Seasonality in molecular and cytometric diversity of marine bacterioplankton: the reshuffling of bacterial taxa by vertical mixing.

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Summary

The 'cytometric diversity' of phytoplankton communities has been studied based on single-

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cell properties, but the applicability of this method to characterize bacterioplankton has been unexplored. Here, we analysed seasonal changes in cytometric diversity of marine bacterioplankton along a decadal time-series at three coastal stations in the Southern Bay of Biscay. Shannon-Weaver diversity estimates and Bray-Curtis similarities obtained by cytometric and molecular (16S rRNA tag sequencing) methods were significantly correlated in samples from a 3.5-year monthly time-series. Both methods showed a consistent cyclical pattern in the diversity of surface bacterial communities with maximal values in winter. The analysis of the highly resolved flow cytometry time-series across the vertical profile showed that water column mixing was a key factor explaining the seasonal changes in bacterial composition and the winter increase in bacterial diversity in coastal surface waters. Due to its low cost and short processing time as compared to genetic methods, the cytometric diversity approach represents a useful complementary tool in the macroecology of aquatic microbes.

Introduction

Marine bacteria are the most diverse component of planktonic communities (Giovannoni and Rappe 2000; Venter et al. 2004) and constitute one of the largest biomass stocks in the ocean (Buitenhuis et al. 2012). Identifying microbial taxa present in the environment and understanding the changes in the composition of microbial communities over spatio-temporal gradients has been a challenge in microbial ecology studies (Pielou 1975; Giovannoni and Stingl 2005; Green et al. 2008; Zinger et al. 2011).

During the last two decades, the use of molecular tools has revolutionized the way ecologists study the distribution and diversity of marine bacteria taxa (Zinger et al. 2012). Different seasonal, spatial and latitudinal patterns in bacterioplankton phylogenetic

composition have been reported using fingerprinting methods such as Denaturing Gradient Gel Electrophoresis (DGGE), Automated Ribosomal intergenic Spacer analysis (ARISA) or Terminal Fragment Length Polymorphysm (TRFLP) (Schauer et al. 2000; Morris et al. 2005; Fuhrman et al. 2008; Chow et al. 2013). While these methods enable a fast comparison of samples, other approaches such as Fluorescence In Situ Hybridization (FISH), have permitted the determination of the abundance of specific target populations or quantitative changes in the phylogenetic composition of bacterial communities with a reasonable accuracy (Cottrell and Kirchman 2000; Pernthaler et al. 2002; Amann and Fuchs 2008; Schattenhofer et al. 2009). Recently, the development of high-throughput sequencing technologies has represented a breakthrough in this field by increasing several orders of magnitude the number of sequences typically obtained per sample (Sogin et al. 2006; Zinger et al. 2011; Vergin et al. 2013; Salter et al. 2014).

While all molecular approaches have their own advantages and disadvantages (e.g. Alonso-Sáez et al. (2007)), a common limitation is that they are time-consuming and/or expensive for large-scale studies requiring the analysis of hundreds to thousands of samples. In early ecological studies, Margalef (1968) proposed other classification criteria, the photosynthetic pigment composition, to study the structure and composition of phytoplankton communities. Later, Li (1997) introduced the concept of 'cytometric diversity' to characterize marine phytoplankton communities, using individual cell properties measured by flow cytometry. In natural and stained samples, cytometric properties such as light scatter and fluorescence reflect changes in cell size, complexity, pigment, and nucleic acid content, and therefore, they can be used to classify planktonic cells. In an initial application of this method in freshwater bacterial communities, its power in differentiating bacterial groups was limited to a few (i.e. < 10) groups, presumably made

up of a variable number of species (Schiaffino et al. 2013). However, the applicability of cytometric diversity based on cytometric bins potentially allows the differentiation of a substantially larger number (> 100) of bacterial groups. Thus, this methodology woud represent an important advance to detect changes in community composition, as flow cytometry is technically simple, requires small volumes, and allows a relatively fast analysis of hundreds to thousands of samples.

Here, we analysed the seasonal dynamics of heterotrophic bacterioplankton in a decadal time-series in the Southern Bay of Biscay using the cytometric diversity approach and compared it with genetic diversity estimated through molecular methods. While seasonal changes in the composition of coastal marine bacterioplankton have been widely reported (Alonso-Sáez et al. 2007 2015; Campbell et al. 2009; Gilbert et al. 2012; Chow et al. 2013), the factors controlling this seasonality are less understood. Using the power of the cytometric method, we performed a large-scale analysis at three continental shelf stations in the Cantabrian Sea (2586 samples in total), at a spatio-temporal resolution not easily achievable with molecular techniques. Our objectives were: (i) to test whether ecological meaningful patterns can be derived from cytometric diversity analysis in marine bacterioplankton, and (ii) to understand the factors responsible for seasonal changes in the diversity and composition of coastal bacterial communities.

Results

Comparison of genetic and cytometric diversities

In the molecular dataset from station E2 (obtained by 16S rRNA tag pyrosequencing over 3.5 years), a cyclical pattern of diversity with maximal Shannon-Weaver index values in winter and minima in early summer was found (using an operational taxonomic unit (OTU)

genetic distance cutoff of 0.03, Figure 1). This seasonality is clear both in the monthly time-series (Figure 1A) and in the average annual cycle (Figure 1B). As expected, the Shannon-Weaver diversity indices were higher when using a more stringent distance cutoff for OTU construction (0.01), but the same monthly and average annual patterns of diversity were observed (Figure S1 and S2).

While the number of taxa obtained by the molecular approach is ultimately defined by the genetic distance cutoff, cytometric diversity relies on the number of bins defined (e.g., 1024 categories in this study). Therefore, the absolute values of diversity indices obtained by the molecular and the cytometric approach cannot be meaningfully compared. Rather, our focus was on comparing spatio-temporal trends. The cytometric approach provided a similar, albeit weaker, seasonal pattern in the diversity of bacterial communities along the 3.5 years (Figure 1A). The largest differences between genetic and cytometric diversity were found during 2011, when relatively high cytometric diversity values were observed as compared to other years, especially from March to June (Figure 1A).

Despite this discrepancy, the average annual cycles obtained with both methodologies were similar, with highest Shannon-Weaver index values during winter and lowest in late spring (Figure 1B). While a significant, although weak correlation was found between individual estimates of Shannon-Weaver diversity obtained by both methods (Figure 2A, $r = 0.46$, $p < 0.01$), monthly averages were highly correlated (Figure 2B, $r = 0.93$, $p < 0.01$). These results indicate that despite some individual discrepancies, cytometric diversity can reliably capture general trends in the bacterioplankton community composition. A Mantel test confirmed a significant relationship between the similarity matrices calculated using the Bray-Curtis metric obtained by both techniques (Figure S3, Mantel statistic $r = 0.59$, $p = 0.001$).

Cytometric diversity patterns in the Cantabrian Sea

Overall, the decadal seasonal dynamics for the 3 stations analyzed (Figure 3) were similar to the pattern found for station E2 during the 3.5-year period (Figure 1), with higher cytometric diversity in early winter and late autumn than in summer (Figure 4). In order to understand the factors leading to temporal changes in surface bacterial cytometric diversity, we performed correlation analyses with different ancillary environmental variables (chlorophyll, temperature, salinity, inorganic nutrients, day length and mixed-layer depth, MLD) using the 10 year time-series. Three variables showed significant correlations of similar strength with the cytometric diversity of surface bacterial communities: day length, inorganic nutrients, (particularly NO_2-NO_3) and MLD. The three variables showed similar correlations at the three stations studied (day length: r = - 0.69, - 0.61 and - 0.54; NO_2-NO_3: r = 0.66, 0.62 and 0.23; MLD: r = 0.61, 0.64 and 0.44, for E1, E2 and E3 respectively), and as they were correlated, it is hard to determine the specific influence of each variable on surface cytometric diversity patterns. Therefore, we explored the influence of these factors on the bacterioplankton composition across another spatio-temporal dimension, the vertical gradient. In particular, for each month and station during the 10 year time-series, we compared the depth profiles of nutrient concentration and potential density with the similarity index calculated between the cytometric diversity of the surface sample and each of the remaining sample depths. We found that the similarity in cytometric diversity always decreased with depth (Figure 4D-4E-4F).

However, during the spring-summer months this decrease in similarity was more pronounced than during late autumn - early winter. This seasonal pattern was found at the three stations but, interestingly, the decay in similarity with depth was sharper at E1 than at

E2 or E3 (Figure 4D-4E-4F). During the summer months the similarity between surface and 20 meters at E1 was around 0.6 while at E2 and E3, the similarity was higher, ca. 0.8 (see Figure 4D and white line in Figure 4E-4F). Similarly, during summer, at E1 there was a sharp change in the cytometric similarity at around 5 meters, while this drastic change occurred at around 20 meters at E2 and E3.

Vertical nutrient profiles varied between stations and seasonally, and the shallowing of the nitracline was coincident in time with the decrease in cytometric similarity between depth and surface samples. Yet, the nitracline was deeper than the depth where the change in bacterial cytometric similarity occurred. The nutrient profiles (NO_2-NO_3) revealed a sharp gradient at ca. 80 and 120 meters at stations E2 and E3 respectively (Figure 4J-4K-4L), while the gradient in density was much shallower (Figure 4G-4H-4I). From April to September, no vertical gradient in nutrients was found at E1.

In contrast to nutrients, the MLD was coincident both in time and vertically with changes in bacterial cytometric composition, suggesting that this variable was the best to explain the cytometric similarity patterns observed at the stations (Figure 4A-4B-4C). During late fall and winter, MLD frequently reached the bottom while during spring-summer conditions it shoaled at all stations resembling the pattern in cytometric similarity. At E1, the MLD during the summer was at around 5-10 meters while at E2 and E3 it shoaled up to only 20 meters, similarly to the patterns found for the decay in cytometric diversity at the same stations (Figure 4D-4E-4F). The agreement between the dynamics of the MLD and cytometric diversity was also supported by the potential density profiles (Figure 4G-4H-4I).

We used the Bray-Curtis similarity metric for all the analyses explained above. Thus, we compared similarity in terms of bacterial community composition based on cytometric categories and their relative abundance (i.e. evenness). In order to clarify whether the

spatio-temporal variation found was mainly caused by changes in the cytometric categories present or by their evenness, we re-estimated the similarity matrix by using a metric based only on presence/absence data (i.e., the Jaccard's index). Hence, we replaced the abundance value in each cytometric category by 1 (when there were cells present) or 0 (when there were no cells in that category). In general, the pattern found using Jaccard's index was similar to that found by using the Bray-Curtis index (Figure S4), suggesting that the trend was due to a change in the presence/absence of particular cytometric categories rather than in their relative abundance. In summary, although we found a high correlation between surface bacterial community composition and day length, nutrients and MLD, the analysis of vertical patterns indicated that the dynamics of the MLD is the factor (of those tested) mostly affecting the composition of bacterioplankton communities

Discussion

Ribosomal phylogenetic markers are the *de facto* standard to analyze compositional and diversity changes in bacterial assemblages (Giovannoni and Rappé 2000). However, high-throughput molecular approaches are still expensive and time consuming when applied on a large number of samples, posing limitations in our knowledge of spatial and temporal patterns in oceanic microbial communities. A fast and easy processing of many microbial samples can be achieved by flow cytometry, a method that records bio-optical information of hundreds to thousands of cells per minute. While the single-cell properties obtained by flow cytometry have been used to study the diversity of phytoplankton communities (Li 1997 2002), its potential for the smallest-sized heterotrophic microorganisms (bacteria and archaea) remains unexplored and, in general, the power of this method to detect ecological patterns in microbial diversity is unclear. Schiaffino et al. (2013) estimated cytometric

diversity of freshwater picoplankton by analysing distinct cytometric populations (clusters) that shared similar properties, rather than the properties of each individual cell, as proposed by Li (1997). In this study we have adapted the methodology proposed by Li (1997) to study bacterioplankton using three cytometric properties to separate the cells falling into each cytometric category instead of the two originally used by Li (1997) for phytoplankton communities. The advantage of incorporating the third parameter is that we have another dimension to separate the cells falling in each cytometric category, thus increasing the resolution of the methodology (see Methods).

Our results show a significant agreement in the temporal pattern of bacterial community diversity using their cytometric properties and a molecular standard approach, opening the door to a promising application of the routine flow cytometry method in aquatic systems. At the compositional level, important changes in the cytometric properties of bacterial communities were also found seasonally and vertically, as commonly found by molecular analyses (Fuhrman et al. 2006; Gilbert et al. 2012; Giovannoni and Vergin 2012). The significant correlation in bacterial community composition based on cytometric and genetic criteria (Figure S3) confirms that, to a certain extent, changes in the cytometric properties of cells in a community reflect changes in microbial community composition. In agreement with this idea, several studies have found that different cytometric groups have a distinct phylogenetic composition. In particular, the two main bacterial groups detected in environmental samples based on their nucleic acid content (HNA and LNA), appear to be taxonomically different (Zubkov et al. 2001; Mary et al. 2006; Vila-Costa et al. 2012) with little overlap between them (Schattenhofer et al. 2011). In particular, the widespread SAR11 clade has been associated with LNA cells in different studies (Mary et al. 2006; Schattenhofer et al. 2011; Morán et al. 2015). Here, we found a drastic decrease in

cytometric diversity in a sample collected in May 2012, when almost no LNA cells were recorded in the samples (Figure 5A) and SAR11 populations were strongly depleted (Alonso-Sáez et al. 2015; Morán et al. 2015). This data point with the lowest cytometric diversity increased the correlation found with the Shannon-Weaver index between both methods (Figure 2) and also in the Mantel test results (Figure S3), although the relationship between both approaches remained significant after removing this value (Mantel test, $r = 0.44$, $p = 0.001$).

Despite the significant correlation found between both approaches, it should be acknowledged that their level of resolution is clearly different. The absolute value of the cytometric diversity index is dependent on the number of cytometric bins which is arbitrarily selected and the degree of spread of cytometric signals. Different taxa can fall in the same cytometric category and the same taxa may occupy different categories due to variations in morphology and cell-size over their growth cycle. The use of regularly-spaced divisions provided by the flow cytometer detection channels is also likely to split cells belonging to the same taxa into multiple bins and/or to create multi-taxa bins. Yet, the evidence presented here is that the cytometric method can pick out large signals in community variability at coarse scales (both over time and across depths, Figure 4). Thus, the utility of the proposed method should be enhanced at detecting large signals over spatial and/or temporal scales. This apparent limitation would be offset by its potential to provide a large coverage of data at a low cost and effort, unfeasible by molecular techniques.

In agreement with previous multiyear time-series of marine bacterioplankton based on molecular data (Fuhrman et al. 2006; Caporaso et al. 2012; Gilbert et al. 2012; Smyth et al. 2014), we found a pattern of increasing diversity during winter. Day length and, more recently, net heat flux have been shown to be the factors best correlated with patterns of

bacterial diversity in the English Channel L4 station, but the mechanistic reason for this correlation remains unclear (Gilbert et al. 2010; Smyth et al. 2014).

Of the variables similarly correlated with cytometric diversity, we found that MLD was the one most closely resembling changes in cytometric diversity along the vertical profile. Moreover, the changes in bacterial cytometric composition occurring in surface waters when the stratification breaks were coincident with increases in diversity at all three stations (Figure 4). Therefore, we hypothesize that the higher values of bacterial diversity in early winter were due to a vertical reshuffling of bacterial taxa during the mixing period. The effects of mixing on the patterns of bacterial diversity have been already highlighted in recent studies (Vergin et al. 2013; Fuhrman et al. 2015). During stratification, taxa adapted to thrive in oligotrophic, warmer waters outcompete other taxa resulting in an overall decrease in diversity. During the winter months, the MLD is deeper and, therefore, the whole water column is well-mixed providing similar environmental conditions at any depth and indirectly causing an increase in the number of bacterial taxa found at the surface able to grow at the surface. The finding of rare taxa typically abundant in the deep ocean such as SAR324 and SAR406, in surface samples from station E2 during winter (Alonso-Sáez et al. 2015) further supports our hypothesis. However, based on our data, we cannot rule out the possibility that the increase in diversity was due to taxa that were originally in surface waters but below the detection limits, which grew favored by other drivers associated with water column mixing not measured in this study (e.g. micronutrients or vitamins).

Changes in community composition following mixing by overturning have been described in the Bermuda Atlantic Time-Series Study (BATS) (Morris et al. 2005). Salter et al. (2015) have recently reported seasonal increases in the diversity of SAR11 in the NW Mediterranean strongly associated with mixing using a 7 year time-series. However, a

mechanistic explanation for the increase of bacterial diversity has been hindered by the lack of high vertical resolution in bacterial composition. The use of flow cytometry to complement molecular techniques has allowed us to increase the spatio-temporal resolution of the time-series analysed and to propose the species reshuffling by vertical mixing hypothesis to explain the late autumn-early winter maxima in bacterial diversity in coastal systems.

In summary, as in the seminal application of Li (1997) to small phytoplankton, our study reveals the utility and the potential of the cytometric diversity to assess heterotrophic bacterioplankton community structure and dynamics in aquatic systems, especially for large-scale studies where the application of molecular techniques is more limited. We took advantage of the high throughput offered by flow cytometry to analyse changes in diversity over spatial, vertical and temporal scales and found that the reshuffling of species by vertical mixing may be an important factor for the recurrent early winter maxima in bacterial diversity in temperate ecosystems of the oceans. The use of flow cytometry for studying the diversity of different components of microbial communities can be a useful complementary tool to molecular approaches to provide new insights in the understanding of global microbial community spatio-temporal distribution.

Experimental Procedures

Sampling sites

Samples were collected monthly off Gijón/Xixón, from April 2002 to December 2012 as part of the programme RADIALES of the Spanish Institute of Oceanography (IEO). This 10-year time-series study includes 3 stations sampled along a transect perpendicular to the coast located in the continental shelf at 43.58 °N, 5.61 °W (Station 1, E1), 43.67 °N, 5.58

°W (Station 2, E2), and 43.78 °N, 5.55 °W (Station 3, E3) (Figure 3). Samples for flow cytometry analysis were collected over the 10-year period at 4 depths at E1 (from the surface to 20 meters depth), 8 depths at E2 (from the surface to 100 meters depth) and 9 depths at E3 (from the surface to 150 meters depth). DNA samples for 454 pyrosequencing were obtained over 3.5 years (July 2009 to December 2012) at the surface (0 meters) at E2.

A SeaBird 25 CTD was used to collect temperature and salinity data. The density (σt) gradient was used to estimate the mixed layer depth (MLD). The MLD was defined as the depth where a change in density of 0.05 kg m^{-3} for a 5 meters interval was first found. Samples for nutrients analyses (NO_2, NO_3 and PO_4) were collected concomitantly with flow-cytometry samples and were frozen until analysis with a Technicon autoanalyzer following the methodology described by Grasshoff (1976).

Flow cytometry analysis and cytometric diversity estimates

Samples for flow cytometry were preserved with 1 % para-formaldehyde + 0.05 % glutaraldehyde and stored at -80 °C until processing, usually within one month of collection. For flow cytometry analysis, 0.4 ml samples were stained with 2.5 µmol L DMSO-diluted SYTO-13 nucleic acid fluorochrome (Molecular Probes) and analyzed with a FACSCalibur flow cytometer (BD/Becton, Dickinson and Company) with a laser emitting at 488 nm. 1.0 µm-diameter fluorescent latex beads (Molecular Probes) were added to each sample as an internal standard. Flow cytometry standard (FCS) files were gated automatically following the methodology described in García et al. (2014). This methodology uses a model-based clustering for automatically detecting the different groups of cells that appears in a sample and allowed us to separate heterotrophic cells from noise or phototrophic cells. Side scatter (SSC), green fluorescence (FL1) and red fluorescence (FL3) are common flow cytometric variables used to identify bacterioplankton groups

(Gasol and del Giorgio 2000). The settings used for detecting these cytometric parameters for bacterioplankton cells were 400 nm for SSC, 511 nm for FL1 and 590 nm for FL3. These variables were used in the flowClust function for the automatic clustering of the heterotrophic bacteria populations.

The methodology used by Li (1997) to estimate phytoplankton diversity was followed and adapted to the analysis of bacterioplankton. For each bacterioplankton FCS file, we selected the clusters corresponding to heterotrophic prokaryotes.

As we specifically targeted heterotrophic bacteria, we analysed cytograms obtained from samples stained with Syto 13. In these samples, the quantification of autotrophs is less reliable than in live-cell samples (Calvo-Díaz and Morán 2006), and thus, we decided to exclude them from the analysis. In any case, the abundance of cyanobacteria was generally low in the samples, contributing to 3.2 ± 4.6 % of total cells. The flow cytometer detection channels allow differentiating the cells according to their optical properties. SSC and FL1 signals are more important for differentiation of heterotrophic bacterioplankton communities than FL3, which results from fluorescence spillover rather than a true signal. Hence, in our analysis we gave more weight to SSC and FL1 by clustering the detection channels into 16 bins each, while FL3 channels were grouped into only 4 bins. The rational of using FL3 was to obtain a 3-dimensional view of the community to separate heterotrophic bacteria from noise or cyanobacteria groups more efficiently (Gasol and del Giorgio 2000). We then estimated the diversity of each sample using the number of cells falling into each of the 1024 (16x16x4) resulting categories. Figure 5 shows an example with two FCS files where SSC and FL1 parameters were divided into 16x16 bins obtained from a sample with relatively low and high cytometric diversity (Figure 5A and 5B, respectively).

Genetic diversity estimates by 454 pyrosequencing

Samples (0.5 to 2 L) for pyrosequencing were collected monthly from surface waters at E2 (Figure 3) using Niskin bottles. Samples were pre-filtered through GF/A filters (Whatman) and filtered onto 0.2 µm diameter polycarbonate filters (Millipore). DNA extraction was performed using Power Water DNA Isolation kit (Mobio). DNA was quantified using a Nanodrop (Thermo) and bacterial sequences were amplified by the use of domain-specific bacterial primers (341F and 805R, Herlemann et al. (2011)) complemented with a 10 bp sample-specific bar-code sequence on the reverse primer. PCR reactions (20 µL) contained a final concentration of 1 µmol L^{-1} of each primer, 0.2 mmol L^{-1} of each dNTP and 0.02 nmol L^{-1} of polymerase Phusion High Fidelity Taq Polymerase. The template DNA concentration varied between 1 and 10 ng per reaction. The PCR cycles started with a 5 min initial denaturation at 98 °C, followed by 25 cycles of 98 °C for 40 sec, 53 °C for 40 sec, and 72 °C for 60 sec and a final 7 min elongation at 72 °C. Triplicate PCR reactions were pooled for each sample. The purified amplicons were subject to pyrosequencing from the reverse primer using a 454 FLX+ system. The raw reads were quality trimmed (with a minimum Phred average quality score of 35 over a 50 bp window) and aligned against a reference SILVA alignment, keeping only those positions that start and stop in the same alignment space. From 488 593 reads initially screened, 248 474 reads were considered of good quality and with a minimum alignment length of 140 bp for further analyses. The resulting sequences were denoised by a preclustering method allowing 1 mismatch (Huse et al. 2010) as recommended (Schloss et al. 2009). Chimeras were removed using Uchime. For OTU construction, reads were clustered at genetic distance cutoffs 0.01 and 0.03 substitution per nucleotide, using the average linkage method. Taxonomic assignment of the

OTUs was obtained by classification with SILVA taxonomy using the Wang approach (Wang et al. 2007) as implemented in Mothur. The average confidence in the taxonomic assignment was 99.6 % ± 3.3% and 98.9 ± 5.6 % for OTUs at the 0.01 and 0.03 cutoff levels. Chloroplasts and cyanobacteria identified by this method were removed from the dataset, and samples were downsized to 4174 reads by random resampling to equal the depth of sequencing of all samples before calculating Shannon-Weaver diversity indices. Sequences of this study have been deposited in the European Nucleotide Archive (accession number PRJEB6399).

It should be acknowledged that due to mismatches of the reverse primer with some members of the SAR11 clade, the recovery of the total diversity of this group may have been affected. However, we consider that in our dataset, the SAR11 group was still fairly well represented as it was the dominant group in our samples (contributing up to 54 % of reads), and indeed, a positive correlation in the abundance of SAR11 by tag-sequencing and Fluorescent In Situ Hybridization using the SAR11-441R probe (Morris et al. 2002) was found (Figure S5, Spearman Rho = 0.73, $p < 0.01$, $n = 12$).

Determination of diversity indices

We calculated Shannon-Weaver indices for the molecular and cytometric dataset to compare the diversity patterns of bacterioplankton communities. For cytometric diversity, this calculation was based on the total number of cytometric categories detected and the relative abundance of cells in each category. In the molecular dataset, the Shannon-Weaver diversity index was calculated based on the total number of OTUs detected (at 0.03 OTU genetic distance cutoff) and their evenness in the samples.

The similarity between samples by each method over a 3.5 y period was calculated using

Bray-Curtis similarity index. A Mantel test was then performed to compare the two similarity matrices. Bacterioplankton flow-cytometry data and molecular data were standardized before similarity analysis. We also calculated pairwise Bray-Curtis similarity matrices between flow cytometry samples across the depth profile for the three sampling stations. For that purpose, we assessed the vertical variability in species bacterial community composition by calculating, for each station and date, the similarity index between the cytometric composition at the surface and each of the other sampled depths. This vertical similarity analysis was applied to every monthly profile along the seasonal cycle during the 10 year time-series.

All the analyses were performed with R (Team 2013) and statistical tests were performed using the R package "vegan" (Oksanen et al. 2012).

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Figure Legends

Figure 1. Trends of genetic (solid line) and cytometric (dashed line) diversity (Shannon-Weaver index) of surface bacterial communities at E2 during a 3.5-year monthly time series (A) and as a mean annual cycle (B).

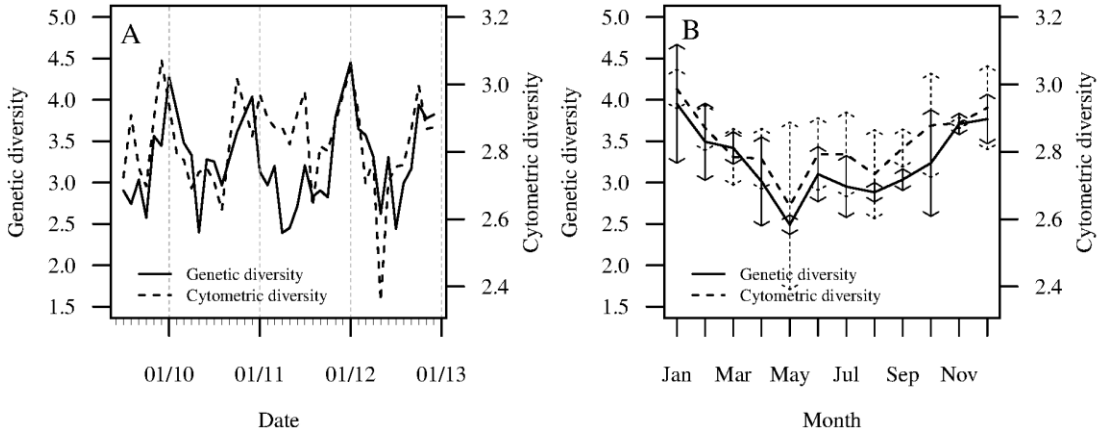
Figure 2. Correlation analysis between genetic and cytometric diversity (Shannon-Weaver index) using the 3.5-year monthly sampled database and mean annual cycle.

Figure 3. Sampling stations along a coastal gradient at the Cantabrian Sea. Samples from stations E1, E2 and E3 were used for cytometric diversity estimates. Genetic diversity analyses were perfomed on surface samples from E2.

Figure 4. Seasonal dynamics along the spatial and vertical gradient of the cytometric diversity and MLD (A, B, C), community similarity (D, E, F), potential density (G, H, I) and nutrient concentration (J, K, L) data estimated from a 10-year monthly time series database collected in the Cantabrian Sea. Community similarity was estimated as the Bray-Curtis ssimilarity between surface data and the different depths. The white line (E, F, H, I,

K, L) represents the 20 m depth. Warm colours represent high similarity with the surface sample and cool colours represent low similarity with the surface sample. Surface similarity value is 1 because it represents similarity with itself.

Figure 5. Comparison between two flow cytometry standard (FCS) files corresponding to May 2012, when a low cytometric diversity was found (A) and January 2012, when a high cytometric diversity was found (B). The vertical axis is green fluorescence (FL1) and the horizontal axis is side scatter (SSC).



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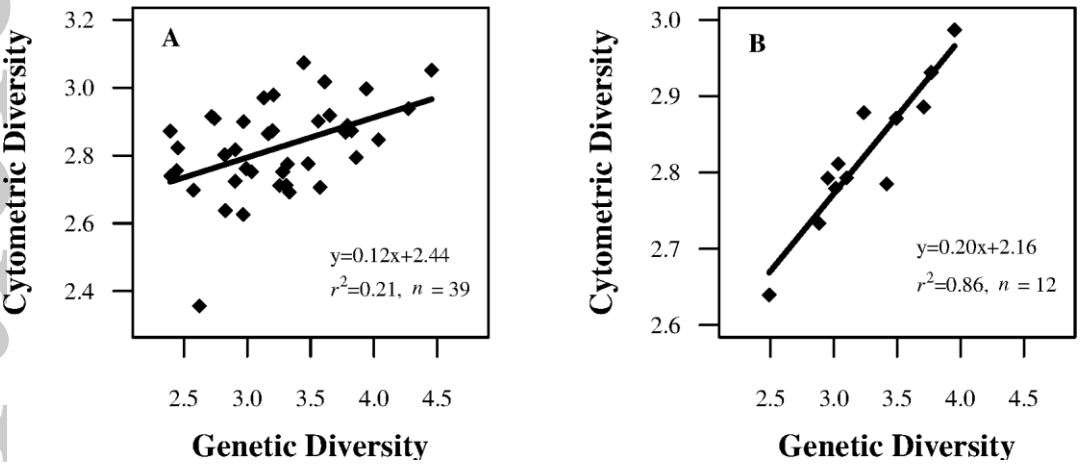

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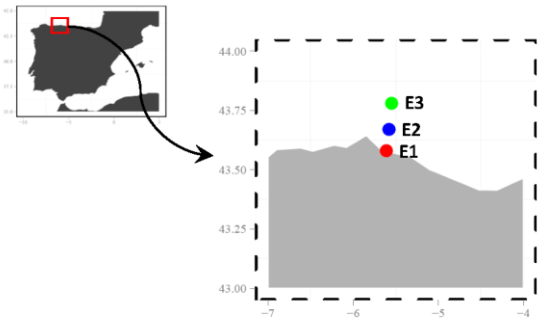

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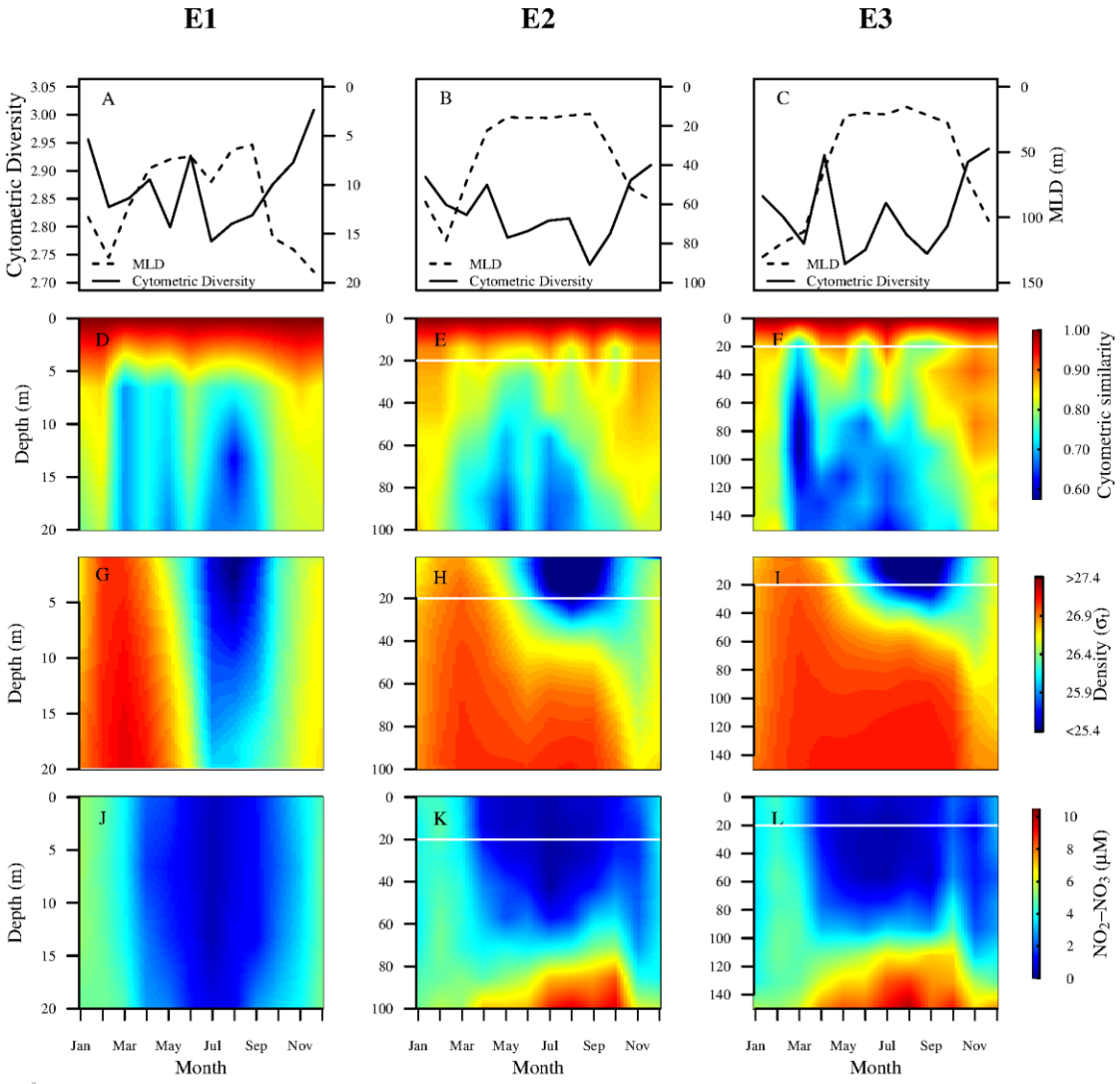

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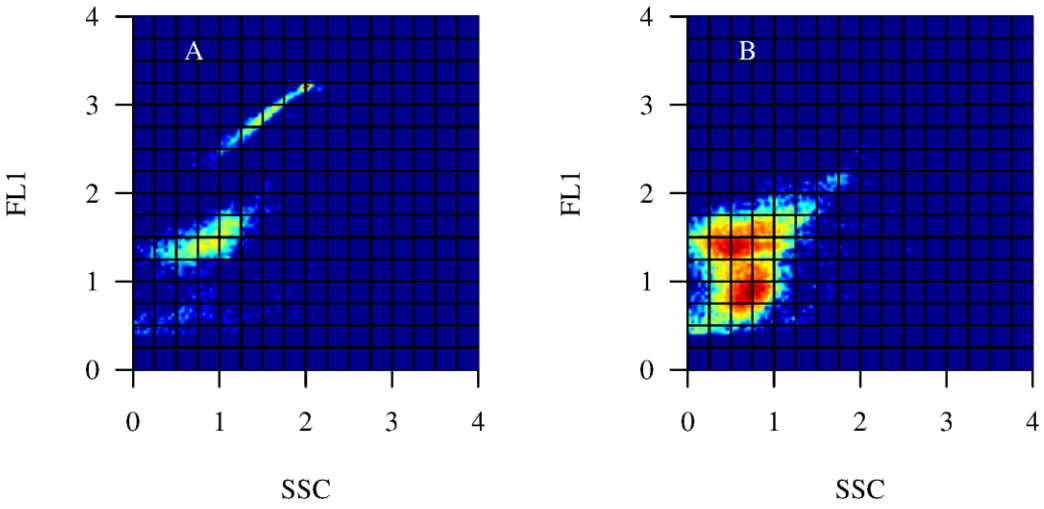

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