Estuarine, Coastal and Shelf Science 154 (2015) 102–110

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss

Factors shaping bacterial phylogenetic and functional diversity in coastal waters of the NW Mediterranean Sea

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ARTICLE INFO

Article history:
Received 13 April 2014
Accepted 29 December 2014
Available online 6 January 2015

Keywords:
bacterioplankton
virus
protozoa
diversity
enzyme
biolog

ABSTRACT

To evaluate the main factors shaping bacterioplankton phylogenetic and functional diversity in marine coastal waters, we carried out a two-year study based on a monthly sampling in Blanes Bay (NW Mediterranean). We expected the key factors driving bacterial diversity to be (1) temperature and nutrient concentration, together with chlorophyll *a* concentration as an indicator of phytoplankton biomass and, hence, a carbon source for bacteria (here called bottom-up factors), and (2) top-down pressure (virus- and protist-mediated mortality of bacteria). Phylogenetic diversity was analyzed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA. Functional diversity was assessed by using monomeric carbon sources in Biolog EcoPlates and by determining the activity of six extracellular enzymes. Our results indicate that the bacterial phylogenetic and functional diversity in this coastal system is shaped mainly by bottom-up factors. A dendrogram analysis of the DGGE banding patterns revealed three main sample clusters. Two clusters differed significantly in temperature, nitrate and chlorophyll *a* concentration, and the third was characterized by the highest losses of bacterial production due to viral lysis detected over the whole study period. Protistan grazing had no effect on bacterial functional diversity, since there were no correlations between protist-mediated mortality (PMM) and extracellular enzyme activities, and utilization of only two out of the 31 carbon sources (N-acetyl-D-glucosamine and α-cyclodextrin) was correlated with PMM. In contrast, virus-mediated mortality correlated with changes in the percentage of use of four carbon sources, and also with specific leu-aminopeptidase and β-glucosidase activity. This suggests that viral lysate provides a pool of labile carbon sources, presumably including amino acids and glucose, which may inhibit proteolytic and glucosidic activity. Our results indicate that bottom-up factors play a more important role than top-down factors (i.e. viral lysis and protistan grazing) in shaping bacterial community structure and activity. Furthermore, they suggest that viruses play a more important role than protists in modifying community structure and functional diversity of bacteria in oligotrophic marine coastal waters.

1. Introduction

Prokaryotes constitute half of the total amount of carbon enclosed in living organisms, and represent the largest pool of nitrogen and phosphorous in the biosphere (Whitman et al., 1998). In aquatic environments, heterotrophic bacteria are an essential part of the food webs, and are the main consumers of dissolved organic matter (DOM) in the ocean (Azam, 1998). The carbon and nutrients contained in bacterial cells can move up along the trophic web through grazing by protists, or return as DOM and particulate organic matter (POM) pools to the water through viral infections and subsequent cell lysis. Thus, viral activity provides an additional input of organic matter and nutrients to ocean waters which, together with the DOM produced by other members of the marine biota, could be available for bacterioplankton (Weitz and Wilhelm, 2012). Although the process of recycling from bacterial cells to the environment can be quantitatively important, little is known of the composition of organic matter released during grazing by protists

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http://dx.doi.org/10.1016/j.ecss.2014.12.039
0272-7714/

or viral lysis (Weinbauer et al., 2011). For protists, Gruber et al. (2006) found little changes in DOM composition during grazing by ciliates in culture. For viruses, several findings, obtained mainly from virus-host systems, suggest that viral lysis changes the composition of DOM: 1) lysis products have been found to be labile and turn over rapidly (Noble and Fuhrman, 1999), 2) lysis products may be an important source of P for marine bacterioplankton (Weinbauer et al., 1995; Riemann et al., 2009) and 3) DOM released during lysis could be mainly composed of dissolved combined amino acids (51%–86%; Middelboe and Jørgensen, 2006).

DOM in aquatic systems is composed mostly of polymeric compounds and therefore has to undergo hydrolysis before bacterial uptake. Hydrolysis is carried out by specific extracellular enzymes mainly synthetized by bacteria, and their activity is an indirect indication of the polymeric molecules available in the environment (Hoppe, 1983). Monomeric DOM is a preferable carbon source for bacteria, since it can be easily taken up and does not require the synthesis of specific extracellular enzymes. The pattern of utilization of sole carbon sources in Biolog plates (mainly monomers) is characteristic for each community, and has been used to show differences in functional diversity of bacterioplankton in contrasting marine environments (Sala et al., 2005a, 2010), among seasons (Sala et al., 2006), and in depth and temporal patterns (Sala et al., 2008). Products of viral lysis provide an additional input of different types of monomers and polymers to the environment. Preferential use of these monomers might suppress the activity of specific bacterial extracellular enzymes and modify the pattern of sole carbon source utilization by the microbial community.

The structure of natural bacterial assemblages can be shaped by a variety of parameters and processes that can be grouped into environmental (bottom-up) factors and predator pressure (top-down) factors. Several studies have shown temporal changes in the dominance of particular bacterial groups in coastal waters (Pinhassi and Hagström, 2000; Ghiglione et al., 2005) and oceanic waters (Morris et al., 2005). These and other studies (Schauer et al., 2003; Alonso-Sáez et al., 2007) suggest that temperature or substrate availability can shape bacterial diversity, and it has been demonstrated that an addition of specific substrates could induce a succession of bacterial species (Pinhassi et al., 1999). Also, changes in the composition of the phytoplanktonic community, e.g. blooms, can influence the shape of the bacterial community (Pinhassi et al., 2004; Ghiglione et al., 2005). Furthermore, activity of predators, protists and phages can also produce changes in bacterial diversity. Grazing by protists has been shown to impact the taxonomic structure of bacterial communities directly (Šimek et al., 1997), for example by selective grazing (Hahn and Höfle, 1999), and indirectly by providing the substrates for bacterial growth (Caron et al., 1988) or by elimination of competitive strains. Bacteriophages can modify bacterial diversity in a variety of ways, e.g. by lysogenic conversion, transduction, resistance induction, or by release of the lysis products to the environment (Weinbauer and Rassoulzadegan, 2004). It has also been hypothesized that through killing bacteria that win the competition for resources, viruses increase or maintain bacterial diversity in the environment (the 'killing the winner' hypothesis; Thingstad and Lignell, 1997; Thingstad, 2000). However, other studies have shown that viruses cause a reduction in the number of bacterial phylotypes (Schwalbach et al., 2004). Microcosm and field studies have shown a great variability of responses of the bacterial assemblage to the presence of viruses (Hewson and Fuhrman, 2006; Bouvier and del Giorgio, 2007), probably partly due to the different impact of viral lysis on different groups of the same microbial community (Winter et al., 2004). This finding shows that the effect of viruses on bacterioplankton diversity is a complex process that may depend on factors such as the presence of binding sites (porins) on the bacterial cell wall (Lenski, 1988), resistance to viral infection (Weinbauer et al., 2007) and the quality of available DOM (Hewson and Fuhrman, 2006).

The work described herein is part of a two-year study performed in the Microbial Marine Observatory of Blanes Bay, NW Mediterranean. The first part of the study focused on evaluating the bacterial mortality caused by viruses and protists and the second part, presented in this paper, on identifying the main factors shaping phylogenetic and functional bacterial diversity. The evaluation of bacterial mortality was broadly described in Boras et al. (2009). Briefly, viruses and grazers together were responsible for an average of 60% of the loss of bacterial production (BP) and viruses were considered a significant source of mortality in Blanes Bay since they removed an annual average of 12%–32% of BP day^{-1}. Based on these results, the second part of the study aims to identify the main factors shaping phylogenetic bacterial diversity and regulating bacterial utilization of monomeric and polymeric carbon sources in this oligotrophic coastal marine environment. These factors were grouped into bottom-up factors (those that derive from supply resources or physical factors: temperature, nutrient concentration, Chl *a* and Chl *a* <3 μm); and top-down factors (those that derive from predator biomass and activity: viral and protist abundances and their bacteria-mediated mortality).

The main aims of the present study were (1) to monitor changes in bacterial community structure during the year, (2) to detect the main factors determining bacterial phylogenetic and functional diversity, the latter based on extracellular enzyme activity and utilization of sole carbon sources, and (3) to evaluate the role of viruses and protists in shaping bacterial phylogenetic and functional diversity. We expected to find (1) changes in bacterial phylogenetic diversity caused by viral lysis and/or protistan grazing, (2) lower specific extracellular enzyme activities with higher viral mortality due to the release of DOM, including monomers during lysis, and (3) higher variability in the utilization of monomeric carbon sources with higher virus-mediated mortality than with protist-mediated mortality since viral lysis releases higher amounts of bacterial DOM.

2. Material and methods

2.1. Study site and sampling strategy

Surface water samples (0.5 m depth) were collected from May 2005 to April 2007 in Blanes Bay, Spain (the Blanes Bay Microbial Observatory, NW Mediterranean, 41°40′N, 2°48′E, 20 m depth), where microbial communities have been investigated for over a decade (Gasol et al., 2012). Samples were collected in 10-L polyethylene carboys once a month, 0.5 miles off the shore, and kept in the dark until they reached the lab (~2 h). Water temperature and salinity were measured in situ with a conductivity, temperature and depth (CTD) profiler.

2.2. Physicochemical and biological parameters

A detailed description of the determination of physicochemical and biological parameters is presented in Boras et al. (2009). Briefly, concentrations of inorganic nutrients (PO_4^{-3} and NO_3), chlorophyll *a* (Chl *a*), and the Chl *a* fraction smaller than 3 μm (Chl *a* <3 μm) were determined using standard methods (Grasshoff et al., 1983 for inorganic nutrients; Yentsch and Menzel, 1963 for Chl *a*). Viral abundances were determined by flow cytometry as described in Brussaard (2004). Bacterial and heterotrophic nanoflagellate (HNF) abundances were obtained by epifluorescence microscopy (Olympus BX40) after staining with DAPI (Porter and Feig, 1980;

Sieracki et al., 1985). HNFs were grouped into four size classes: ≤ 2 μm, 2–5 μm, 5–10 μm and 10–20 μm.

Losses of BP due to protists (protist-mediated mortality PMM) were evaluated following the fluorescent labeled bacteria (FLB, Spanish Type Culture Collection, http://www.cect.org/index2.html) disappearance method (Sherr et al., 1987; Vázquez-Domínguez et al., 1999), and losses due to viruses (virus-mediated mortality [VMM]) were evaluated by the virus reduction approach (Weinbauer et al., 2002; Wilhelm et al., 2002). A detailed description of the mortality experiments and results can be found in Boras et al. (2009).

2.3. DGGE and phylogenetic analysis

2.3.1. Nucleic acid extraction

DGGE of polymerase chain reaction (PCR)–amplified 16S rRNA gene fragments was used to compare bacterial communities from each sampling. To collect microbial biomass, 20 L of seawater was filtered immediately after arriving at the lab through a 3.0-μm pore size filter (Millipore) to eliminate larger organisms, and through a 0.2-μm Sterivex filter (Durapore, Millipore) to collect bacterial biomass. The biomass retained in the Sterivex filter was then processed in order to assess bacterial diversity. The Sterivex units were filled with 1.8 ml of lysis buffer (40 mM EDTA, 50 mM Tris–HCl pH = 8.3, 0.75 M sucrose) and stored at a temperature of −80 °C. The nucleic acids were treated with lysozyme, proteinase K and sodium dodecyl sulfate, extracted with phenol and concentrated in a Centricon-100 (Millipore) (Massana et al. 1997). The quality of the DNA was checked by agarose gel electrophoresis.

2.3.2. DGGE gel analysis

The DGGE was carried out as described in Schauer et al. (2003). The PCR amplification of bacterial 16S rRNA was done using the bacterial specific primer 358f and the universal primer 907rM. The PCR was performed with a thermal cycler using the following program: initial denaturation at 94 °C for 5 min, 10 touchdown cycles of denaturation at 94 °C for 1 min, annealing at 65 °C–55 °C for 1 min (decreasing 1 °C each cycle), and extension at 72 °C for 3 min and 20 standard cycles under the same conditions (annealing at 55 °C) and a final extension at 72 °C for 5 min. The PCR products were verified by agarose gel electrophoresis with a standard in the gel (Low DNA Mas Ladder, Gibco BRL).

To perform the DGGE, 800 ng of PCR product for each sample was loaded onto the 6% polyacrylamide gels with a DNA-denaturant gradient ranging from 40% to 80%. The 24 samples were run in three DGGE gels. Identical samples were run on the outer bands of consecutive gels as standards to allow comparison between them (Sep-05 and Sep'-05 for gels I and II, and Jun'-06 and Jun'-06 for gels II and III). The DGGE gels were run at 100 V for 16 h at 60 °C in 1× TAE running buffer using the DGGE-2000 system (CBS Scientific Company), as previously described for the Blanes samples (Alonso-Sáez et al., 2007). The gels were stained with 3 μl of SYBR Gold in 20 ml of TAE 1× and analyzed using the Fluor-S MultiImager (Bio-Rad) with the Multi-Analyst software (Bio-Rad).

Digitized DGGE images were analyzed using the Diversity Data software (Bio-Rad) described previously (Schauer et al. 2000; Díez et al. 2001). A matrix was constructed taking into account the relative contribution of each band (as a percentage) to the total intensity of the band line and the number of bands detected in each sample. A ranked matrix of similarities among samples was constructed using the Bray–Curtis similarity measure (Bray and Curtis, 1957). Based on this matrix a hierarchical clustering was performed, resulting in a dendrogram.

2.4. Functional diversity

Functional diversity was assessed by determining the utilization of carbon sources in Biolog EcoPlates and by the activity of a set of selected extracellular enzymes.

2.4.1. Biolog EcoPlates

Microplates were used to assess the differences in the functional diversity of the bacterioplankton assemblage (Preston-Mafham et al., 2002). Each of the 96 wells of the microplate contained a carbon source (31 carbon sources, in triplicates and 3 blanks). Together with the carbon source, tetrazolium violet is included in each well to indicate substrate catabolism. Each sample provides a unique pattern of utilization of carbon sources. After inoculation of 150 μl of sample in each well, samples were incubated in the dark for 6 days and then kept at −20 °C until the measurement of the absorbance at 590 nm wavelengths in a spectrophotometric microplate reader (ELX800 BIOTEK Instruments, Inc. Winooski, Vermont, USA). The mean color development of the three replicate wells for each substrate was calculated and the mean absorbance of the blanks (with only water) was subtracted. Absorbance of each substrate was standardized to total absorbance of the plate to avoid the effect of the inocula size. Further details can be found in Sala et al. (2005b).

2.4.2. Extracellular enzyme activities

We determined the activity of six extracellular enzymes (α-glucosidase, β-glucosidase, xylosidase, esterase, alkaline phosphatase and leu-aminopeptidase) using fluorogenic substrates (Hoppe, 1983) (methylumbelliferyl-4-α-glucosidase, methylumbelliferyl-4-β-glucosidase, methylumbelliferyl-4-β-xylosidase, methylumbelliferyl-4-butyrate, methylumbelliferyl-4-phosphate, and leucine 7-amido-4 methylcoumarin, respectively). We followed the methodology of Sala et al. (2001). Briefly, substrates were added at a final concentration of 100 μM to 0.9 ml of sample in duplicates. Fluorescence of the sample was read immediately after addition of the substrate and after an incubation time of between 15 and 120 min, depending on the substrates, on an RF-540 Shimadzu spectrofluorometer at 365 nm excitation and 446 nm emission wavelengths. The increase of fluorescence units during the incubation was transformed to activity with a standard curve of the end product of the reactions. Specific activities were calculated by dividing activities by bacterial cell counts. For statistical analysis, activities of each enzyme were standardized: first, we calculated the percentage of the highest activity of each enzyme during the cycle, and then we calculated the percentage of the sum of specific activities of the six enzymes on a sampling day.

2.5. Statistical analysis

For the purpose of statistical analysis, the monthly samples were grouped by seasons: spring (April, May, June); summer (July, August, September); fall (October, November, December); and winter (January, February, March).

The normality of data was checked using the Shapiro–Wilk W test, and data were logarithmic transformed when necessary. Mortality rates (VMM and PMM) were arcsine square root transformed to achieve normality of distributions. Differences in values of parameters among clusters were tested using one-way ANOVA in the case of normally distributed parameters, and Mann–Whitney U-test for non-normally distributed parameters. The Pearson correlation coefficient was used to establish the relationships between pairs of parameters. These statistical analyses were performed using the JMP program. Samples similarity matrices and a dendrogram were constructed and analyzed using the PRIMER v.6 program

(Plymouth Marine Laboratory). Similarity between pairs of data matrices (phylogenetic, based on Biolog, enzymatic, biotic and abiotic data) was checked by a Mantel-type test (PRIMER v.6).

Canonical correlation analysis (CCA) was performed to evaluate multivariate patterns in the data, using the XLSTAT-ADA software. CCA is a multivariate statistical technique for examining the relationships of a matrix of biological or response variables to their environment. The method is based on obtaining environmental gradients ('main axes' in the graphical representation) from a set of environmental factors or predictors in the data matrix and relating these gradients to biological variables. Bottom-up factors, i.e. temperature, nutrient concentrations, Chl *a* and the Chl *a* <3 μm fraction, were used to build the predictor (bottom-up) matrix, and bacterial apparent richness, diversity (as Shannon–Wiener index), and specific extracellular enzyme activities (activity per cell) were used to build the response (diversity) matrix. The two-dimensional graphical representation (ordination diagram) of the CCA results allows us to visually correlate the environmental variables (represented as arrows) contributing to the main axes with the centroids of biological variables (represented as filled triangles) in an intuitive way: the influence of each environmental variable on a diversity variable is related to the length of the arrow and the proximity of the arrow tip to the centroid. The permutation test based on the F-statistic (Legendre and Legendre, 1998) was used to assess the significance of the environmental matrix in explaining the patterns observed in the diversity matrix, and the diversity matrix was considered correlated to the environmental matrix when $p < 0.05$. A second CCA consisted in using the top-down factors: abundances of viruses and HNF, as well as VMM and PMM as predictor (top-down) matrix, vs. the same response (diversity) matrix as above.

3. Results

3.1. Physicochemical and biological parameters

Water temperature followed a seasonal trend, with the highest temperatures in summer (25.0 ± 1.0 °C), and the lowest in winter (13.4 ± 1.2 °C; Table 1). Among inorganic nutrients, only nitrate concentration showed significant seasonal changes, with the highest values in winter (1.49 ± 0.75 μM), and the lowest in summer (0.29 ± 0.17 μM; ANOVA, $F_{3,19} = 4.4$, $p = 0.02$; Table 1). Concentrations of Chl a and Chl a <3 μm followed the same trend as nitrate concentration (ANOVA, $F_{3,20} = 5.9$, $p = 0.005$ and $F_{3,20} = 3.2$, $p = 0.04$, respectively; Table 1). Abundance of viruses reached significantly higher values in spring (3.3 ± 1.5 × 107 viruses ml^{-1}) than in the rest of the year (ANOVA, $F_{3,20} = 5.3$, $p = 0.003$; Table 1).

Among HNF, the 2–5 μm size fraction, which contains most of the bacterivorous HNFs, reached the highest abundances in summer (7.4 ± 4.3 × 10^2 cells ml^{-1}) and the lowest in autumn (1.8 ± 1.4 × 10^2 cells ml^{-1}). VMM and PMM of bacteria, expressed as the removal of BP per day, reached average values of 12.3 ± 12.3% d^{-1} and 33.9 ± 24.3% d^{-1}, respectively, in the first year of study, and 40.9 ± 20.7% d^{-1} and 32.4 ± 20.0% d^{-1}, respectively, in the second year. The values of VMM and PMM are summarized in Table 2, and are presented in detail in Boras et al. (2009).

3.2. Bacterial community composition

The analysis of the DGGE gel yielded a total of 58 band positions (operational taxonomic units, OTUs) detected in 24 samples. The number of OTUs ranged between 8 (Jun 05 and Jul 05) and 27 (Dec 06) per sample. Twelve of the 58 detected bacterial taxa (21% of total OTUs) were restricted to single samples (8 samples). Only one OTU (2% of the bacterial phylotypes) was observed in all samples during the two years. Samples analyzed in the same DGGE gel did not show a particularly high similarity to each other. Samples used as standards of comparability of gels showed a similarity of 89.5% (Sep-05 and Sep-05′) and 100% (Jun'-06 and Jun'-06), taking into account the number of detected bands.

The number of bands per sample was significantly different between seasons (ANOVA, $F_{3,20} = 9.0$, $p < 0.01$), increasing from spring to winter from 11 to 20 bands. Also, significant differences were detected between the spring-summer and autumn-winter periods (ANOVA, $F_{1,22} = 16.9$, $p < 0.01$), with a higher number of bands in the second period (20 ± 4 bands) than in the first one (13 ± 4 bands). No significant differences in the number of OTUs or relationships with any top-down factor were found between the two years.

Hierarchical clustering of samples (Fig. 1) based on the DGGE gel bands revealed two main clusters, which separated the samples

Table 1
Average seasonal values of selected physicochemical and biological parameters, as well as extracellular enzyme activities determined during a two-year study in Blanes Bay, NW Mediterranean ($n = 24$; SD, standard deviation). Chl *a*, chlorophyll *a*; HNF, heterotrophic nanoflagellates; VMM, virus-mediated mortality, as losses of bacterial production; PMM, protist-mediated mortality, as losses of bacterial production; OTUs, operational taxonomic units. Statistical significance of differences between the seasons is presented as a *p*-value. As a p value and significant values ($p < 0.05$) are highlighted in bold.

Parameter	Average value ± SD				*p*-value
	Spring	Summer	Autumn	Winter	
Bacteria (× 10^5 cells ml^{-1})	10.6 ± 3.7	9.0 ± 3.3	7.5 ± 1.5	6.4 ± 1.6	0.0798
Bottom-up parameters					
Water temperature (°C)	17.7 ± 3.2	25.0 ± 1.0	18.0 ± 2.6	13.4 ± 1.2	**<0.0001**
NO_3^- (μM)	1.08 ± 1.23	0.29 ± 0.17	1.18 ± 1.01	1.49 ± 0.75	**0.0158**
Chl *a* total (μg l^{-1})	0.97 ± 0.79	0.23 ± 0.12	0.59 ± 0.11	0.77 ± 0.38	**0.0054**
Chl *a* <3 μm fraction (μg l^{-1})	0.13 ± 0.07	0.11 ± 0.09	0.23 ± 0.07	0.30 ± 0.15	**0.0440**
Top-down parameters					
Viruses (× 10^7 viruses ml^{-1})	3.3 ± 1.5	1.5 ± 0.4	1.6 ± 0.6	1.7 ± 0.8	**0.0031**
HNF (× 10^2 cells ml^{-1})	10.10 ± 8.3	10.50 ± 5.0	4.1 ± 2.8	6.4 ± 2.4	0.0623
VMM (% day^{-1})	23.5 ± 16.4	21.8 ± 14.7	36.7 ± 32.8	24.3 ± 23.2	0.9104
PMM (% day^{-1})	29.2 ± 27.3	44.6 ± 26.9	35.9 ± 10.9	22.9 ± 16.9	0.6409
Response parameters					
OTUs	11 ± 2	15 ± 4	19 ± 5	20 ± 3	**0.0006**
α-glucosidase (nmol l^{-1} h^{-1})	10.2 ± 9.6	10.8 ± 18.1	5.8 ± 12.3	3.7 ± 5.7	0.2297
β-glucosidase (nmol l^{-1} h^{-1})	29.4 ± 24.3	5.9 ± 4.1	6.8 ± 6.4	6.6 ± 2.3	**0.0193**
β-xylosidase (nmol l^{-1} h^{-1})	16.9 ± 8.5	24.1 ± 21.5	34.6 ± 57.2	35.6 ± 54.7	0.9619
Esterase (nmol l^{-1} h^{-1})	3186 ± 1360	2872 ± 1368	1978 ± 628	3404 ± 4653	0.1628
Alkaline phosphatase (nmol l^{-1} h^{-1})	263.7 ± 202.0	194.7 ± 106.1	229.3 ± 268.4	153.4 ± 152.8	0.3659
Leu-aminopeptidase (nmol l^{-1} h^{-1})	1234 ± 907	283 ± 220	258 ± 156	261 ± 193	**0.0136**

Table 2
Virus-mediated mortality (VMM) and protist-mediated mortality (PMM) of bacteria expressed as a % of the bacterial production (BP) detected during the monthly sampling in the two-year study in Blanes Bay, Mediterranean. nd, not detected.

Sampling month	VMM (%)	PMM (%)
May 2005	47.74	9.62
June 2005	5.92	34.09
July 2005	15.75	63.81
August 2005	12.19	nd
September 2005	16.59	77.64
October 2005	5.91	49.61
November 2005	4.01	36.4
December 2005	16.94	46.79
January 2006	3.77	9.65
February 2006	3.12	38.45
March 2006	9.12	3.9
April 2006	5.97	36.5
May 2006	22.96	77.04
June 2006	35.18	18.09
July 2006	27.45	43.76
August 2006	48.96	33.07
September 2006	9.58	49.22
October 2006	48.31	27.7
November 2006	84.07	34.02
December 2006	60.82	21.02
January 2007	59.51	35.38
February 2007	42.3	40.56
March 2007	28.23	9.36
April 2007	23.14	nd

collected in months with low water temperature (November to March, 'Group I' cluster) from samples collected in months with high water temperature (April to October, 'Group II' cluster; Table 3) at a 35% similarity level. Other bottom-up parameters—nitrate concentration and Chl *a* <3 μm—were significantly higher in Group I samples than in Group II samples (Table 3). In contrast, HNF abundance, one of the top-down parameters, was significantly lower in Group I samples than in Group II samples. Other top-down parameters did not differ significantly between the clusters (Table 3). One sample, from Nov 06, shared only 20% similarity with the rest of the samples and formed the third cluster. This sample showed significantly higher VMM than the other samples (ANOVA, $F_{1,22} = 9.7$, $p < 0.01$).

3.3. Functional diversity

The pattern of extracellular enzyme activities changed during the annual cycles. Mean seasonal values of β-glucosidase, alkaline phosphatase and leu-aminopeptidase activity were higher in spring than in the rest of the year (Table 1). Seasonal differences were significant for leu-aminopeptidase (ANOVA, $F_{3,\ 20} = 4.5$, $p < 0.05$) and β-glucosidase (ANOVA, $F_{3,\ 20} = 4.2$, $p < 0.05$). In contrast, β-xylosidase and esterase activity showed the highest mean values in autumn or winter. Among the 31 carbon sources in the Biolog-EcoPlate, none showed significant differences between seasons.

Comparison of extracellular enzyme activities between the 'Group I' and 'Group II' clusters yielded significant differences between them: α-glucosidase, alkaline phosphatase and aminopeptidase showed higher mean activity values in 'Group II' samples than in 'Group I' samples (Table 3). Also, significant differences between the two main clusters in substrate utilization in the Biolog plates for 11 carbon sources were observed. Four of them were higher in the 'Group II' than in the 'Group I' periods (L-arginine, L-serine, D-mannitol and D-malic acid), and seven in the 'Group I' than in the 'Group II' periods (D,L-α-glycerol phosphate, phenylethylamine, α-D-lactose, γ-hydroxybutyric acid, itaconic acid, 2-hydroxy benzoic acid, and tween 80).

Significant correlations were found between VMM and extracellular enzyme activities: β-glucosidase, leu-aminopeptidase and β-xylosidase (Table 4). In contrast, none of the enzymatic activities was significantly correlated with PMM. Out of the 31 substrates in the Biolog EcoPlates, four showed correlations with VMM (glucose-1-phosphate, L-asparagine, I-erythritol and D-mannitol) and only two with PMM (N-acetyl-D-glucosamine and α-cyclodextrin) (Table 4).

3.4. Relation between parameters

The Mantel test showed a significant positive relationship between phylogenetic data (based on the DGGE) and functional data (based on Biolog) ($r = 0.345$, $p < 0.01$).

The CCA analysis showed a correlation between bottom-up data and bacterial apparent richness (S), diversity (H′) and extracellular

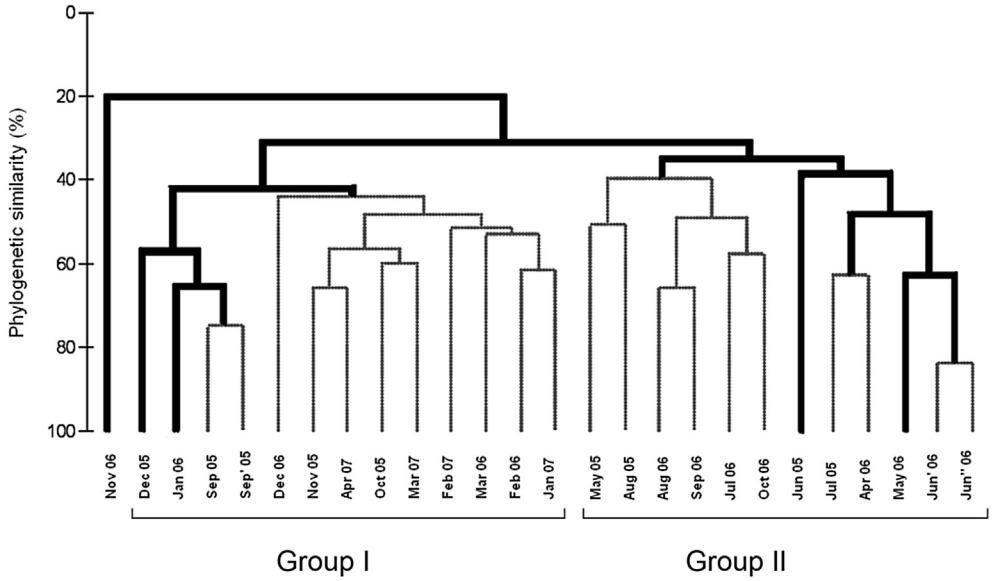

Fig. 1. Dendrogram for hierarchical clustering of 24 samples from Blanes Bay, NW Mediterranean, using group-average linking of Bray–Curtis similarities. Samples are grouped into three main clusters: the 'Group I' cluster, the 'Group II' cluster, and the sample from November 06. The bold line means the significantly different branches at the 5% significance level.

Table 3
Differences in selected parameters between two main clusters of samples obtained by hierarchical clustering of DGGE results, assessed by ANOVA analysis. Chl *a*, chlorophyll *a*; HNF, heterotrophic nanoflagellates; VMM, virus-mediated mortality, as losses of bacterial production; PMM, protist-mediated mortality, as losses of bacterial production; OTUs, operational taxonomic units. Statistical significance of differences between the seasons is presented as a *p*-value. As a p value and significant values ($p < 0.05$) are highlighted in bold.

Parameter	Mean value for Group I (range)	Mean value for Group II (range)	Differences between "Group I" and "Group II" clusters
			p
Bacteria ($\times 10^5$ cells ml^{-1})	7.09 (4.70–12.60)	9.79 (4.63–15.60)	**<0.05**
Bottom-up parameters			
Water temperature (°C)	16.0 (12.0–26.0)	21.2 (13.5–26.0)	**<0.01**
NO_3^- (μM)	1.30 (0.27–2.48)	0.64 (0.14–3.23)	**<0.01**
Chl *a* (μg l^{-1})	0.66 (0.27–1.46)	0.63 (0.02–2.45)	0.09
Chl *a* <3 μm fraction (μg l^{-1})	0.25 (0.05–0.52)	0.12 (0.01–0.23)	**<0.05**
Top-down parameters			
Viruses ($\times 10^7$ viruses ml^{-1})	1.71 (0.89–3.24)	2.43 (1.21–5.99)	0.06
HNF ($\times 10^2$ cells ml^{-1})	5.64 (1.10–12.60)	10.40 (2.71–24.80)	**<0.05**
VMM (% day^{-1})	22.79 (3.12–60.82)	25.46 (5.92–48.96)	0.36
PMM (% day^{-1})	30.73 (nd-77.64)	35.72 (nd-77.04)	0.52
Response parameters			
OTUs	19 (9–27)	14 (8–24)	**<0.01**
α-glucosidase (nmol l^{-1} h^{-1})	6.0 (0.0–30.8)	11.6 (0.0–47.3)	**<0.05**
Alkaline phosphatase (nmol l^{-1} h^{-1})	181 (28–712)	265 (76–648)	**<0.05**
Leu-aminopeptidase (nmol l^{-1} h^{-1})	263 (72–619)	779 (44–2734)	**<0.05**
D,L −α-glycerol phosphate (%)	0.97 (0.80–1.09)	0.89 (0.80–0.96)	**<0.05**
L-arginine (%)	0.79 (0.70–0.89)	0.77 (0.70–0.87)	**<0.05**
L-serine (%)	0.93 (0.86–1.06)	1.06 (0.86–1.38)	**<0.05**
Phenylethylamine (%)	1.06 (0.93–1.17)	0.95 (0.81–1.11)	**<0.05**
D-mannitol (%)	1.03 (0.95–1.17)	1.17 (0.92–1.62)	**<0.05**
α-D-lactose (%)	1.00 (0.93–1.12)	0.91 (0.78–1.12)	**<0.05**
γ-hydroxybutyric acid (%)	1.12 (1.02–1.21)	0.99 (0.84–1.20)	**<0.05**
Itaconic acid (%)	1.10 (0.87–1.29)	0.98 (0.79–1.15)	**<0.05**
D-malic acid (%)	1.03 (0.78–1.20)	1.12 (0.88–1.48)	**<0.05**
2-hydroxy benzoic acid (%)	1.13 (0.95–1.25)	1.01 (0.81–1.19)	**<0.05**
Tween 80 (%)	0.98 (0.85–1.13)	0.89 (0.80–1.00)	**<0.01**

Table 4
Pearson correlations observed between the virus-mediated mortality (VMM) and the protist-mediated mortality (PMM) of bacteria (expressed as % of bacterial production BP) and extracellular enzymatic activities and carbon source utilization by bacteria detected during the two-year study in Blanes Bay, Mediterranean. nd, not detected; r, correlation coefficient; n, number of samples; *p*, significance level.

Parameter	VMM (%)			PMM (%)		
	r	n	*p*	r	n	*p*
Extracellular enzyme activities						
β-glucosidase	−0.426	23	<0.05	nd		
Leu-aminopeptidase	−0.417	23	<0.05	nd		
β-xylosidase	0.569	23	<0.01	nd		
Carbon source utilization on Biolog plates						
Glucose-1-phosphate	−0.508	22	<0.02	nd		
L-asparagine	0.601	22	<0.01	nd		
I-erythritol	0.542	22	<0.01	nd		
D-mannitol	0.481	22	<0.05	nd		
N-acetyl-D-glucosamine	nd			0.492	22	<0.03
α-cyclodextrin	nd			−0.449	22	<0.05

enzyme activity ($p = 0.05$, Fig. 2A). The first ordination axis accounted for 45% of the variance in the biological matrix, while the second axis accounted for 31%. The first axis was mainly negatively related to a combination of Chl *a*, nitrate and phosphate, while the second axis was positively defined by Chl *a* <3 μm and negatively by temperature. The Chl *a* <3 μm fraction was the main factor shaping bacterial richness and phylogenetic diversity. Activities of leu-aminopeptidase and β-glucosidase were mainly determined by phosphate and nitrate concentrations, and α-glucosidase activity was strongly determined by temperature.

The top-down variables showed a less clear relationship with the evaluated parameters (Fig. 2B). The ordination was mainly determined by the first canonical axis, accounting for 70% of the variance in the biological data. This first axis was negatively related to VMM. The second axis was determined mainly by viral abundances, but explained a small proportion of the variance (17%). The main factor that determined bacterial richness, phylogenetic diversity, and xylosidase and leu-aminopeptidase activity was VMM (Fig. 2B). Virus abundance strongly determined the second ordination axis, but as it accounted for a low proportion of the variance explained, species richness, phylogenetic diversity and most enzymatic activities are probably not determined by virus abundance.

4. Discussion

This study is one of the few that have investigated the effect of both bottom-up and top-down factors controlling structural and functional diversity of bacterioplankton. In our study, bottom-up factors (temperature, nutrient concentration, Chl *a* and Chl *a* <3 μm) seemed to shape both phylogenetic and functional diversity, whereas top-down factors (viral abundance and mortality caused by bacteria and by viruses) had a minor effect.

4.1. Changes in community structure

The number of bands on the DGGE gels detected during this two-year study changed gradually over the year, with clear differences between the two DGGE clusters. According to Schauer et al., 2003), the main factors that affected bacterial diversity in Blanes Bay during the year were the changing DOM supply and the temperature. These authors also suggest that top-down factors do not impact the bacterial diversity in this particular system. Furthermore, Alonso-Sáez et al. (2007) found a succession between SAR11 and *Roseobacter* groups over seasons, which was apparently due to changing nutrient concentrations in the water. The results of our study suggest that the bottom-up factors Chl *a* <3 μm, water temperature and nutrient concentration had an effect on phylogenetic richness and diversity of bacterioplankton over the study period, as indicated by the low number of OTUs detected during months with low Chl *a* <3 μm, high temperatures and low nitrate concentrations. Autotrophic organisms, here presented as Chl *a* <3 μm and Chl *a*, are a large potential source of carbon for bacteria, as POM and DOM are released to the environment during sloppy feeding and lysis of these cells.

Previous studies on the factors driving microbial diversity (e.g. Schauer et al., 2003; Alonso-Sáez et al., 2007) did not evaluate the activity of predators, viruses and protists, which can also modify bacterial community structure. Other studies have shown that viral activity typically causes a reduction in the number of bacterial phylotypes detected (Schwalbach et al., 2004; Winter et al. 2004) and affects individual members of the same marine bacterial community differently (Winter et al., 2004). Rare bacterial groups may be more susceptible than abundant ones (Bouvier and del Giorgio, 2007). Like viruses, protists can also change the

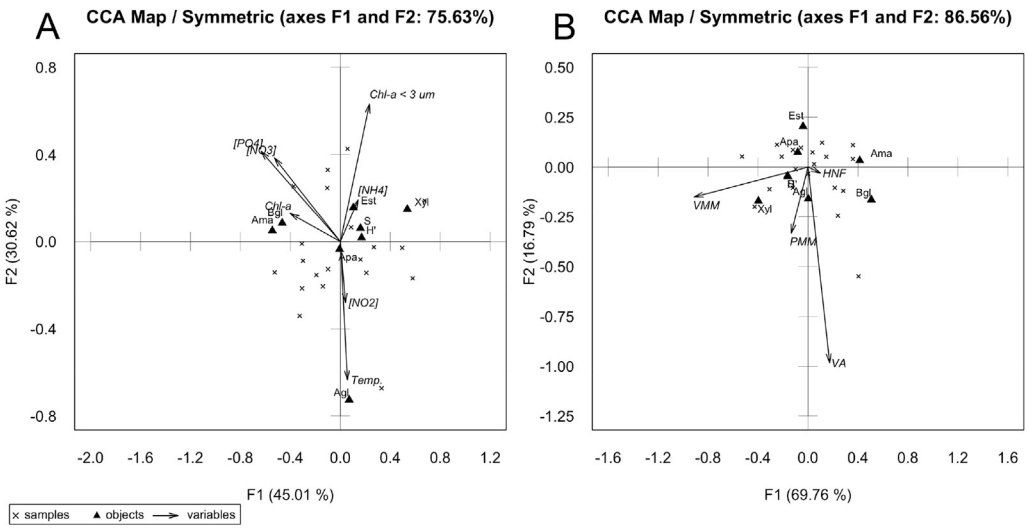

Fig. 2. Ordination diagram with the results of the canonical correspondence analysis (CCA) showing A) multivariate patterns among the bacterial diversity data (apparent richness S, diversity as Shannon–Wiener index H′, and specific extracellular enzyme activities, as activity per cell) and the bottom-up factors (temperature [Temp.]; phosphate, nitrate, nitrite, and ammonium concentrations; chlorophyll *a* [Chl *a*] and Chl *a* <3 μm concentrations); and B) top-down factors (virus- and protist-mediated losses of bacterial production [VMM and PMM, respectively]; abundance of viruses [VA], and of heterotrophic nanoflagellates [HNFs]). Extracellular enzymes: Est, esterase; Apa, alkaline phosphatase; Ama, leu-aminopeptidase; Xyl, xylosidase; Agl, α-glucosidase; Bgl, β-glucosidase. Bottom-up (A) or top-down (B) variables are shown as arrows; the centroids ("objects") of the diversity variables are shown as filled triangles. Crosses correspond to the centroid of the samples in the ordination diagrams.

taxonomic composition of bacterial communities, leading to the development of rare bacteria (Suzuki, 1999) or the replacement of strains (Hahn and Höfle, 1999). Finally, Zhang et al. (2007) found that viral lysis and protistan grazing could act additively, substantially increasing the apparent richness of the studied bacterial communities in eutrophic marine waters. In our study no clear relationship between top-down factors and phylogenetic and functional diversity of bacteria was found. We can, however, state some important conclusions. Using the CCA analysis we showed that the effect of viral activity on bacterial diversity is more pronounced than the effect of protistan grazing in the studied marine system. We also observed that viral lytic activity was a more important factor in shaping bacterial diversity than viral abundance alone. Finally, we showed that virus-induced mortality might occasionally be important for determining phylogenetic and functional bacterial diversity. This was clearly seen in the November '06 sample that formed the third cluster on the DGGE dendrogram, with a completely different pattern of bands to the other samples. In this month the only factor that was significantly different from the rest of the samples was the VMM, which was the highest of the whole two-year study period (84% d^{-1}; Boras et al. 2009). This finding suggests that in conditions of severe viral predation the effect of phages on bacterial community can be more pronounced than the effect of the bottom-up factors.

4.2. Changes in functional diversity

Previous studies on functional diversity in Blanes Bay have shown clear seasonal patterns in the utilization of carbon sources in contrast to eutrophic coastal stations (Sala et al., 2006). In the present study, the pattern of utilization of carbon sources in the Biolog plates was correlated with the patterns of phylogenetic diversity, contrary to what was observed in another highly eutrophic and stable system of the NW Mediterranean coast (Sala et al., 2005a). Similarly to previous findings in Blanes Bay (Alonso-Sáez et al., 2008) that showed peaks of β-glucosidase and leu-aminopeptidase in spring and summer coinciding with high BP, the highest mean values of several extracellular enzyme activities, and especially leu-aminopeptidase activity, were found in spring. The CCA shows that the patterns of extracellular enzyme activity of bacteria in our study were found to be driven mainly by the bottom-up factors temperature, Chl *a* <3 μm and nutrient concentrations. The fact that Chl *a* <3 μm was more correlated with extracellular enzyme activity than Chl *a* might be related to the different taxonomic composition of autotrophic species in each fraction. Becker et al. (2014) showed differences in the composition of the DOM released by different groups such as cyanobacteria or diatoms, which might have an effect on bacterial functional diversity.

The top-down factors, i.e. viral and protistan abundances and mortality of bacteria induced by both predators, showed no clear relationship with enzymatic activity of the bacterial community. It could be observed that VMM was the main top-down factor determining the pattern of activities of xylosidase (positive), and leu-aminopeptidase and β-glucosidase (negative). In contrast, PMM showed a positive relationship only with α-glucosidase. It has been observed in other studies that viruses regulate bacterial metabolic activity by repressing metabolically active bacteria with high nucleic acid content (Xu et al., 2013). Furthermore, viral lysate has been suggested to be a significant source of P for marine bacteria (Weinbauer et al., 1995; Riemann et al. 2009) and in the NW Mediterranean viral infection has been suggested as a route for phosphate recycling (Noble and Fuhrman, 1999). Middelboe et al. (1996) found increased alkaline phosphatase and leu-aminopeptidase in a model system of *Vibrio* and viruses, indicating bacterial degradation of polymeric DOM containing N and P molecules. Our study does not provide evidence for a relationship between viral infection and alkaline phosphatase increase, though we sampled in the generally P-limited NW Mediterranean (Sala et al., 2002; Pinhassi et al., 2006). However, our results point towards a negative correlation between VMM and specific leu-aminopeptidase activity. This suggests that DOM in the lysate might be partly composed of amino acids, as found recently by Ankrah et al. (2014), who observed a two-fold increase in the concentration of several amino acids in the lysate of a marine bacterium. Specific leu-aminopeptidase activity reflects more

accurately the activity of the bacterial cells than bulk leu-aminopeptidase activity, since the enzyme can be partly constitutive and respond in part to changes in bacterial biomass or abundance. For this reason, our results do not contrast with those of Middelboe et al. (1996), showing an increase in bulk leu-aminopeptidase in model systems with viruses.

Additionally to the role of extracellular enzymes in the utilization of organic matter, Noble and Fuhrman (1999) postulated those enzymes as a defense mechanism against viruses since proteases could destroy the capsid protein of living particles. In our study, we found a negative correlation between viral-mediated mortality and leu-aminopeptidase (Table 4). It is unclear whether proteases are produced for the purpose of resisting viruses or are just a beneficial side effect of the extracellular enzymes synthesized to hydrolyze peptides. Our results provide slight evidence in the former direction but further research is needed in order to clarify this point.

Analysis of the relationship between the utilization of carbon sources in the Biolog plates and bacterial mortality also showed more cases of correlation between substrate utilization and VMM than PMM. PMM correlated with the utilization of two substrates: N-acetyl-D-glucosamine and α-cyclodextrin (Table 4). VMM, in contrast, correlated with the utilization of a higher number of substrates (L-asparagine, I-erythritol, D-mannitol and glucose-1-phosphate; Table 4). Viral lysis provides bacterioplankton with a highly labile DOM source (Noble and Fuhrman, 1999), whose composition is still relatively unknown. The correlations among the utilization of four substrates with VMM might be an indication that the changes in the utilization of carbon sources could be a response to the new input of DOM from lysed cells.

5. Conclusions

We conclude that the bottom-up factors water temperature, inorganic nutrients, and DOM sources (Chl *a* <3 μm) shaped the phylogenetic and functional diversity of the bacterioplankton community in the studied oligotrophic system during the seasonal cycle. Among predators, viruses probably had a stronger effect than protists on functional and phylogenetic diversity. Our study suggests that, while bottom-up factors are typically controlling factors of bacterial diversity, the effect of viral lysis can prevail episodically in conditions of strong (mass) lysis events.

Acknowledgments

Funding for this research was provided by projects funded by the Spanish Ministry of Science and Innovation (MICINN): MICROVIS, FLAME and MEFISTO (CTM2007-62140 and CGL2010-16304, CTM2013-43767) to D. Vaqué; and STORM, ÍCARO and DOREMI (CTM2009-09352, CTM2008-30I120, CTM2012-34294) to M.M. Sala. The work of J.A. Boras was supported by a PhD fellowship from the Spanish Ministry of Education and Science (an FPU grant). The authors thank J.M. Gasol for his thoughtful comments, and V. Balagué for her help in the molecular and digital analysis of the samples. Sampling, CTD data and chlorophyll *a* analyses were provided by colleagues involved in the Blanes Bay Microbial Observatory net.

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