Environ Monit Assess (2012) 184:471–485
DOI 10.1007/s10661-011-1981-2

Impact of water quality on bacterioplankton assemblage along Cértima River Basin (central western Portugal) assessed by PCR–DGGE and multivariate analysis

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Received: 16 April 2010 / Accepted: 24 February 2011 / Published online: 24 March 2011

Abstract The information on bacterial community composition (BCC) in Portuguese water bodies is very scarce. Cértima River (central western Portugal) is known to have high levels of pollution, namely organic. In the present work, the BCC from a set of 16 water samples collected from Cértima River Basin and its main tributaries was characterized using 16S rDNA–denaturing gradient gel electrophoresis, a culture-independent molecular approach. Molecular data were related to environmental parameters through multivariate analysis to investigate potential impact of water pollution along the river. Principal component analysis using environmental data showed a water quality gradient from more pristine waters (at the mountain tributaries) to waters with increasingly eutrophic potential (such as Fermentelos Lake). This gradient was mainly defined by factors such as organic and inorganic nutrient sources, electrical conductivity, hydrogen carbonate concentration, and pH. Molecular results showed variations in BCC along Cértima River Basin but in the main river section, a *Bacteroidetes* phylotype (*Flavobacterium* sp.) proved to be dominant throughout the river course. Multivariate analysis suggests that spatial variation of BCC along the Cértima River Basin depended mainly on parameters such as Chl *a*, total suspended solid (TSS), total organic carbon, electrical conductivity, and HCO_3^- levels. *Bacteroidetes* phylotypes were all related to higher electrical conductivity and HCO_3^- levels although some of these were also correlated with high SO_4^{2-} and others with high soluble reactive phosphorus, nitrate, TN, and Kjeld-N levels. The *Gammaproteobacteria* occurrence was correlated with high SO_4^{2-} levels. One of the *Betaproteobacteria* phylotypes showed to correlate with low redox potential (E_h) and high temperature, pH, TSS, and Chl *a* levels while another one showed a negative correlation with Chl *a* values.

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Keywords Riverine bacterioplankton diversity · 16S rDNA–DGGE · Organic pollution · Nutrients · CCA analysis

Introduction

The impact of natural (climatic change) and anthropogenic (industrial and domestic effluents) stressors over freshwaters has led to the increase of water pollution worldwide and the enhancement of the eutrophication process (Ducharne et al. 2007; Tong et al. 2007). This has major impacts on the aquatic communities such as the bacterioplankton (Paerl et al. 2003; Hall and Cotner 2007; Zeng et al. 2009), which may endanger the quality and safety of water used for human purposes (de Figueiredo et al. 2004; Zaitlin and Watson 2006).

Cértima River (central western Portugal) is an excellent case study. In spite of the effort for implementation of wastewater management plans, Cértima River is still suffering from considerable pollution levels due to inputs from domestic wastewater, runoffs from agriculture fertilizers, and effluents from industry and animal farming (Cerqueira et al. 2005; Ferreira 2007). The presence of high levels of contamination, not only from organic and inorganic sources of nutrients but also from pesticides used in agriculture and heavy metals from industrial activity, has been reported over the last two decades (Rino and Gil 1987; Calado 1990; Calado et al. 1991; Pereira 1999; Almeida 2001; Teles et al. 2007). Although the Cértima River Basin has been a topic for important investigation on phytoplankton occurrence over the past 20 years (Rino and Gil 1987; Almeida 2001; Calado et al. 2005), studies on its bacterioplankton diversity are very scarce and focused on Fermentelos Lake (located downstream Cértima River; de Figueiredo et al. 2007, 2010).

The main purpose of the present study was to assess the bacterioplankton diversity shifts along the Cértima River Basin, using the culture-independent molecular methodology 16S rDNA PCR–denaturing gradient gel electrophoresis (DGGE; Muyzer et al. 1993; Lyautey et al. 2005). The impact of the water physical and chemical parameters on spatial BCC diversity was investigated through multivariate analysis.

Materials and methods

Sampling

The Cértima River is a relatively small river—approximately 43 km long—and a tributary of Águeda River. The river source is at the Buçaco (or Bussaco) Mountain (central Portugal), and the river mouth is an enlargement area (Fermentelos Lake) of about 5 km^2. The main tributaries include Serra and Levira Rivers and Ribeira do Pano. The Cértima River Basin is markedly impacted by agriculture but also industrial activity and domestic discharges (Rino and Gil 1987; Cerqueira et al. 2005). Geomorphologically, this basin shows heterogeneity between sandy lowlands with altitudes ranging from 8 to 70 m and highlands (on the eastern margin of the main river course) where altitudes are always above 200 m and characterized by marked relief and cliffs (Pinho et al. 1988). The sampling sites were determined based on previous published studies about the Cértima River Basin (Cerqueira et al. 2005). Their codes, location, and description are presented in Fig. 1 and Table 1. In late May 2007, during three consecutive days, the samples were taken sub-superficially at about 1 m from the shore using sterile bottles and assuring that sediment was not collected. Samples were placed at 4°C in the dark until further treatment within 12 h after collection. Table 1 shows the results for hydrogeochemical variables (Ferreira 2007) which were determined according to standard procedures (APHA 1995).

DNA extraction and PCR amplification of bacterial 16S rDNA fragments

Total DNA was extracted from water samples after filtering 100 to 200 mL (depending on the water transparency) through 0.22 μm polycarbonate sterile filters; cells and particles retained on the filter were resuspended in 2 mL of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and then centrifuged. After resuspension in 200 μL

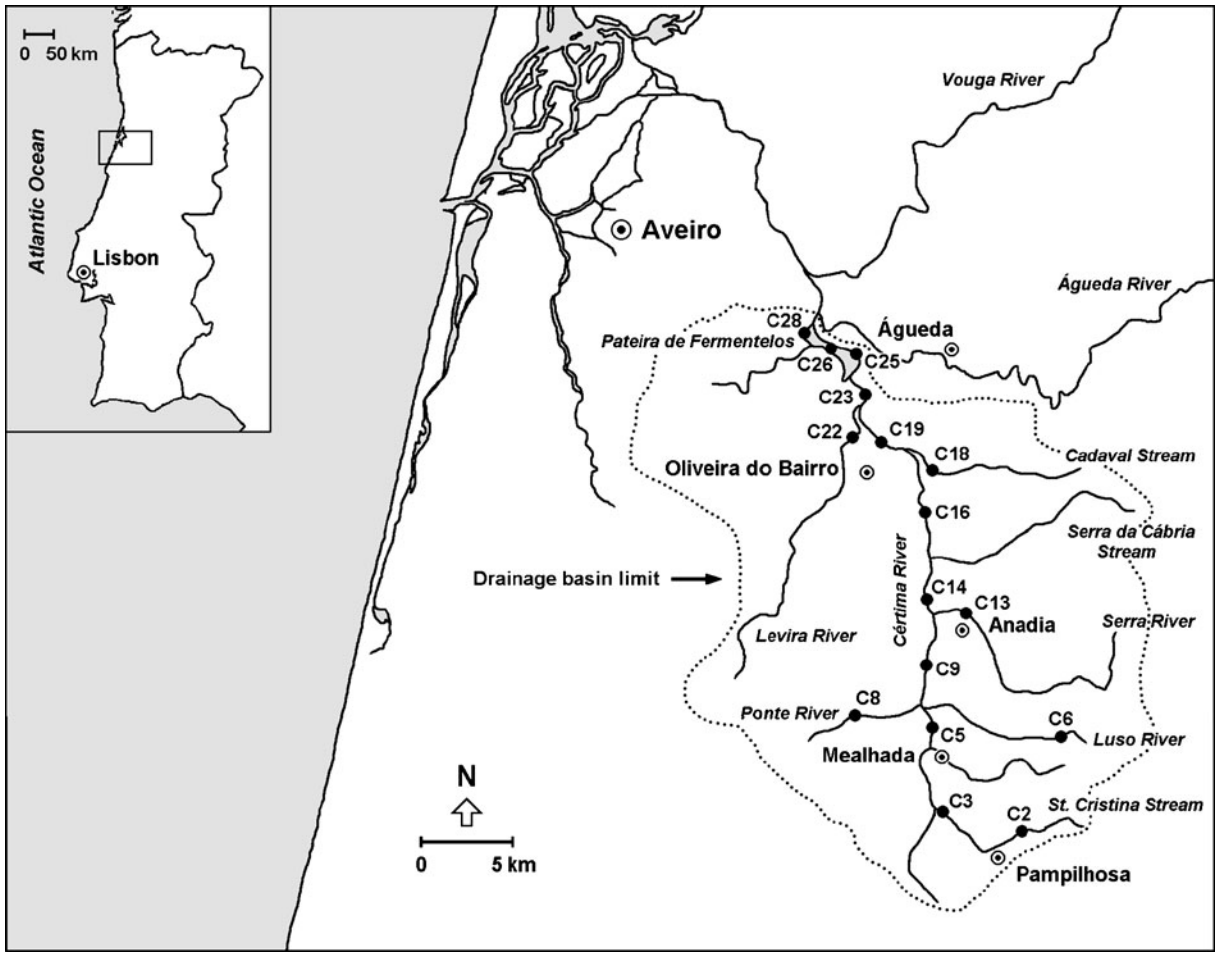

Fig. 1 Location of sampling sites along the Cértima River Basin (see sample codes in Table 1)

of TE, lysozyme was added and incubation was performed at 37°C for 1 h. The subsequent DNA extraction and purification steps were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). DNA was finally suspended in TE buffer and stored at −20°C. The *16S rRNA* gene fragments for DGGE analysis were amplified using the universal primers for bacteria 338F-GC/518R (Muyzer et al. 1993). Primers were synthesized by STABVida (Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with 50 μL reaction mixtures each containing 3 mM $MgCl_2$, 200 μM of each nucleotide, 1× PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide, 15 pmol of each primer, 1 U of *Taq* DNA polymerase, and 50–200 ng template DNA. The PCR program had an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 s at 92°C, 30 s at 55°C, and 30 s at 72°C and a final extension step at 72°C for 30 min. Negative control reactions without template DNA were performed simultaneously. The quality of the resulting PCR amplicons was confirmed by electrophoresis in 1.5% agarose gels using a molecular weight marker (GeneRuler™ 1 kb DNA ladder), after staining with ethidium bromide and visualization on a UV transilluminator.

Denaturing gradient gel electrophoresis

PCR products were analyzed through DGGE, using a 35–60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized

Table 1 Characterization and environmental data recorded for Cértima River Basin samples in May 2007 (where maxima and minima values are in bold)

Sample code	Sampling site location	Original water body	pH	Water temp (°C)	Cond. (µS cm^{-1})	E_h	Chl *a* (µg L^{-1})	O_2 sat. (%)	Diss. O_2 (mg L^{-1})	TOC	BOD_5	TSS	SO_4^{2-}	HCO_3^-	NO_2^- -N	NO_3^- -N	NH_4^+ -N	Kjeld -N	TN	Org -N	SRP
C2	Póvoa do Loureiro (Vacariça, Mealhada)	St. Cristina stream	7.45	18.8	175	352	3.0	91	8.5	2.3	0.75	1.2	**14**	48	<0.1	0.79	**0.04**	**0.29**	1.08	0.25	<0.01
C3	Viadores (Pampilhosa, Mealhada)	Canedo stream	7.42	20.0	441	324	5.1	94	8.6	5.6	2.50	7.0	40	111	<0.1	2.37	1.09	1.67	4.04	0.58	<0.01
C5	Lagoa Seca (Antes, Mealhada)	Cértima River	7.39	20.5	**690**	343	3.4	66	5.9	8.1	**12.20**	14.8	40	**230**	<0.1	11.3	**10.45**	**12.38**	**23.68**	**1.93**	**1.30**
C6	Várzeas (Luso, Mealhada)	Luso River	7.20	**16.7**	**132**	361	6.5	98	9.6	**1.6**	**0.67**	**1.0**	14	**25**	<0.1	**0.61**	0.07	0.30	**0.91**	**0.23**	0.03
C8	Ponte (Ventosa do Bairro, Mealhada)	Ponte River	7.39	17.0	317	**394**	**2.2**	91	8.8	6.7	0.91	4.3	26	93	<0.1	6.09	0.07	0.69	6.78	0.62	<0.01
C9	Curia (Tamengos, Anadia)	Cértima River	7.50	19.5	608	362	**2.2**	78	7.2	5.0	5.96	5.9	55	189	<0.1	4.88	1.50	2.16	7.04	0.66	0.26
C13	Famalicão (Arcos, Anadia)	Serra River	7.04	18.7	206	299	**2.2**	126	11.8	2.9	2.14	2.1	20	62	<0.1	1.88	0.68	1.30	3.18	0.62	0.07
C14	Malaposta (Mogofores, Anadia)	Cértima River	7.57	18.6	622	326	11.7	109	10.2	4.1	4.16	5.6	94	185	<0.1	4.72	1.68	2.47	7.19	0.78	0.53
C16	São João da Azenha (Sangalhos, Anadia)	Cértima River	7.62	20.9	650	335	12.7	114	10.2	4.7	2.73	12.4	**116**	170	<0.1	3.63	0.61	1.32	4.95	0.71	<0.01
C18	Landiosa (Aguada de Baixo, Águeda)	Cadaval stream	**6.44**	17.7	220	347	3.7	87	8.3	2.5	5.34	6.3	15	30	<0.1	4.60	1.28	1.83	6.43	0.54	<0.01
C19	Repolão (Barrô, Águeda)	Cértima River	7.38	18.9	561	324	6.7	80	7.5	3.1	3.22	12.1	98	141	<0.1	3.32	0.25	0.78	4.10	0.53	<0.01
C22	Amoreira do Repolão (Oliveira do Bairro)	Levira River	7.52	17.0	519	363	3.7	69	6.6	4.8	2.18	7.8	65	114	0.1	**6.36**	0.34	0.94	7.40	0.60	<0.01
C23	Perrães (Espinhel, Águeda)	Cértima River	7.34	18.4	541	**295**	5.8	**58**	**5.4**	4.8	5.05	18.5	85	128	<0.1	4.22	0.29	0.94	5.16	0.64	<0.01
C25	Eastern lake (Óis da Ribeira, Águeda)	Fermentelos Lake	**8.77**	**23.7**	509	318	29.6	**165**	**13.9**	**9.3**	7.37	**40.0**	73	111	<0.1	2.48	0.12	1.84	4.32	1.72	<0.01
C26	Middle lake (Requeixo, Aveiro)	Fermentelos Lake	8.57	20.4	496	304	27.0	93	8.4	6.2	6.80	26.2	70	99	<0.1	1.91	0.05	1.18	3.09	1.13	<0.01
C28	Western lake (S. Paio, Requeixo, Aveiro)	Fermentelos Lake	8.12	21.6	503	301	**70.0**	93	8.2	6.2	4.23	9.3	64	95	**0.2**	1.80	0.03	1.08	3.08	1.05	<0.01

formamide) in 1 mm vertical polyacrylamide gels (8% (wt/vol) acrylamide in 0.5× TAE buffer). Electrophoresis was performed in a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA) using 0.5× TAE buffer containing 20 mM Tris, 10 mM acetic acid, and 0.5 mM EDTA (pH 8.0) during 16 h at 75 V, with an initial step at 20 V for 15 min. The gel was then stained for 5 min in an ethidium bromide solution (5%) and then gently distained with agitation in distilled water for 15 min before image digitalization in a Molecular Imager FX™ system (Bio-Rad Laboratories, Hercules, CA, USA). The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5 mL Eppendorf tubes and washed in 10 μL of sterile milli-Q-purified water, from which 5 μL of the eluted DNA was used for PCR amplification with the original primer pair. The isolation and identity of each DNA band was confirmed through DGGE, and if necessary, the extraction procedure was repeated until the targeted band was clearly isolated. Each band was then cloned using the TA cloning kit from Invitrogen. Prior to cloning, an A tailing for PCR products was performed according to manufacturers' instructions. The migration point of each cloned sequence was checked through DGGE after PCR amplification with 338F-GC/518R.

Sequencing, nucleotide sequence accession numbers, and phylogenetic analysis

The nucleotide sequence of the cloned DGGE bands was made taking advantage of the vector primers M13R/T7. The sequences determined were deposited in the GenBank database under the accession numbers GU908476 to GU908486. A BLAST search (http://www.ncbi.nlm.nih.gov) was used to explore similarity against sequences deposited in the GenBank database. The sequences' alignment was carried out using the CLUSTAL X software version 1.8 (Thompson et al. 1994). A phylogenetic tree of the 16S rDNA gene fragments was built using the neighbor-joining method (Saitou and Nei 1987). Bootstrap analyses were based on 1,000 replicates. TreeView version 1.6.6 (Page 1996) was used to display the trees.

Multivariate analysis

The distribution of samples according to environmental parameters was assessed through principal component analysis (PCA) after standardization of environmental data (by subtracting the mean from each observation and dividing by the corresponding standard deviation). A cluster analysis of samples according to environmental parameters was executed using the unweighted pair group method with mathematical averages (UPGMA). The dendrogram was created with the similarities calculated using the Pearson correlation coefficient (95% probability) and the PRIMER 6 software (Clarke and Gorley 2006). Pearson's correlation coefficient was also used to assess relationships between environmental parameters and phylotype occurrence and band intensity. The DGGE profiles were analyzed using the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories, Hercules, CA, USA), and bands with a relative intensity of less than 0.5% in each lane were not considered for statistical analyses.

For DGGE data, the presence or absence of co-migration points was converted to a binary matrix (0/1), and cluster analysis was performed using also UPGMA but based on the Bray–Curtis similarity coefficient. Co-migration points of DGGE profiles were also used to build a matrix based on the relative band intensity in each lane after log transformation. Canonical correspondence analysis (CCA; ter Braak and Verdonschot 1995; ter Braak 1995) was performed with CANOCO 4.5 software (Scientia Software) to extract relationships between the distribution of the dominant phylotypes and environmental variables. We used an a priori forward selection of significant environmental parameters ($P < 0.05$) using a Monte Carlo permutation test (499 unrestricted permutations). Environmental data were standardized (as above) to reduce the relative influence of scale. The relation between bacterial phylotype data and explanatory variables (reduced model) was tested with a Monte Carlo unrestricted permutation test.

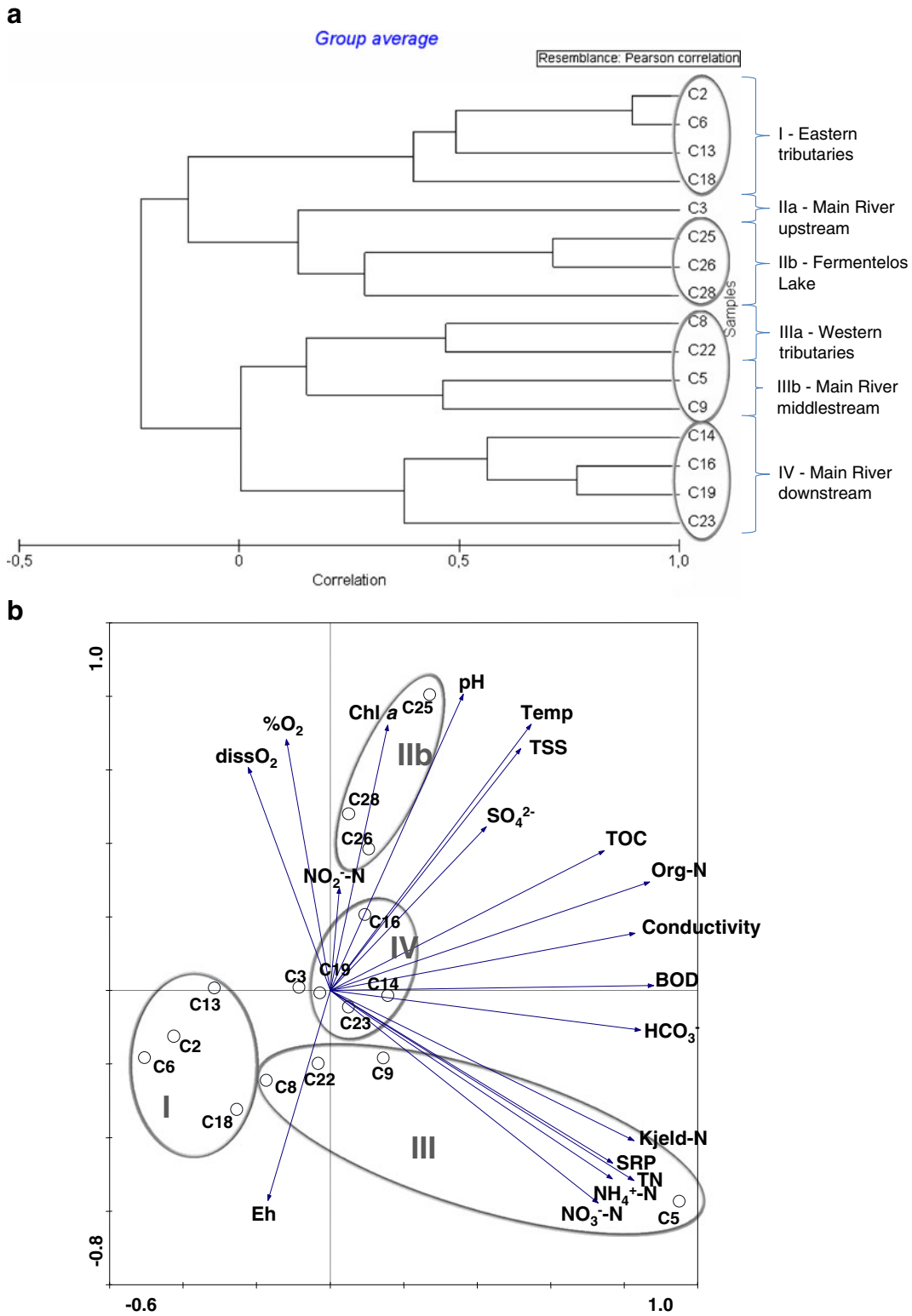

Fig. 2 **a** Cluster dendrogram and **b** PCA ordination biplot of Cértima River Basin samples according to environmental parameters recorded in May 2007 (see sample codes in Table 1)

Results

Environmental parameters

The environmental parameters recorded for each sample are summarized in Table 1. The cluster analysis of samples according to these parameters (Fig. 2a) resulted in two main clusters (negatively correlated) which showed to be related to inorganic nutrient sources and organic pollution along with electrical conductivity and HCO_3^- levels as shown in the PCA (Fig. 2b). This suggests trophic and mineralization gradients, the latter related to the geological features of the sampled locations. Cluster I included samples from the eastern mountain tributaries (C2, C6, C13, and C18), with the lowest HCO_3^- concentration and the highest water quality (lowest electrical conductivity, total organic carbon (TOC), total suspended solid (TSS), SO_4^{2-}, and low nitrate, org-N, and Chl *a*), and clusters II included the upstream sample C3 plus Fermentelos Lake samples (C25, C26, and C28), which, in spite of the high pH and Chl *a* levels, have relatively low nitrate and HCO_3^- concentrations. This cluster had intermediate electrical conductivity levels between the high water quality sampling sites (cluster I) and clusters III and IV. Clusters III included samples from the Western sandy lowland tributaries (C8 and C22) and the middle river section samples C5 and C9, all characterized by the highest nitrate concentrations and high electrical conductivity levels; cluster IV included samples from downstream sites before reaching Fermentelos Lake (C14, C16, C19, and C23), and they were mainly characterized by the highest electrical conductivity and SO_4^{2-} levels along with high HCO_3^- and nitrate concentrations.

The PCA biplot puts in evidence this gradient (Fig. 2b): Samples C2, C6, and C13 (with the highest water quality) appear on the negative side of the first axis, and sample C5 (characterized by a strong organic charge) appears on the positive side. This axis showed to be mainly defined by organic pollutants, electrical conductivity, and HCO_3^- levels, but also inorganic nitrogen sources (Fig. 2b). The second axis was mostly related to pH, Chl *a*, water temperature, TSS, and oxygen levels; this led to a clear separation between extreme samples C25 (on the positive side) and C5 (on the negative side). The first two axes of the PCA accounted for 67% of the total variance of samples distribution.

DGGE band patterns and CCA analyses

A total of 299 bands could be detected in the DGGE profiles obtained for Cértima River Basin samples (Fig. 3) corresponding to 56 different band migration points. The number of bands per sample showed an average of 19 ± 4 ($n = 16$). DGGE band patterns showed variability between the bacterial assemblages along the Cértima River Basin, although strong common bands could be detected among samples belonging to the main river section (see Fig. 3). Band 28 was ubiquitous in all samples while unique phylotypes were detected at C3 (bands 4 and 39), C6 (bands 1 and 2), C8 (band 7), C13 (band 3), C14 (bands 6 and 19), C18 (band 52), C22 (band 40), C25 (band 16), and C28 (band 20). Interestingly, in spite of the spatial

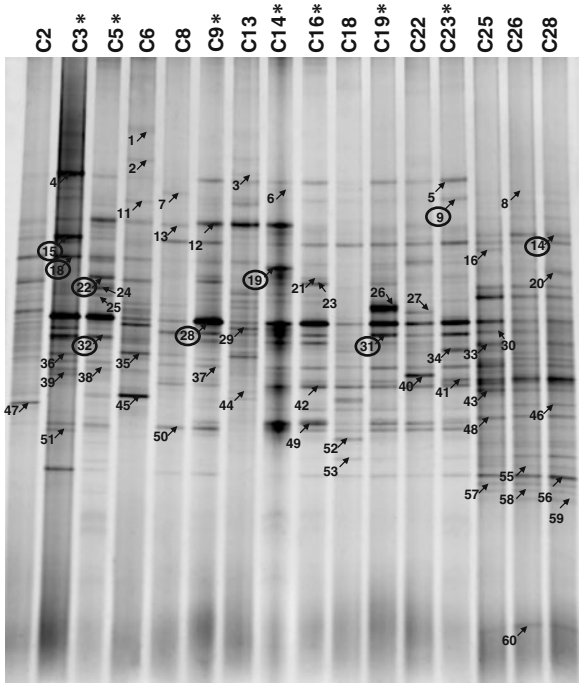

Fig. 3 DGGE 16S rDNA band profiles for samples obtained along Cértima River Basin in May 2007. The *code above each lane* refers to each sample (see Table 1), and the bands numbering corresponds to the different migration positions considered for the analyses

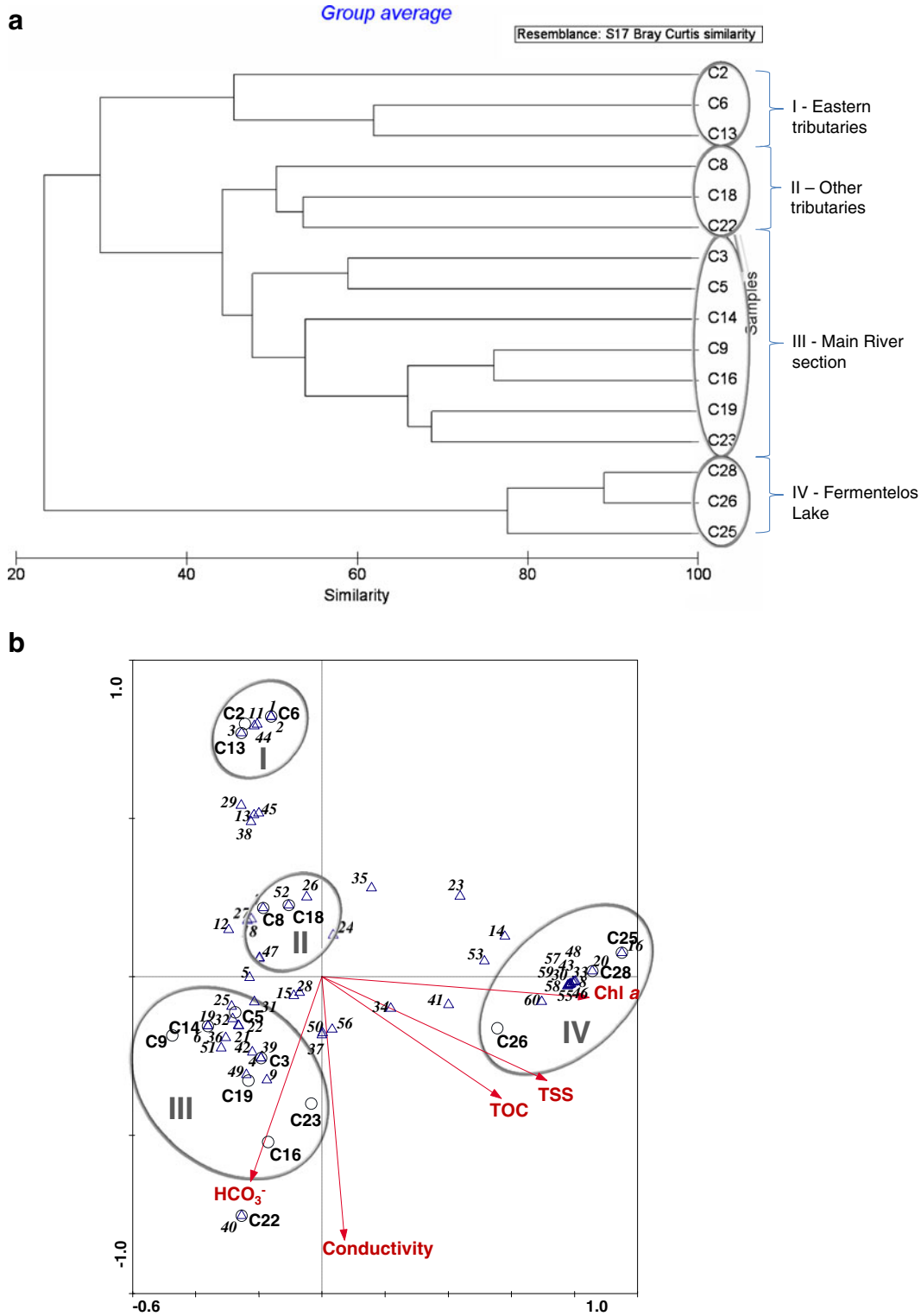

Fig. 4 **a** Dendrogram and **b** CCA ordination triplot of Cértima River DGGE band patterns according to the environmental parameters recorded during May 2007. DGGE bands numbering and samples coding are described in Fig. 2 and Table 1, respectively

Table 2 Sample, accession number, closest relative (after a BLAST search), and corresponding percentage similarity for the 16S rDNA bacterial partial sequences from excised bands

Sample	Band	NCBI accession no.	Phylogenetic affiliation	Closest relatives (accession no.)	Origin	Percentage (%) similarity
C23	9_cl8	GU908476	*Gammaproteobacteria*	Gammaproteobacterium MH154 (EU052746)	Microalgae culture association	96
				Gammaproteobacterium IMCC1704 (DQ664237)	Freshwater pond, Republic of Korea	
C28	14_ cl51	GU908477	*Betaproteobacteria*	Uncultured bacterium clone 3C002569 (EU801309)	Chesapeake Bay, MD, USA	99
				Betaproteobacterium MWH-C5 (AJ938026)	Freshwater Lake Mondsee, Austria	98
C16	15_cl24	GU908478	*Betaproteobacteria*	Uncultured betaproteobacterium clone IRD18H03 (AY947977)	Ipswich River, Massachusetts, USA	97
C3	18_cl53	GU908479	*Bacteria* (*Bacteroidetes*)	Uncultured bacterium BAx5 (AF087086)	Activated sludge, Germany	100
C14	19_ex1	GU908480	*Bacteria*	Uncultured bacterium clone PL14-3B (EU409545)	Wastewater, Palestine	99
				Uncultured candidate division TM7 bacterium clone Skagenf60 (DQ640706)	Activated sludge, Denmark	
C5	22_cl50	GU908481	*Bacteria* (*Bacteroidetes*)	Uncultured bacterium clone M0111_73 (EU104070)	Activated sludge, New Zealand	99
C5	28_cl38	GU908482	*Bacteroidetes*	*Flavobacterium* sp. AKB-2008-TE19 (AM988929)	Freshwater lake, Finland	100
C9	28_ex3	GU908483	*Bacteroidetes*	*Flavobacterium* sp. AKB-2008-TE19 (AM988929)	Freshwater lake, Finland	100
C16	28_ex9	GU908484	*Bacteroidetes*	*Flavobacterium* sp. AKB-2008-TE19 (AM988929)	Freshwater lake, Finland	100
C16	31_cl21	GU908485	*Betaproteobacteria*	Uncultured betaproteobacterium clone GC1m-1-96	Lake Michigan, Wisconsin, USA	100
C5	32_cl56	GU908486	*Bacteria* (*Bacteroidetes*)	Uncultured bacterium clone D5-43 (DQ113698)	Dog duodenum	98

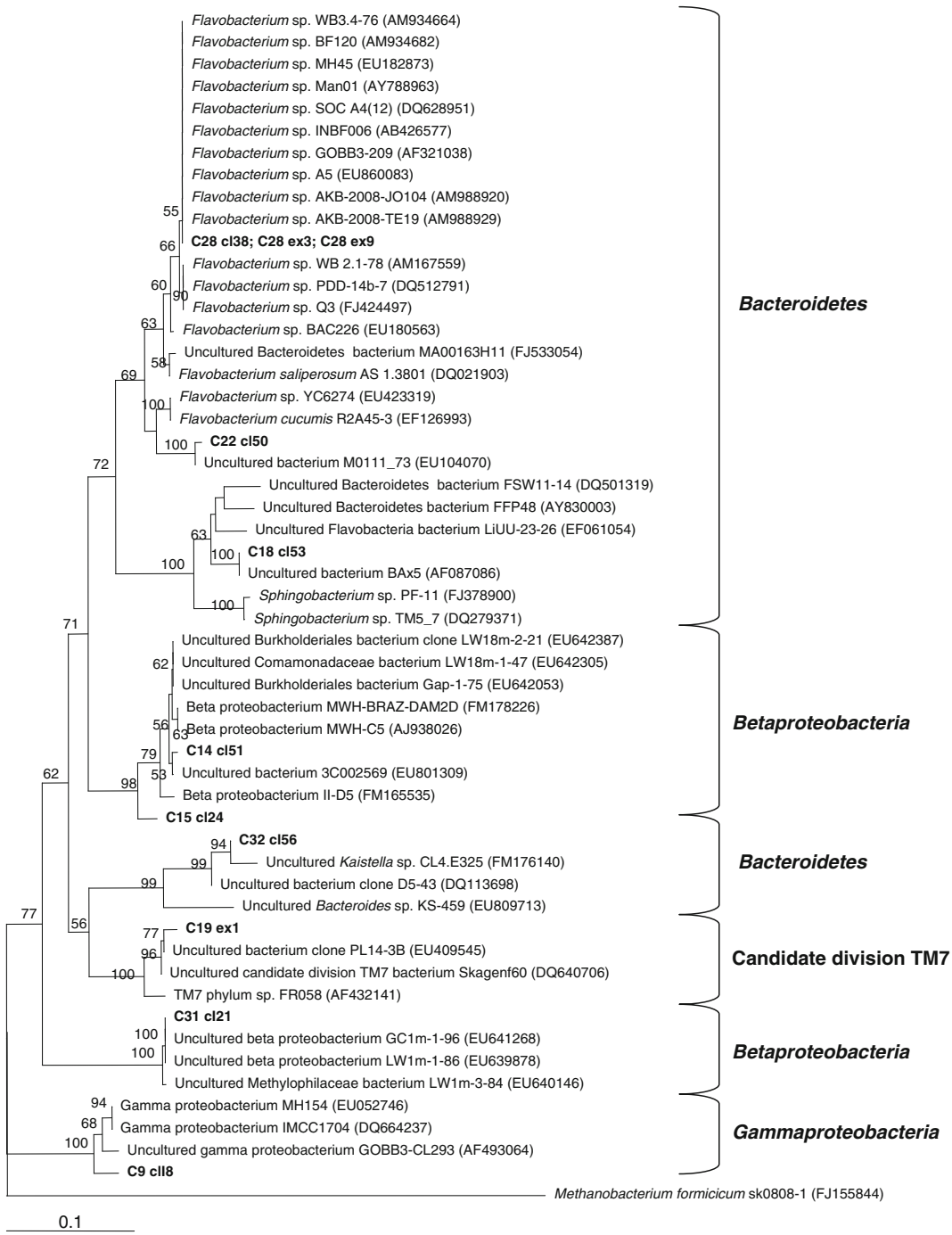

Fig. 5 Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel of Cértima River Basin samples (Fig. 3). The archaeal sequence of *Methanobacterium formicicum* strain sk0808-1 (FJ155844) was used as outgroup. *Scale bar* indicates 0.1 substitutions per site. Bootstrap values (1,000 replicates) that were >50 are placed at the nodes of the branches

gradient for environmental parameters inside the lake, there were only very slight variations in the bacterial assemblage structure among the sampled spots.

Cluster analysis based on band patterns showed a clear grouping of samples according to water quality and trophic status (Fig. 4a). Two main clusters were obtained: one including the lotic samples and the other included the Fermentelos Lake samples (cluster IV). This appears to be related to the hydrodynamic features of the river and lake (lotic and lentic, respectively) and associated trophic differences (related to parameters such as TSS and Chl *a* levels), as shown by the CCA triplot graph (Fig. 4b). Within the Cértima River main cluster, cluster I included the most unpolluted water samples from the eastern tributaries, while cluster II incorporated the remaining tributaries and cluster III included the main River section samples (where high inorganic nutrient and HCO_3^- levels were recorded—see Fig. 4b). The variance explained by CCA analysis was 59% (from which 47% could be explained by the first two axes), and the relationship between phylotypes and the environmental data matrix was significant ($P < 0.05$, Monte Carlo permutation test). The first axis was defined by bands 6, 19, and 51 on the negative area while the positive side was defined by band 16, with Chl *a* (0.82) and TSS (0.69) as the main factors behind this distribution. The second axis was mainly related to electrical conductivity (−0.76) and HCO_3^- concentration (−0.59), and bands 40 (on the negative side) and 1 and 2 (on the positive side) were well segregated along the axis.

Sequencing and phylogenetic affiliation of dominant phylotypes in DGGE profiles

A total of 25 DGGE bands were excised from the gel—but only ten bands gave clear results in the sequencing reactions; those are shown in Table 2. The phylogenetic affiliation of the sequenced bands (see Figs. 3 and 5) corresponded to the *Bacteroidetes* (bands 18, 22, 28, 32 and 28), *Betaproteobacteria* (bands 14, 15 and 31) and *Gammaproteobacteria* (band 9) groups. The most dominant phylotype present along the main river course (band 28, as shown by sequencing more than one band corresponding to this same migration point) showed total match with partial 16S rDNA sequences from *Flavobacterium* sp. strains (Fig. 5).

Discussion

Trophic status and pollution along the Cértima River Basin

In this study, the water quality of the river presented signs of degradation from the upstream tributaries to the downstream part of the main river body. This was related to the increase of biochemical oxygen demand (BOD), electrical conductivity, nutrient levels, and Chl *a* (suggesting a gradient based on pollutants and trophy). Nevertheless, an increase of pH and HCO_3^- (as consequence of water–rock interaction from carbonate rock dissolution Appelo and Postma 2005) downstream was also recorded. According to nutrients and Chl *a* levels, the upstream tributaries (C2, C6, and C13) were within the range of the oligotrophic to mesotrophic status, while all other samples in general fell into the descriptions for eutrophic and hypereutrophic state (Nürnberg 1996). The upstream eastern mountain tributaries are located in drainage areas with low population pressure and no relevant sources of pollutants (Cerqueira et al. 2005), and this was reflected in the observed low levels of electrical conductivity, TOC, TSS, SO_4^{2-}, and low nitrate, org-N, and Chl *a*, as usually observed for pristine waters (Cortecci et al. 2002; Saksena et al. 2008). The similarity found between C3 (Canedo stream) and Fermentelos Lake samples may be related to the water retention (by a small weir) in this river section (creating common hydrological features with the lake) and the organic pollution attributed to sewage discharge (Rino and Gil 1987; Cerqueira et al. 2005). At C5 (Lagoa Seca), TOC and BOD levels further increased related to urban untreated wastewater and animal farming effluents (Rino and Gil 1987; Calado 1990; Cerqueira et al. 2005); the high ammonium levels also suggest a weak oxidation potential of the water as corroborated by the low oxygen levels. Electrical conductivity, nitrate, soluble reactive phosphorus (SRP),

and ammonium levels were much higher than previous records (Rino and Gil 1987). However, at C9 (Curia), these values tended to decrease after the contribution of Luso and Ponte Rivers; this highlights the importance of the tributaries on the water quality maintenance of Cértima River. Interestingly, samples from the western sandy lowland tributaries (C8 and C22) shared high electrical conductivity levels and the highest nitrate concentrations with samples C5 and C9. Levira River (C22) suffers the pressure from a dense population along their margins and receives effluents from ceramics industry, distilleries, and animal farms (Rino and Gil 1987). However, at C8 (Ponte River), no previously pollution sources have been reported that justify the high nitrate concentrations recorded; agriculture runoffs constitute a potential candidate. The downstream Cértima River samples were mainly characterized by the highest values of electrical conductivity and SO_4^{2-} (indicative of industrial pollution or, eventually, from agricultural activities as described by Cortecci et al. 2002) although HCO_3^- and nitrate concentrations tended to decrease. At C14 (Malaposta), the discharge of effluents from wine industry has been previously related to high BOD levels and oxygen depletion (Rino and Gil 1987; Calado 1990). However, at C16 (São João da Azenha), BOD decreased and oxygen levels increased, lowering the ammonium and pollution levels due to water auto depuration and/or the contribution of small clean tributary streams that increase the river's width and depth (Rino and Gil 1987). Nevertheless, SO_4^{2-} levels achieved the maximum of 116 mg L^{-1} as recorded in polluted waters (Cortecci et al. 2002). Therefore, tributaries may have a dual impact over the main river, by simultaneously helping depuration of some pollutants and adding new ones. At C19 (Repolão), the water becomes shallower and its flow is reduced by floodgates; this enhances the impact of domestic and industrial effluents usually discharged just upstream this spot (Rino and Gil 1987). At C23 (Perrães), the water depth rises again but nitrate, BOD, and TSS levels increased after the affluence of the polluted Levira River. Fermentelos Lake samples (C25, C26, and C28), in spite of the high pH, electrical conductivity, and Chl *a* levels, have relatively low nitrate and HCO_3^- concentrations, when comparing to samples from the main river section. Inside the lake, a spatial gradient could be observed for some parameters such as oxygen levels, TSS, TOC, Chl *a*, BOD, and ammonium. Fermentelos Lake is known to be eutrophic since decades ago (Gil 1988; Calado et al. 1991) due to its high nutrient levels which have as main sources the runoffs from surrounding fields and Cértima River (Cerqueira et al. 2005). In retrospective, a general trend for the increase of pH, electrical conductivity, and nitrate levels has also been recently recorded (de Figueiredo et al. 2007, 2010).

Bacterial assemblage and trophic gradient of the Basin

The phylogenetic affiliation of the most intense bands corresponded to phylogenetic groups commonly found in freshwater bodies such as *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* (Cottrell et al. 2005; Lindström et al. 2005; Van Der Gucht et al. 2005; Allgaier and Grossart 2006). The clustering analysis based on the DGGE band patterns suggest the hydrodynamic features (lotic or lentic) and trophic status of the sampling sites as the main BCC modulators. Multivariate analysis showed Chl *a*, TSS, TOC, electrical conductivity, and HCO_3^- levels were the most important parameters to determine the BCC variation. Temperature, pH, and redox potential (E_h) as well as total phosphorus, nitrogen sources, and organic matter have proven to be important factors for BCC variation (Lindström et al. 2005; Rooney-Varga et al. 2005; Haukka et al. 2006; Hall and Cotner 2007; Berggren et al. 2009), along with the water flow and retention time (Crump and Hobbie 2005; Lindström et al. 2005), Chl *a* (Muylaert et al. 2002; Allgaier and Grossart 2006; Šimek et al. 2008) and sulfate levels (Awadallah et al. 1998; Bacelar-Nicolau et al. 2003). However, the impact of HCO_3^- concentration is not usually reported although here it showed to be an important BCC modulator.

The BCC showed variations along Cértima River Basin but among most samples common bands could be detected such as band 28, affiliated with *Flavobacterium* spp. from freshwater lakes (Berg et al. 2008), whose representation was

stronger in samples with higher HCO_3^-, electrical conductivity, and SO_4^{2-} levels (according to Pearson correlation). In fact, the *Cytophaga–Flavobacterium* group is well represented in organic-rich rivers (Brümmer et al. 2000). Band 32, affiliated with *Bacteroidetes*, was correlated with higher electrical conductivity and HCO_3^- levels and was similar to bacterial sequences from animal gastrointestinal tract suggesting a relation with untreated effluents from domestic wastewater and/or animal farming, which are known to exist near C5 (Cerqueira et al. 2005). Bands 22 and 18 showed the highest similarity with partial sequences from uncultured bacteria isolated from activated sludge samples; they showed to be related with high ammonium levels although band 22 was also related with high electrical conductivity, HCO_3^-, SRP, nitrate, TN, and Kjeld-N levels. In fact, the *Bacteroidetes* group, in general, is known to appear abundantly at mesotrophic and eutrophic water bodies (Riemann and Winding 2001; Van Der Gucht et al. 2005; de Figueiredo et al. 2007), and it usually correlates with high nutrient levels (Brümmer et al. 2000; de Figueiredo et al. 2007, 2010; Xi et al. 2007). At C14, band 19 matched partial 16S rDNA sequences from uncultured bacteria also found in wastewater and activated sludge (Kong et al. 2007). Actually, at C14, a strong pollution from wine industry effluents has been reported and related to high organic charge and oxygen depletion (Rino and Gil 1987; Calado 1990). The *Gammaproteobacteria* phylotype (band 9) showed to be correlated with higher SO_4^{2-} levels suggesting a preference for polluted waters. The highest sequence similarities were found with freshwater bacteria; however, this subdivision is not very abundant in freshwaters although it has been reported in several lakes and rivers (Zwart et al. 2002; Allgaier and Grossart 2006) as well as in wastewater treatment plants (Kong et al. 2007). *Betaproteobacteria* are very abundant in freshwaters (Zwart et al. 2002; Cottrell et al. 2005; Van Der Gucht et al. 2005; Allgaier and Grossart 2006). Bands 14 and 15 were affiliated with the family *Comamonadaceae* (*Burkholderiales*; *Betaproteobacteria*) from lakes and rivers (Crump and Hobbie 2005; Mueller-Spitz et al. 2009). In fact, members of this family have been recorded at Fermentelos Lake (de Figueiredo et al. 2010) and are abundant in rivers with organic pollution levels such as Cértima River (Brümmer et al. 2003). Band 14 correlated with low E_h and high temperature, pH, TSS, and Chl *a* levels. Band 31 was affiliated with members of the family *Methylophilaceae* (*Methylophilales*; *Betaproteobacteria*) isolated from a freshwater lake (Mueller-Spitz et al. 2009), and its occurrence showed a negative correlation with chlorophyll *a* levels.

Conclusions

The results obtained in this study showed that the water quality of Cértima River Basin suffered degradation from the upstream tributaries and along the river main body. Parameters such as BOD, electrical conductivity, pH, Chl *a*, HCO_3^-, and nutrient levels were the main modulators of this water quality gradient suggesting the influence of eutrophication, pollution but also of hydrogeological features (mineralization gradient). Samples from Lagoa Seca (Mealhada) and Curia (Anadia) but also from the tributary Levira River showed to have the highest pollution levels; this was associated with discharge of wastewaters and effluents from animal farming and industrial activity. Nevertheless, in general, Cértima River tributaries showed to play an important role for the river pollution depuration.

The variation of the bacterial assemblage along the Cértima River Basin showed to depend mainly on parameters such as Chl *a*, TSS, TOC, electrical conductivity, and HCO_3^- levels. *Bacteroidetes* phylotypes were all related to higher electrical conductivity and HCO_3^- levels. Some of these were also correlated with high SO_4^{2-} and others with high SRP, nitrate, TN, and Kjeld-N levels. The occurrence of a *Gammaproteobacteria* phylotype was correlated with high SO_4^{2-} levels. One of the *Betaproteobacteria* phylotypes showed to correlate with low E_h and high temperature, pH, TSS, and Chl *a* levels while another showed a negative correlation with Chl *a* values. Overall, the bacterioplankton assemblage was a good indicator of water quality, namely of anthropogenic inputs, and the dominant phylotypes along the Cértima Basin

were typically associated with organic-enriched waters.

Acknowledgements The authors would like to thank the contributions made by Raquel Silva and Alexandra Moura during the development of the present work. The research has been supported by Fundação para a Ciência e a Tecnologia in the form of a Ph.D. grant to Daniela de Figueiredo (SFRH/BD/23864/2005) and the project EcOwEt (POCI/CTE-GEX/58951/2004).

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