FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Effects of large river dam regulation on bacterioplankton community structure

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Received 6 September 2012; revised 11 December 2012; accepted 12 December 2012.
Final version published online 14 January 2013.

DOI: 10.1111/1574-6941.12063

Editor: Gary King

Keywords
aerobic anoxygenic phototrophic bacteria; bacterioplankton community composition; CARD-FISH; reservoirs; river regulation.

Abstract

Large rivers are commonly regulated by damming, yet the effects of such disruption on prokaryotic communities have seldom been studied. We describe the effects of the three large reservoirs of the Ebro River (NE Iberian Peninsula) on bacterioplankton assemblages by comparing several sites located before and after the impoundments on three occasions. We monitored the abundances of several bacterial phylotypes identified by rRNA gene probing, and those of two functional groups (picocyanobacteria and aerobic anoxygenic phototrophic bacteria-AAPs). Much greater numbers of particles colonized by bacteria were found in upstream waters than downstream sites. Picocyanobacteria were found in negligible numbers at most sites, whereas AAPs constituted up to 14% of total prokaryotes, but there was no clear effect of reservoirs on the spatial dynamics of these two groups. Instead, damming caused a pronounced decline in *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* from upstream to downstream sites, whereas *Alphaproteobacteria* and *Actinobacteria* significantly increased after the reservoirs. Redundancy analysis revealed that conductivity, temperature and dissolved inorganic nitrogen were the environmental predictors that best explained the observed variability in bacterial community composition. Our data show that impoundments exerted significant impacts on bacterial riverine assemblages and call attention to the unforeseen ecological consequences of river regulation.

Introduction

Prokaryotes are essential players in aquatic ecosystems, catalyzing significant biogeochemical reactions and holding central roles in aquatic food webs (Cotner & Biddanda, 2002; Pernthaler, 2005). Natural assemblages of bacterioplankton are highly diverse and can undergo shifts in composition in response to spatial and temporal environmental gradients across ecosystems (Kirchman *et al.*, 2004; Crump & Hobbie, 2005; Alonso-Sáez *et al.*, 2007; Comte & del Giorgio, 2010), fluctuations that may lead to changes in the functional roles of bacterial communities in the biogeochemical cycles. However, our current knowledge of the dynamics of freshwater bacterioplankton diversity is almost entirely based on lake studies (Zwart *et al.*, 2002; Newton *et al.*, 2011) with much less attention having been paid to the structure of bacterial communities in rivers.

Recent studies using culture-independent approaches (e.g. fluorescent *in situ* hybridization, FISH, and 16S rRNA gene sequencing) have revealed that typical riverine bacterioplankton assemblages are dominated by taxa belonging to *Betaproteobacteria*, *Bacteroidetes* and *Actinobacteria* (Glöckner *et al.*, 2000; Crump *et al.*, 2009; Portillo *et al.*, 2012). However, these major bacterial groups and the phylotypes within them show diverging abundances at different temporal and spatial scales. Recent work on the composition of riverine bacterial communities has shown shifts according to seasonal variations in discharge, temperature, nitrate concentration, dissolved organic matter (DOM) and conductivity, and even episodic events such as freshets caused by rain, ice or

snow melting (Leff *et al.*, 1999; Crump & Hobbie, 2005; Crump *et al.*, 2009; Portillo *et al.*, 2012). Spatial changes in bacterial assemblages along river systems have also been reported and related to phytoplankton development, changes in land use, variations in nutrient and DOM concentration, and quality and intensity of grazing pressure, among other factors (Leff, 2000; Levine & Crump, 2002; Winter *et al.*, 2007).

Given that the composition of bacterioplankton communities often seems to change slowly and gradually along rivers (Sekiguchi *et al.*, 2002; Winter *et al.*, 2007), the magnitude of the seasonal variation may exceed that of spatial changes, as observed elsewhere (Leff *et al.*, 1999). However, many rivers are highly regulated for hydropower or water supply purposes, which generates important hydrological disturbances at the spatial scale. Reservoirs constitute a discontinuity for the river system as they regulate water flow circulation, modify water residence time, and affect riverborne nutrient and matter loads through retention of a great fraction of the suspended material transported by the river (Batalla & Vericat, 2011). As a result, the upstream and downstream sections of the reservoirs tend to differ greatly in their physico-chemical properties (Sabater *et al.*, 1989; Pozo *et al.*, 1997) and, consequently, changes in the planktonic communities could also be expected. Seston sedimentation due to damming has been shown to cause shifts in the proportion of free-living vs. particle-attached bacteria (e.g. Kondratieff & Simmons, 1985), which might imply changes in the composition and metabolic capabilities of the bacterial assemblages from both reaches (Karner & Herndl, 1992; DeLong *et al.*, 1993; Besemer *et al.*, 2005). Moreover, studies performed within reservoirs have reported large longitudinal shifts in bacterioplankton community structure that can ultimately be attributed to the extended residence time of water within the impoundment (Mašín *et al.*, 2003). As such, we would expect a clear differentiation between up- and downstream bacterial assemblages, yet given that the response of bacteria to environmental changes is not only due to replacement of the existing phylotypes, but also to functional adjustments of the existing taxa (Comte & del Giorgio, 2011), phylogenetically different communities before and after the reservoirs may not always be encountered. Indeed, the very few available studies so far comparing the communities before and after the reservoirs show contrasting results. Whereas clearly different bacterial assemblages were found before and after the reservoir at the small Sinnamary River (Dumestre *et al.*, 2001), large impoundments in the Danube River caused undiscernible effects on bacterial communities (Winter *et al.*, 2007). Our own objective was thus to determine the influence of damming on the spatial and seasonal patterns of bacterial community composition in the large regulated Ebro River (NW Spain).

The Ebro is the third largest river system in the Mediterranean basin in terms of watershed area, and has been strongly regulated since 1940. Its largest reservoirs (Mequinenza, Ribarroja and Flix) are located in the mid-lower part of the river and can cause significant changes in the discharge pattern (Ibáñez *et al.*, 2008). Rivers in Mediterranean climate regions are physico-chemically and biologically shaped by sequential, predictable seasonal events of flooding and drying over the annual cycle (Armengol *et al.*, 1991; Gasith & Resh, 1999). Under natural unaltered conditions, plankton densities in these river systems tend to increase from mid to lower river sections and from winter to summer, when slower, warmer and well-lit waters allow maximal phytoplankton development (Vis *et al.*, 2007). Increased discharge during wet periods decreases water residence time, homogenizing water quality conditions and diluting planktonic biomass. These seasonal and longitudinal patterns, though, are dramatically disrupted by the presence of reservoirs. Long-term data in the Ebro River reveal that more than 99% of the original sediment load is retained by impoundments along its course (Batalla & Vericat, 2011). Abrupt decreases in turbidity, conductivity, chlorophyll *a* (Chl *a*) and changes in the concentrations of some nutrients are equally associated with the presence of reservoirs, features that seem to trigger the development of differentiated phytoplankton communities between both reaches as well as the massive growth of macrophytes in downstream sites (Roura, 2004; Sabater *et al.*, 2008). Moreover, reservoirs in the river have also been shown to cause a change in the use of inorganic and organic phosphorus (Artigas *et al.*, 2012), leading to strong phosphorus (P)-limitation during periods of low water flow in waters upstream of the reservoirs. Such wide range of environmental conditions co-occurring in the Ebro River would likely affect bacterioplankton community structure as well. Exploring these relationships may also contribute to the understanding of the mechanisms driving bacterial community composition in complex river systems.

We analyzed the changes in bacterioplankton communities in 12 sites located upstream and downstream of the largest reservoir system of the Ebro River. We addressed such variability both from a phylogenetic perspective (through rRNA gene probing) and a functional point of view, distinguishing among heterotrophic, autotrophic (picocyanobacteria) and photoheterotrophic bacteria (aerobic anoxygenic phototrophs, AAPs, Kolber *et al.*, 2000), providing the first quantitative evidence of significant AAP numbers in rivers. We hypothesized that the two river sections partitioned by the reservoirs would develop distinct heterotrophic microbial assemblages with varying

biogeochemical roles, and that the magnitude of the reservoir-driven changes would also change seasonally, being lower in winter due to higher discharge and homogenization of water characteristics (Artigas *et al.*, 2012). The contrasting environmental conditions between the sections located up- and downstream of the reservoirs offer a good opportunity to explore relationships between the dynamics of these phylogenetic and functional groups and their physico-chemical environment.

Materials and methods

Study area

The Ebro River is located in the northern third of the Iberian Peninsula. With a length of 910 km and a basin surface of 85 000 km^2, it is the largest Iberian river draining to the Mediterranean Sea. Along the course to its delta, its watershed encompasses diverse climate regimes, landscapes, and land uses (Sabater *et al.*, 2009). The Ebro River is characterized by high precipitation and discharge periods in autumn and spring, while summer rainfall decreases from the NW to the SE parts of its basin. The river shows a highly variable water discharge at the Ebro River mouth (monthly means ranged between 19.5 and 2470 $m^3\ s^{-1}$ from 1912 to 2008; Sabater *et al.*, 2008). The basin has been strongly regulated since the 1940s, and nearly 187 reservoirs impound 57% of the mean annual runoff. The largest ones (Mequinenza, Ribarroja and Flix) interrupt the hydraulic continuity in the mid-lower part of the river (Fig. 1). This reservoir system is ca. 140 km long, has a maximum depth of 60 m, and presents a relatively long water residence time oscillating between 1 and 5 months (Roura, 2004).

Sampling design

The study was done in the main mid-low course of the Ebro River. We sampled six sites upstream (Zaragoza, Pina de Ebro, Quinto, La Zaida, Sástago, Escatrón) and five downstream (Flix, Ascó, Móra d'Ebre, Benifallet, Xerta) of the Mequinenza-Ribarroja-Flix reservoir system, as well as one intermediate site located at the Ribarroja reservoir (Almatret). The studied transect extended for 330 km, reaching up to 30 km from the river mouth (Fig. 1). Samplings were carried out in three occasions in 2011 during summer (July and September) and winter (December). Water flow (Table 1) was provided by the 'Confederación Hidrográfica del Ebro' (CHE) from one upstream site (Zaragoza), one reservoir site (Mequinenza) and one downstream site (Ascó). Surface water samples were collected from the free water zone with 10-L polyethylene buckets. At each station, water temperature,

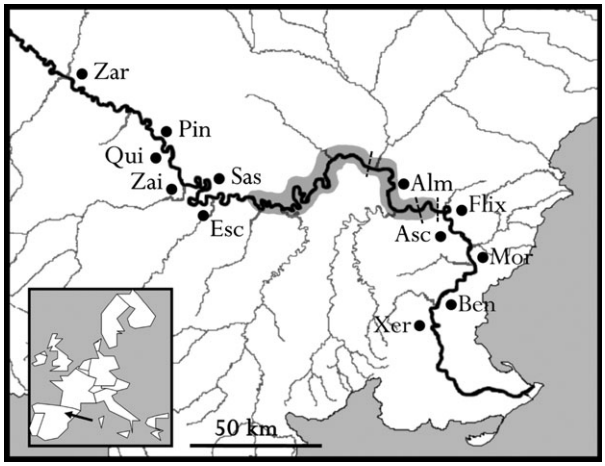

Fig. 1. Map of the Ebro watershed showing the 12 sampled sites. The presence of the reservoirs is indicated by the shaded area. Upstream sites: Zaragoza (Zar), Pina de Ebro (Pin), Quinto (Qui), La Zaida (Zai), Sástago (Sas), Escatrón (Esc). Reservoir site: Almatret (Alm). Downstream sites: Flix, Ascó (Asc), Móra d'Ebre (Mor), Benifallet (Ben), Xerta (Xer). The arrow indicates the location of the sampling area within the Iberian Peninsula. Dashed lines indicate the dams separating the three reservoirs (Mequinenza, Ribarroja and Flix).

conductivity, pH and dissolved oxygen were determined *in situ* by means of appropriate probes. Samples for all other parameters were collected in triplicate and processed in the lab.

Chemical analyses

Triplicate samples for dissolved nutrient analyses were filtered through 0.2-μm-pore nylon filters and frozen at 20 °C until analysis in the laboratory. Concentrations of nitrate, ammonia, reactive phosphorus, dissolved organic and inorganic carbon (DOC and DIC), and total dissolved nitrogen and phosphorus were determined by standard methods as explained in Artigas *et al.* (2012). Suspended solids were estimated after filtration of 0.2–2.5 L of water and heating in a muffle furnace at 450 °C for 4 h to obtain their ash-free dry weight.

Chl *a* determination

Chl *a* concentration was determined in triplicate by filtering 0.2–3 L of water on GF/C filters and extracting the pigment in acetone (90% v/v) for 12–20 h in the dark at 4 °C. Absorbance of the pigment was measured with a Shimadzu UV-1800 spectrophotometer.

Prokaryote abundance and biomass

Heterotrophic prokaryote abundances were quantified in triplicate catalyzed reporter deposition-fluorescence *in*

Table 1. Averaged water characteristics in the upstream, reservoir and downstream sections during the three samplings (July, September and December 2011)

Sampling	River section	Discharge (m^3 s^{-1})	Temp (°C)	DO (mg L^{-1})	Cond (µS cm^{-1})	pH	Seston (mg L^{-1})	SRP (µg L^{-1})	DIN (µg L^{-1})	DOC (mg L^{-1})	Chl *a* (µg L^{-1})	Prok (10^6 cells mL^{-1})
July 2011	Upstream	64.1 (4.6)	25.1 (0.4)	8.2 (0.4)	2203 (68)	8.1 (0)	26.7 (18)	67.4 (6.1)	930 (44)	4.7 (0.6)	13.8 (3.4)	6.2 (0.3)
	Reservoir	51.3 (11.0)	25.9	9.3	846	8.2	2.5	10.4	358	3.4	7.9	5.8
	Downstream	160.4 (1.6)	25.4 (0.6)	9.4 (0.5)	1010 (9)	8.5 (0.1)	2.8 (1.5)	48.6 (2.3)	578 (39)	3.3 (0.2)	1.7 (0.3)	3.8 (0.5)
September 2011	Upstream	34.3 (4.2)	24.2 (0.6)	7.6 (0.6)	2069 (35)	8.2 (0.1)	30.1 (13.4)	48.2 (9.8)	1118 (52)	3.1 (0.4)	15.1 (5.3)	3.7 (0.5)
	Reservoir	57.6 (7.2)	24.8	9.0	1173	8.6	6.2	6.5	480	4.9	6.7	4.9
	Downstream	129.7 (5.2)	26.6 (0.6)	8.6 (0.6)	1363 (12)	8.4 (0.1)	4.6 (2.5)	82.8 (3.9)	519 (14)	3.1 (0.5)	2.2 (0.6)	3.3 (0.3)
December 2011	Upstream	52.5 (0.8)	11.0 (0.1)	9.3 (0.2)	1813 (35)	8.3 (0.02)	32.5 (6.0)	74.1 (3.1)	4312 (35)	4.2 (0.2)	2.3 (0.1)	3.6 (0.3)
	Reservoir	77.7 (3.1)	12.8	8	1256	8.3	9.4	53.0	2621	3.2	1.6	3.4
	Downstream	134.39 (2.1)	14.2 (0.5)	9.7 (0.2)	1247 (10)	8.4 (0.02)	5.4 (1.3)	55.0 (2.8)	2098 (24)	3.3 (0.1)	1.2 (0.2)	3.2 (0.2)

Values are means ± standard errors of the sites considered (n = 6 for upstream and n = 5 for downstream sites, respectively). Only one site was located at the reservoir. Mean discharge values were obtained from three stations (see Methods) and averaged for the days of sampling. Temperature (Temp), dissolved oxygen (DO), conductivity (Cond), pH, suspended matter (Seston, mg dry weight L^{-1}), SRP, DIN, DOC, Chl *a* and prokaryote abundances (Prok).

situ hybridization (CARD-FISH) filters (see below) by epifluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI, 1 µg mL^{-1}). A minimum of 10 fields (500–1200 DAPI stained cells) per filter were manually counted in an Olympus BX61 epifluorescence microscope. The presence of filamentous bacteria and particles intensely colonized by bacteria was also quantified in these filters from transects across the section of the filters.

Bacterial cell size and biomass were estimated by flow cytometry. Samples of 5 mL were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and kept frozen at −80 °C until analysis with a Becton-Dickinson FACSCalibur flow cytometer after staining with SYTO-13 (Molecular Probes, Eugene, OR). Bacterial cell size was estimated using the relationship between the average bacterial size and the average fluorescence of the SYTO-13 stained bacteria relative to that of standard beads (Gasol & del Giorgio, 2000). Bacterial carbon content was further calculated with the carbon to volume relationship described in Norland (1993). Total bacterial biomass (in µg C L^{-1}) was calculated by multiplying bacterial carbon content by their abundances.

CARD-FISH

Triplicate samples of 10 mL were fixed with paraformaldehyde (1% final concentration) at 4 °C in the dark for the determination of the *in situ* abundances of different bacterial populations by CARD-FISH (Pernthaler *et al.*, 2002). Aliquots of 2–3 mL were filtered through 0.22-µm polycarbonate filters (GTTP, 25 mm diameter, Millipore), rinsed with milliQ water, air dried and stored at −20 °C until processing. For the characterization of the bacterial community, we used a suite of seven horseradish peroxidase (HRP)-probes: Eub338-II-III for most *Eubacteria* (Daims *et al.*, 1999), Beta42a and Gam42a for *Betaproteobacteria* and *Gammaproteobacteria*, respectively (Manz *et al.*, 1992), Alf968 for *Alphaproteobacteria* (Neef, 1997), CF319 for many clades belonging to the *Bacteroidetes* group (Manz *et al.*, 1996), HGC96a for *Actinobacteria* (Roller *et al.*, 1994) and CYA339 for the photosynthetic cyanobacteria (Nübel *et al.*, 1997). Prior to hybridization, cells were permeabilized with lysozyme (37 °C, 1 h) and achromopeptidase (37 °C, 30 min). Hybridizations were carried out on sections of the filters at 35 °C overnight, and specific hybridization conditions were established by addition of different proportions of formamide to the hybridization buffers (30% for *Actinobacteria*, 45% for *Alphaproteobacteria*, and 55% for the rest of probes). Counterstaining of CARD-FISH filters was done with DAPI

(1 μg mL^{-1}) and a minimum of 10 fields (500–1200 DAPI-stained cells) were manually counted in the epifluorescence microscope.

Enumeration of AAP bacteria

In September and December, samples were additionally collected for the quantification of AAPs. Samples were fixed with 1% paraformaldehyde and 2-mL aliquots were filtered onto 0.22-μm polycarbonate black Nucleopore filters (Whatman). Cells were stained with DAPI and counted using an Olympus BX51TF fluorescence microscope equipped with the Olympus UPlanSApo 100°/1.40 Oil objective as described previously (Mašín *et al.*, 2006). Briefly, three fluorescence images were acquired for each frame: one of the cells stained with DAPI in the blue part of the spectrum, one of the fluorescence of Chl *a* in the red part of the spectrum and finally, both bacteriochlorophyll *a* (BChl *a*)- and Chl *a*-containing organisms were recorded in the infra-red region of the spectrum (> 850 nm). The red image was used to subtract Chl *a*-containing organisms from the infrared counts. For each sample, eight to 10 frames were recorded (> 500 DAPI cells) and analyzed semimanually with the CELL F Software (Olympus) to distinguish between heterotrophic bacteria, picocyanobacteria and AAP bacteria.

Statistical analyses

Differences in physico-chemical and biological variables were analyzed through two-way multivariate analysis of variance (MANOVA). Samplings (July, September and December) and river sections (upstream and downstream of the reservoirs) were considered as the fixed factors Time (T) and Site (S), respectively. To fulfill the normality assumptions of this test, variables were log-transformed when necessary. Correlations between variables were calculated using Pearson's correlation coefficient. These statistical analyses were performed using JMP software (SAS Institute). The ordination of the bacterial groups in relation to environmental data was examined by means of multivariate analyses. Transformed data (log- or square root-transformation) were included in a detrended correspondence analysis to determine the length of the gradient for the first two axes. This indicated that the gradient length was lower than 3 standard deviation units (0.5), so the use of linear ordination techniques was appropriate (ter Braak & Smilauer, 2002). Redundancy analysis (RDA) was applied to find the environmental predictors that best explained the distribution of the different bacterial groups and samples. These analyses were performed using CANOCO version 4.5.

Results

Environmental conditions

Strong differences in physico-chemical and Chl *a* concentrations existed between the sections upstream and downstream of the reservoirs, as well as between the periods examined (Table 1). Water flow was lower from July to September and increased again in December, although differences were not very great (Table 1). Downstream discharge values were on average 2.5–3.8 times greater than those upstream, although they followed similar discharge patterns. July and September were characterized by higher water temperature and conductivity, and much lower dissolved inorganic nitrogen (DIN) concentrations. Temperature ranged from 10 °C in winter to 27.5 °C in summer (July and September) and was relatively constant throughout the studied river stretch at each sampling period. In winter, however, the temperature in the section downstream of the reservoirs was 2–5 °C higher than that of the upstream sites. The reservoirs also caused an abrupt decrease in conductivity that was maintained in downstream waters; this decrease was smaller in the winter period (Table 1). Soluble reactive phosphorus (SRP) and DIN differed significantly among sections and periods (Time × Site effect, *P*-values < 0.0001–0.05). Whereas DIN concentrations were always greater in winter than in summer, and upstream rather than downstream, SRP showed greater upstream concentrations in July and December and the opposite trend in September, but differences were small. The lowest SRP concentrations occurred at the reservoir site (10.4 and 6.5 μg L^{-1} in July and September, respectively).

Suspended matter consistently decreased at the reservoirs by sedimentation, leading to downstream waters of increased transparency. DOC concentrations were often higher in upstream waters, but values were highly variable among sites (Time × Site effect, $P > 0.05$). Chl *a* concentration varied greatly among sites, sections and periods (Time × Site effect, $P < 0.0001$). The lowest values occurred in winter downstream of the reservoirs, and the highest in summer upstream of the reservoirs (Table 1, Fig. 2a). Chl *a* was positively correlated to conductivity (Pearson's $r = 0.60$, $P < 0.0001$, $n = 36$) and to suspended organic matter ($r = 0.58$, $P < 0.0005$, $n = 36$).

Impact of reservoirs on bacterial abundances

Bacteria occurred as free-living cells as well as attached to particles. Abundances of total bacteria ranged from 2.1 to 7.2 × 10^6 cells mL^{-1} and tended to be higher in the sections upstream of the reservoirs (Table 1, Fig. 2b). The greatest differences occurred in July, when the upstream

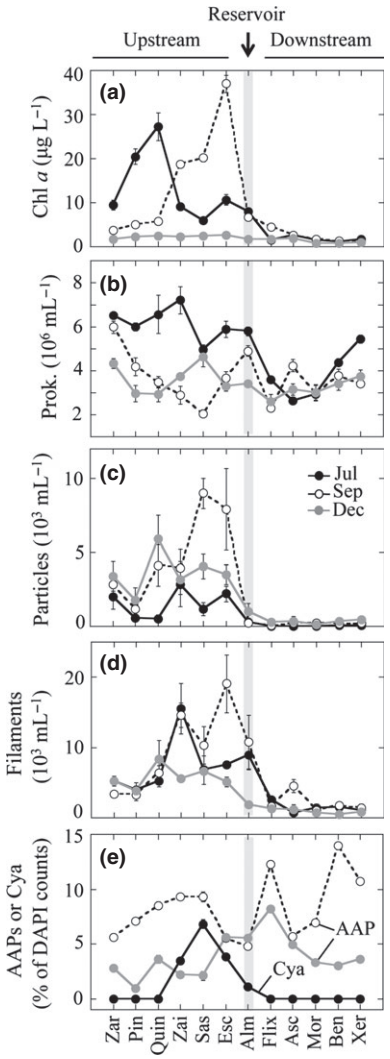

Fig. 2. Temporal and longitudinal patterns in Chl *a* concentration (a), prokaryote abundances (Prok., b), particles densely colonized by bacteria (c) and filamentous bacteria (d) during the three studied periods. The abundance of AAPs (e) was not determined in July, and unicellular picocyanobacteria (Cya, e) showed negligible numbers in September and December. Values are means ± standard errors of triplicate samples. Shaded areas indicate the reservoir site. Site acronyms as in Fig. 1.

abundances were 40% higher than those below the reservoirs. In September and December, however, total abundances could have been underestimated due to high numbers of bacteria colonizing particles (Fig. 2c). We quantified the number of particles colonized by bacteria, as it was not possible to accurately estimate the number of bacteria attached to each particle because not all of them were visible in the 3-D aggregate structure. Much higher numbers of particles colonized by bacteria (sized from 5 to 100 μm in diameter) occurred in upstream waters than in downstream sites. The number of colonized particles increased from July (range 0–2900 particles colonized by bacteria mL^{-1}) to September (up to 9000 particles mL^{-1} at Sástago), and then decreased till December (range 65–5900 particles mL^{-1}). The inverse correlation between the abundance of such bacterial aggregates and total bacterial numbers in upstream sites ($r = -0.62$, $P < 0.01$, $n = 18$) supports the concept that bacteria could actually be more abundant than quantified at some locations, in particular in September and December. Lower concentrations of aggregates (< 450 particles mL^{-1}) occurred downstream of the reservoirs, where most prokaryotes were free-living bacteria. Colonized particle numbers covaried with the concentration of suspended matter over the three periods ($r = 0.76$, 0.83 and 0.92 for July, September and December, respectively, $P < 0.005$, $n = 12$) exhibiting the highest abundances between Quinto and Escatrón (Fig. 2c).

Bacterial size estimates indicated that prokaryotic cells were on average larger in upstream sites (0.077–0.085 μm^3) than downstream (0.066–0.077 μm^3, $P < 0.05$ for the three samplings) and, accordingly, mean bacterial biomass was higher upstream (0.072–0.117 μg C L^{-1}) than after the reservoirs (0.069–0.064 μg C L^{-1}). These differences in cell size detected with the flow cytometer were visually confirmed under the microscope. However, in upstream waters we also found significant numbers of filamentous bacteria (10–20 μm in size, Fig. 2d). This morphotype was nearly absent downstream of the reservoirs, suggesting that the differences in bacterial volume between both sections of the rivers could be even greater, as these large bacteria are not well quantified by flow cytometry. These filaments, most of which hybridized with the probe for *Bacteroidetes* (see below), covaried significantly with increasing Chl *a* concentrations over the three sampling campaigns ($r = 0.69$, 0.89, and 0.88 for July, September and December, respectively, all $P < 0.02$, $n = 12$).

Effect of reservoirs on functional bacterial groups

Besides heterotrophic bacteria, we targeted other two functional groups, the autotrophic picoplanktonic cyanobacteria, and the photoheterotrophic AAPs. Unicellular coccoid cyanobacteria were only observed in July (Fig. 2e), peaking in Sástago (up to 8% of DAPI counts) and showing negligible numbers in most sites. In contrast, filamentous cyanobacteria such as *Planktothrix* sp. and *Geitlerinema* sp. were present in microphytoplankton samples at abundances ranging from 400 to 1900 cells mL^{-1}, and peaking generally at Almatret (M.C. Pérez-Baliero, unpublished data). On the other hand, AAPs, which were quantified in September and December but not in July (Fig. 2e), were

detected in all sites inspected and showed higher abundances in September than in December (Time effect, $P < 0.005$). The differences were not statistically significant between up- and downstream sites, although the highest percentages of AAPs were found downstream from the reservoirs. In September, AAPs ranged from 5% to 14% of total prokaryotes. Values were highest at both Flix and Benifallet, whereas AAPs contributed only to 5% of total prokaryotes at the reservoir site. Lower proportions of AAPs were found in winter, when the amount of these photoheterotrophic bacteria gradually increased from Pina de Ebro (< 1% of total prokaryotes) to the maximum at Flix (8%) and gradually declined afterwards till Xerta.

AAP abundance was negatively related to DIN ($r = -0.73$, $P < 0.0001$, $n = 24$) and positively to total dissolved P ($r = 0.58$, $P < 0.005$, $n = 24$). AAPs in September decreased with higher Chl *a* concentrations ($r = -0.60$, $P < 0.05$, $n = 12$), and in December were negatively related to particulate organic matter ($r = -0.78$, $P < 0.005$, $n = 12$). No one single bacterial group was correlated with AAP relative abundances; however, when upstream and downstream sites were considered separately, a relationship emerged between *Betaproteobacteria* and AAP percentages ($r = 0.86$, $P < 0.0005$, $n = 12$ for upstream sites, and $r = 0.75$, $P < 0.01$, $n = 12$ for downstream sites, details not shown).

Overall, the contribution of autotrophic and photoheterotrophic bacteria to total prokaryote abundance ranged from 0% to 7% for the former and from 1% to 15% for AAPs, indicating that the river contained a largely heterotrophic bacterial community.

Effects of reservoirs on bacterial community composition

The phylogenetic composition of the prokaryotic community was assessed by CARD-FISH (Figs 3 and 4). Most prokaryotic cells hybridized with the eubacterial probes EUB338-II-III (range 91–98% of total DAPI counts, Fig. 3a) indicating a basic absence of archaeal groups. Only in winter downstream waters were there lower numbers of *Bacteria* (66–72% of total DAPI counts). Typically, the sum of cells hybridized with group-specific probes matched well with the total detected *Bacteria* (Fig. 4).

Hybridization with specific probes showed a different composition of the bacterial communities in the upstream and downstream river sections, as well as some temporal variability (Figs 3 and 4). The presence of reservoirs was associated with changes in the dynamics of most groups, yet the most noticeable effects were detected in the summer periods. Overall, bacterial communities were dominated by *Actinobacteria*, which accounted for 22–57% (average 37%) of total DAPI counts (Fig. 3e), and by *Betaproteobacteria* (5–43%, average 22%, of total DAPI counts, Fig. 3c). *Alphaproteobacteria* often showed relatively lower percentages (2–18%, average 10%, Fig. 3b) except in downstream waters in July, where they comprised up to 33% of the total community. Both *Bacteroidetes* (Fig. 3f) and *Gammaproteobacteria* (Fig. 3d) ranged between 1% and 23% of total DAPI counts (average 12% and 6% for *Bacteroidetes* and *Gammaproteobacteria*, respectively) but showed different spatial and temporal dynamics. Nearly all the filamentous bacteria observed (Fig. 2d) hybridized with the probe for *Bacteroidetes*.

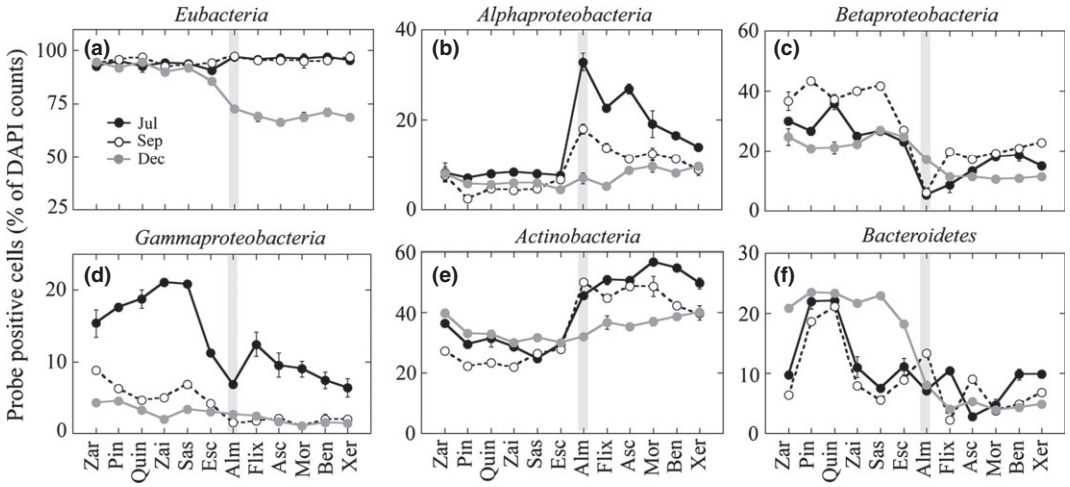

Fig. 3. Temporal and longitudinal dynamics of the relative abundances of the different bacterial groups detected by CARD-FISH probes for *Eubacteria* (a), *Alphaproteobacteria* (b), *Betaproteobacteria* (c), *Gammaproteobacteria* (d), *Actinobacteria* (e) and *Bacteroidetes* (f). Shaded areas indicate the reservoir site, located between upstream and downstream sites. Values are means ± standard errors of triplicates. Site acronyms as in Fig. 1.

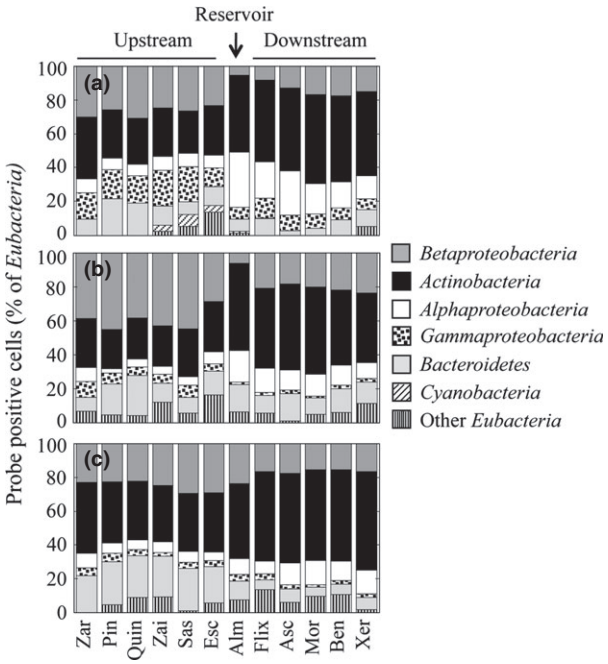

Fig. 4. Composition of the bacterial assemblages present at each site during the three samplings: (a) July, (b) September and (c) December 2011. The relative abundances of each group were calculated with respect to total bacteria. Site acronyms as in Fig. 1.

Despite the high cell abundance of *Actinobacteria*, they likely accounted for only a small proportion of total bacterial biomass due to their small size in comparison with the much larger *Betaproteobacteria* (details not shown). Indeed, the abundance of the latter was positively related with total bacterial biomass over the three sampling campaigns ($r = 0.83$, 0.61, and 0.90 in July, September and December, respectively, $P < 0.05$, $n = 12$) but *Actinobacteria* only showed a positive relationship in December ($r = 0.75$, $P < 0.05$, $n = 12$), when they dominated the whole river section (Fig. 4c).

The presence of the reservoirs clearly influenced the longitudinal distribution of the bacterial groups, and the changes in community along each section were smaller than the changes between up- and downstream reaches (Fig. 3). *Alphaproteobacteria* and *Actinobacteria* strongly increased their relative abundances at the reservoir site and at all downstream sites maintained percentages higher than at upstream sites (Table 2, Fig. 3b and e). *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* showed larger proportions in upstream communities and decreased from the reservoirs onwards (Table 2, Fig. 3c, d and f). On some occasions, however, this decrease started before the reservoirs, at Escatrón, as was the case for *Betaproteobacteria* in September (Fig. 3c) and *Gammaproteobacteria* in September and December (Fig. 3d).

Table 2. Averaged percentages of hybridized cells in the upstream, reservoir and downstream sections considering the three samplings together

	Fraction (%) of total DAPI counts					
	Eub	Alph	Bet	Gam	Act	Bctd
Upstream	93 (0.6)	6 (0.4)	30 (2)	9 (2)	29 (1)	16 (2)
Reservoir	89 (8)	19 (8)	10 (4)	4 (2)	43 (5)	10 (3)
Downstream	87 (3)	13 (2)	15 (1)	4 (1)	45 (2)	8 (1)

Values are means ± standard errors of the sites considered ($n = 18$ for upstream, $n = 3$ for reservoir, and $n = 15$ for downstream sites, respectively).
Eub, *Eubacteria*; Alph, *Alphaproteobacteria*; Bet, *Betaproteobacteria*; Gam, *Gammaproteobacteria*; Act, *Actinobacteria*; Bctd, *Bacteroidetes*.

The variations between upstream and downstream sections further depended on the period considered (Time × Site effect, P values < 0.05–0.0001 for all groups except *Betaproteobacteria*). *Alphaproteobacteria* showed the largest differences among sections in July (an average 2.5 increase from upstream to downstream sites), *Gammaproteobacteria* decreased 70% after the reservoirs in September, and *Bacteroidetes* decreased by a factor of 4.9 in December. Only *Betaproteobacteria* presented a similar magnitude of change among sections regardless of the month considered (47–53% mean decrease between up- and downstream sites, Time × Site effect, $P > 0.05$).

Summer upstream communities showed a greater contribution of *Betaproteobacteria* (23–43% of total prokaryotes) and *Gammaproteobacteria* (5–21%), whereas downstream assemblages were largely dominated by *Actinobacteria* (40–51%) and presented higher proportions of *Alphaproteobacteria* (9–22%). Instead, *Bacteroidetes* only showed significant differences between the two sections in winter ($P < 0.05$), although in July and September the greatest abundances were reached in Pina and Quinto, both upstream sites (Fig. 3f).

Most bacterial groups covaried significantly with each other. The relative abundances of *Alphaproteobacteria* and *Actinobacteria* correlated significantly ($r = 0.84$, $P < 0.0001$, $n = 36$), as did *Betaproteobacteria* with *Bacteroidetes* ($r = 0.47$, $P < 0.005$, $n = 36$) and with *Gammaproteobacteria* ($r = 0.36$, $P < 0.05$, $n = 36$). On the other hand, *Betaproteobacteria* were inversely correlated to both *Actinobacteria* ($r = 0.69$, $P < 0.0001$, $n = 36$) and *Alphaproteobacteria* ($r = 0.70$, $P < 0.0001$, $n = 36$).

Distribution of bacterial assemblages in relation to environmental variables

To summarize the environmental variables influencing the composition of the bacterial communities, a RDA was performed with all bacterial taxa except *Cyanobacteria*

(Fig. 5). In the RDA model, temperature, conductivity, and DIN and DOC concentrations were the environmental variables that statistically best explained the variations in the distribution of the bacterial groups among samples. The explanatory power of the model did not significantly improve when phosphorus was included with the environmental variables, and none of the phosphorus sources (SRP, total dissolved P, dissolved organic P) was selected among the best environmental variables.

The RDA model accounted for 64% of the variation in bacterial community composition data. The first two axes explained up to 45% (axis 1) and 14% (axis 2) of the variation. The variables that correlated most strongly with these axes were conductivity, DIN, and temperature. *Alphaproteobacteria* and *Actinobacteria* were associated with lower conductivity and DIN concentrations (typical of downstream sites, Fig. 5a). *Gammaproteobacteria* occurrence was correlated with higher temperatures, DOC, and lower DIN concentrations. *Betaproteobacteria* was associated to higher conductivities and DOC, and *Bacteroidetes* to higher conductivities and higher nitrogen.

Pairwise correlation analyses supported these observations (Table 3). For instance, the relative abundance of all bacterial groups was related to conductivity and nitrate either positively (*Beta- and Gammaproteobacteria* and *Bacteroidetes*) or negatively (*Actinobacteria* and *Alphaproteobacteria*). Abundances of *Beta-* and *Gammaproteobacteria* were also associated with higher concentrations of suspended solids, whereas *Bacteroidetes* only in December. Significant correlations between groups and DOC or Chl *a* were more evident in December than in summer (Table 3).

These patterns resulted in a clear distribution of the different samples regarding site (upstream vs. downstream) and period of the year (Fig. 5b). The first axis of the RDA separated the bacterioplankton communities characteristic of downstream sites (right part of the graph) from those upstream of the reservoirs. All upstream sites clustered

Table 3. Correlation coefficients for significant ($P < 0.05$) relationships between group relative abundances of *Eubacteria* (Eub), *Alphaproteobacteria* (Alph), *Betaproteobacteria* (Bet), *Gammaproteobacteria* (Gam), *Actinobacteria* (Act), *Bacteroidetes* (Bctd), and several environmental variables

	Eub	Alph	Bet	Gam	Actino	Bctd
July						
Conductivity	−0.821	−0.940	0.824	0.848	−0.941	ns
Nitrate	−0.869	−0.948	0.855	0.799	−0.885	ns
SRP	ns	−0.616	0.746	ns	ns	ns
DOC	ns	ns	ns	0.750	−0.776	ns
Chl *a*	ns	−0.642	ns	0.650	−0.795	0.641
Suspended matter	−0.870	−0.758	0.611	0.577	−0.714	ns
September						
Conductivity	ns	−0.904	0.869	ns	−0.966	ns
Nitrate	−0.581	−0.876	0.909	0.891	−0.933	ns
SRP	ns	ns	ns	ns	ns	ns
DOC	0.687	ns	ns	ns	ns	ns
Chl *a*	ns	ns	ns	ns	−0.579	ns
Suspended matter	ns	ns	0.586	0.850	−0.769	ns
December						
Conductivity	0.948	−0.638	0.910	0.676	−0.637	0.964
Nitrate	0.895	−0.669	0.966	0.663	−0.736	0.941
SRP	0.799	ns	0.756	ns	ns	0.800
DOC	0.745	−0.679	0.712	0.694	−0.593	0.766
Chl *a*	0.699	−0.810	0.797	0.739	−0.778	0.800
Suspended matter	0.970	−0.577	0.914	0.780	ns	0.986

ns, Not significant results, $P > 0.05$, $n = 12$ for all cases.

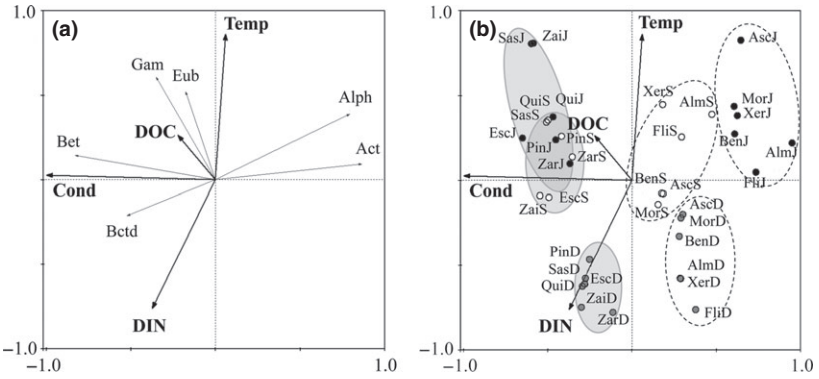

Fig. 5. RDA biplots. (a) Different bacterial groups [*Betaproteobacteria* (Bet), *Alphaproteobacteria* (Alph), *Gammaproteobacteria* (Gam), *Bacteroidetes* (Bctd), *Actinobacteria* (Act) and *Eubacteria* (Eub)] in relation to the gradient of the strongest environmental variables: DIN, DOC, Temp (temperature) and Cond (Conductivity). (b) Different samples in relation to the strongest environmental variables. Axis 1 and 2 explain 45% and 13% of the variance, respectively. Site names (as in Fig. 1) contain the season (J: July, black dots; S: September, open dots; D: December, gray dots). Upstream sites (gray circles) and downstream sites (dashed circles) are also indicated.

together and were associated with high conductivity, DIN and DOC concentrations, and downstream sites grouped together towards the opposite conditions. The reservoir site (Almatret) was included in the analysis and it often grouped with the downstream sites. The second RDA axis was mostly related to temperature and DIN and separated summer samples (upper part of the graph) from winter samples. Finally, the magnitude of the differences between upstream and downstream communities also varied depending on the month considered, and mainly due to differences in the conductivity values, being larger in July, followed by September and December.

Discussion

River regulation through damming has been shown to affect the water physico-chemical conditions, the sediments transported, and the composition of phytoplankton in the sections before and after the dams in major rivers (Roura, 2004; Bi *et al.*, 2010; Dang *et al.*, 2010). Waters upstream of the Ebro River reservoirs were characterized by lower velocities, higher conductivity and greater concentrations of particulate matter, DIN, DOC and Chl *a* in comparison with downstream sites, as previously reported for this system (Roura, 2004; Sabater *et al.*, 2008; Batalla & Vericat, 2011). These different characteristics between up- and downstream waters have been attributed to changes in water residence time (Sánchez-Cabeza & Pujol, 1999), as well as to not fully understood processes within the reservoirs (Roura, 2004; Batalla & Vericat, 2011). The presence of hypolimnetic dam outlets may also influence the magnitude of these differences over seasons, depending on the degree of mixing between the water from the river and that of the reservoir (Roura, 2004).

Our study shows evidence that these large impoundments also produce considerable effects in bacterial communities. These effects concern bacterial size, the occurrence of free- and particle-attached bacteria, as well as the longitudinal and temporal patterns of community composition. Thus far, the only study reporting significant effects of damming on bacterioplankton between river sections is that of Dumestre *et al.* (2001), where different bacterial populations (identified as DGGE fingerprints) occurred between sites upstream and downstream of an equatorial reservoir. Given that different bacterial groups display diverse functional roles (e.g. Cottrell & Kirchman, 2000; Kirchman *et al.*, 2004), the occurring changes in bacterial community structure should have implications for biogeochemical processes in the river.

Bacteria attached to particles may constitute as much as 90% of total bacterial numbers and production in riverine and estuarine systems (Bell & Albright, 1981; Crump & Baross, 2000). In the Ebro, much higher numbers of particles colonized by bacteria were found at upstream sites than in downstream waters, presumably due to sedimentation of suspended particles. Hence, the reservoirs provoked a shift in the proportion of attached vs. free-living bacteria, so that downstream bacterial communities mostly comprised free-living cells, as observed elsewhere (Kondratieff & Simmons, 1985). This is relevant as free-living and particle-attached bacteria are known to differ both phylogenetically and functionally across ecosystems (Karner & Herndl, 1992; DeLong *et al.*, 1993; Besemer *et al.*, 2005). For example, whereas groups such as *Bacteroidetes*, *Gammaproteobacteria* and *Betaproteobacteria* have often been found associated to particles, *Alphaproteobacteria* usually comprise free-living bacteria (DeLong *et al.*, 1993; Crump *et al.*, 1999; Böckelmann *et al.*, 2000). It was not obvious which particular group dominated the bacterial aggregates in our study, although the abundance of particles was positively correlated with *Beta-*, *Gammaproteobacteria* or *Bacteroidetes*. In contrast, *Alphaproteobacteria* and *Actinobacteria* were negatively correlated to particles, in accordance with their known dominant free-living lifestyle. Several studies have proved that bacteria associated with aggregates exhibit higher ectoenzymatic hydrolysis rates, and sometimes they can account for most of bacterial production (Crump *et al.*, 1998; Crump & Baross, 2000). The high abundance of particle-attached bacteria in the section upstream of the reservoirs in the Ebro may have importance for the cycling and flux of elements and energy, yet the fine phylogenetic affiliation of these aggregate-associated bacteria and their biogeochemical role in the ecosystem are still unknown.

Overall, upstream water bacteria were larger than downstream cells and the presence of filamentous bacteria was widespread upstream, where up to six times more filaments occurred in comparison to downstream waters. Intense grazing by protists triggers the development of large and filamentous (non-edible) morphotypes of varying phylogenetic affiliations as shown in mesocosm and field studies (Pernthaler *et al.*, 1997, 2004; Šimek *et al.*, 1999). Nearly all filaments in our study were identified as *Bacteroidetes* in accordance with the reported ability of this group of organisms to form filaments under high grazing pressure (Pernthaler *et al.*, 2004; Salcher *et al.*, 2005). Different authors have also shown that protozoa, in particular heterotrophic flagellates, can control bacterial production, abundances and community composition in rivers and reservoirs (Carlough & Meyer, 1991; Šimek *et al.*, 1999; Servais *et al.*, 2000). Should the abundance of these filaments in the Ebro River be related to bacterivory, it would indicate a higher grazing pressure on upstream rather than on downstream communities (and

consequently, differences in the amount of carbon flowing to higher trophic levels). However, the morphology of very tiny coccoid cells of the *Actinobacteria* group has also been considered a defense strategy against bacterivory (Pernthaler *et al.*, 2001; Jezbera *et al.*, 2006), and their increase downstream of the reservoirs might indicate an enhanced grazing pressure on downstream bacteria. Finally, prey selectivity might in turn be affected by nutrient availability (Šimek *et al.*, 2003; Jezbera *et al.*, 2006), so it is likely that the changes in bacterial community composition observed between sections might be partially explained by different top-down controlling factors.

Bacterial community composition in terms of functional groups

AAP bacteria were observed in the Ebro in what is the first quantification in riverine systems after Mašín *et al.* (2008), who found minimal numbers of these photoheterotrophs (< 1% of total bacteria) in two low altitude rivers. In the Ebro, AAPs ranged from < 1% to 14% of DAPI-positive cells and were more abundant in summer than in winter. These percentages fall within the range previously reported for other freshwater systems (Mašín *et al.*, 2008, 2012). Even though this group is known to be widely distributed across different aquatic environments, very little is known about their ecological preferences. In a pioneering paper, Kolber *et al.* (2000) speculated that the capacity to harvest light could be beneficial in nutrient-poor environments, yet diverse studies, most of them from marine environments, have found greater AAP abundances in mesotrophic and eutrophic environments (e.g. Jiao *et al.*, 2007; Hojerová *et al.*, 2011). Freshwater environments have been under-studied compared with marine sites for the enumeration of AAPs and so far most studies have been carried out in lakes. Although a tendency for higher abundances towards more oligotrophic conditions was first documented (Mašín *et al.*, 2008), the opposite trend has been found recently (Mašín *et al.*, 2012). Thus, the relationship between AAP abundance and lake trophic status remains unsolved and even less is known about this relationship in riverine systems. In the Ebro River, AAPs were more abundant in downstream waters, yet their values greatly varied among individual sites and AAP abundances and proportions could not be related to the measured environmental variables, indicating that other environmental factors likely determined the survival of particular AAP groups. Light attenuation could be among those, as it is remarkably higher in the section upstream of the reservoirs and this could affect the occurrence of these groups in comparison with the more transparent downstream waters. Top-down factors, such a bacterivory, could also influence AAP numbers, given that these bacteria have been shown to be fast-growing cells subjected to high grazing pressure (Ferrera *et al.*, 2011).

AAPs in freshwater ecosystems have been mainly associated to *Alpha-*, *Gamma-* and *Betaproteobacteria* (Salka *et al.*, 2011), the last often being dominant. Although we did not find a general correlation between AAP abundances and that of any other groups, when upstream and downstream data were considered separately a relationship emerged between *Betaproteobacteria* and AAP relative abundances. *Betaproteobacteria* are phylogenetically diverse, so this might suggest that AAP from up- and downstream waters belong to different *Betaproteobacteria* types adapted to different environmental conditions. Further studies on the abundance, function and phylogenetic composition of these bacteria are required to understand their role in freshwater ecosystems.

Small unicellular cyanobacteria were only detectable with the CARD-FISH probe in July in upstream waters, and in most sites and seasons they constituted a negligible proportion of prokaryotic communities. Picocyanobacteria are widely distributed in marine and freshwater environments (Stockner *et al.*, 2000; Newton *et al.*, 2011) and can dominate phytoplankton communities in rivers as well (Sorokin & Sorokin, 1996; Portillo *et al.*, 2012). However, Sabater & Muñoz (1990) did not find planktonic chroococcoid cyanobacteria when studying the dynamics of phytoplankton over a period of 1 year in the Ebro River. Hence, it seems that picocyanobacteria in this system are not major contributors to total primary production, and that the majority of prokaryotes very likely display a heterotrophic lifestyle.

Bacterial community composition in terms of phylogenetic structure

The influence of reservoirs on bacteria was reflected not only in changes in their abundances, morphotypes, cell sizes and the proportion of attached vs. free-living bacteria, but also in the relative contribution of the different groups considered within the bacterial assemblages.

The use of six oligonucleotide probes targeting four phyla (*Actinobacteria*, *Bacteroidetes*, *Cyanobacteria and Proteobacteria)* and three classes within the last phylum (*Alpha-*, *Beta-* and *Gammaproteobacteria*), identified 84–100% of the bacterial community in the Ebro River, providing the first characterization of the bacterioplankton in this system and one of the few for large rivers. Most of the taxonomic groups enumerated have been shown to be prevalent in freshwater ecosystems (Kenzaka *et al.*, 1998; Kirchman *et al.*, 2004; Fortunato *et al.*, 2012). In particular, the high percentages of *Actinobacteria* and *Betaproteobacteria* in our samples are in agreement with the well

documented numerical dominance of these two groups in freshwater ecosystems (Stepanauskas *et al.*, 2003; Newton *et al.*, 2011; Warkentin *et al.*, 2011). *Bacteroidetes*, *Alpha-* and *Gammaproteobacteria*, less abundant on average, also showed proportions similar to those described previously (Kirchman, 2002; Stepanauskas *et al.*, 2003).

The reservoirs generated a clear shift in bacterial communities, yet their composition was also affected by the period considered. Total detected *Bacteria* remained fairly constant across the entire transect in July and September, but showed an average 35% decrease in downstream sites in December. This might indicate that bacteria in these sites and period were less active and thus were not visually detected by our probe. Groups such as *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* showed higher proportions in upstream than in downstream waters, whereas *Actinobacteria* and *Alphaproteobacteria* increased sharply at the reservoir site and maintained higher percentages in downstream sites. In winter, however, these patterns homogenized and only *Betaproteobacteria* and *Bacteroidetes* maintained significantly higher percentages upstream than downstream.

Factors determining the composition of bacterial communities

The RDA analysis indicated that the temporal segregation of the bacterial communities was mainly driven by changes in temperature, and also by DIN concentrations, which were higher in December. As such, winter communities separated from those of July and September. On the other hand, the clearly differentiated upstream and downstream assemblages co-occurred with strong changes in conductivity, DIN and, to a lesser extent, DOC. The higher variability associated with the longitudinal gradient, indicates that the spatial differences generated by these reservoirs were more important than the temporal variations. It is important to note, however, that the long water retention time within the reservoir system (1–5 months, Roura, 2004) might play a relevant role in shaping these differences by allowing new communities to become established, as reported elsewhere (Mašín *et al.*, 2003). Supporting this idea, the reservoir-driven changes were largest in July and smallest in December, when water characteristics along the river start to homogenize because of the greater discharge and shorter water residence times (Artigas *et al.*, 2012). Later in the season, however, when higher rainfall occurs and the patterns of distribution of variables such as conductivity and nitrate are homogenized or even reversed [*Confederación Hidrográfica del Ebro* (CHE), unpublished data], we would expect greater similarity among bacterial assemblages along the entire reach.

Betaproteobacteria, *Gammaproteobacteria* and *Bacteroidetes* were positively related to conductivity, whereas *Actinobacteria* and *Alphaproteobacteria* showed negative correlations to this variable. High conductivity values upstream of the reservoirs are attributed mostly to the large inputs of chlorides and sulfates weathered from tertiary substrata (Torrecilla *et al.*, 2005), and their decrease after the reservoirs is associated to dilution by tributaries and to biogeochemical processes taking place in the reservoirs (Roura, 2004). Although large gradients in salt concentration has been shown to be a major environmental determinant for bacterial community composition across diverse environments (Kirchman *et al.*, 2005; Lozupone & Knight, 2007; Fortunato & Crump, 2011), the observed relationship between riverine bacterial groups and conductivity (this work, Rubin & Leff, 2007) may not be due directly to mineral composition or the abundance of total salts.

DIN also appeared to have an influence in structuring up- and downstream bacterial communities. In freshwater systems, a relationship between specific phylotypes and DIN has sometimes been observed. Gao *et al.* (2005), for example, found that *Beta-* and *Gammaproteobacteria* from stream biofilms tended to be most abundant at sites with high DOC and nitrate concentrations, whereas *Alphaproteobacteria* were more abundant in environments with low DOC and nitrate load, in accordance with our findings. In contrast, none of the chemically different forms of phosphorus influenced the abundance or composition of bacterial communities, despite the previous observations that suggested that summertime plankton communities were strongly limited by P in upstream sites (Artigas *et al.*, 2012). In view of our results, it would appear that in this system, nitrate was a more important factor structuring bacterial communities than phosphorus.

DOC also covaried with the presence of some bacterial groups, particularly with *Gammaproteobacteria*, their abundances increasing with greater DOC concentrations. However, DOC concentration alone is a poor predictor of bacterial diversity, as different groups respond differently depending on DOM quality and lability (e.g. Pérez & Sommaruga, 2006). *Gammaproteobacteria* often comprises large and fast-growing cells that respond quickly to increases in labile DOM (e.g. Pinhassi & Berman, 2003). Algal lysates are presumably rich in labile organic compounds and different bacterial phylotypes are known to prefer exudates from certain phytoplankton species (Sarmento & Gasol, 2012). Hence, the decrease in gammaproteobacterial numbers from July to December, and from above- to downstream waters, might be partially associated to changes in the availability or origin of DOC derived from seasonally changing phytoplankton

assemblages. Downstream bacteria might in turn rely on DOM of macrophyte origin. In this river section the low abundance of phytoplankton is balanced by the mass development of macrophytes. Should downstream bacterial communities depend to some extent on plant primary production, macrophyte loss in winter might explain the decrease in the number of positively hybridized *Bacteria* (which were also related to DOC in the RDA analysis) found in downstream winter waters. Indeed, submersed macrophytes were shown to be a key factor structuring bacterial community composition in a subtropical lake in China (Wu *et al.*, 2007). In any case, riverine bacterioplankton carbon demand is known to be dependent on carbon sources other than primary production, such as terrestrial inputs (Kirschner & Velimirov, 1997), and hence correlations between the whole DOC pool and specific bacterial groups should not always be expected.

Overall, the contrasting ecological preferences of different bacterial groups translated into negative correlations between taxa from up- and downstream sites. In particular, the antagonistic relationship consistently observed in our samples between the two dominant clades *Betaproteobacteria* and *Actinobacteria* has been also reported by other authors (Glöckner *et al.*, 2000; Pérez & Sommaruga, 2006), who suggested that the two groups inhabit separate functional niches defined by DOM quality, water temperature regimes, and grazing pressure (Pérez & Sommaruga, 2006, 2011). In the Ebro, groups harboring larger and presumably fast-growing bacteria such as *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were related to upstream waters of elevated concentrations of nutrients, DOC and suspended matter. This is in accordance with the classification of soil *Betaproteobacteria* and *Bacteroidetes* as *r*-strategists (Fierer *et al.*, 2007), i.e. taxa able to grow rapidly under conditions of high resource availability. Other groups with typically smaller cell sizes, such as *Actinobacteria* and *Alphaproteobacteria*, presumably more efficient at lower nutrient and DOC concentrations, seem to prefer more oligotrophic and/or colder conditions (Jürgens *et al.*, 1999; Pinhassi & Berman, 2003; Šimek *et al.*, 2006), potentially explaining their dominance in downstream sites and in winter. In any case, other structuring factors not considered here, such as water retention time, viral lysis, bacterivory or the presence of submerged macrophytes, could certainly play a role in shaping the bacterial communities of the Ebro River.

In summary, our results suggest that river regulation has a significant influence on the phylogenetic composition of riverine bacterial assemblages. Reservoirs in the river cause an abrupt interruption in most physico-chemical parameters, leading to a niche partition, with the development of clearly differentiated bacterial assemblages adapted to such contrasting conditions. Variables such as temperature, conductivity and DIN had an impact on the abundance of major phylogenetic groups, supporting the idea that these major taxonomic groups may share some ecological traits, as suggested elsewhere (Fierer *et al.*, 2007; Philippot *et al.*, 2009, 2010). In any case, owing to the limitations of the current methods for detecting the entire diversity of microbial communities, it is likely that the patterns observed mostly reflect variations in the dominant taxa and that new ecological trends would certainly emerge if bacterial taxa were targeted at a finer resolution. Future studies using high throughput pyrosequencing of PCR-amplified 16S rRNA genes are necessary for a deeper understanding of the bacterial diversity, the factors explaining their temporal and spatial dynamics along the river, and the potential biological and biogeochemical consequences of river regulation.

Acknowledgements

This study was funded by the Confederación Hidrográfica del Ebro. Additional funds were provided by the project SCARCE (Consolider-Ingenio 2010, CSD2009-00065). We acknowledge the support of Concha Duran throughout the study, and the help from Elisabet Tornes and Carmen Gutiérrez in the field and laboratory.

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