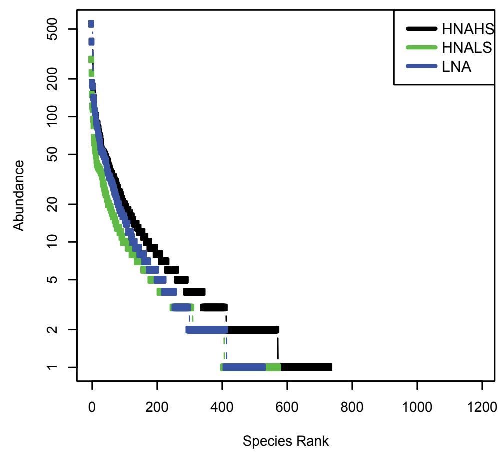

Fig. S4. Rank abundance curves for HNA-HS, HNA-LS and LNA fractions combining summer and winter samples.

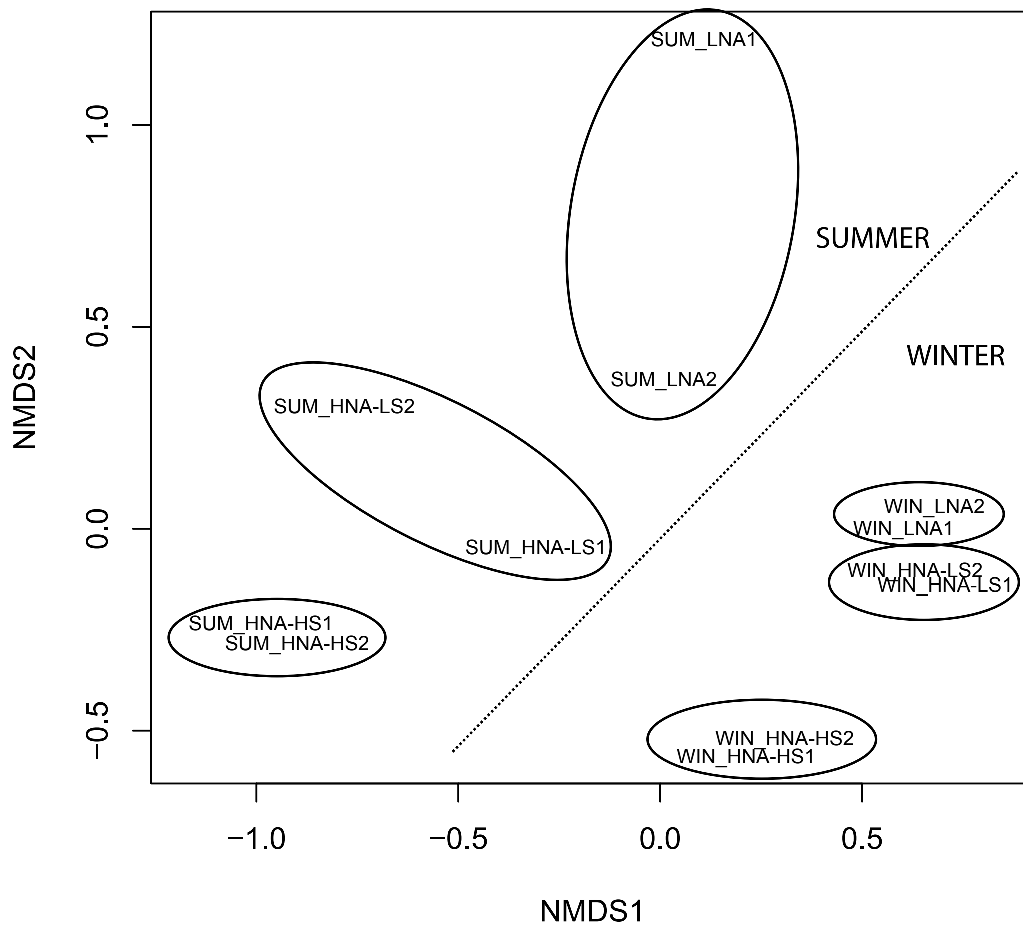

Fig. S5. Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distance matrices comparing bacterial community composition of HNA-HS, HNA-LS and LNA fractions in summer and winter samples.

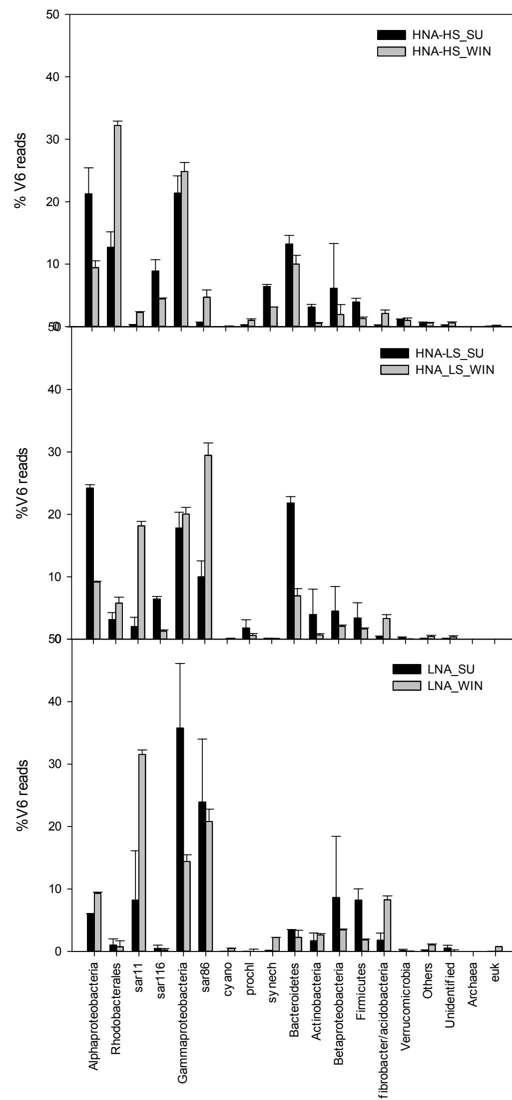

Fig. S6. Phylogenetic composition of HNA-HS, HNA-LS and LNA fractions. Abbreviations of taxonomic groups are as follows: cyano, *Cyanobacteria*; prochl, *Prochlorococcus*; synech, *Synechococcus*; euk, eukaryotes.

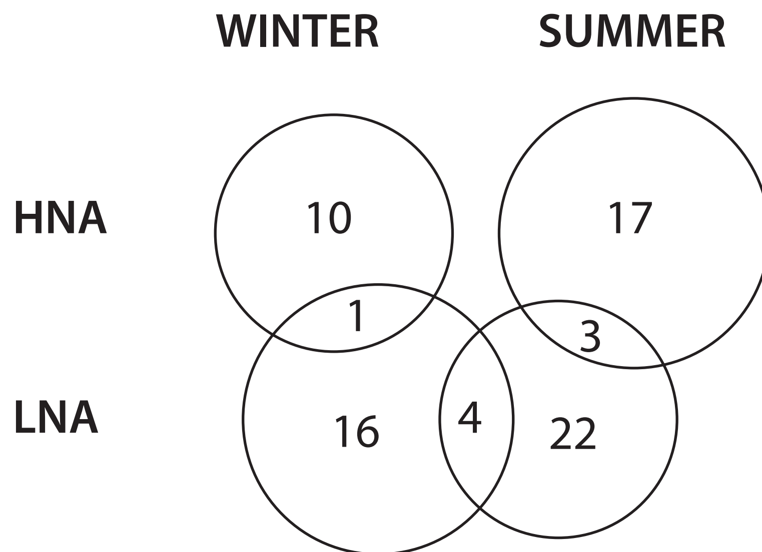

Fig. S7. Venn diagram of abundant OTUs (> 1%) in the HNA (HNA-LS plus HNA-HS) and LNA populations in summer and winter. The position and size of the spheres approximate the position and size of the populations in flow cytometry plots (as in Fig. 1).