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Seasonal changes in the functional diversity of bacterioplankton in contrasting coastal environments of the NW Mediterranean

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ABSTRACT: To understand the seasonal and intersite variations in the functional diversity of coastal bacterioplankton assemblages, their utilization of 31 different carbon sources was analyzed with Biolog-Ecoplates™ in waters from 3 harbours and 2 oligotrophic coastal environments of the NW Mediterranean. Polymers (α-cyclodextrin and glycogen) and carbohydrates (D-cellobiose and *N*-acetyl-D-glucosamine) were most utilized in the harbours, while carboxylic acids were mainly used in the coastal areas. Seasonal differences in the patterns of carbon source utilization (the so-called 'functional diversity') were investigated in 2 spatially close, but contrasting, coastal stations: the oligotrophic coastal site of Blanes Bay, and the Barcelona inner harbour. The existence of a possible seasonal trend in functional diversity of bacterioplankton in the oligotrophic coastal station, but not in the harbour, suggests that the bacterial assemblage of oligotrophic environments can adapt to changing inputs of nutrients and DOC. In contrast, the low water exchange in the harbour provides a pool of DOC of relatively stable composition throughout the year which could allow few potential bacterial metabolisms to persist. We considered the quantity of substrates used (of all those provided in the Biolog plate) as an index of potential functional diversity. The index calculated for the harbour and the coastal station samples was negatively correlated with chlorophyll *a* concentration, suggesting that the bacterial assemblages of oligotrophic systems have a higher number of metabolic pathways in order to be able to exploit a wide variety of DOC molecules present at low concentrations.

KEY WORDS: Bacterioplankton · Biolog · Coastal carbon · Functional diversity · Carboxylic acids

INTRODUCTION

Heterotrophic bacteria play a crucial role in the biogeochemical processes of aquatic ecosystems, since they are the major consumers of dissolved organic matter (DOM), the largest pool of organic carbon in natural waters (Azam 1998). DOM availability and composition strongly influences bacterioplankton activity and phylogenetic diversity (Eiler et al. 2003, Kirchman et al. 2004, Malmstrom et al. 2005) and might influence the functional diversity of the bacterial assemblages.

Data on DOM molecular composition in the ocean are difficult to obtain due to a lack of methods with suitable sensitivity for characterizing DOM in seawater (Benner 2002). For this reason, work on the characterization (Amon & Benner 2003, Kramer et al. 2005) and bacterial utilization (Jørgensen et al. 1993, Keil & Kirchman 1999, Grossart & Simon 2002, Pérez et al. 2003, Rosenstock et al. 2005, Sannigrahi et al. 2005) of identifiable compounds within the DOM pool has focused mainly on monosaccharide and amino acid analyses. Furthermore, the methodological constraints in the measurement of individual DOM molecule fluxes have led to the application of indirect methods for studying bacterial utilization of DOM compounds, such as the characterization of ectoenzymatic activities (Christian & Karl 1995, Unanue et al. 1999, Misic et al. 2002, Arnosti et al. 2005), or nutrient addition experi-

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ments (Jørgensen et al. 1993, 2003, Carlson & Ducklow 1996, Foreman et al. 1998, Church et al. 2000). However, these nutrient addition experiments are time-consuming and, therefore, the potential amount of carbon sources tested simultaneously is limited. Biolog-Ecoplates are a miniaturized version of these experiments and provide information on the simultaneous utilization of 31 carbon sources. Similar to the addition experiments, the main shortcoming of the utilization of the Biolog microplates for community profiling is the fact that this is a culture-based approach, in which opportunistic bacteria might dominate. Another drawback is that the substrates in the Biolog plate are not necessarily representative of those available in the natural environments (Konopka et al. 1998). This has been improved with the introduction of the Biolog-Ecoplates, which contain a set of carbon sources more relevant for ecological studies (Choi & Dobbs 1999).

In spite of these shortcomings, Biolog plates have provided useful information on the functional diversity of bacterial communities from soils and sediments (see references in Preston-Mafham et al. 2002), freshwater (Grover & Chrzanowski 2000, Sinsabaugh & Foreman 2001, Worm et al. 2001) and marine plankton systems. For example, studies in marine ecosystems have shown (1) that functional diversity was strongly influenced by temperature and salinity in the York River estuary (Schultz & Ducklow 2000); (2) that there was a lack of relationship between phytoplankton abundance and bacterioplankton functional diversity in Antarctic waters (Sala et al. 2005a), suggesting that bacteria could utilize carbon sources other than freshly released phytoplanktonic DOC in such waters; and (3) that phylogenetic and functional diversity can be uncoupled during blooms of harmful dinoflagellates (Sala et al. 2005b). Additionally, Biolog plates may provide information on the bacterial utilization of dissolved organic nitrogen sources in aquatic environments (Sala et al. 2006), and have been useful for characterizing bacterial isolates from several marine environments (Tan 1997, Tan & Rüger 1999, Frette et al. 2004).

While the Catalan coast (NW Mediterranean) is relatively oligotrophic, with low nutrient and chlorophyll *a* (chl *a*) concentrations, the harbours offer contrasting environments characterized by high nutrient concentrations (Vila & Masó 2005) and lower water exchange, which generally support high phytoplankton biomass and occasionally harmful algal blooms (Vila et al. 2001). Previous studies indicated a distinct composition of the bacterial assemblage in these contrasting environments, i.e. coastal waters and harbours, in the Catalan Coast (Schauer et al. 2000). A similar study revealed clear seasonal changes in the taxonomic composition of bacterioplankton in the oligotrophic coastal station of Blanes Bay (Schauer et al. 2003). Since changes in composition are often linked to changes in the metabolic potential of the bacterioplankton assemblage (Martínez et al. 1996, Riemann & Azam 2002, Kirchman et al. 2004), we expect to find differences in the functional diversity of bacterioplankton assemblages of the harbours and coastal waters, and seasonal changes at the Blanes station.

The aims of the present study were (1) to compare the single carbon source utilization profiles (CSUPs) of the surface bacterial assemblages of 5 different sites of the Catalan coast (NW Mediterranean), including harbours and coastal stations, and (2) to examine the seasonal pattern of CSUPs in the Barcelona harbour and the coastal station in Blanes Bay and relate them to environmental factors. We hypothesized that bacterioplankton in the harbours would show a higher utilization of complex carbon sources, perhaps caused by the presence of oil or other waste products from boats, and that, due to the lower water exchange, the bacterial assemblage would be less dynamic than in coastal waters.

MATERIALS AND METHODS

Sampling. Surface seawater was collected from 5 stations along the Catalan coast (NW Mediterranean): 3 harbours and 2 coastal stations (see Table 1). Seawater was kept in 25 l clean plastic carboys and water was transported within 2 h in the dark to the laboratory.

The Barcelona harbour was sampled 24 times during June 2001 to October 2002. The Tarragona harbour, 100 km south of Barcelona, was sampled 4 times in June 2001 during a bloom of the dinoflagellate *Alexandrium catenella*. The Arenys de Mar harbour, 40 km north of Barcelona, was sampled 6 times during a bloom of *Alexandrium minutum* in January–February 2002.

The 2 coastal stations Masnou and Blanes were both sampled by boat 400 m offshore. Waters from the Microbial Observatory of Blanes Bay, 70 km north of Barcelona, were sampled monthly during January 2003 and December 2004, and 4 additional samples were collected during 2001 and 2002. The Masnou station, 20 km north of Barcelona was sampled on 2 occasions in April and November 2002.

Water was collected with a bucket at all sites, at approximately 0 to 50 cm depth. In Blanes, Barcelona harbour and Masnou, water was sampled from a boat while in Arenys de Mar and Tarragona harbours it was collected from the pier.

Environmental parameters. Chl *a* concentrations were determined fluorimetrically following Yentsch & Menzel (1963). Samples between 100 and 150 ml were

filtered through Whatman GF/F filters. After keeping the filters frozen at –20°C for at least 2 h, pigments were extracted in 6 ml 90% acetone in the dark for 24 h. Inorganic nutrient concentrations of soluble reactive phosphorus (SRP), nitrate, nitrite and ammonia were measured according to Grasshoff et al. (1983). Total inorganic nitrogen (TIN) represents the sum of nitrate, nitrite and ammonia. Bacterial numbers were obtained using flow cytometry for the Blanes samples and epifluorescence microscopy for those from the other stations. For flow cytometry, we followed the method of Gasol & del Giorgio (2000) and for epifluorescence microscopy, a modification of the method of Porter & Feig (1980) was used. Five ml of seawater fixed with formaldehyde (2% final conc.) were filtered onto 0.2 µm black polycarbonate filters and stained with DAPI (0.5 mg ml^{-1} final conc.) onto 0.2 µm black polycarbonate filters. Bacteria on filters were counted under a Zeiss Axioplan epifluorescence microscope.

Biolog plates. Biolog-Ecoplate™ microplates were used to determine differences in the metabolic potential of the bacterioplankton assemblages following the method of Sala et al. (2005a). Biolog-Ecoplates are 96-well microtiter plates containing 31 carbon sources in triplicate. Each well contains the redox dye tetrazolium violet in a dry film together with the carbon source. As the carbon source is oxidized, formazan is formed which can be quantified spectrophotometrically.

Each well was inoculated with 150 µl of sample water and the plates were then incubated at room temperature for 6 d. After incubation, absorbance of the plates was measured at a wavelength of 590 nm using a spectrophotometric microplate reader (ELX800 BIOTEK Instruments).

The utilization of each carbon source was expressed as the average substrate color development (ASCD). This was calculated after subtracting the mean absorbance of the control wells (without a carbon source) from the absorbance of each well and then calculating the mean absorbance of each substrate of the 3 replicate wells for each substrate. The ASCD was then computed as a percentage of the absorbance of each substrate over the sum of absorbance of all substrates.

Hierarchical cluster analysis. Hierarchical cluster analysis (HCA) was used to detect differences among the patterns of substrate utilization at the 5 stations sampled and also to differentiate the seasonal means of ASCD for each substrate. The program STATISTICA 6.0 with City Block and Ward's method were used in this analysis. Data from HCA were used to construct a similarity dendrogram. Similarity was scaled to 100%.

RESULTS

Differences between coastal stations and harbours

Samples from the harbours always showed higher mean values of chl *a*, bacterial abundance and inorganic nutrient concentrations than coastal samples (Table 1). Mean chl *a* concentrations were 4 times higher in Barcelona than in Blanes; they were especially high in the Arenys de Mar (10.2 µg l^{-1}) and Tarragona (140.2 µg l^{-1}) harbours, since samples had been collected during *Alexandrium* spp. blooms. Nutrient concentrations of both TIN and SRP were up to 6 times higher in the harbours than at the coastal station.

HCA of the substrate use in the Biolog plates revealed a root discrimination of most of the harbour samples from those of coastal waters (Fig. 1). Samples from coastal waters were separated in 2 main clusters, whereas 82% of

Table 1. Average properties of the samples used to investigate differences in bacterioplankton functional diversity among the studied areas. Results show mean (coefficient of variation in parentheses). n: number of samples; BACT: heterotrophic bacterial concentration; SRP: soluble reactive phosphorus; DIN: dissolved inorganic nitrogen

Stn	Date	n	Temp. (°C)	Salinity	Chl *a* conc. (mg l^{-1})	BACT ($\times 10^6$ ml^{-1})	SRP (µM)	DIN (µM)
Harbours								
Barcelona	Jun 2001–Oct 2002	24	18.0 (0.70)	37.6 (0.03)	4.47 (1.41)	2.51 (0.62)	0.97 (0.81)	14.45 (0.86)
Arenys de Mar	Jan–Feb 2002	6	12.5 (0.01)	–	10.15 (0.37)	1.72 (0.58)	0.33 (0.72)	20.98 (1.99)
Tarragona	Jun 2001	4	22.5 (0.02)	37.5 (0.00)	140.2 (1.18)	9.81 (0.41)	0.40 (1.14)	14.41 (0.44)
Coastal waters								
Blanes	May, Jun, Nov 2001 Nov 2002 Jan 2003-Dec 2004	27	18.0 (0.25)	37.5 (0.02)	0.84 (0.81)	1.00 (0.30)	0.16 (0.25)	2.55 (0.9)
Masnou	Apr, Nov 2002	2	–	–	2.89	1.20	–	–

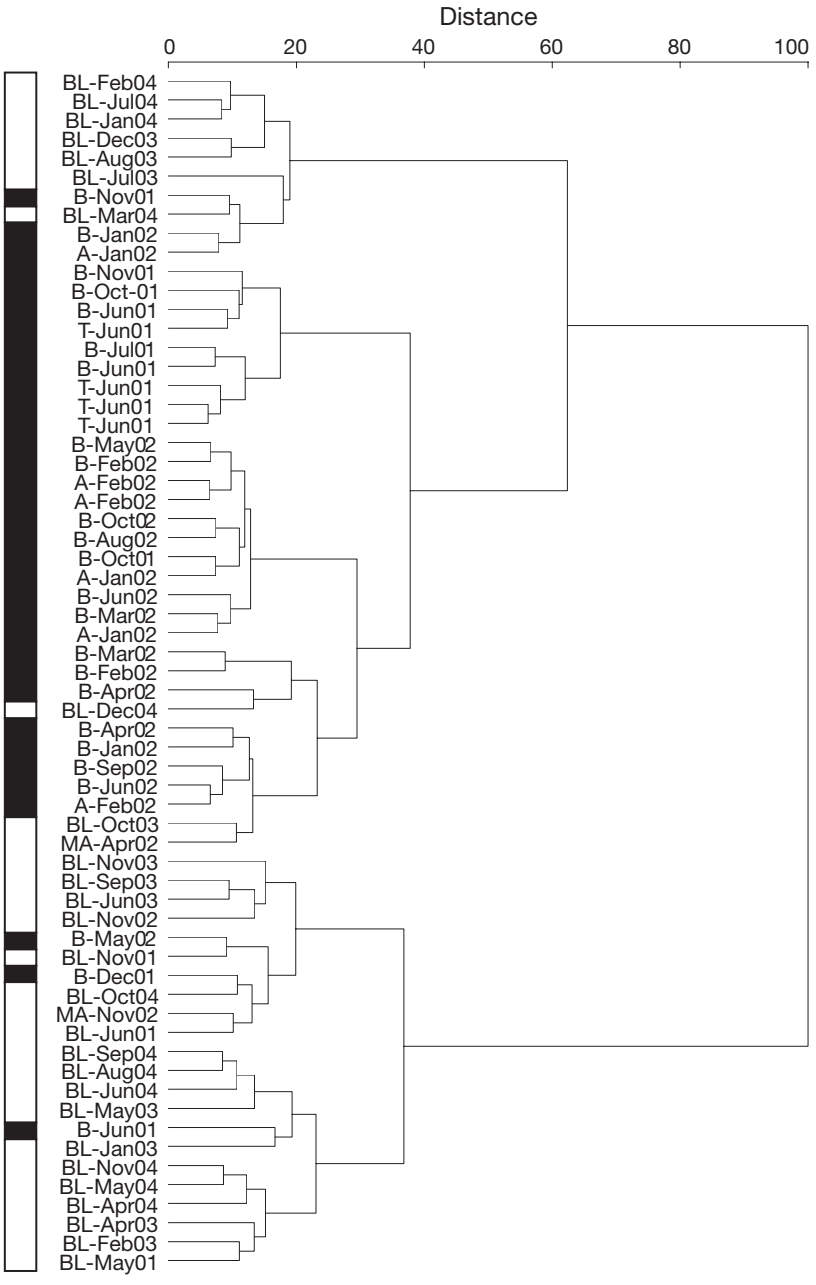

Fig. 1. Hierarchical cluster analysis of the ASCD (average substrate color development) in the Biolog-Ecoplates obtained at 5 stations (3 harbour stations and 2 coastal stations) of the NW Mediterranean coast—B: Barcelona harbour; T: Tarragona harbour; A: Arenys de Mar harbour; BL: Blanes; M: Masnou. The bar shows the origin of the sample—black: harbours; white: coastal stations

harbour samples could be found in one single branch. Several substrates were differentially used in both sampling areas (Table 2). The harbours were characterized by higher (*t*-test, $p < 0.0001$) utilization of 4 substrates, 2 polymers and 2 carbohydrates: α-cyclodextrin, D-cellobiose, *N*-acetyl-D-glucosamine and glycogen. In contrast, 4 carboxylic acids, γ-hydroxybutyric acid, D-glucosaminic acid, D-malic acid and itaconic acid, and 1 amino acid (L-phenylalanine) were used significantly more in the coastal waters than in the harbours ($p < 0.001$).

Seasonal patterns

We investigated the seasonal variability at 2 stations, Barcelona harbour and the coastal station of Blanes (Fig. 2), and tested the differences using a 1-way ANOVA and a post-hoc Tukey test after aggregation of the samples in seasons. In Blanes, significant variablility was found for chl *a* concentration ($F = 7.90$, $p = 0.001$), with higher concentrations in winter than in summer ($p = 0.002$), in autumn ($p = 0.005$) and in spring ($p = 0.037$). TIN also varied significantly among seasons ($F = 4.03$, $p = 0.024$), and was higher in winter than in summer ($p = 0.014$). This led to substantial differences in the N:P ratio ($F = 41.80$, $p < 0.001$) between winter (mean N:P = 29.5) and summer (mean N:P = 6.2).

In the Barcelona harbour, both inorganic nutrients showed marked differences in concentrations among seasons. SRP concentration ($F = 8.866$, $p = 0.0003$) was higher in autumm than in summer ($p = 0.004$) or spring ($p = 0.015$), and in winter than in summer ($p = 0.03$). TIN also exhibited clear differences among seasons ($F = 12.94$), with higher concentrations in winter than in spring ($p = 0.0008$) or summer ($p = 0.0002$), and in autumm than in spring (0.015) or summer ($p = 0.0016$). However, these differences in both inorganic nutrient concentrations led to no significant differences in the N:P ratios of the dissolved pools, which varied between 11.0 in spring and 23.2 in winter.

A statistical comparison of the average values of utilization of each carbon source for each season is shown in Fig. 3. Coastal samples clustered mainly according to the sampling site. Samples from Barcelona clustered more closely than those taken off Blanes. A much looser affiliation with the Blanes cluster could be observed in winter.

Seasonal means of the ASCDs of each carbon source in the Biolog-Ecoplate are shown in Fig. 4. In this study, we regarded 2% absorbance of the total absorbance per plate as the threshold for substrate utilization. In general, we found that a lower number of substrates was used in Barcelona harbour (17 to 19),

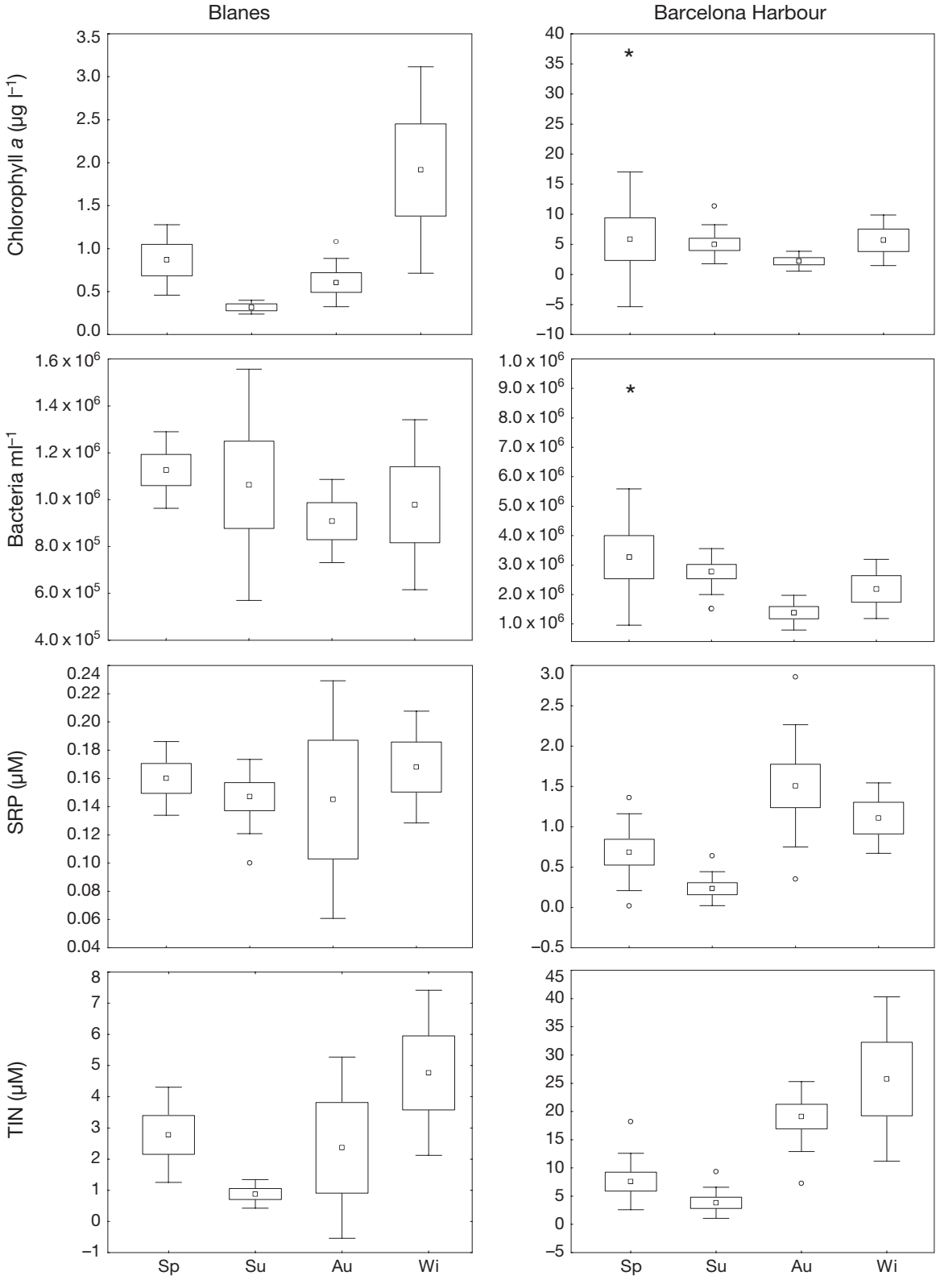

Fig. 2. Distribution of chlorophyll *a*, bacterial abundance, soluble reactive phosphorus (SRP) and total inorganic nitrogen (TIN) concentrations over the 4 seasons (Sp: spring; Su: summer; Au: autumn; Wi: winter). For Barcelona harbour, n = 5 to 10, and for Blanes, n = 5 to 7. Square: mean; Box: standard error; Whisker: ±SD; Circles: outliers (values higher/lower than: the upper/lower value of the box + 1.5 times the height of the box); asterisks: extremes (values higher/lower than: the upper/lower value of the box + 3 times the height of the box)

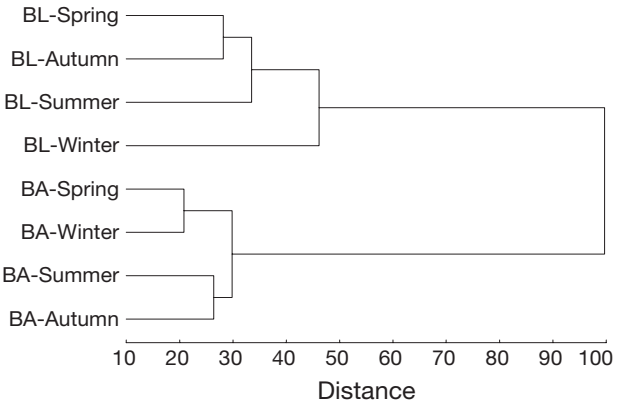

Fig. 3. Hierarchical cluster analysis of the mean ASCD for each substrate, season and station. BA: Barcelona harbour; BL: Blanes

Table 2. Differences in substrate utilization in samples collected at the 2 coastal stations and the 3 harbours. *t*-test with: $^{*}p < 0.001$, $^{**}p < 0.0001$, $^{***}p < 0.00001$

Substrate	Category
Coastal waters	
γ-hydroxybutyric acid**	Carboxylic acid
D-glucosaminic acid**	Carboxylic acid
Malic acid*	Carboxylic acid
Itaconic acid*	Carboxylic acid
L-phenylalanine*	Amino acid
Harbours	
α-cyclodextrin****	Polymer
D-cellobiose****	Carbohydrate
N-acetyl-D-glucosamine***	Carbohydrate
Glycogen**	Polymer

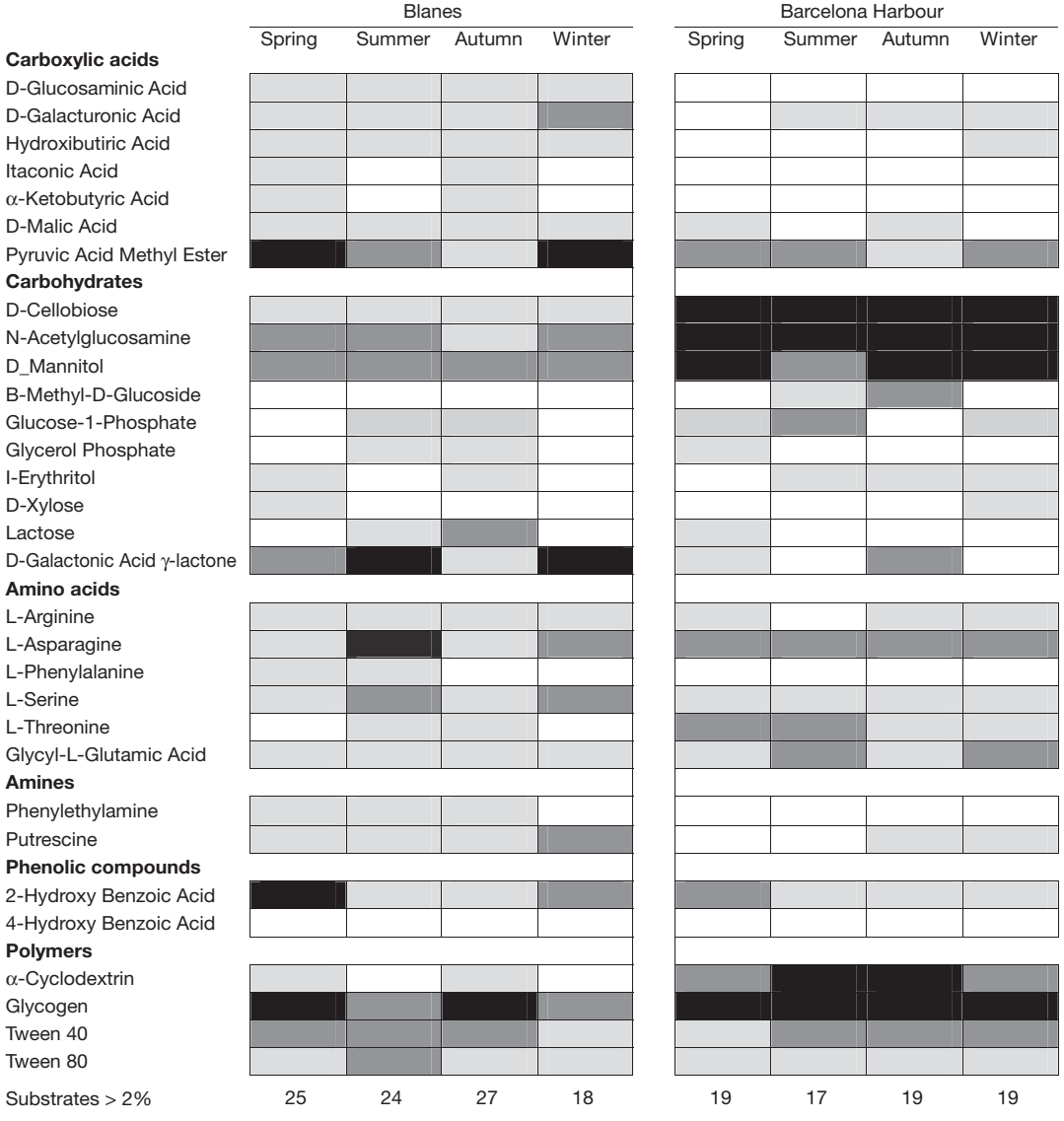

Fig. 4. Mean ASCD for each season for the samples taken at Blanes and in Barcelona Harbour. Shading in the boxes indicates the range of percentage absorbance of the total absorbance of the plate. White: <2%, light grey: 2 to 4%, dark grey: 4 to 6%, black: >6%. Below each column, the number of substrates with >2% absorbance for each season is indicated

whereas in Blanes this value was significantly higher (18 to 27, $p < 0.05$). The number of utilized substrates varied considerably in Blanes, with a lower number in winter (18) compared to the rest of the year (24 to 27).

The breakdown of the percent utilization of the carbon sources into the main categories listed in Fig. 4 showed no significant seasonal trends for substrate use at both sites. However, some differences could be observed for the Blanes station: a higher mean percentage of utilization of amino acids in summer (24%) than the other seasons (17 to 19%) and a lower utilization of polymers in winter (12%) than during the rest of the year (17 to 19%).

DISCUSSION

Differences between harbours and coastal stations

Functional diversity, understood in this study as the utilization of single carbon sources in Biolog plates, showed clear differences between harbours and coastal ecosystems. Generally, harbour samples were characterized by a higher utilization of 2 polymers and 2 carbohydrates. The 2 carbohydrates, D-cellobiose and *N*-acetyl-D-glucosamine (NAG), have been shown to be important sources for bacterial growth in aquatic systems (Riemann & Azam 2002, Sala & Güde 2004). NAG is a major component of structural polymers in bacteria, algae and zooplankton and fungi, in the form of the homopolymer of NAG, chitin. D-cellobiose is a disaccharide subunit of cellulose and a primary structural component of algae. Both substrates were among the most used in Biolog-Ecoplate samples from western Antarctic seawaters (Sala et al. 2005a). The 2 polymers used mainly in harbours are also carbon substrates likely to be found in nature and probably of autochthonous origin: α-cyclodextrin (analog of amylose, a component of starch, the main storage molecule of algae), and glycogen (a storage molecule in animals and bacteria).

In contrast, in coastal waters, the most used carbon sources were carboxylic acids. Carboxylic acids can be considered to be part of the labile pool of organic matter, which is an important carbon source for bacterioplankton in the Mediterranean Sea (Obernosterer et al. 1999) and other marine environments (Pullin et al. 2004). Few studies compare the lability of DOC in harbours and open waters, and only Fry et al. (1996) observed a larger recalcitrant portion of DOC in harbours compared to the DOC released during phytoplankton blooms.

Chl *a* and nutrient concentrations in the harbours were often 1 order of magnitude higher than at the coastal stations. Phytoplankton are the main producers of DOC in marine environments, and DOC derived from phytoplankton is efficiently utilized by bacteria (Jensen 1983, Lancelot 1984). The high inorganic nutrient concentrations, particularly in phosphorus, probably enhance bacterial utilization of carbohydrates and polymers (cellobiose, glycogen, α-cyclodextrin and *N*-acetyl-D-glucosamine) in the harbours. The presence of complex molecules in waste products of boats or sewage in the harbours would probably also favour bacteria capable of using polymeric carbon sources. These reported differences in metabolic abilities between the harbours and coastal stations appear to be associated with the differences in phylogenetic composition observed for the bacterial assemblages among these stations (Schauer et al. 2000).

Seasonal patterns

No clear seasonal patterns of single carbon source utilization were found in Barcelona harbour and the coastal station of Blanes. The mean seasonal values shown in Fig. 3 indicated that samples clustered depending on the sampling site. However, within each cluster, the samples from Barcelona harbour appeared more closely related than the Blanes cluster, with winter being the most loosely associated. In Blanes, winter was characterized by significantly higher chl *a* and TIN concentrations, leading to higher N:P ratios. The different environment in winter probably led to a bacterial assemblage with distinct functional diversity, with a higher utilization of carboxylic acids and lower use of polymers. In contrast, no seasonal trends could be observed in the samples from the Barcelona harbour for any of the parameters measured. This relatively stable environment corresponds with a less dynamic bacterial community in which only small differences were found in single carbon source utilization among different seasons, i.e. a lower utilization of polymers in spring. In contrast to coastal environments, harbours are characterized by calm waters with low water exchange, probably favoring an environment characterized by low changes in DOC and nutrients. These conditions might select for only a small number of metabolic pathways persisting throughout the year. Grover & Chrzanowski (2000) found also seasonal trends in the functional diversity of bacteria, analyzed with Biolog plates, in 4 lakes: a higher response to amino and carboxylic acids in colder seasons and strong relative responses to carbohydrates in warm seasons. They related this pattern to seasonal events among phytoplankton.

The number of substrates used in each season and station showed similar trends as the seasonal mean values of ASCD. In Barcelona harbour, the number of substrates used was lower than at Blanes and relatively constant throughout the year. Only the number of sub-

strates used in winter at Blanes (18) was markedly lower than the annual mean and within the range of the number of substrates used in Barcelona harbour (17 to 19). Similarly, results on bacterial clone libraries constructed for each season (L. Alonso-Sáez et al. unpubl. data) show a lower phylogenetic diversity in winter than during the rest of the year, when α-Proteobacteria clearly dominated. These results suggest a possible coupling between functional and phylogenetic diversity in Blanes Bay.

Cottrell & Kirchman (2000) observed that bacteria of the Bacteroidetes group were related to the utilization of NAG, whereas members of the α-Proteobacteria dominate amino acid uptake. The contribution of α-Proteobacteria and Bacteroidetes to total bacterial abundance was determined by fluorescent *in situ* hybridization in the samples from Blanes (L. Alonso-Sáez et al. unpubl. data). They found positive correlations, although not significant, between the percentage of α-Proteobacteria and the utilization of each of the 6 amino acids in the plate, and between the percentage of Bacteroidetes and utilization of NAG in the Biolog plate.

Both chl *a* and TIN showed higher concentrations in winter at Blanes, and high concentrations in Barcelona harbour throughout the year, and these were the periods and stations characterized by the utilization of a low number of substrates. We, therefore, investigated the influence of both parameters on the low number of substrates used. Only chlorophyll showed a significant correlation with the number of substrates used (log:log $r = -0.34$, $n = 43$, $p = 0.026$). If we consider the number of substrates used as an estimation of functional diversity, our results suggest a negative relationship between chl *a* concentration and bacterial functional diversity. These results support the ecological theory of a high diversity in oligotrophic systems and lower diversity in richer systems (Frontier 1985), although Horner-Devine et al. (2003) claim that primary productivity has no clear effects on bacterial phylogenetic richness.

The differences in the number of substrates used among different seasons at Blanes might suggest that the bacterial assemblage in oligotrophic environments is perhaps forced to adapt to the changing inputs of organic matter and nutrients. Therefore, the bacterial assemblages in oligotrophic areas express a higher plasticity in metabolic pathways in order to be able to exploit the changing and limited carbon sources available for growth.

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