Vertical stratification of bacterial communities driven by multiple environmental factors in the waters (0-5000 m) off the Galician coast (NW Iberian margin)

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ABSTRACT

The processes mediated by microbial planktonic communities occur along the entire water column, yet the microbial activity and composition have been studied mainly in surface waters. This research examined the vertical variation in bacterial abundance, activity and community composition and structure from surface down to 5000 m depth following a longitudinal transect off the Galician coast (NW Iberian margin, from 43°N, 9°W to 43°N, 15°W). Community activity and composition changed with depth. The leucine incorporation rates decreased from the euphotic layer to the bathypelagic waters by three orders of magnitude, whereas prokaryotic abundance decreased only by one order of magnitude. The relative abundance of SAR11 and *Alteromonas*, determined by catalyzed reported deposition fluorescence in situ hybridization (CARD-FISH), decreased with depth. Meanwhile, the contribution of SAR 202 and SAR324 was significantly higher in the deeper layers (i.e. NEADW, North East Atlantic Deep Water and LDW, Lower Deep Water) than in the euphotic zone. Bacterial community structure, assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA), was depth-specific. A distance based linear model (DistLM) revealed that the variability found in bacterial community structure was mainly explained by temperature nitrate, phosphate, dissolved organic matter (DOM) fluorescence, prokaryotic abundance, leucine incorporation and to a lesser extent salinity, oxygen, CDOM absorbance and dissolved organic carbon. Our results displayed a bacterial community structure shaped not only by depth-related physicochemical features but also by DOM quality, indicating that different prokaryotic taxa have the potential to metabolize particular DOM sources.

KEY WORDS: Bacteria, community composition, fluorescent in situ hybridization (FISH), Automated Ribosomal Intergenic Spacer Analysis (ARISA), DOM, deep waters, NW Iberian margin

INTRODUCTION

Prokaryotes are an important component of marine plankton, accounting for a major fraction of the total planktonic biomass in marine waters (Giovannoni and Rappé, 2000). Moreover, they play an important role as mediators of the main biogeochemical cycles (Furhrman et al., 2015). Prokaryotic abundance and activity decrease one and two orders of magnitude, respectively, from the euphotic to the bathypelagic zone in the ocean (Nagata et al. 2000, Aristegui et al., 2009). Such pattern is determined by the vertical variability in the physical and chemical features of the pelagic environment, which also contribute to the vertical stratification of the bacterial communities associated to water masses (De Long et al., 2006). Recently, Agogué et al. (2011) showed that a considerable fraction of the bacterial taxa is present throughout the water column, whereas some operational taxonomic units (OTUs) are specific for the different water masses, overall leading to a water mass-specific clustering of bacterial communities in the dark realm of the boreal to tropical Atlantic. Similar results were obtained by Lekunberri et al. (2013) in the subtropical Atlantic and Mapelli et al. (2013) in the Mediterranean Sea by using T-RFLP (Terminal Length Polymorphism Restriction Fragment) and ARISA (Automated Ribosomal Intergenic Spacer Analysis), respectively.

Prokaryotic community composition in the ocean is depth-stratified (DeLong et al., 2006; Brown et al., 2009), potentially linked to depth related changes in the quality and quantity of available energy sources. Previous studies in the Atlantic have shown that the abundance of the SAR11 cluster (Alphaproteobacteria), a k-strategist slow growing (Yilmaz et al., 2016), decreases with depth. By contrast the abundance of clusters like SAR202 (Cloroflexi), which is well adapted to scarce food availability and to utilize recalcitrant organic compounds (Yilmaz et al., 2016), or SAR324 (Alphaproteobacteria) and SAR406 (Deltaproteobacteria), with genes associated with a particle attached lifestyle (Yilmaz et al., 2016), increases with depth (Varela et al. 2008a; Lekunberri et al. 2013). Other phylogenetic groups, such as *Alteromonas* (Gammaproteobacteria), show more variable and patchy distributions. While Lekunberri et al. (2013) found that

the relative contribution of *Alteromonas* increased with depth in the tropical Atlantic, Schattenhofer et al. (2009) showed that *Alteromonas* was more abundant in the euphotic layer than in mesopelagic waters in a latitudinal Atlantic transect, likely associated with the capability of feeding phytoplankton storage molecules (Yilmaz et al., 2016).

The vertical variability in the bacterial community composition has been shown to correlate with changes in biotic and abiotic environmental factors (Ghiglione et al., 2012), indicating their control on bacterial populations. Recent studies have revealed that physical forces (e.g temperature, hydrostatic pressure, salinity) are correlated with spatial variation in abundance, activity and diversity of marine microbial communities (reviewed in Fuhrman et al., 2015). Also, previous studies have provided some evidences that bacterial community composition varies with dissolved organic matter (DOM) sources supporting the potential structuring effect of DOM composition (Kirchman et al., 2004). Despite these advances, however, our knowledge on the sources of DOM in the meso- and bathypelagic waters and the link between the composition and diversity of DOM and bacterial communities in the dark ocean is still limited.

Thus, the aim of this study was twofold: (i) to characterize the vertical variability of the abundance, activity, composition and structure of the bacterial communities from surface down to 5000 m depth along a latitudinal section off NW Spain and (ii) to identify the environmental factors, including physico-chemical variables and optical indices of the DOM, that best explain the variation of the bacterioplankton community structure.

MATERIALS AND METHODS

Study site and sampling

The sampling of the epi- meso- and bathypelagic waters was carried out along a longitudinal transect off Galicia during the oceanographic cruise BIOPROF-2 (September 2012) on board the R/V Cornide de Saavedra. In total, 22 stations were sampled from 43°N, 9°W to 43°N, 15°W off Cape Finisterre (NW Spain) for the physicochemical characterization, and 6 stations were sampled for the organic matter variables as well as bacterial community analyses (see Fig. 1). At each station, water samples were collected with Niskin bottles mounted on a CTD (conductivity-

temperature-depth) rosette sampler from several “core” depths, on the basis of different temperature and salinity characteristics from each CTD cast: the euphotic zone (EZ, 0-100m) the Eastern North Atlantic Central Water (ENACW, 250-900m), the layer of the Oxygen Minimum Zone (OMZ); the Mediterranean Water (MW, $\approx$ 1000m); the Labrador Sea Water (LSW, 1800-2000m); the Eastern North Atlantic Deep Water (ENADW, 2450-2900m) and the Lower Deep Water (LDW, $\geq$ 4000m).

Environmental parameters

Dissolved oxygen samples were taken in pyrex “iodine titration” flasks with flared necks and ground glass stoppers, with a nominal volume of about 115 mL, and measured, on board after sampling collection, following the Winkler potentiometric method after Langdon (2010). Aliquots for inorganic nutrients determination (nitrate, nitrite, ammonium, phosphate and silicate) were collected in rinsed polyethylene bottles and frozen at -20°C until further analysis by standard colorimetric methods with a Bran-Luebbe segmented flow analyser.

Water samples for the analysis of dissolved organic carbon (DOC) were collected in glass ampoules, and acidified with H₃PO₄ to pH < 2 before heat sealing. Samples were measured in a Shimadzu TOC-CSV analyser by high-temperature Pt-catalytic oxidation. Fluorescence intensity was measured at two fixed excitation/emission wavelengths of 320 nm/410 nm (FDOM-M), characteristic of marine humic-like substances, and of 280 nm/350 nm (FDOM-T), characteristic of protein-like materials using a Perkin Elmer LS55 and following Nieto-Cid et al. (2006). The absorption spectra of the chromophoric DOM were acquired on a Beckman Coulter DU800 spectrophotometer equipped with 10 cm quartz cells. Spectral scans were collected from 250 to 700 nm, providing the following indexes (Green and Blough, 1994): aCDOM 254 (absorption coefficient at 254 nm), aCDOM 340 (absorption coefficient at 340 nm), aCDOM 365 (absorption coefficient at 365 nm), and sCDOM 275/295 (slope of the absorption spectrum between 275 and 295 nm).

Prokaryotic abundance

The prokaryotic abundance (PA) was quantified by flow cytometry following Gasol et al. (1999). Briefly, water samples (1.8 mL) were fixed with paraformaldehyde and glutaraldehyde (2-4% final concentration), flash-frozen in liquid N₂ for 10 min and stored at -80°C until further analysis. Prior to analysis, the samples were thawed to

room temperature and the prokaryotic cells stained with Syto13 in the dark for 10 min. Subsequently, fluorescent latex beads (approximately $1 \times 10^5 \text{ mL}^{-1}$) (Molecular Probes, Invitrogen, Carlsbad, CA) were added to all the samples as internal standard. The prokaryotes were enumerated using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) according to their signature in right angle light scatter and green fluorescence.

Prokaryotic leucine incorporation rate

^3H -Leucine incorporation rate (Leu incorp.) was determined as a proxy for prokaryotic production (Kirchman, 2001). Subsamples from the euphotic and lower meso-pelagic (down to 500 m) were amended with 20 nmol L^{-1} [^3H]-leucine (final concentration, specific activity $160 \text{ Ci mmol L}^{-1}$, GE Healthcare, Amersham, Bucks, UK). Triplicate 1.2 mL samples and duplicate TCA (trichloroacetic acid)-killed blanks (50% final concentration) (Simon and Azam, 1989) were incubated in the dark at *in situ* temperature-controlled chambers for 2-6 h depending on the expected activity. Incubations were terminated by adding TCA (50% final concentration) to the samples. Bacterial proteins were precipitated by two successive centrifugations (12000 rpm, 10 min), including a washing step with 1 mL of 5% TCA according to the method of Kirchman (1985) with slight modifications by Smith and Azam (1992).

Leu incorp. of the samples from the bathy- and mesopelagic waters (below 1000m) was measured by adding 5 nmol L^{-1} [^3H]-leucine (final concentration, specific activity $160 \text{ Ci mmol L}^{-1}$, GE Healthcare, Amersham, Bucks, UK) to duplicate 40 mL samples and duplicate formaldehyde-killed blanks (2% final concentration) (Simon and Azam, 1989). Samples and blanks were incubated in the dark at *in situ* temperature in temperature- controlled chambers for 10-24 h depending on the expected activity. Incubations were terminated by adding formaldehyde (2% final concentration) to the samples. After 10 min, the samples and the blanks were filtered onto $0.2 \mu\text{m}$ polycarbonate filters (25 mm filter diameter, Millipore). Subsequently, the filters were rinsed three times with 10 mL of 5% TCA. Thereafter, the filters were transferred into scintillation vials and dried at room temperature.

Finally, liquid scintillation cocktail was added to all samples and after 18 h, the radioactivity was determined in a scintillation counter (LKB Wallac). The mean disintegrations per minute (DPM) of the blanks were subtracted from the mean DPM of

the respective samples and the resulting DPM converted into leucine incorporation rates.

Enumeration of specific groups of Bacteria by CARD-FISH and FISH

CARD-FISH was used to determine the abundance of specific groups of bacteria (Table 1) following the method described by Pernthaler et al. (2002). Immediately after collecting the samples from the Niskin bottles, 20-80 mL of water were fixed with paraformaldehyde (2% final concentration) and stored at 4°C in the dark. After 12-18 h, the samples were filtered through 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 processing in the laboratory. Filters were cut in sections and hybridized with specific horseradish peroxidase (HRP)-labelled oligonucleotide probes for the following groups: Eubacteria, SAR11 (Alphaproteobacteria), SAR324 (Deltaproteobacteria), SAR406, and *Alteromonas* (Gammaproteobacteria). Signal was amplified by adding tyramide-Alexa488. FISH with Cy3-labeled probes was used to enumerate the members of the SAR202 (Chloroflexi) cluster following the protocol of Morris et al. (2004). Filter sections were carefully mounted in slides and counterstained with a DAPI-mix [5.5 parts of Citifluor (Citifluor), 1 part of Vectashield (Vector Laboratories) and 0.5 parts of phosphate-buffered saline (PBS) with 4',6-diamidino-2-phenylindole (DAPI) (final concentration 2 µg mL⁻¹)]. The probes and hybridization conditions for the individual groups are given in Table 1. Enumeration of DAPI-stained cells and cells stained with the specific probes was performed under a Nikon Eclipse 80i epifluorescence microscope equipped with a Hg lamp and appropriate filter sets for DAPI, Cy3 and Alexa448. A minimum of 500 DAPI-stained cells was counted per sample.

Bacterioplankton community structure by ARISA fingerprinting

A volume of 10-15 L of water was filtered through sterile Sterivex 0.22 µm pore size filters (Millipore, USA). Subsequently, 1.8 mL of lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) was immediately added to the filters before storing them at -80°C until extraction. The DNA extraction began by adding lysozyme (1 mg mL⁻¹, final concentration (FC); SIGMA-ALDRICH) to the Sterivex, followed by 30 min incubation at 37°C. Then, proteinase K (0.5 mg mL⁻¹, FC; SIGMA-ALDRICH) and sodium dodecyl sulphate (SDS) (1%, FC) were added, followed by 2 h incubation at

55°C. Lysate was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1; saturated with 10 mM Tris, pH 8.0, 1 mM EDTA; SIGMA-ALDRICH) and once with chloroform-isoamyl alcohol (24:1; 99% purity for molecular biology; SIGMA-ALDRICH) at 4500 rpm 10 min. The aqueous phase was concentrated in microconcentrator (Amicon with Ultracel-100 membrane; Millipore), washed twice with sterile water, and reduced to approximately 200 μ L. The recovered DNA was quantified in a Nandrop spectrophotometer. All DNA extractions were diluted to working concentrations of 10 ng μ L⁻¹ of template DNA, and stored at -20°C. ARISA-PCR was conducted on a standard amount of DNA on each sample by using the primer set ITSf, 5'-GTC GTA ACA AGG TAGGCC GTA-3' and ITSReub, 5'-GCC AAG GCA TCC ACC 3', as previously described (Cardinale et al., 2004). PCR reactions (40 μ L) contained final concentrations of 1x PCR buffer (Genecraft), 2.5 mM MgCl₂ (Genecraft), 250 μ M of each dNTP (Genecraft), 250 nM of each primer, 40 ng μ L⁻¹ bovine serum albumin, 3.5 U of BioThermD-TM Taq DNA Polymerase (GeneCraft) and 0.13 ng μ L⁻¹ of template DNA. The reaction mixture was held at 94°C for 2 min, followed by 32 cycles of amplification at 94°C for 15s, 55°C for 30 s and 72°C for 3 min, with a final extension of 72°C for 10 min. ARISA fragments were separated using the ABI Prism 3730XL (Applied Biosystems) genetic analyzer applying the internal standard LIZ 1200 (20-1200 pb, Applied Biosystems). Peaks showing a height value <20 fluorescence units were removed from the output peak matrix before binning. Profile peaks were binned and rearranged by operational taxonomic units (OTUs), by using R automatic and interactive binning scripts as follows. The total peak area per sample was normalized to one in order to account for run-to-run variations in signal intensity (Yannarell and Triplett, 2005) and each ARISA peak was defined as a different operational taxonomic unit (OTU).

Statistical analysis of bacterial communities

The microbial community dataset was correlated with the physico-chemical and the DOM-related dataset (see below for variables included in each data set) by a comparative Mantel type test, using the RELATE function of the primersoftware. Hierarchical Cluster Analysis was carried out to explore the bacterioplankton community structure (similarities between samples), based on the resemblance matrix generated using Bray Curtis similarity on the presence/absence of the OTUs within each sample. Significant differences in microbial community structure among water masses

were investigated by permutational analysis of variance (PERMANOVA, Anderson 2001). Furthermore, parsimonious models were built to identify the best factors explaining variation in the community structure (DistLM, Anderson et al., 2008). Three sets of variables were considered to study the bacterioplankton community structure: (i) physico-chemical variables: temperature, salinity, oxygen, nitrate, phosphate, silicate; (ii) DOM-related variables: DOC, FDOM-T, FDOM-M, aCDOM 254, aCDOM 340, aCDOM 365, sCDOM 275/295; and (iii) microbial community variables: PA, Leu incorp. Previously, all variables were analyzed to test the collinearity between them using a Pearson correlation matrix, eliminating the variables with $r^2 > 0.95$. In order to assign the contribution of each variable and each set of variables taken alone (physico-chemical, organic matter-related and microbiological), an “all specified” selection procedure was carried out using the “ r^2 ” as selection criterion. The contribution of each variable was assessed using “marginal tests” to assess the statistical significance and percentage contribution of each variable and each set of variables taken alone. Finally, all variables were introduced in the model using the “step wise” selection procedure of the DistLM and the “Akaike” information criterion (AIC). Such procedure allowed us to find the best combination of environmental variables that explained the highest proportion of variability found in the bacterioplankton community structure resemblance matrix. A “sequential test” was employed to evaluate the cumulative effect of each variable once the previous variable (s) had been accounted for. A distance-based redundancy analysis (dbRDA) was used for graphical visualization of the DistLM results. All analyses were performed, using the software packages PRIMER6 & permanova+ (Anderson et al., 2008).

RESULTS

Environmental characteristics

Physical and chemical characteristics of the main water masses are summarized in Table 2. The LDW was found below 4000 m depth, characterized by low salinity (34.9), low temperature (2.5°C) and high silicate concentrations (32.8-44.9 $\mu\text{mol kg}^{-1}$). The ENADW was clearly identifiable between 2450 and 2900 m depth. ENADW differed from LDW in showing slightly higher temperature (2.5-3.5°C) and higher oxygen content (Table 2). Two types of intermediate waters were found; the LSW (1800-2000 m) that showed a minimum of salinity (35.0-35.4) and relatively high oxygen

concentration ($197.5\text{--}262.8\ \mu\text{mol kg}^{-1}$), and MW (1000 m) that was clearly identifiable by high salinity ($35.0\text{--}36.2$). OMZ was located at about 900 m displaying oxygen concentrations around $180\text{--}241\ \mu\text{mol kg}^{-1}$. Regarding DOC concentrations, the average values decreased progressively from the shallower depths (68.8 and $55.1\ \mu\text{mol L}^{-1}$, for the EZ and ENACW-OMZ, respectively) to the deeper water masses (50.8 , 48.5 , 46.1 , $42.7\ \mu\text{mol L}^{-1}$ for MW, LSW, ENADW and LDW, respectively) (Fig. 2)

Depth distribution pattern of DOM-related variables

Dissolved organic carbon ranged from $\sim 70\ \mu\text{mol C L}^{-1}$ at the surface waters to $40\text{--}45\ \mu\text{mol C L}^{-1}$ in the deeper water masses (Fig. 2A). In the same way, FDOM-T decreased from ~ 1.0 to 0.4 QSU (Fig. 2C). On the contrary, FDOM-M increased with depth, displayed lower values at surface (~ 0.7 QSU) than at the deep layers (1.0 QSU, Fig. 2B). Regarding the optical indexes of the coloured DOM, in general they exhibited a decrease with depth, more significantly for the aCDOM 254 (Fig. 2).

Depth distribution pattern of prokaryotic variables

Prokaryotic abundance (PA) ranged from 1.1×10^5 to $4.7 \times 10^5\ \text{cell mL}^{-1}$ in the euphotic zone, and decreased exponentially with depth at all stations. The minimum values were found in the ENADW (ranging from 1.5×10^4 to $2.7 \times 10^4\ \text{cell mL}^{-1}$; Fig. 3A). The leucine incorporation rate (Leu incorp.) showed a similar vertical trend, decreasing three orders of magnitude from the EZ ($14.2 \pm 8.3\ \text{pmol Leu L}^{-1}\ \text{h}^{-1}$) to the LDW ($6.7 \pm 3.8 \times 10^{-3}\ \text{pmol Leu L}^{-1}\ \text{h}^{-1}$; Fig. 3B). Cell-specific activity (prokaryotic Leu incorp. divided by PA) decreased also with depth. Maximum cell-specific activity was found in the euphotic layer ($1.9 \pm 1.3 \times 10^{-3}\ \text{fmol cell}^{-1}\ \text{d}^{-1}$) (Fig 3). In the meso- and bathypelagic the minimum cell-specific activity was measured at the LDW ($4.7 \times 10^{-6}\ \text{fmol cell d}^{-1}$) and the maximum at the MW ($1.7 \pm 1.0 \times 10^{-3}\ \text{fmol cell d}^{-1}$).

Relative abundance of specific groups of Bacteria

Specific phylogenetic groups of Bacteria showed different patterns of distribution according to the water mass (Table 3). While SAR 11 and *Alteromonas* contribution to total bacterial abundance was significantly higher in the EZ (ANOVA-Tukey, $p < 0.0001$) than in deeper waters, SAR202 relative abundance was significantly higher in the LDW, LSW and ENADW (Tukey, ANOVA, $p < 0.0001$) than in the EZ. The abundance of SAR324 increased slightly with depth (ANOVA-Tukey, $p > 0.05$).

Conversely, the abundance of SAR406 varied between 12.6-17.0% and we did not find significant differences among water masses (ANOVA-Tukey, $p < 0.05$).

Bacterial community structure determined by ARISA fingerprinting

The ARISA pattern of the bacterial community revealed a total of 268 polymorphic peaks, with fragments ranging from 102.9 to 846.9 bp (data not shown). 158 OTUs were found in the EZ, 168 OTUs in the ENACW-OMZ, 148 OTUs in the MW, 153 OTUs in the LSW, 148 OTUs in the ENADW and 88 OTUs in LDW. 12.3% of the 268 OTUs were present in all the water masses while 26.1% were unique to specific water masses. Most unique OTUs (38.5%) were found in the EZ, 17.5% in the OMZ, 5.7% in ENADW and 8.5% in LDW. These 26.1% of unique OTUs led to a clear separation of bacterial communities in three main clusters (PERMANOVA, $p < 0.01$). The first cluster comprised the bacterial communities of EZ, the second cluster consisted mainly of the bacterial communities of the ENACW-OMZ and MW, and the third cluster comprised mostly the bacterial communities of the LSW, ENADW and LDW (Fig. 4).

Relationship between biotic and abiotic environmental variables with bacterial communities

A significant correlation was obtained between the physico-chemical and microbiological characteristics (PA and Leu incorp.) of the different depth levels (Mantel test, $r = 0.433$, $p < 0.05$). Also these microbiological variables were related significantly to the DOM-related variables ($r = 0.511$, $p < 0.05$).

A principal component analysis (PCA) was applied to the available biotic and abiotic environmental data to illustrate the distribution of the samples according to their physico-chemical and microbiological variables (Fig. 5A). Inorganic nutrients, temperature, and microbiological variables determined the segregation between euphotic-intermediate (EZ, ENADW-OMZ, MW) and deep (LSW, LDW, ENADW) samples, whereas the oxygen concentration and the absorption coefficient at 340 nm and 365 separated the intermediate samples from the deep ones.

In order to determine which combination of environmental variables better explained the variability in bacterioplankton community structure we used a distance linear-based model (DISTLM). The output of marginal test on each set of variables showed that the physico-chemical, the DOM-related and the microbiological variables explain 50.4%, 38.2% and 28.3%, respectively, of the bacterial community structure variability (Table

4). Temperature account for 29% of the variance observed. Other main contributors include inorganic nutrients as nitrate (32%), FDOM-M (28%) and PA (26%). The same analysis was performed by choosing the best fitted DistLM model. As a result, only nine variables were retained in the model and remain significant in the sequential test of the best model, together accounting for 70.4 % of the variability found in the bacterial community resemblance matrix (Table 5, Fig 5B). Four of the nine variables were physico-chemical: temperature, salinity, silicate and nitrate. Furthermore, the influence of three DOM-related variables (sCDOM 275/295, aCDOM 365 and FDOM-T) and two microbiological (PA and Leu incorp.) act in synergy to explain bacterial community structure. The first two dbRDA axes accounted for 81.3 of the fitted variability, corresponding to 46.3 of the total variability of the bacterioplankton resemblance matrix. This model based only on nine variables clearly represented the community patterns shown in the PCO plot of Figure 5A, demonstrating that the main structuring forces were included in the best DistLM model.

DISCUSSION

The different DOM variables presented different vertical profile patterns (Fig 2). Thus, DOC and FDOM-T displayed a clear decrease with depth due to the labile organic mater respiration (Nieto-Cid et al., 2006). These processes promoted the generation of humic-like substances, which, in general and as expected, exhibited higher fluorescence intensities in the deep layers and lower at the upper levels, due to photodegradation processes (Nieto-Cid et al., 2006). Waters from the Mediterranean Sea (MW) slightly diverge from this patter due to the special conditions, for example high temperature and salinity, of this enclosed sea (Martínez-Pérez et al., submitted). In the coast off Galicia this water mass is easily differenced by the highest salinity values (Table 2) which correspond with relative low values of DOC and FDOM-T, as expected due to the low values of DOM found in the Mediterranean Sea (Martínez-Pérez et al., submitted). The decrease with depth of aCDOM 254 also supported the idea of the microbial oxidation of labile organic materials, as this coefficient is a tracer of molecules containing double bonds of carbon (Helms et al., 2008). In addition, the decline of the optical slope indicates an increase of the molecular weight of the DOM with depth (Helms et al., 2008).

The distinct physical and chemical features across the water column off the Galician coast sustain unique environments with specific abundance, biodiversity and microbial activity. The prokaryotic abundance and the leucine incorporation rates in the euphotic meso- and bathypelagic waters measured in this study were comparable to previous observations in the deep waters of the North Atlantic (Varela et al., 2008a; Aristegui et al., 2009), tropical Atlantic (Lekunberri et al., 2013), and other deep oceanic environments, as the Mediterranean Sea (Yokokawa et al., 2010; Mapelli et al., 2013), in spite of the fact that it has very different features compare to the Atlantic. Also cell-specific activity rates were in the same order of magnitude than those measured in the meso- and bathypelagic waters of the subtropical Atlantic (Baltar et al. 2009, 2010). However, it must be taken into account that all activity rates measured in this study were done at atmospheric pressure, and a recent review displayed that activity measurements made using decompressed samples underestimate in situ activity (Tamburini et al., 2013). By contrast, ratios of in situ prokaryotic leucine incorporation to atmospheric pressure decreased with increasing depth indicating that hydrostatic pressure retards overall heterotrophic activity in the deep sea (Amano-Sato, pers. comm.). Thus, more specific analysis are needed to decipher microbial activity in the water column as a function of pressure. Some environmental factors have been described to co-vary with the prokaryotic heterotrophic activity, such as temperature and inorganic nutrient concentrations (Ghiglioni et al. 2008; Yokokawa et al., 2010; Mapelli et al., 2013). In this research work, we found that the microbiological variables (prokaryotic abundance and activity) were significantly correlated with the physical (e.g. temperature) and chemical (e.g. inorganic nutrients) dataset, as well as with specific characteristic of the organic matter. These findings agree with Yokokawa et al., (2010), who showed that the vertical variation of the prokaryotic activity in the Mediterranean Sea might be explained not only by the depth, but also by a set of covarying physical variables. On the other hand, depth may be a proxy for the amount of substrate available as the concentration and chemical composition of DOM change with depth (McCarthy et al., 1995), as labile compounds are degraded along the water column while humic substrates are produced as sub-product of this bacterial respiration. This pattern may influence the heterotrophic bacterial activity, which is also highly dependent on the bioavailability and composition of the DOM (Carlson, 2002). The significant correlation found between DOM quality-related variables and prokaryotic abundance and activity suggests a clear link between DOM composition (Kawasaki and

Benner, 2006) and the potential utilization of DOM by the bacterial communities inhabiting the deep sea waters off the Galician coast.

Vertical distribution patterns in the abundance of specific bacterial groups

Our study clearly showed different vertical distribution patterns of the specific phylogenetic groups of bacteria in waters off the Galicia coast. The SAR11 relative abundance decreased with depth, a pattern previously observed in a wide range of oceanic regions (Mary et al., 2006; Schattenuhofer et al., 2009; Alonso-Saez et al., 2007; Carlson et al., 2009). This group is very abundant and cosmopolitan, and the general pattern of decreasing abundance with depth suggests that this plays an important role in the oxidation of organic carbon in surface oceanic waters (Carlson et al., 2009), as also demonstrated by radiolabeled organic substrates uptake experiments (Malmstrom et al., 2004; Mou et al., 2007). Despite its dominance in the euphotic zone, SAR11 was found throughout the whole water column, which is consistent with other works based on DNA sequencing (Rappé et al., 2002; Giovannoni et al., 2005). The contribution of *Alteromonas* was also particularly high in the euphotic zone, probably related to the availability of labile organic substrates, derived from phytoplankton activity (Schattenuhofer et al., 2009), suggesting that they are preferentially heterotrophic.

By contrast, SAR234 and SAR202 were more abundant in deep waters off the Galician coast, suggesting that these groups are well adapted to the available DOM in the dark ocean. SAR202 cells are, on average, larger than other bacterioplankton cells at the bathypelagic ocean, suggesting that this group may be a highly active member of the bathypelagic bacterial community (Varela et al., 2008b). Metagenomic studies have recently revealed high abundance of bacterial genes related to chemotaxis and motility in the deep waters (DeLong et al., 2006), suggesting adaptive advantages of deep-water relative to surface-water bacteria to overcome DOM shortage in the dark ocean. Thus, SAR202 could be regarded as an opportunistic group, with the ability to detect and quickly exploit nutrient patches in the dark ocean (Varela et al., 2008b). The percentage of SAR324 slightly increased with depth in the Galician waters, as observed by Lekunberri et al. (2013). The high abundance of SAR324 in deep waters has a particularly high ecological relevance in the “dark ocean”, since genomic studies have shown that members of this group have genes related to the chemolithoautotrophy (Swan et al., 2011). Finally, the SAR406 did not show any trend with depth in the waters off

the Galician coast. Our results differ from those obtained by Lekunberri et al. (2013) and Agogu et al. (2011) in Atlantic water, which found that the contribution of this group to the total prokaryotes increased with depth.

Linking biotic and abiotic environmental variables with bacterial communities structure

Both analysis of qualitative ARISA fingerprints and the quantification of specific bacterial groups by CARD- FISH indicated a vertical stratification of the bacterial communities across the water column off the Galician coast. Furthermore, this vertical stratification of the bacterial community structure was related to several environmental factors. Studies by Agogu et al. (2011) and Lekunberri et al. (2013) on the composition of the bacterial community in major water masses of the tropical Atlantic suggest a water mass-specificity of bacterial communities driven, not only by a few abundant OTUs, but especially by the less abundant OTUs, which constitute the "rare biosphere". Therefore, the use of molecular fingerprinting techniques based on PCR amplification of the ribosomal intergenic region, which detect, not only the abundant, but also some less abundant OTUs, seems to be effective for describing changes in the structure of the bacterial communities of marine ecosystems (Mapelli et al., 2013).

The driving force that determines community structure variation down the vertical profiles can arise from a complex mixture of biotic and abiotic factors. We have found that temperature and inorganic nutrient concentrations were the most important factors explaining the variability in the distribution of bacterial communities off the Galician coast. Several recent studies provided similar correlations between the prokaryotic community composition and the physico-chemical features of the water column (Galand et al., 2009; Agogu et al., 2011; Ghiglione et al., 2012; S et al., 2014). Interestingly, in the present research work the observed changes in bacterial community structure were also related to the quantity and quality of the organic matter (Table 4). FDOM-M and FDOM-T, indicative of humic- and protein-like substances, respectively, were the primary DOM-related variables driving the bacterial community structure, explaining, 27 and 17% of the variation respectively (Table 4). Both, labile and refractory compounds, relate differently with the bacterial communities in the deep ocean: while protein-like molecules are consumed by the microbial communities humic-like substrates are generated by these microbes through these respiration processes

(Aparicio et al., 2015). The best linear model displayed that FDOM-T remain significantly in the sequential test after the effect of the other variables, whereas FDOM-M was no longer significantly correlated with bacterioplankton community structure, which may indicate a higher dependence of the microbial communities on the original DOM along the investigated vertical profile off the Galician coast. The best linear model also included aCDOM 365 and sCDOM 275/295 as significant explicative variables. Although aCDOM 365 does not represent any specific kind or group of molecules, it is related to the aromaticity of the DOM and, for this set of samples, it is significantly correlated with the DOC concentration (data not shown) which could account for the importance of this variable, at interpreting the observed variation of the bacterial community structure along the depth gradient off the Galician coast. sCDOM 275/295, related to DOM molecular weight and aromaticity (Helms et al., 2008), also contributed at explaining the vertical stratification of the bacterial communities, indicating that the molecular weight of the DOM (very likely associated to DOM ageing in the deeper water masses) may also drive changes in the bacterial communities in the dark waters off the Galician coast.

In conclusion, we have shown clear vertical spatial patterns of bacterial abundance, activity and community composition and structure in the pelagic realm off the Galician coast. A DistLM model selected 9 environmental variables which explain up to 70.4 % of the vertical variation of the bacterial community. Our study evidenced that standard physico-chemical variables were the main factor responsible for the observed vertical patterns, however specific variables of the optical characterization of DOM further explained variability in the bacterial community structure throughout the water column. The remaining 29.6 % unexplained possibly represent one or several specific variables not included in the regression model displayed in this investigation.

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FIGURE LEGENDS

Figure 1. Bathymetric map of NW of Iberian Peninsula and location of sampling stations off Cape Finisterre during the oceanographic cruise BIOPROF-2. Numbers show the stations where the abundance, leucine incorporation and bacterial community composition and structure were determined (microbiological stations).

Figure 2. Average vertical profiles of dissolved organic carbon (A), humic-like fluorescence (B), protein-like fluorescence (C), absorption coefficient at 254 nm (D), absorption coefficient at 365 nm (E), and optical slope between 275 and 295 nm (F) found in microbiological stations along the Finisterre section. Samples were grouped by water masses.

Figure 3. Average vertical profiles of prokaryotic abundance (A), leucine incorporation rate (B) and cell-specific activity (C) found in microbiological stations along the Finisterre section. Samples were grouped by water masses. Error bars represent SE of 2-14 measurements.

Figure 4. Clustering of individual samples based on Bray Curtis similarity matrix obtained by ARISA fingerprinting of bacterial communities. Different symbols denote water masses; filled squares corresponding to EZ samples, triangles corresponding to ENACM-OMZ samples, circles corresponding to MW samples, crosses corresponding to LSW samples, plusses signs corresponding to ENADW samples and asterisks corresponding to LDW samples. For water mass abbreviations, see Table 2. Different line-boxes delineate different clusters. Different lines and letters denote groups.

Figure 5. (A) Principal component analysis performed on the biotic and abiotic environmental data. (B) Distance-based redundancy analysis (dbRDA) ordination for the fitted model of bacterial community structure based on Bray-Curtis similarity of ARISA presence/absence dataset overlaid with the partial correlations of the tested biotic and abiotic environmental variables explaining the cluster of samples. For water mass abbreviations see Table 2.

TABLES

Table 1. Probe and the hybridization conditions used in the (CARD-) FISH

Probe	Organism	Sequence (5' – 3')	% FA	Reference
Eub 338	Bacteria	GCT GCC TCC CGT AGG AGT	55	Amann et al., 1990
Eub 338 II	Bacteria	GCA GCC ACC CGT AGG TGT	55	Daims et al., 1999
Eub 338 III	Bacteria	GCT GCC ACC CGT AGG TGT	55	Daims et al., 1999
NON 338	Negative control	ACT CCT ACG GGA GGC AGC	55	Wallner et al., 1993
SAR 11 -152	SAR 11	ATT AGC ACA AGT TTC CYC GTGT	45	Morris et al., 2002
SAR 11 -441	SAR 11	TAC AGT CAT TTT CTT CCC CGAC	45	Morris et al., 2002
SAR 11 -542	SAR 11	TCC GAA CTA CGC TAG GTC	45	Morris et al., 2002
SAR 11 -732	SAR 11	GTC AGT AAT GAT CCA GAA AGYTG	45	Morris et al., 2002
SAR 202 -104	SAR 202	GTT ACT CAG CCG TCT GCC	35	Morris et al., 2004
SAR 202 -312	SAR 202	TGT CTC AGT CCC CCT CTG	35	Morris et al., 2004
SAR 324 -1412	SAR 324	GCC CCT GTC AAC CTC CAT	35	Schattenhofer et al., 2009
SAR 406 -97	SAR 406	CAC CCG TTC GCC AGT TTA	65	Fuchs et al., 2005
Alt 1413	<i>Alteromonas</i> , <i>Colwellia</i>	TTT GCA TCC CAC TCC CAT	55	Eilers et al., 2000

% FA, percent formamide

Table 2. Physical and chemical characteristics of the water masses sampled along the Finisterre section (43 ° N 9.3 ° W to 43 ° N 14.9 W). The maximum and minimum values are showed. LDW, Lower Deep Water; ENADW, Eastern North Atlantic Deep Water; LSW, Labrador Sea Water; MW, Mediterranean Water; ENACW-OMZ, Eastern North Atlantic Central Water- Oxygen Minimum Zone; Temp, temperature; Sal, salinity; Oxy, oxygen; NO3, nitrate; PO4, phosphate; SiO3, silicate.

Water mass	Depth (m)	Temp. (°C)	Sal.	Oxy ($\mu\text{mol kg}^{-1}$)	NO3 ($\mu\text{mol kg}^{-1}$)	PO4 ($\mu\text{mol kg}^{-1}$)	SiO3 ($\mu\text{mol kg}^{-1}$)
EZ	≤ 100	12.8-19.9	35.7-35.9	189.8-269.2	0.1-8.7	0.1-0.7	0.2-4.4
ENACW-OMZ	250-900	10.1-12.8	35.6-36.1	180.5-241.8	7.3-18.2	0.5-1.0	2.1-7.9
MW	1000	3.6-11.4	35.0-36.2	180.6-261.7	13.0-19.2	0.7-1.3	6.6-12.7
LSW	1800-2000	3.6-6.3	35.0-35.4	197.5-262.8	15.5-19.5	1.0-1.3	10.8-16.6
ENADW	2450-2900	2.5-3.5	34.9-35.0	235.1-253.4	14.6-23.0	1.0-1.5	10.6-34.1
LDW	≥ 4000	2.5	34.9	232.0-236.5	18.3-23.2	1.4-1.6	32.8-44.9

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Table 3. Average $\pm$ SD (mean $\pm$ standard deviation) of the contribution of Bacteria to the total prokaryotic community (% of DAPI counts) and specific Bacterial groups to the total bacterial abundance (% of Eubacteria counts) determined by (CARD)-FISH in the different water masses of the Finisterre section. (EZ, n = 8; ENACW-OMZ, n=8 MW, LSW and ENADW,n=4; LDW, n = 2).

Water mass	Eubacteria	SAR 11	SAR324	SAR406	<i>Alteromonas</i>	SAR 202
	(% DAPI counts)					
EZ	65.0 $\pm$ 10.2	31.8 $\pm$ 5.6	12.3 $\pm$ 4.4	12.6 $\pm$ 2.9	31.4 $\pm$ 3.1	7.4 $\pm$ 4.0
ENACW-OMZ	54.6 $\pm$ 4.3	27.2 $\pm$ 9.2	14.7 $\pm$ 4.1	13.5 $\pm$ 3.2	28.7 $\pm$ 5.0	13.3 $\pm$ 5.6
MW	50.1 $\pm$ 2.1	20.5 $\pm$ 5.6	16.1 $\pm$ 2.0	14.3 $\pm$ 3.4	21.5 $\pm$ 6.8	26.0 $\pm$ 5.5
LSW	45.6 $\pm$ 1.8	12.3 $\pm$ 2.1	22.3 $\pm$ 7.2	16.1 $\pm$ 2.7	13.3 $\pm$ 2.2	33.7 $\pm$ 7.6
ENADW	42.1 $\pm$ 5.8	12.0 $\pm$ 5.2	19.2 $\pm$ 8.0	17.0 $\pm$ 4.4	13.8 $\pm$ 1.7	36.5 $\pm$ 4.1
LDW	49.1 $\pm$ 8.0	7.7 $\pm$ 1.4	23.4 $\pm$ 0.3	15.0 $\pm$ 5.6	12.8 $\pm$ 4.5	40.5 $\pm$ 12.3

* For water mass abbreviations see Table 2

Table 4. Results of DistLM model obtained with “all specified” variables as selection procedure and R² as selection criterion, showing the marginal tests on each variable, with the significance (*p*-value), the proportion of variability explained for each variable (Prop) alone, and the total variation explained for each set of variables (% Var).

Set	Variable	Marginal test		
		p - valor	Prop	% Var
Physico-chemical	Temp	0.001	0.2934	50.4
Physico-chemical	Sal	0.001	0.1560	
Physico-chemical	Oxy	0.001	0.1257	
Physico-chemical	NO ₃	0.001	0.3196	
Physico-chemical	SiO ₃	0.001	0.1868	
Physico-chemical	PO ₄	0.001	0.3020	
Organic matter	FDOM-M	0.001	0.2781	38.1
Organic matter	FDOM-T	0.001	0.1701	
Organic matter	aCDOM 254	0.001	0.0889	
Organic matter	aCDOM 340	0.007	0.0416	
Organic matter	aCDOM 365	0.020	0.0364	
Organic matter	sCDOM 275/295	0.001	0.0718	
Organic matter	DOC	0.001	0.0230	28.3
Microbiological	PA	0.001	0.2635	
Microbiological	Leu incorp.	0.001	0.1933	

Table 5. Results of DistLM model obtained with the best explanatory environmental variables on the basis of AIC as selection criterion, showing the sequential tests, with the significance (*p*-value), the proportion of variability explained for each variable (Prop) and the cumulative percentage variance (Cumul).

Variable	Sequential test			
	Pseudo-F	p-value	Prop.	Cumul.
NO3	33.833	0.001	0.3196	0.3196
SiO3	12.423	0.001	0.1013	0.4209
Temp	5.0704	0.001	0.0802	0.5011
PA	3.3167	0.001	0.0513	0.5524
Sal	2.6607	0.008	0.0502	0.6026
FDOM-T	2.2483	0.047	0.0489	0.6515
Leu incorp.	2.1611	0.012	0.0209	0.6724
aCDOM 365	2.0241	0.020	0.0208	0.6932
sCDOM275/295	1.3583	0.024	0.0102	0.7034

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5