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Author’s Statement

Tania Montes is the main author of the manuscript. This work is in partial fulfillment of the requirements for a bachelor’s degree from the University of A Coruña.

Elisa Guerrero-Feijoo was involved in the bioinformatic analysis to obtain the bacterial OTUs Table

Victor Moreira Coello was involved in the hydrographic information obtained by using a microstructure profiler for estimation dissipation of turbulent kinetic energy and the calculation of the squared Brunt Vaisala frequency.

Antonio Bode was supporting the manuscript with the environmental information: abiotic (temperature, salinity, Chla, nitrate+nitrite, phosphate, silicate, PAR) and biotic (diatoms, dinoflagelates, ciliate) factors.

Manuel Ruiz Villareal was actively involved in scientific discussions prior to the writing of the manuscript, particularly those related to hydrography. He was also the chief of the Hercules cruise.

Beatriz Mouriño Carballido was the supervisor of the hydrographic information obtained by using a microstructure profiler for estimation dissipation of turbulent kinetic energy and the calculation of the squared Brunt Vaisala frequency. She was also actively involved in all discussions during the writing of the manuscript.

Marta M Varela is the corresponding author of the manuscript, she was developing the main idea of the manuscript (hypothesis and objectives), she was also involved in the sampling during the Hercules cruise, she was also highly involved in the writing of the manuscript and she did the peer review.
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Abstract

We combined flow cytometry, CARD-FISH, and 16S rRNA gene tag pyrosequencing to investigate bacterioplankton dynamics along a transect in shelf waters off A Coruña (Galicia, NW Spain). Over five days (16-20 July 2012) we sampled a situation of summer upwelling relaxation, providing an opportunity to examine the impact of pulses of cold nutrient-rich water into coastal microbial communities. The hydrographic conditions, characterized by intense density stratification of surface waters and the presence of a deep chlorophyll maximum (DCM) at 20-30 m, were relatively maintained over the sampling period. Indeed, bacterial abundance and composition displayed low day to day variation. Alpha diversity analysis suggested that species richness and diversity increased from coastal to shelf stations and from the surface down to the DCM, which could be caused by the mixing of upwelled bacteria with the coastal surface waters. SAR11, SAR86, and Roseobacter were the most abundant bacteria detected in the samples by using CARD-FISH. The assemblages observed by pyrosequencing displayed a strong vertical zonation along the transect. Rhodobacteraceae (under class Alphaproteobacteria) and Bacteriodetes dominated the surface waters and decreased during the upwelling pulse, while SAR 86 (under class Gammaproteobacteria), Actinobacteria and SAR11 clade increased their relative abundance at the DCM with upwelling relaxation, particularly at the shelf stations. Bacterial assemblages from surface waters were associated with higher temperature and light conditions, while DCM assemblages were rather associated to salinity, inorganic nutrients and a diatom-bloom leading to high chlorophyll-a. Our findings suggest that the vertical variability in environmental conditions induced by the intense density stratification, the exportation of warmer and less saline surface water from the rias to the adjacent shelf, and the fertilizing effect of recently upwelled water at the deeper layer, determined the composition of distinct bacterial assemblages at the subsurface and DCM layers.

Keywords: Bacteria, CARD-FISH, 454 pyrosequencing, microplankton; summer upwelling relaxation, upwelling pulse, NW Spain.
Introduction

Ecological and biogeochemical processes in the ocean are dependent on a diverse assemblage of microbes including members of Bacteria (Glöckner et al., 2012). These diverse bacterial assemblages fulfill a wide range of ecological roles in the marine system including the carbon biogeochemical cycle (Falkowski et al., 2008) and the energy transfer to higher trophic levels (Fuhrman et al., 2015). The relatively recent applications of new molecular techniques have revolutionized microbial research and have considerably contributed to an increase in our knowledge of the vast marine microbial diversity. High-Throughput Sequencing (HTS) and bioinformatic analyses offer a sensitive and high resolution approach to study diversity and composition of microbial assemblages. Indeed, these methods have been used in global research surveys (i.e. GOS and Sorcerer cruises, and recently Tara Oceans and Malaspina expedition) aiming at better understanding composition and distribution patterns of microbial communities at the global scale and at detecting significant trends (Sunagawa et al. 2015, Salazar et al. 2016). For instance, two recent studies in the global open ocean have illustrated that community composition of phytoplankton (Estrada et al., 2016) but also of bacterioplankton (Sunagawa et al., 2015) in the upper and the lower parts of the euphotic zone contrasted significantly. Previous regional oceanic studies have identified that the vertical stratification of microbes is likely associated to the differences in the physicochemical properties of the water column (i.e. DeLong et al., 2006; Hewson et al., 2006; Treusch et al., 2009). The study of bacterioplankton in coastal zones is also of critical importance, and in particular there is a need for microbial studies in highly dynamic coastal ecosystems (such as upwelling systems). Microorganisms inhabiting these systems have the ability to function under extremely variable conditions, and thus likely play a disproportionately important role in the microbial-mediated cycling of marine nutrients. However, relatively few studies on the genomic composition of bacteria have been carried out in upwelling zones (Allen et al. 2012, Bergen et al. 2015, Gregoracci et al. 2015, Zancker el al. 2018, Bachmann et al. 2018) and the role that bacterioplankton plays in these highly variable zones still not well understood (i.e. Bachman et al. 2018).

The northwestern Iberian coast, located at the northern limit of the eastern boundary upwelling ecosystem of the Canary current, is a temperate region where alternation between water column stratification and mixing, driven by the seasonal variations in
solar irradiance, is modified by wind-driven upwelling-downwelling events (Aristegui et al., 2006). Furthermore, the region is characterized by the influence of freshwater outflows and the presence of the “Rías”, coastal inlets that amplify the nutrient inputs provided by the upwelling by the export of regenerated nutrients and organic matter (Álvarez-Salgado et al., 2000). Rapid increases in phytoplankton growth typically occur during early spring and through the summer in this region (Varela et al. 2001, 2005). Notwithstanding the frequent fertilization of surface waters by upwelling, transient situations of stratification occur during periods of upwelling relaxation, leading to the formation of a defined deep chlorophyll maximum (DCM). The persistence and position of this DCM, generally composed by small diatoms and dinoflagellates, is related with the frequency of upwelling and the renewal of the nutrients consumed in the surface layer (Figueiras and Pazos, 1991; Casas et al., 1997).

Investigations of the role of bacteria in this region have focused on bulk biological measurements of abundance, biomass and single-cell activity (e.g. Valencia et al., 2003; Bode et al., 2006; Teira et al., 2017). In these studies, the variation in bacterioplankton was related to diverse environmental factors, unveiling hydrography and phytoplankton dynamics as the main driving forces. However, the composition of bacterial assemblages has been comparatively less studied and previous analysis of upwelling induced-changes on bacterial composition were limited by low taxonomic resolution (Alonso-Gutierrez et al. 2009, Teira et al. 2009, Dobal-Amador et al. 2016). The present study represents the first attempt to study the genomic patterns of 16S rRNA gene sequence in the coastal wind-driven upwelling section off A Coruña (NW Iberian Peninsula) within the RADIALES Long-Term Time-Series (http://www.seriestemporales-ieo.com/). Over one week we conducted a survey along a transect in the shelf waters of A Coruña (NW Spain) to investigate the trends in bacterioplankton communities under the influence of upwelling. The aims of this paper are twofold (i) to determine bacterioplankton abundance, diversity and community composition distribution patterns and (ii) to examine the environmental variables that could best drive those bacterioplankton variations.

**Material and methods**

*Study site and sampling*
Seawater samples were collected on board the R/V Lura during HERCULES-0712 cruise (from 16\textsuperscript{th} to 20\textsuperscript{th} July 2012, with the exception of the 19\textsuperscript{th}) at six shelf stations off A Coruña from the coast in the ria de A Coruña to the adjacent shelf (Figure 1). At each station, vertical profiles of temperature, salinity, fluorescence and photosynthetically active radiation (PAR) were obtained with a Seabird-25 conductivity temperature depth (CTD) sensor attached to a rosette sampler, and a Licor spherical PAR sensor deployed to a maximum depth of 100m. Seawater samples were collected for the determination of inorganic nutrient concentration, chlorophyll-a concentration (Chl-a) and bacterial abundance at up to 7 discrete depth levels, and for bacterial diversity and community composition at 2 depth layers: surface (at 1m depth) and the deep chlorophyll maximum (DCM, at 20-30m). Seawater samples from six stations (1C, 2, 3A, 3C, 3B and 4, Figure 1) were sampled for hydrography while microbial analysis (including abundance, activity and bacterial community composition) were included only in four of those stations (1C, 2, 3C, and 4).

Aliquots for inorganic nutrient analysis (nitrate, nitrite, phosphate and silicate) were collected in polyethylene bottles and frozen at -20°C until analysis by standard colorimetric methods with a Bran-Luebbe segmented flow analyser (Grasshoff et al., 1983). Chl-a was determined from acetonic extracts of plankton retained by GF/F filters and measured by the fluorimetric method (Neveux and Panouse, 1987).

Additionally, hydrographic information at higher spatial resolution was obtained by using a microstructure profiler (MSS, Prandke and Stips, 1998) with microstructure shear and temperature sensors for estimating dissipation of turbulent kinetic energy that was equipped with a high-precision CTD probe. Ten sets of 3-5 profiles each were carried out between stations 2 and 4 on the 17\textsuperscript{th} July, and 16 sets between stations 1C and 4 on the 18\textsuperscript{th} and 20\textsuperscript{th} July. The squared Brunt Väisälä frequency ($N^2$), a proxy for water column stratification, was computed from the CTD profiles according to the equation:

$$N^2 = \left(-\frac{g}{\rho_w}\right)\left(\frac{\partial \rho}{\partial z}\right)(s^{-2})$$

where $g$ is the acceleration due to gravity (9.8 m s$^{-2}$), $\rho_w$ is seawater density (1025 kg m$^{-3}$), and $\partial \rho/\partial z$ is the vertical potential density gradient.
Fluorometrically determined Chl-a was used to calibrate the fluorometer attached to the CTD-rosette (Chl a = fluorescence /1.153; \( r^2 = 0.961, n = 16 \)), which was used to plot the chlorophyll a distribution on the 16th July, and also to calibrate the fluorometer sensor included in the MSS profiler (Chl a = 2.255 × fluorescence–0.527; \( r^2 = 0.859, n = 134 \)).

Abundance of microphytoplankton was estimated in 50 mL aliquots of a sample collected with a plankton net (30 cm diameter, 40µm mesh size) deployed between the surface and 50m or 5m near the bottom, according to the bathymetry. The sample was preserved in glutaraldehyde (25% final concentration) and cells by counted using a FlowCAM® system (Fluid Imaging Technologies). Particles in the sample were digitized at x200 to obtain reliable counts of cells a range of 3 to 50µm (Álvarez et al., 2012). Prior to the analysis, the samples were screened with a 100 µm nylon mesh to prevent clogging of the FlowCAM cell. Cell identification was made on the digitized images with an emphasis in the identification of major groups (diatoms, dinoflagellates and other small flagellates). Ciliates were counted in Lugol-preserved samples under an inverted microscope. In this study, the abundance of diatoms, dinoflagellates and ciliates are reported as the number of cells per volume of seawater.

The upwelling index (Iw) was estimated by calculating the Ekman transport from surface winds data. In this study we employed the Ekman transport computed as estimates every 6 h and provided by the Instituto Español de Oceanografía (http://www.indicedeafloramiento.ieo.es) in two cells of 1º x1º centred at 43.5ºN, 9ºW, using data from atmospheric pressure at sea level, derived from the WXMAP model (González-Nuevo et al., 2014).

**Microbial abundance by flow cytometry**

Microbial abundance was determined in up to 7 depths at each station by flow cytometry following Gasol & Del Giorgio (2000). A volume of 1.8 mL per water sample was preserved with 1% paraformaldehyde (final concentration), shock-frozen in liquid nitrogen for 10 min and stored at -80 °C. Samples were thawed to room temperature and stained with Syto13 (Life Technologies) in the dark for 10 min. Subsequently, 1 µm fluorescent latex beads (approximately 1x10^5 mL^-1) (Molecular Probes, Invitrogen, Carlsbad, CA) were added to all the samples as internal standard.
Prokaryotes were counted using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) according to their signature in right angle light scatter and green fluorescence. Two types of bacteria were distinguished according to their high or low relative content of nucleic acids: HNA and LNA bacteria, respectively (Morán et al., 2007).

Abundance of the most abundant groups of Bacteria by CARD-FISH

CARD-FISH analyses were carried out to quantify the abundance of the specific bacterial groups following the method described by Pernthaler et al. (2002). Immediately after collecting the samples from the Niskin bottles of surface and DCM layers, a volume of 10-20 mL of seawater was fixed with paraformaldehyde (2% final concentration) and stored at 4 °C in the dark. About 12–18 h later, the samples were filtered onto 0.2 µm polycarbonate filters (Millipore GTTP, 25 mm filter diameter) supported by nitrocellulose filters (Millipore, HAWP, 0.45 µm), washed twice with 10 mL Milli-Q water, dried and stored in a microfuge vial at -20 °C until further analysis in the laboratory. Filters were cut in sections and hybridized with horseradish peroxidase (HRP)-labelled oligonucleotide probes: Eub338I-III (Bacteria) (Daims et al., 1999); Gam42a (Gammaproteobacteria) (Manz et al., 1992); CF319a (Bacteroidetes) (Manz et al., 1996); Ros537 (Roseobacteria) (Eilers et al., 2001); SAR11-732 (Morris et al., 2002); SAR86-1249 (Eilers et al., 2000) and Non338 (negative control) (Wallner et al., 1993), followed by tyramide-Alexa488 signal amplification. Cells were stained with DAPI-Mix [4',6-diamidino-2-phenylindole (DAPI, concentration 2 µg mL\(^{-1}\)) phosphate buffer (PBS, 0.5 mg mL\(^{-1}\)), Vectashield (1 µg mL\(^{-1}\)) and Citifluor (5.5 mg mL\(^{-1}\))]. Quantification of DAPI-stained cells and cells stained with the specific probes were performed with an epifluorescence microscope (Nikon Eclipse 80i) equipped with a mercury lamp (130 W) and a set of filters appropriate for DAPI and Alexa488 stains. For each sample at least 20 fields (equivalent to \(\approx 500\) cells) were counted.

DNA extraction, PCR amplification and sequencing of the 16S rRNA gene fragments

Samples for DNA pyrosequencing were obtained by filtration of \(\approx 5\) L of seawater through sterile Sterivex cartridge 0.22 µm pore size filters (Millipore, USA) and stored at -80°C until DNA extraction could be performed. DNA extraction was performed
using the PowerWater DNA Isolation Kit (Mobio, Carlsbad, CA, USA). DNA extraction and quantification (via Nanodrop, Thermoscientific) was carried out for a total of 17 samples. A subsample of DNA extracted was used for pyrosequencing at the Research and Testing Laboratory (Lubbock, TX, USA: http://www.medicalbiofilm.org/) using 454 GL FLX technology. The primer set 341F (5’-CCTACGGGNGGCWGCAG-3’) and 805R (5’-GACTACHVGGGTATCTAATCC-3’) (Herlemann et al., 2011) were used to generate amplicons spanning the V3 to V4 regions of the bacterial 16S rRNA gene. The raw sequence data were processed using Quantitative Insights into Microbial Ecology (QIIME) pipeline (https://www.qiime.org) (Caporaso et al., 2010). A quality check was performed to minimize low quality pirotags. These sequences were removed from the subsequent analyses if they were shorter than 100 bp, had an average quality score < 25 calculated in sliding windows of 50 bp, and contained primer mismatches. The remaining sequences were run using Denoiser implemented in QIIME to reduce the impact of pyrosequencing errors (Reeder and Knight, 2010). The curated sequences were grouped into operational taxonomic units (OTUs) with 97% similarity threshold. A representative sequence from each phylotype was chosen by selecting the most abundant sequence within that particular phylotype and was used for taxonomic identification. The chimeric sequences were removed using ChimeraSlayer implemented in MOTHUR (Schloss, 2009; Haas et al., 2011) based on the alignment file SILVA119 (http://www.arb-silva.de). Blast Classifier (Wang et al., 2007) implemented in QIIME determined the identity of 16S rRNA phylotypes. The chloroplast, mitochondria or archaeal members identified by this method were removed from the dataset. To enable comparisons between samples, the OTU table was downsized to 1552 sequences to ensure an equal number of sequences in each sample. All further analyses, alpha and beta diversity estimations implemented in QIIME, were performed with the subsampled OTU table. In addition, the rarefaction curves were plotted to verify that the sequences obtained in each sample showed a tendency of plateauing for the most samples of Bacteria (Supplementary Figure 1A, B). Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under SRP133560 bioproject number.

*Statistical Analysis*
One-way ANOVA was performed to verify significant differences between subsets of variables (inorganic nutrients, phytoplankton biomass, microbial abundance, CARD-FISH counts) for the different days, stations and/or depths. These statistical analyses were performed with SPSS software package. Hierarchical cluster analysis based on Bray-Curtis distance matrix, was carried out to explore similarities in the taxonomical composition between samples. Significant differences in bacterioplankton community composition between samples were determined by a permutational analysis of variance (PERMANOVA). Non-metric multidimensional scaling (nMDS) was conducted to illustrate the associations among the relative abundance of OTUs, and environmental or taxonomic group variables by vector overlays. The correlations between the different environmental parameters and the relative abundance of bacterial taxa were measured with the Spearman ρ coefficient. Cluster, PERMANOVA and NMDS statistical tests were performed with PRIMER6 & PERMANOVA+ (Anderson et al., 2008).

Results

Hydrographic conditions

During the five day sampling the situation was of relaxation of upwelling favorable conditions observed during the 16th and 17th July (Figure 2), which induced an accumulation of diatoms after the pulse (Table 1). An intense vertical density gradient (evident in T and S and quantified by N²) was observed over the sampling period in the upper layers (Figure 3, Table 1), with relatively warm (20.20 ± 0.37 ºC) and fresher waters (33.95 ± 5.48) at the surface, and cooler (13.79 ± 1.01°C) and saltier waters (35.77 ± 0.01) below (Figure 3, Table 1). The base of the pycnocline (20-30m) coincided with the location of a distinct DCM, where Chl-a ranged between 2.03—9.86 mg m⁻³ versus 0.48-0.81 mg m⁻³ quantified at surface waters (Figure 3, Table 1). The intense vertical density gradient acted as a barrier for the transport of nutrients from deep to surface waters (Figure 4, Table 1). Surface concentrations of nitrate+nitrite, phosphate and silicate at surface waters (1.52 ±0.17, 0.19 ±0.01 and 1.30 ±0.09 µmol L⁻¹, respectively) were lower than those quantified at the DCM (2.60 ±0.54, 0.31 ±0.02, 2.27±0.19 µmol l⁻¹, respectively). (Figure 4, Table 1). The DCM layer, got thinner vertically forced by the upwelling of deep waters, and got broader again when upwelling relaxed on 18th July and water restratified in the area between stations 2 and 3B.
**Microplanktonic abundance by flowCAM and light microscopy**

A bloom of diatoms occurred during the cruise in response to the upwelling pulse. Diatom concentration in cell per liter increased throughout the week in stations 2, 3C and 4 both at the surface and the DCM in response to the nutrients introduced by upwelling (Table 1). Diatom concentrations were lower in surface waters, where several dinoflagellates (mainly *Lingulodinium polyedrum*) and small unidentified flagellates dominated. In contrast, at the DCM there was a drastic shift to a ciliate- and diatom-dominated community (*Leptocylindrus danicus*, *L. minimus* and *Chaetoceros socialis*) at all stations, which resulted in maximum Chl-a concentrations at a DCM around 20-30 m depth. (Table 1)

**Microbial abundance by flow cytometry**

Microbial abundance values were, in general, higher in surface waters (2.05-6.92 x 10^5 cells mL^{-1}) and decreased significantly with depth (ANOVA, p<0.05), showing minimum values at the shelf stations below 60 m (1.22-3.33 x 10^5 cells mL^{-1}) (Figure 5). Similarly, the contribution of HNA bacteria to total abundance was on average, slightly higher in surface waters (46 ± 9.5 %) than in the DCM (42 ± 9.5 %) and/or below that depth layer (Figure 5). It is remarkable that microbial abundance at stations 2 and 3C increased throughout the week and was highest on 20th (i.e. in a later stage of the diatom bloom). At the DCM in 3C on 18th, where nutrients were high, the HNA% was high. Average vertical profiles of the more coastal stations (St. 4 and 3C) consistently showed the highest values of microbial abundance and HNA Bacteria, particularly during the sampling days of the 17th and 18th July, when nutrients were lowest, and progressively decreased towards the shelf stations (St. 1C and 2) (ANOVA, p<0.05) (Figure 5). Overall, no significant differences were found from day to day for microbial abundance and for HNA Bacteria considering the whole data set (ANOVA, p>0.05).

**Relative abundance of specific groups of bacteria by CARD-FISH**

The relative abundance of Bacteria showed similar values throughout the water column, on average, the recovery was 71 ± 12 % of total DAPI counts (Table 2). Overall, SAR11 (Alphaproteobacteria) and Bacteroidetes phylum showed, on average, the highest relative abundance (28 ± 4 % and 31 ± 4 % respectively) of total Bacteria (Table
2). By contrast, SAR86 (Gammaproteobacteria) displayed the lowest contribution of the total Bacteria (12%) (Table 2). No significant differences were found from day to day samples for any of the bacterial groups (ANOVA, p>0.05), with the only exception of *Roseobacter*, which was significantly reduced throughout the week (ANOVA, p<0.01). Similarly, no significant differences were found for any of these bacterial groups between stations (ANOVA, p>0.05). Moreover, no significant differences were found between the surface and the DCM waters for 5 of the 7 groups targeted (ANOVA, p>0.05).

*Diversity and composition of bacterial community by NGS (454 pyrosequencing)*

A total number of 69220 pyrosequencing reads of V3-V4 region of the 16S rRNA gene were obtained from 8 and 9 samples of surface water and DCM layer, respectively. Sequences were clustered into a total number of 554 OTUs. Shelf stations (i.e. St.1C and 2) showed in general higher richness values, estimated by the number of OTUs and Chao1 indices, than coastal stations (Table 3). Similarly, the Shannon diversity index indicated that the latter stations had the lowest bacterial diversity, while the highest diversity values were found at the DCM of station 2 on 17th July (3.77) and on 20th July (3.36) (Table 3), during upwelling relaxation. The number of OTUs and Chao 1 index values were generally higher in the DCM than in the surface (Table 3), while Shannon diversity values of surface were equivalent to those of DCM samples, ranging from 2.69 to 3.77 (Table 3). No clear trend of day to day variation either in richness and/or diversity was found (Table 3).

The relative abundance of bacterial sequences, determined at different taxonomic levels, indicated that bacterial assemblages in the water column varied considerably between the surface and DCM (Figure 6). Furthermore, some phyla showed slightly differences in their relative contribution to the total Bacteria along with the distance from the coastline and throughout the week. Samples were dominated by Proteobacteria, Bacteroidetes, Actinobacteria, and Deferribacteres phyla (80%, 10%, 7% and 5% of total Bacteria, respectively) (Figure 6A). Other less abundant members accounting for ≈1% of Bacteria include members of SAR202 (Chloroflexi), Acidobacteria, Verrucomicrobia, Planctomycetes, and Cyanobacteria (Figure 6A). Among Proteobacteria, Alphaproteobacteria and Gammaproteobacteria were the most abundant classes accounting for 35% and 30% of total abundance of Proteobacteria, respectively (Figure
6B). The most abundant bacterial groups, as SAR11 and Rhodobacteraceae belonging to Alphaproteobacteria, showed an opposite distribution pattern with depth and distance from the coastline. The SAR11 clade was relatively more abundant in the DCM layer near the coast, while Rhodobacteraceae dominated in surface waters at the shelf St. 2. Gammaproteobacteria was largely represented by Oceanospirillales, mainly SAR86 clade (25%), JL-ETNP-Y6 (≈5%) and Oceanospirillaceae (<5%), while Alteromonadales mainly consisted of Alteromonadaceae (5%) (Figure 6B). The SAR86 clade showed high abundance at the DCM of the shelf stations, while Oceanospirillaceae and Alteromonadaceae prevailed in surface waters of the coastal stations. Flavobacteriaceae and Cryomorphaceae (phyla Bacteroidetes) were more abundant at the surface (≈15%) than at the DCM (≈5% of total Bacteria), while OCS-155 (phyla Actinobacteria) showed a relatively higher abundance in the DCM (10%) than in surface waters (<5%). SAR406 (phyla Deferribacteres) accounted for ≈5% of total Bacteria and showed a patchy distribution (Figure 6B).

The clustering of samples showed that the main bacterial assemblages were those associated with the two different water layers considered in this study (Figure 6C). At a similarity level of 50%, one cluster grouped the samples belonging to the surface, and a second cluster included the DCM samples (Figure 6C; PERMANOVA, p <0.05). Surface and DCM assemblages had a large overlap, with most of the shared-OTUs belonging to the more abundant bacterial phyla, which included Rhodobacteraceae, Cryomorphaceae, Flavobacteriaceae, Alteromonadaceae, SAR86 and SAR 116. The number of non-shared OTUs was higher in DCM compared to surface layer. Several bacterial phylotypes such as Ulvibacter and Litoricola were exclusive of the surface layer, while SAR202 clade and SAR 324 were some of the non shared-OTUs belonging to the DCM. As for the environmental variables, the variability in bacterial community composition was not significantly different from day to day for any of the stations (Figure 6C, PERMANOVA, p>0.05). However, our results showed that DCM samples in the inner most station 4 changed from the surface cluster on 18th to the DCM cluster on 20th in response to the influence of upwelled nutrient-rich waters in deeper layers of station 4 introduced by the upwelling pulse.

*Relationship between bacterial community composition and environmental variables*
Surface samples were characterized by higher temperature and PAR values (Figure 7A) and higher number of reads of Rhodobacteraceae, Flavobacteriaceae and Alteromonadaceae than in DCM samples (Figure 7B). By contrast, the high nutrient concentrations observed in the DCM layer, particularly at shelf stations (i.e. St. 2 and 1C), along with the high Chl-a and high salinity (Figure 7A) were associated to the occurrence of SAR86, SAR11, OCS155 and JL-ETNP-Y6 (Figure 7B). Overall, the relative abundance of Bacteria displayed a significant correlation with temperature, Chl-a and nitrate + nitrite concentrations but not with PAR or salinity (Supplementary Table 1).

Discussion

In this investigation we have generated baseline information on the taxonomic diversity of bacteria in an exploratory survey off A Coruña (NW Spain), as part of a multidisciplinary research examining short-term hydrodynamic variability and marine plankton in the Galician shelf during upwelling events in the Artabro gulf (i.e. Otero-Ferrer et al. 2018). We have characterized hydrography, nutrient concentration and microbial abundance and composition in a section off A Coruña (from the ría to the adjacent shelf) during an upwelling pulse and its relaxation. Each of the approaches employed in this study for characterizing the microbial assemblages has limitations, yet combined they identified the diversity of microbial communities at the coastal upwelling off Galicia. Flow cytometry only provided total microbial abundance and a very general characterization of two physiological groups of organisms (HNA and LNA populations), while CARD-FISH identified the dominant groups of bacteria and 454-pyrosequencing allowed us to further investigate the bacterial diversity including also those bacterial phylotypes that had very low abundance. However, only the latter technique provided enough sensitivity for revealing spatial and temporal changes in diversity that could be related to changes in the environmental conditions.

The distribution pattern of bacterial assemblages showed a marked difference between surface and DCM layers, but was remarkably uniform across stations and throughout the week, in spite of the variability of hydrographical conditions. Our sampling occurred during the relaxation of a summer upwelling pulse on 16th and 17th (Figure 3). The pulse introduced cold, saltier and nutrient rich in deep layers and exported warmer and less saline surface waters from the rías to the adjacent shelf. This situation is typical
during the summer in the Galician coast, where periods of upwelling that introduce nutrients alternate with situations of stratification when wind relaxes (Casas et al., 1997; Álvarez-Salgado et al., 2000). Because of the presence of continental water near the coast and the thermal stratification caused by warming of the surface, the upwelled waters only reach the surface when upwelling pulses last for several days, which was not the case in our study. However, the upwelled water with relatively high concentration of nutrients influenced deep layers of the water column (DCM and below). For instance, the characteristics of the water below 50m at the shelf stations ($t<14^\circ C$, $S>35.8$, $NO_3+NO_2> 7 \mu mol L^{-1}$) agree with the presence Eastern North Atlantic Central Water, which is modified by warming and remineralization on the shelf (Álvarez-Salgado et al., 2000).

Two distinct clusters of bacterial assemblages along the transect were present, possibly associated with these two distinctive oceanographic features. Furthermore, this vertical zonation of bacterioplankton could also be linked to the abundance and quality of phytoplankton, since DOC originating from different phytoplankton species differentially stimulated heterotrophic prokaryotes (Sarmento and Gasol 2012). Interestingly, there were also marked changes in the composition of microplankton in the two layers, with a marked dominance of ciliate and diatoms in the DCM and an increase of diatoms throughout the week. Diatoms are well adapted to the DCM, generally associated to the nutricline, because of their ability to capture nutrients from the deep layer (Latasa et al., 2017). For instance, upwelling pulses can provide nitrate that can be stored by diatoms and other microphytoplankton groups for later use in biosynthesis (Bode et al., 1997; Lomas et al., 2000). Daily vertical displacements of phytoplankton, notably from dinoflagellates but also from diatoms, can influence the quantity and quality of the organic matter synthesized, as illustrated by the excess of carbohydrates observed in the surface layer during periods of upwelling relaxation reported for the Ria de Vigo, and which contrasts with the more balanced composition of the organic matter in the layers below the DCM where nutrients were abundant (Fraga et al., 1999).

**Abundance of major bacterial groups: CARD–FISH 454 versus 454 pyrosequencing**

The diversity of microplanktonic assemblages, including phytoplankton and ciliates has been well studied by conventional sampling methods and microscope techniques in the
shelf waters off A Coruña (Varela et al., 2003; Bode et al., 2004b; Bode et al., 2006, Varela et al. 2017). However, these techniques only detect a minor part of the microplanktonic diversity (Rodríguez-Ramos et al., 2014), and do not allow to study of the taxonomic diversity of bacteria. In contrast, the approach employed in the present study is based on a combination of two molecular techniques (i.e. Korlević et al., 2015; Guerrero-Feijóo et al., 2017): the CARD-FISH (accounting for the dominant microbial groups) and the high resolution 454 pyrosequencing (for specifically addressing the identification of both abundant and rare bacterial taxa). A direct correlation between the results obtained by CARD-FISH and pyrosequencing analysis could not be performed because these different techniques target different regions of the 16S rRNA gene. However, in general, this investigation has found a good correspondence between the relative abundance of the major bacterial groups by both methodologies. For instance, pyrosequencing sequences for Alphaproteobacteria or Gammaproteobacteria yielded a percentage of contribution similar to that obtained by CARD-FISH counts (see Table 2), indicating that this cluster was recovered by both methodologies similarly. By contrast, the relative abundance of SAR11 and Bacteroidetes was higher in CARD-FISH counts (see Table 2) than pyrosequencing. It is well known that the use of different primers complicates the comparison of microbial community composition and diversity between different studies. In this regard, we conducted our study prior to the report of Apprill et al. (2015) and it is possible that the primers we have used here underrepresent the SAR11 clade. In contrast, SAR86 cluster contributed more to the total bacterial abundance in pyrosequencing than in CARD-FISH counts (see Table 2). These different patterns could be due to the different number of samples analyzed, and also to the PCR bias associated with the pyrosequencing approach compared to CARD-FISH, where the probes target directly the 16S rRNA.

**Bacterial diversity and composition by 454 pyrosequencing**

Alpha diversity analysis suggested that species richness and diversity increased from coast to shelf stations and from the surface down to the DCM, which could be associated to the mixing of upwelled bacteria with the coastal surface waters and probably also reflecting the marked functional diversity encompassed by bacteria. Consistent with previous studies of the relative richness of dominant phyla, the variability in bacterioplankton community composition in the shelf waters of the
Galician coast showed some characteristics in common with oceans globally (i.e. GOS, Yooseph et al. 2007 and/or Tara Oceans expedition, Sunagawa et al. 2018). It is well established that Proteobacteria and Bacteroidetes, and to a lesser extent Actinobacteria are the dominant Bacteria phyla in seawater (DeLong et al., 2006; Agogué et al., 2011; Walsh et al., 2016; Guerrero-Feijóo et al., 2017) and, consequently, they displayed the highest relative abundance in our study. However, as it was described in the previous section, the distribution of the bacterial assemblages at different taxonomic levels was driven by the differences induced by upwelling and its relaxation in the area.

The main sequences within Alphaproteobacteria comprised SAR11 and Rhodobacteria, and their distribution varied in surface and DCM layers along the sampling days of the cruise. As expected, a considerable portion of 16S rRNA amplicons belong to SAR 11 (under class Alphaproteobacteria), an ubiquitous clade at the world ocean. We found a significant positive correlation between the abundance of the SAR11 clade and Chl-a, supporting previous findings describing that the abundance of SAR11 is significantly higher in epipelagic waters and correlated with dissolved organic matter produced by phytoplankton (Carlson et al., 2009; Morris et al., 2012; Eiler et al., 2009) and thus suggesting that this clade actively responds to organic matter released by the phytoplanktonic bloom found at the DCM. This is illustrated by the observed increase during the diatom bloom (from 18th to 20th) of the contribution of SAR11 at the DCM in St. 3C, where the increase of microbial abundance in time was also apparent. In contrast to the SAR11 distribution, the Rhodobacteraceae family (also under class Alphaproteobacteria) was observed to be relatively abundant in the surface layer shelf. Members of the Rhodobacteraceae family have been described to prevail in areas of relatively low nutrient concentrations (Gilbert et al., 2012), which may explain its relative dominance at the surface layer in our study. One of the dominating genera found in this study within the Rhodobacteraceae family, *Roseobacter*, a strong competitor thriving under low-nutrient conditions (Pinhasi and Beman, 2003), which in our case appears to be at the surface. Overall, Rhodobacteraceae was reduced throughout the week at the DCM in stations 4 and 3C, in response to the nutrient enrichment at these stations with the upwelling pulse and the further evolution with relaxation. However, Rhodobacteraceae contribution was high at the DCM in 3C on the 18th, where nutrients and also HNA% were high. The presence of Gammaproteobacteria in upwelling systems has been reported in several studies (Bergen et al. 2015, Zhou et
al. 2018). The main sequences under the Gammaproteobacteria class comprised SAR86 and Alteromonadaceae. In our study, SAR86 dominated in the DCM at the shelf stations, suggesting that this group could be stimulated by macronutrients such as nitrate of upwelled water since it has been identified as a typical taxa of open ocean waters in other regions (Treusch et al., 2009; Beman et al., 2011; Walsh et al., 2015). The efficiency of SAR86 in exploiting nutrient pulses is suggested by the increase of its contribution throughout the week (being maximum on 20th) at all stations and depths, excluding Station 3C at the surface. Furthermore, this group is often defined as opportunistic and has a broad substrate spectrum (Skerratt et al. 2002), thus suggesting substrate utilization of algal derived organic matter (Teeling et al. 2012, Buchan et al. 2014). Hence, SAR86 may have been linked to the DCM bloom of *Leptocylindrus* species, which could produce and release compounds that serve as chemoattractants for these bacterial groups. Conversely, Alteromonadaceae (another Gammaproteobacteria family) was relatively more abundant in surface waters, contrasting with the dominance of SAR11 at the DCM, which might be attributed to competition for nutrients and/or to a response to the contrasted environmental conditions of surface versus DCM layers found during our study.

The Bacteroidetes consisted mostly of members of the Flavobacteria class, including Cryomorphaceae and Flavobacteriaceae families. Flavobacteria has been reported to be an important component of the microbial loop in coastal phytoplankton blooms (Williams et al. 2013, Tully et al. 2014). In this study, Flavobacteria were relatively more abundant at the surface waters of the coastal stations, especially the innermost coastal station 4 (under the influence of water from continental origin), perhaps reflecting their relationship with the availability and quality of organic substrates, since they have been identified as powerful degraders of complex organic matter (Arnosti 2011, Goméz Pereira et al. 2012). In particular, sequences from the Flavobacteriaceae (mainly belonging to genera *Formosa*, *Polaribacter*, *Croceitalea* and to the uncultured marine groups NS3, NS4, NS5) and Cryomorphaceae families (mainly genus *Owenweeksia*) were found at all stations of the transect but exhibited pronounced abundance fluctuations, maybe related with the fact they are highly diverse, displaying distinct niches and different life strategies (Gómez-Pereira et al., 2010; Abell et al., 2005). In this respect, the abundance of Flavobacteria at station 4 both at the DCM and the subsurface and its considerable reduction throughout the week suggest that before
the pulse that introduced nutrients in this station, the Flavobacteria community was adapted to nutrient depleted waters. Additional support to this is the fact that some Flavobacteriaceae sequences, such as *Formosa* and *Polaribacter* and NS5, were more abundant at station 4 before the pulse. Conversely, NS4 increased during the diatom bloom (from 18th to 20th), while *Croceitalea* and NS3 abundances remained relatively constant throughout the week. We must note that station 4 is periodically fertilized by upwelled waters during pulses, and during relaxation the bacterial community might depend on the recycling of organic matter, that most likely would increase the variability in its taxonomic composition.

Another widespread and commonly found class is Actinobacteria, which in our study mainly belonged to OCS155 sequences. Recent studies found unique sequences of OCS155 associated to the upwelling in the South Pacific Ocean (West et al., 2016) and Actinobacteria clones were also recovered from the DCM in the South Atlantic gyre (Morris et al., 2012). Indeed, the presence of Actinobacteria in the shelf waters off A Coruña (relatively more abundant at the DCM than at the surface) could be associated with the phytoplankton biomass accumulated at the DCM. Actinobacteria helps to decompose organic matter (i.e. senescent algae) for uptake by phytoplankton, and several investigations have correlated Actinobacteria with phytoplankton blooms (Brown et al., 2005, Eckert et al. 2012, Bunse et al. 2016). Deferribacteres, dominated by the SAR406 clade, showed a patchy distribution, although generally being more abundant in the DCM layer at the shelf stations. In the marine environment SAR406 clade is ubiquitously distributed, and it is also known that is positively correlated with Chl-a (Gordon and Giovannoni, 1996; Cram et al., 2014). SAR 406 tags were more abundant on days 18th and 20th at the DCM of shelf stations 2 and 1C, where Chl-a reached the highest values of the study.

*Environmental factors regulating microbial structure*

Past studies of plankton community in the area identified that bacterial abundance and production was maximum in periods of upwelling relaxation with a time-lag with the phytoplankton bloom fueled by nutrient-rich upwelled waters (Valencia et al. 2003, Barquero et al. 1998). In our study we have described the variability of hydrography and nutrients in a section from the coast to the mid-shelf during an upwelling-induced diatom bloom and its relaxation concomitant to the characterization of bacterial
abundance and community composition. The correlation analysis showed that T, Chl-a and inorganic nutrient concentrations had a significant impact on bacterial distribution, especially on its vertical zonation, as illustrated in the two clusters of the nMDS. The surface cluster comprised samples in warm and less saline water (located at the mouth of the ria and advected to the adjacent coastal zone), low Chl-a, dominance of dinoflagellates and high PAR. At this surface layer we found high relative abundance of OTUs affiliated with Rhodobacteraceae, Flavobacteriaceae, and Alteromonadaceae, which have been reported to be abundant in upwelling areas (Baltar et al. 2007). The DCM cluster was characterized by blooming phytoplankton at ≈20-30 m with high abundance of diatoms and dominance of ciliates, high concentration of inorganic nutrients, and low PAR. The richness and phylogenetic diversity of the bacterial community was a higher at the DCM than at the surface. SAR86 and SAR-11 were present at the DCM and increased their contribution with relaxation of upwelling and senescence of diatom bloom. The coexistence of these two assemblages, suggests that they could be specialized in utilizing different DOM compounds released during the DCM phytoplankton bloom and thus avoid direct competition for resources. This is further supported by the presence of specialist taxa, such as OCS155 and JL-ETNP-Y6 sequences, that displayed high abundances in the DCM layer.

In summary, this study provides the first attempt to study the bacterial diversity and community composition at the coastal upwelling off Galicia using 16S rRNA gene sequencing. This technique was more sensitive than CARD-FISH and flow cytometry for the detection of changes in community composition during the upwelling-relaxation transition. Sampling of surface and DCM depths allowed us to show that while the bacterial communities in each depth layer showed a high day to day similarity across 14 km section, the surface and DCM communities were significantly different. The separation of these communities can be due to dispersal limitation due to differential water densities acting as a physical barrier, but may also be due to the selection of communities adapted to the contrasted environmental conditions. Temperature, phytoplankton composition and nutrient concentrations might have acted as the main drivers of the observed vertical zonation of marine Bacteria, with enhanced diversity and presence of specialist taxa in the DCM compared to the surface waters.

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Table 1. Physical, chemical and biological characteristics of the surface (1m) and the deep chlorophyll maximum (DCM) from different sampling days and stations along a transect in the shelf waters in front of A Coruña during the Hercules III cruise. Depth (m); T: temperature (ºC); Sal: salinity; PAR: photosynthetically active radiation (µmol photons m$^{-2}$ s$^{-1}$); Chl-a: Chlorophyll-a concentration (µg L$^{-1}$); Diatoms: Diatoms concentration (cells L$^{-1}$); Dinoflag: dinoflagellate concentration (cells L$^{-1}$); Ciliates: ciliate concentration (cells L$^{-1}$); NO$_2$+NO$_3$: nitrate + nitrite concentration (µmol L$^{-1}$); PO$_4$: phosphate concentration (µmol L$^{-1}$); SiO$_4$: silicate concentration (µmol L$^{-1}$)
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<th>PAR</th>
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<th>Diatoms</th>
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Table 2. Percentage of relative abundance (mean ± standard deviation) of the main bacterial groups determined by CARD-FISH along a transect in the shelf waters in front of A Coruña during the HERCULES-0712 cruise. DCM: deep chlorophyll maximum.

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Table 3. Number of OTUs, richness and diversity estimated by 454-pyrosequencing of Bacterial 16S rRNA gene. The samples marked with an asterisk (*) are outliers. DCM: deep chlorophyll maximum.

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Figure 1. Map of the study area and location of stations sampled during the oceanographic cruise HERCULES-0712 in the shelf waters in front of A Coruña. The numbers between brackets indicate the depth of each station.

Figure 2. Distribution of six-hourly (0:00, 06:00, 12:00, and 18:00 GMT) offshore upwelling index (Iw) during the sampling period of the oceanographic cruise HERCULES-0712 in the shelf waters in front of A Coruña.

Figure 3. Daily evolution of physical and biological variables (temperature, salinity, chlorophyll-a and water column stratification, \( N^2 \)) sampled during the oceanographic cruise HERCULES-0712 in the shelf waters in front of A Coruña.

Figure 4. Inorganic nutrients during the oceanographic cruise HERCULES-0712 in the shelf waters in front of A Coruña. \( \text{NO}_2 + \text{NO}_3 \): nitrate + nitrite concentration; \( \text{PO}_4 \): phosphate concentration; \( \text{SO}_4 \): silicate concentration.

Figure 5. (A) Microbial abundance (MA, cells mL\(^{-1}\)) and high nucleic acid content bacteria (% HNA) in the shelf waters in front of A Coruña.

Figure 6. Comparison of bacterial diversity determined by 454-pyrosequencing among different samples collected in the shelf waters in front of A Coruña (A) Stacked column graph representing the relative distribution of the dominant phyla (B) Stacked column graph representing the taxonomic composition at the order and family level (C) Cluster analysis of bacterial diversity. Sample codes consist of the sampling date (day followed by the month and year), station and depth of sampling (1m: surface samples, DCM: depth chlorophyll maximum samples). Diamond symbols: station 4. Triangle symbols: station 3C; Circle symbols: station 2; Square symbols: Station 1C. Open and close symbols correspond to Surface and DCM horizons, respectively. Samples of 17\textsuperscript{th} July and 18\textsuperscript{th} July of the DCM of station 4 and 2, respectively, are outliers.

Figure 7. Non-metric multidimensional scaling (NMDS) analysis showing the correlation between (A) different environmental variables and (B) specific taxonomic phyla and the relative abundance of bacterial OTUs determined by 454-pyrosequencing in the different samples in the shelf waters in front of A Coruña. Sampling points that are close together are more similar in their bacterial composition than those that are far apart. For sample labels see Figure 6.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Vertical zonation of bacterial assemblages attributed to physical stratification during the summer relaxation of the coastal upwelling off Galicia (NW Spain)

Tania Montes¹⁻², Elisa Guerrero-Feijóo², Víctor Moreira-Coello³, Antonio Bode², Manuel Ruiz-Villarreal², Beatriz Mouriño-Carballido³, and Marta M. Varela².

Highlights:

(i) no previous studies have been found in the literature showing how bacterial community composition evolves during an episode of relaxation of an upwelling, by characterizing bacterial assemblages (using different diversity measurement techniques) along with hydrography, nutrient concentration and microbial abundance in a section off A Coruña (from the ría to the adjacent shelf).

(ii) the three different approaches (flow cytometry, CARD-FISH and Illumina MiSeq) provided complementary information on the abundance, diversity, community composition and distribution patterns of bacterial communities showing up robustness and solidity to our results and

(iii) we have been able to show a coupling between bacterial communities and the hydrodynamics, due mainly to temperature, phytoplankton composition and nutrient concentrations, shaping the observed vertical zonation of marine Bacteria.
Vertical zonation of bacterial assemblages attributed to physical stratification during the summer relaxation of the coastal upwelling off Galicia (NW Spain)

Tania Montes\textsuperscript{1,2}, Elisa Guerrero-Feijóo\textsuperscript{2}, Víctor Moreira-Coello\textsuperscript{3}, Antonio Bode\textsuperscript{2}, Manuel Ruiz-Villarreal\textsuperscript{2,3}, Beatriz Mouriño-Carballido\textsuperscript{3}, and Marta M. Varela\textsuperscript{2}.

The authors do not declare any conflict of interest.