Universidade de Aveiro Departamento de Biologia
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Caracterização molecular de cianobactérias de sistemas aquáticos portugueses

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor António Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, e Doutor Mário Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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Aos que adoçam o meu lar e a minha vida...

o júri

presidente

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Sistemas dulçaquícolas, bacterioplâncton, *blooms* cianobacterianos, métodos moleculares, análise multivariada.

resumo

As alterações climáticas favorecem a ocorrência global de episódios de precipitação e seca extremas, colocando em risco a qualidade da água em sistemas aquáticos usados consumo humano ou recreação. O fenómeno de seca, em particular, será mais frequente e severo, alterando toda a hidrodinâmica dos sistemas aquáticos e, consequentemente, a ecologia das comunidades aquáticas. A ocorrência de *blooms* de cianobactérias intensificar-se-á sob este novo cenário climático. Em Portugal, estudos parcelares em rios e barragens têm sido realizados com enfoque em cianobactérias tóxicas e outras bactérias patogénicas, mas não há trabalhos publicados acerca da composição da comunidade bacteriana (CCB). O presente trabalho pretende colmatar esta falha, com particular atenção para a ocorrência de *blooms* cianobacterianos, em vários sistemas aquáticos portugueses lóticos e lênticos. Este objectivo foi alcançado utilizando metodologias moleculares, como a técnica rDNA 16S-DGGE (Denaturing Gradient Gel Electrophoresis), independente do cultivo, e a sequenciação. Dados ambientais foram também determinados para correlacionar com as variações sazonais ou espaciais da diversidade da CCB. O impacto da seca na distribuição espacial da CCB foi também investigado. A lagoa da Vela é um caso de estudo especial, devido à vasta documentação sobre a ocorrência de *blooms* de cianobactérias durante os últimos anos, e várias estirpes isoladas de *blooms* foram estudadas em mais detalhe. Os resultados mostraram, em geral, perfis de DGGE típicos de verão *vs.* inverno nos sistemas aquáticos estudados. Nos sistemas lênticos, os filótipos dominantes afiliaram com *Cyanobacteria* (formas unicelulares, coloniais e filamentosas), eucariotas fototróficos e *Actinobacteria*, enquanto nos rios, *Bacteroidetes* e *Betaproteobacteria* foram dominantes. Nos sistemas lênticos, os factores mais significativos para a sazonalidade da CCB incluíram a temperatura da água, a condutividade e a clorofila *a*, apesar da variação extrema dos níveis de precipitação, sugerindo que a BCC poderá resistir a mudanças severas causadas pela seca. Nos rios, a sazonalidade da CCB foi principalmente definida pela temperatura e os níveis de amónia. No verão seco de 2005, as barragens do Alentejo (Sul de Portugal) mostraram similaridade na CCB, com filótipos comuns de *Cyanobacteria*, A*ctinobacteria* e *Alphaproteobacteria*. No entanto, os perfis de DGGE sugerem filótipos ubíquos em sistemas portugueses geograficamente distantes. Na Lagoa da Vela, a seca conduziu à redução drástica do nível da água e à variação na diversidade espacial da CCB (e cianobactérias dominantes) e potencial tóxico, o que pode ter impacto directo nos utilizadores da lagoa. Os resultados também mostraram a presença de estirpes tóxicas de *Microcystis* na lagoa e um *bloom* não clonal de estirpes de *Aphanizomenon aphanizomenoides*, com diferentes morfótipos, genótipos e ecótipos.

keywords

Portuguese freshwater bodies, bacterioplankton, cyanobacterial blooms, molecular approaches, multivariate analysis.

abstract

Global climatic changes are increasing the occurrence of extreme precipitation or drought episodes worldwide and putting at risk the water quality in freshwater bodies used for drinking water supply or recreation. Phenomena such as droughts will become more frequent and severe, changing the all hydrodynamics of water bodies and, ultimately, the ecology of aquatic communities. Cyanobacterial blooms are expected to become more frequent under these new climatic scenarios. In Portugal, parceled studies on rivers and reservoirs have been made targeting toxic cyanobacteria and other bacterial pathogens but no investigation has been conducted on the bacterial community composition (BCC). The present work intends to fulfill this gap, with a particular focus on cyanobacterial blooms occurrence, for several Portuguese lotic and lentic freshwater bodies. This was achieved by using molecular approaches such as the culture-independent methodology 16S rDNA-DGGE (Denaturing Gradient Gel Electrophoresis) and sequencing. Environmental data were also determined for correlation with shifts in the seasonal or spatial BCC diversity. The impact of drought in spatial distribution of BCC was also investigated. Vela Lake is a special case-study due to the vast documented occurrence of cyanobacterial blooms over the past decades and several bloom-forming cyanobacterial strains were studied in more detail. Results showed that, in general, there could be established typical winter *vs.* summer DGGE profiles in every studied water body but the BCC differed and the occurrence of cyanobacterial blooms was related to lentic conditions. In lentic water bodies, dominant phylotypes affiliated with *Cyanobacteria* (unicellular, colonial and filamentous forms), phototrophic eukaryotes and *Actinobacteria*, whereas in riverine systems BCC showed to be dominated by *Bacteroidetes* and *Betaproteobacteria*. In lentic systems, the most significant factors behind the BCC seasonality included water temperature, conductivity and chlorophyll *a*, in spite of the severe variation of precipitation levels throughout the study period, which suggests BCC may resist severe shifts caused by drought. In riverine systems, BCC seasonality was mostly defined by water temperature and ammonium levels. Under a drought scenario, in summer 2005, a clear segregation of Alentejo (Southern Portugal) reservoirs was observed, where the BCC was dominated by *Cyanobacteria*, but also *Actinobacteria* and *Alphaproteobacteria*, and common phylotypes were recorded. Nevertheless, DGGE patterns suggest there may be ubiquitous phylotypes throughout geographically distant Portuguese water bodies. At the shallow Vela Lake, drought led to a drastic reduction in the water level and spatial differences in the BCC (and dominant bloom-forming cyanobacteria) and in the toxin-producing potential were recorded; this may have direct impact on the health of the lake users. Results also reported toxic *Microcystis* strains at Vela Lake and a non-clonal bloom of the invasive *Aphanizomenon aphanizomenoides*, showing different morphotype, genotypes and ecotypes.

List of publications from the present work

Published papers

de Figueiredo D. R., Pereira M. J. & Correia A. (2010). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26: 1067-1077.

de Figueiredo D. R., Alves A., Pereira M. J. & Correia A. (2010). Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). *Journal of Plankton Research* 32: 239-252.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology and Ecology* 59: 638–650.

Submitted papers

de Figueiredo D. R., Gonçalves A. M. M., Castro B. B, Gonçalves F., Pereira M. J. & Correia A. (submitted). Effects of different nutrient levels on growth of bloom-forming *Aphanizomenon* strains isolated from a eutrophic temperate shallow lake – importance of intra-specific variation. *Hydrobiologia.*

de Figueiredo D. R., Ferreira R. V., Cerqueira M., Condesso de Melo T., Pereira M. J., Castro B. B. & Correia A. (submitted). Impact of water quality on bacterioplankton assemblage along Cértima River Basin (Central Western Portugal) assessed by PCR-DGGE and multivariate analysis. *Environmental Monitoring and Assessment.*

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General introduction

Scope of the thesis

Climatic changes and eutrophication – impact of drought on freshwater bodies

The concern with the global climatic changes is increasing worldwide since episodes such as extreme precipitation or drought are becoming more frequent and the maintenance of water quality in freshwater bodies may be endangered, particularly in water bodies used for drinking water supply. Drought conditions lead to increased water retention times during summer months and water quality is degraded, with the risk of waterborne diseases outbreaks (Charron et al., 2004; Bond et al., 2008a) which makes the establishment of water management strategies indispensable (Bond et al., 2008a). The occurrence of summer droughts can also have repercussions for recreational users of shallow lakes, as these are very vulnerable to drought conditions which cause a rapid decrease in the water level and lead to important changes in water physical and chemical characteristics as well as in the composition of the aquatic communities (Mitraki et al., 2004). The bacterial community, in particular, can rapidly change its composition and the development of groups such as *Cyanobacteria* and *Actinobacteria* (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; Wiedner et al., 2007) is enhanced, which may put at risk the water quality (Zaitlin & Watson, 2006; Smith et al., 2008). More, *Cyanobacteria* can produce toxins and the development into blooms that can lead to toxic outcomes and endanger human health safety (de Figueiredo et al., 2004b; Codd et al., 2005b; Mazur-Marzec, 2006). More, pathogenic bacteria including *Aeromonas*, *Vibrio*, *Acinetobacter* and *Pseudomonas* have been also related to the development of cyanobacterial blooms, which increases even further the human health risk (Stewart et al., 2006; Berg et al., 2008). Therefore, the establishment of water management strategies requires previous knowledge on the ecological dynamics of target communities in the water body.

Interestingly, at a large spatial scale, the composition of bacterial assemblages seems to be mainly explained by differences in environmental conditions rather than biogeography (Van Der Gucht et al., 2007), which suggests there may be a transversal global pattern for similar freshwater bodies under common environmental contexts. If this happens between Portuguese water bodies from Northern and Southern Portugal, the development of national management strategies to mitigate drought outcomes would become easier to develop and establish.

In 2005, Portugal suffered one of the most intense droughts over the last fifty years, according to time series data made available by the Water Resources Information National System (http://snirh.pt/). In Alentejo region (Southern Portugal), drought conditions are recurrent (Caetano et al., 2001) but the summer of 2005 brought severe consequences with some reservoirs becoming dry, as reported by National Commission for Drought in 2005 (http://www.inag.pt/inag2004/port/divulga/actualidades/seca/relatorios/RelatorioParlamento.pdf). However, the impact of that drought on the whole bacteriplankton community of Portuguese water bodies has not been

assessed. Although several reports on *Cyanobacteria* occurrence have been published over the last decade (Vasconcelos et al., 1996; Vasconcelos, 1999; Vasconcelos, 2001; Saker et al., 2003; Valério et al., 2005; de Figueiredo et al., 2006), the information on the shifts of the whole bacterial community composition (BCC) dynamics at the Portuguese freshwater bodies is very scarce or inexistent, which restricts the development of effective water management planning. Thus, it is urgent to obtain data and make public studies on the seasonal bacterial diversity, as the present one.

Eutrophication, bacterioplankton diversity and cyanobacterial blooms

The variation of the BCC is known to depend on parameters such as water temperature and pH (Crump & Hobbie, 2005; Lindström et al., 2005; Yannarell & Triplett, 2005; Wu et al., 2007b) as well as the water retention time or water flow rate (Crump & Hobbie, 2005; Lindström et al., 2005) and the levels of nitrogen (Crump & Hobbie, 2005; Wu et al., 2007b; Wei et al., 2008; Zeng et al., 2009), phosphorus (Boucher et al., 2006; Xing & Kong, 2007) and chlorophyll *a* (Muylaert et al., 2002; Allgaier & Grossart, 2006b; Xing & Kong, 2007; Šimek et al., 2008).

Groups such as *Bacteroidetes*, *Betaproteobacteria, Alphaproteobacteria, Cyanobacteria* and *Actinobacteria* are usually dominant in eutrophic water bodies (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007; Wu et al., 2007b). The co-dominance of *Actinobacteria*, *Alphaproteobacteria*, *Cyanobacteria* as well as *Betaproteobacteria* phylotypes has been recorded before (Xi et al., 2007; Pope & Patel, 2008) as well as the desynchronizing dominance of *Cyanobacteria* and *Alphaproteobacteria* in summer and *Bacteroidetes* in winter (Xi et al., 2007). In shallow eutrophic and hypereutrophic lakes, in particular, a dominance of the *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes* and *Actinobacteria* groups is usually recorded (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006a).

The *Bacteroidetes* group, in general, is known to appear abundantly at mesotrophic and eutrophic water bodies (Riemann & Winding, 2001; Zwart et al., 2002; Van Der Gucht et al., 2005; Wu et al., 2007b) and it usually correlates with high nutrient levels (Brümmer et al., 2000; Xi et al., 2007) and high conductivities (Van Der Gucht et al., 2005). *Flavobacterium* spp., in particular, are frequently dominant in eutrophic rivers from Northern Europe (Brümmer *et al.*, 2000) and have been related to high nutrient levels, as they are nitrate-reducing bacteria (Nijburg & Laanbroek, 1997). In general, *Actinobacteria* are recorded across seasons in lentic eutrophic water bodies (De Wever et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006b; Newton et al., 2007; Wu et al., 2007b; Xi et al., 2007). *Betaproteobacteria* are very abundant in freshwaters (Zwart et al., 2002; Cottrell et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006b), namely in rivers with high organic pollution levels (Brümmer et al., 2003). The dominance of *Betaproteobacteria* has been also associated with short water retention time (Lindström et al.,

2005) but high nitrogen levels (Brümmer et al., 2000; Lee et al., 2002). Members of the *Betaproteobacteria* can be found as part of denitrifying populations in environments with high nitrate concentrations (Lee et al., 2002) as well as performing ammonia oxidization (Altmann et al., 2003). *Alphaproteobacteria* dominance may be associated with long water retention time (Lindström et al., 2005) and high pH values as well as high nutrient and TSS concentrations (Zwart et al., 2002).

The development of cyanobacterial blooms in freshwater shallow lakes occurs worldwide (Dokulil & Teubner, 2000) and is usually enhanced by low N:P levels, water stability, reduced transparency and increased water temperature, pH and conductivity (Codd, 2000; Dokulil & Teubner, 2000; Jacoby et al., 2000; Oliver & Ganf, 2000; De Wever et al., 2005; Van Der Gucht et al., 2005; de Figueiredo et al., 2006). Nevertheless, the eutrophication process is known to lead to dominance of *Cyanobacteria* even in almost oligotrophic conditions (Pearce et al., 2005) and different species may have distinct preferences to enhance their growth (Mischke, 2003; Nixdorf et al., 2003). Some characteristics allow cyanobacteria to develop successfully under low nitrogen sources concentrations, such as diazotrophy which is the capability of to fix atmospheric nitrogen (e.g. in *Anabaena* and *Aphanizomenon* spp.) in specialized cells (Flores & Herrero, 2010) or alternating with photosynthesis (as nitrogenase is sensitive to oxygen). Yet, N-fixing filamentous cyanobacteria do not have much success under phosphorus limitation or unavailability (Lehtimaki et al., 1997; Rapala et al., 1997b; Saadoun et al., 2001; de Figueiredo et al., 2004c; Degerholm et al., 2006). Planktonic cyanobacteria can also regulate their buoyancy allowing vertical movement in the water column in a way to optimize nutrient availability and light conditions (Oliver & Ganf, 2000), involving production and collapse of small intracellular cylindrical structures – gas vesicles. Therefore, calm conditions may lead to rapid and unexpected development of surface blooms due to massive migration to surface of pre-existing cyanobacteria dispersed in water and not to a rapid population growth. The loss of buoyancy regulation (by photo-oxidation, for example) may lead to a dense accumulation of cells at surface forming the called scum. Hence, cyanobacteria that produce gas-vesicles are the main responsible for surface blooms or scum. They can be filamentous (*Anabaena*, *Aphanizomenon*, *Anabaenopsis*, *Nodularia*, *Cylindrospermopsis*, *Gloeotrichia*, *Oscillatoria/ Planktothrix*, *Spirulina*) or globular colonies (*Microcystis*, *Gomphosphaeria*, *Coelosphaerium*) (Oliver & Ganf, 2000). Other characteristics that can give competitive advantage for cyanobacteria over the other algae are the low grazing rate by zooplankton (Dokulil & Teubner, 2000; de Figueiredo et al., 2004a). In spite planktonic surface blooms with scum formation being the most concerning in terms of animal and human health, some benthic cyanobacteria can homogeneously develop in oligotrophic waters with sunlight reaching the bottom of the lake or reservoir (Oliver & Ganf, 2000).

It has been recently proven that the growth of diazotrophic filamentous cyanobacteria, in particular, will be enhanced by the expected global warming, with nutrient inputs playing a major role (Markensten et al., 2010). Species such as *Cylindrospermopsis raciborskii*, *Aphanizomenon* (*Cuspidothrix*) *issatschenkoi* and *Aphanizomenon aphanizomenoides* have already been considered invasive in Europe (Stüken et al., 2006; Wiedner et al., 2007; Kaštovský et al., 2010). Allelopathy seems also to be important to explain this expansion of invasive nitrogen-fixing filamentous species (Figueredo et al., 2007) which highlights the need for more studies on these species ecology in order to better understand and control their development into blooms. The phytoplankton community, in particular, is strongly affected by cyanobacterial blooms, not only due to the advantageous competition of cyanobacteria over microalgae (by nitrogen fixation, phosphorus storage and/or buoyancy regulation) but also to the effect of cyanotoxins' release (Dokulil & Teubner, 2000; Kearns & Hunter, 2001).

Although cyanobacteria are more frequent and form blooms in lakes and reservoirs, they can also be recorded in riverine systems worldwide (Cadel-Six et al., 2007; Anderson-Glenna et al., 2008; Amer et al., 2009), including in Portugal (Caetano et al., 2001). Even surface blooms can also occur in rivers with high flow rates and turbulence (Codd et al., 1995). The study of the bacterial community composition (BCC) in rivers is not very explored by comparing to lakes since its variation is not as predictable as in lentic systems due to the high oscillations in the water flow and contribution of allochthonous bacteria from soil, groundwater and wastewater (Brümmer et al., 2000; Crump & Hobbie, 2005). However, predictable seasonal changes for the BCC within a river but also among similar rivers have been reported (Crump & Hobbie, 2005), having the river flow rate and temperature as major modulators.

Impact of cyanobacterial blooms occurrence

The occurrence of cyanobacterial blooms causes a deterioration of the water quality, with implications for the ecology of the water body and surrounding area as well as for the economy of the region (Steffensen, 2008) but also for public health, with potential toxic outcomes involved (de Figueiredo et al., 2004b; Codd et al., 2005b; Mazur-Marzec, 2006). From an ecological point of view, specific biodiversity is reduced at all trophic levels and there is a habitat deterioration, with increased turbidity, a decrease in oxygen concentration and production of substances that give a bad taste and odor to water, or toxins that are noxious to a great variety of organisms (de Figueiredo et al., 2004b; Smith et al., 2008). Cyanobacterial blooms occurrence in drinking water reservoirs have significant economical consequences resulting from the deterioration of water quality (with bad taste and odour (Smith et al., 2008)) as well as water treatment filter blockage and requirement for additional and more effective water treatment processes (Rapala et al., 2006; van Apeldoorn et al., 2007) to remove cyanotoxins that may endanger consumers health. In recreational waters, the

occurrence of surface scum causes a decrease in local tourism economy due to the loss of water quality and cyanotoxins hazard, hindering the water sports practice and bath contact due to potential outcome of animal and human illness (WHO, 2003). Cyanobacterial blooms have been recorded in marine, brackish and freshwaters worldwide and a great percentage (50 a 90 %) of them have been considered toxic (Codd, 2000; WHO, 2003).

Cyanobacterial toxins differ in its chemical structure and toxicity. Generally they are classified as neurotoxins, hepatotoxins, cytotoxins, irritants and gastrointestinal toxins, according to the toxic effects in mammals (Kaebernick & Neilan, 2001; Codd et al., 2005b). Chemically, neurotoxins include alkaloids such as anatoxin-a and homoanatoxin-a (which are neuromuscular blocking agents that interfere with acetylcholinesterase activity) or saxitoxins (which block the sodium channels), and the guanidin methyl phosphate ester (which ihnibits acetylcholinesterase) (Codd, 2000; Kaebernick & Neilan, 2001); hepatotoxins, the most frequent and dangerous cyanotoxins, include cyclic peptides such as nodularin and microcystins which cause major damage in liver by inhibiting protein phosphatases and disrupting membrane integrity and tumour promoters (Kaebernick & Neilan, 2001; de Figueiredo et al., 2004b; van Apeldoorn et al., 2007); cytotoxins include the alkaloid cylindrospermopsin that ihnibits protein synthesis which leads to necrotic injury in several organs and is genotoxic (Codd et al., 2005a; Falconer & Humpage, 2006); irritants and gastrointestinal toxins include aplysiatoxin, debromoaplysiatoxin and lyngbyatoxin (which are tumour promoters and cause skin irritation) and also lipopolysaccharide endotoxins (LPS) that may enhance inflammatory and gastrointestinal incidents (Codd et al., 2005b).

Anatoxin-a is mainly produced by *Anabaena flos-aquae*, but it can be also synthesized by *Anabaena circinalis*, *Aphanizomenon issatchenkoi*, *Aphanizomenon flos-aquae* and some species of *Oscillatoria/Planktothrix*, *Cylindrospermum*, *Microcystis* and *Phormidium* (Codd et al., 1995; Codd, 2000; Wood et al., 2007). Homoanatoxin-a has been identified in *Oscillatoria formosa* (Dow & Swoboda, 2000) and *Anabaena flos-aquae* is the main producer of anatoxin-a(s) (Codd et al., 1995; Dow & Swoboda, 2000; Kaebernick & Neilan, 2001). Saxitoxins are usually produced by marine dinoflagellates but can also occur in freshwater cyanobacteria such as *Anabaena circinalis*, *Anabaena lemmermanni* and *Aphanizomenon flos-aquae*, as well as in species from the genera *Lyngbya*, *Cylindrospermopsis* and *Planktothrix* (Codd et al., 1995; Codd, 2000; Dow & Swoboda, 2000; Kaebernick & Neilan, 2001). The brackish species *Nodularia spumigena* is responsible for nodularins production (Codd, 2000; Dow & Swoboda, 2000; Kaebernick & Neilan, 2001). The main microcystin variants (or microcystins) synthesizers include *Microcystis* spp., *Anabaena* spp., *Planktothrix* (or *Oscillatoria*) *agardhii* and *P. rubescens*, and species of *Anabaenopsis* and *Nostoc*, but microcystins were also found in *Aphanizomenon flos-aquae* and terrestrial *Hapalosiphon* (Codd et al., 1995; Dow & Swoboda, 2000; Kaebernick & Neilan, 2001). Cylindrospermopsin is mainly produced by *Cylindrospermopsis raciborskii*, a cyanobacterium that has been increasingly found in

tropical and temperate regions (Neilan et al., 2003), but also by *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae* and *Umezakia natans* (Codd, 2000; Dow & Swoboda, 2000; Kaebernick & Neilan, 2001; Preußel et al., 2006). Lyngbyatoxins are mainly produced by marine cyanobacteria belonging to the genera *Lyngbya* (*Lyngbya majuscula*), *Schizothrix* and *Oscillatoria* (Codd, 2000; Kaebernick & Neilan, 2001) and lipopolysacharides are synthesized by many brackish and freshwater species of the genera *Anabaena, Aphanizomenon, Nodularia, Oscillatoria, Gloeotrichia* (Codd, 2000; WHO, 2003).

During cyanobacterial blooms, there are major impacts on aquatic and terrestrial organisms, from bacteria and microalgae to fish, birds and humans (de Figueiredo et al., 2004b; Wiegand & Pflugmacher, 2005; Stewart et al., 2006; Valdor & Aboal, 2007; van Apeldoorn et al., 2007) that depend on the water quality maintenance of the affected aquatic system. More, there is also the effect of bioaccumulation of cyanotoxins either by consuption of food supplements made from natural cyanobacterial blooms (Saker et al., 2005b) or through the food chain (Codd et al., 1999; van Apeldoorn et al., 2007), endangering the human safety. However, the most common route of exposure to cyanotoxins is direct, through drinking water consumption. Acute intoxication (with hepatotoxicity and gastroenteritis) usually occurs after bloom degradation or after cyanobacterial lysis by treatment processes (e.g. copper sulphate) when the toxins are released from the cells (Dow & Swoboda, 2000). The long exposure to low levels of the toxins poses great concern due to cancer promotion potential of some cyanotoxins such as microcystins (Ueno et al., 1996; Zhou et al., 2002). Nodularins are not very common in drinking water, but shouldn't be forgotten because they are also tumour liver promoters and cylindrospermopsin has already shown to be dangerous through drinking water exposure (Fitzgerald, 2001). In recreational waters, activities like taking a bath, swimming or playing water sports during cyanobacterial blooms lead to direct exposure of skin, eyes and ears to the water but can also lead to accidental water ingestion, aspiration or inhalation of cyanobacterial cells. There has never been reported a human fatal case due to recreational exposure but usually it results in allergies and irritation of external and internal revestment tissues (gastrointestinal and respiratory organs, eyes, ears, mouth and throat) due to irritants, in spite hepatotoxic and neurotoxic situations may also occur (Dow & Swoboda, 2000; Chorus & Fastner, 2001; Fitzgerald, 2001). The chronic effects due to recreational exposure should be also considered because long periods of exposure can occur during summer vacancies with regular swimming in a water body with a hepatotoxic bloom, for example. The lethal dose of contaminated water depends on factors such as toxin type and its content in cyanobacterial cells, toxin producing cyanobacterial biomass, exposure route and victims' susceptibility to the toxins (age, sex, weight and species) (Dow & Swoboda, 2000). Nevertheless, for humans, there are more sensitive groups that require special attention such as B-hepatitis patients, hypersensitive persons and children (Chorus & Fastner, 2001). Moreover, the places that children choose to play are

shallow waters near the shore were the scum usually accumulates. Over the past years, experimental studies have been conducted concerning the attenuation of human intoxication by microcystins (Gehringer et al., 2003) but for neurotoxins that is difficult due to his rapid action, and only procedures such as artificial respiration, lavage and activated carbon are applied to reduce the toxin absorption when dose is not lethal (Fitzgerald, 2001). This is why cyanotoxins such as microcystins, anatoxin-a and saxitoxins are listed as biological warfare agents and considered water threats as they are biotoxins tolerant to chlorine that may be weaponized and used for massive contamination of water bodies and human populations (Burrows & Renner, 1999).

Cyanobacterial blooms are frequent in Portuguese water bodies, mainly lentic systems, with the dominance of potentially toxin-producing cyanobacteria such as *Microcystis* spp., *Aphanizomenon flos-aquae*, *Aphanizomenon gracile*, *Planktothrix* sp., *Anabaena flos-aquae*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii* (Vasconcelos et al., 1993a; Saker et al., 2003; de Figueiredo et al., 2006; Galvão et al., 2008; Valério et al., 2008).

Over the last years, studies have reported that the toxicity of a bloom depends on the dominant strains, as there are toxic and non-toxic strains of a same species (e.g. *Microcystis* spp. (Yoshida et al., 2008)), sometimes co-existing in a same water body and even within a same bloom (Kardinaal et al., 2007). Classical techniques based on observation of morphological characters may not be sufficient to correctly identify *Microcystis* spp., particularly if these are cultured for a long time (Komárek, 1991; Komárek & Anagnostidis, 1999; Bittencourt-Oliveira et al., 2001), and certainly are not suitable to assess the strains' toxicity. Thus, molecular approaches have been increasingly used due to its accuracy and fastness in determining the toxic potential of an isolated strain as well as adding in its taxonomical identification (Bittencourt-Oliveira et al., 2001; Innok et al., 2005; Saker et al., 2005a; Wilson et al., 2005; Komárek, 2006).

DNA-based approaches as important tools to study the ecology of bacterioplankton community and cyanobacterial blooms

The assessment of microbial communities' diversity, contextualized in a particular habitat, is one of the most important (but challenging) foundations in microbial ecology. Molecular approaches have shown to be important tools to study biodiversity in aquatic microbial communities (Dorigo et al., 2005). Culture-independent molecular methodologies, such as PCR-DGGE (polymerase chain reaction - denaturing gradient gel electrophoresis) (Muyzer et al., 1993), have proven over the last years to be suitable for a general and rapid characterization of bacterial assemblages in natural aquatic environments, allowing to compare temporal or/and spatial patterns (Muyzer & Smalla, 1998; Lyautey et al., 2005; Marzorati et al., 2008; Tian et al., 2009). This method takes advantage of PCR amplification of highly conserved domains within the 16S rRNA gene and takes also into account the diversity of microorganisms not cultivable at the present time

(Bent & Forney, 2008). The method is based on the assumption that amplified fragments having the same length but at least one nucleotide different will be separated, by electrophoresis, in a polyacrylamide gel having a denaturing gradient of urea and formamide, as the double stranded DNA molecule has a specific melting temperature to separate both strains, depending on the hydrogen bonds between the complementary base pairs (e.g. GC-rich domains melt at higher temperatures) and on the attraction between neighboring GC stretches on the same strand; at the time of electrophoresis, the molecule mobility is retarded when the first melting domain is reached in the increasing denaturing gradient, resulting in partial dissociation of the fragment; however, the presence of a high melting domain (a GC clamp added to one primer) prevents the complete strand separation. At the end of the electrophoresis, a DGGE pattern is obtained which allows to retrieve qualitative but also semi-quantitative estimations of diversity of predominant phylotypes in target communities (Dorigo et al., 2005). Moreover, sequences of bands can be recovered directly by excising them from the gel, reamplifying (or cloning it) and sequencing. In spite of all the advantages of using DGGE, there have been recognized some limitations associated with this methodology associated with DNA extraction method, PCR amplification of 16S rRNA gene, shortness of migration fragments (which may not allow discrimination between species), and representiveness of the microbial community due to multiple copies of rRNA genes within the genome of many organisms (Muyzer & Smalla, 1998; Fromin et al., 2002; Marzorati et al., 2008). Nevertheless, most of these limitations can be minimized (Marzorati et al., 2008). In adition, if one is specifically interested in a particular group within the bacterial community, it becomes difficult to take conclusions from the complex DGGE profile obtained by using bacterial universal primers. Thus, hybridization analysis with taxonspecific oligonucleotides probes is possible, but specific primer sets for DGGE analyses have also been developed, targeting groups such as *Cyanobacteria* (Nübel et al., 1997), *Actinobacteria* (Allgaier et al., 2007), *Bacteroidetes* (Possemiers et al., 2004) or *Betaproteobacteria* (Brümmer et al., 2003). By avoiding the amplification of other bacteria present in the sample, the DGGE profiles become simpler, also allowing the visualization of less abundant phylotypes and preventing the contamination in sequencing with DNA from those other nontarget bacteria. Moreover, DGGE fingerprinting data associated with statistical analysis (Fromin et al., 2002; Ramette, 2007; Marzorati et al., 2008) may provide important ecological information on target bacterial communities from different temporal or spatial environmental samples, allowing improving the development of microbial resource management for water bodies.

Microcystins, in particular, are secondary metabolites produced non-ribosomally which have proven to be potent hepatotoxins with serious risks for many organisms (Wiegand & Pflugmacher, 2005; Valdor & Aboal, 2007), including humans (Ueno et al., 1996; Pouria et al., 1998; de Figueiredo et al., 2004b). Thus, the monitoring for microcystin-producing potential is very important during the occurrence of cyanobacterial blooms. This can be easily and rapidly

performed by PCR, using primers targeting regions of the microcystin-synthetase gene cluster (*mcy* operon) (Kaebernick & Neilan, 2001; Tooming-Klunderud et al., 2008). Currently, there are also primers available to screen for the cylindrospermopsin-production potential (Schembri et al., 2001) during blooms of *Aphanizomenon ovalisporum* or the invasive *Cylindrospermopsis raciborskii* and *Aph. aphanizomenoides* species (Stefaniak & Kokociński, 2005).

Classical vs. modern cyanobacterial taxonomy – implications of intra-specific genotypic variability on identification and characterization of cyanobacterial strains

Presently, five orders of *Cyanobacteria* are recognized: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales e Stigonematales (Komárek & Anagnostidis, 1999). Over the half century, after the improvement of molecular approaches based on PCR and 16S rRNA gene sequencing, there have been major changes in the cyanobacterial taxonomy (namely at the genera and species concepts) but even more are expected (Komárek, 2010a; Komárek, 2010b). Speciation is enhanced by factors such as the rapid adaptation of cyanobacteria to different environmental conditions (Komárek & Kaštovský, 2003) and the horizontal transfer of genetic material, often associated with the toxicity potential (Lodders et al., 2005; Christiansen et al., 2008). Modern cyanobacterial taxonomy is based on the assumption that different classical "genera" correspond to different molecular genotypes (clusters with a similarity index of 95% or less, using the 16S rRNA gene sequence) (Komárek, 2010b). However, at the subgeneric level, 16S rRNA sequences may not be sufficiently discriminatory to distinguish among clearly different morphotypes and ecotypes (Komárek, 2010b). At the light of plylogenetic affiliation results, many filamentous cyanobacteria belonging to the monophyletic clade of Nostocales were reviewed concerning their classical morphological characterization (currently including cytomorphological information) (Komárek, 2006). Particularly the family Nostocacean (order Nostocales) has been suffering many changes over the past years (Rajaniemi et al., 2005a; Zapomelová et al., 2009). A revision of the nostocacean genera has been recently published by Komárek et al. (2010a; 2010b). For several genera, such as *Aphanizomenon*, a correct identification through phenotypical characters is often compromised due to the ambiguity of morphological features (Hindák, 2000; Li et al., 2000; Komárek & Komárková, 2006), especially when handling strains maintained under laboratory conditions for a long time (Palinska *et al.*, 1996; Nübel *et al.*, 1997; Zapomělová, 2006). Spontaneous mutants are known to occur among cultured cyanobacteria under certain media conditions (Jeeji-Bai, 1976; Singh, 1976; Das & Singh, 1977) and cases of misidentifications of *Aphanizomenon* spp. based on morphological features have been reported (Li *et al.*, 2000; Li *et al.*, 2003; Wood *et al.*, 2007). There were also found differences between populations from geographically and ecologically distant ecosystems which highlights the need to a better characterization of cyanobacteria through complex studies combining not only molecular and

phenotypic analyses, but also exploring cytomorphological, biochemical and ecological features (Komárek, 2006; Komárek, 2010a). Fingerprinting based on repetitive sequences such as short tandemly repeated repetitive (STRR) (Mazel *et al.*, 1990), and highly iterated palindrome (HIP1) (Smith *et al.*, 1998) sequences, which are present in many cyanobacteria, has been also a very useful technique to assess genetic variability among cyanobacterial strains, even using non-axenic cultures (Rasmussen & Svenning, 1998; Lehtimäki et al., 2000; Bruno et al., 2006). For example, over the last years, studies have reported intra-specific genetic variation within *Microcystis* spp. (Yoshida et al., 2008) and the problem associated with the fact that there are toxic and non-toxic strains, sometimes co-existing in a same water body and even within a same bloom, determining its toxicity (Kardinaal et al., 2007). Methods based on PCR amplification and the use of cyanobacteria-specific primers targeting highly conserved domains inside the 16S rRNA gene (Nübel *et al.*, 1997) have also contributed to a more objective identification and discrimination of cultured cyanobacterial strains and serve as basis for the reorganization of present taxonomy. The use of specific primers is important since it can be very difficult to obtain axenic cultures from cyanobacteria, particularly filamentous strains (Abed & Köster, 2005; Bruno et al., 2006). Complementary to this, partial sequences of protein-coding genes such as *hetR*, coding for a serine type protease essential in heterocyst differentiation (Janson *et al.*, 1998; Schiefer *et al.*, 2002; Zhang *et al.*, 2006), and *nifH*, coding for the dinitrogenase reductase (Zehr & McReynolds, 1989), also give relevant information for phylogenetic differentiation (Dyble *et al.*, 2002; Haande *et al.*, 2008). By the exposed above, intra-specific genetic variability may play an important role in the cyanobacterial blooms dynamics and toxicity, which makes it a major issue for the study of bloom-forming strains.

Aims and structure of the thesis

Climatic changes are leading to an increase and severity of drought episodes in Southern Europe during summer months, which must have major impacts on aquatic communities. The information on the background bacterial community composition (BCC), in particular, is almost inexistent for Portuguese freshwater bodies, hindering the evaluation of the drought impact on that community, although an enhancement of cyanobacterial blooms usually occurs in eutrophic systems. In this context, the main goal of the present work was to screen the bacterioplankton community in several Portuguese water bodies with eutrophic potential and try to contextualize the occurrence of cyanobacterial blooms in the overall bacterial community. For that purpose, the following specific objectives were established for this thesis:

- Characterization of bacterioplankton community in lentic freshwater bodies with eutrophic potential, including Crestuma reservoir and Fermentelos and Vela lakes (chapter I);
- Characterization of bacterioplankton community in riverine freshwater bodies with eutrophic potential, including Cértima, Sousa and Antuã rivers (chapter II);
- Characterization of bacterioplankton community from Portuguese freshwater bodies under severe drought conditions – increased risks associated with cyanobacterial blooms (chapter III);
- Characterization of bloom-forming cyanobacterial strains isolated from Vela Lake, including genetic and ecological features of *Aphanizomenon* strains and genetic characteristics of *Microcystis* strains (chapter IV);
- Associate most important environmental parameters to the dominance of target bacterial phylotypes at the studied water bodies and thus contribute to a better understanding of the ecology of the bacterioplankton community in temperate aquatic systems.

The present thesis begins with a general introduction to contextualize the aims of the work and ends with a section for general conclusions in order to systematize and resume the results obtained during all the work. The remaining thesis is structured in chapters, which correspondingly incorporate most of the especific objectives described above. Inside each chapter, there are more than one issue discussed, corresponding to independent subchapters, with their own Introduction, Materials and methods, Results, Discussion and References.

References

Abed R. M. M. & Köster J. (2005). The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. *International Biodeterioration and Biodegradation* 55: 29-37.

Allgaier M., Brückner S., Jaspers E. & Grossart H. P. (2007). Intra- and inter-lake variability of free-living and particle-associated Actinobacteria communities. *Environmental Microbiology* 9: 2728-2741.

Allgaier M. & Grossart H. P. (2006a). Diversity and seasonal dynamics of *Actinobacteria* populations in four lakes in Northeastern Germany. *Applied and Environmental Microbiology* 72: 3489-3497.

Allgaier M. & Grossart H. P. (2006b). Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

Altmann D., Stief P., Amann R., Beer D. d. & Schramm A. (2003). *In situ* distribution and activity of nitrifying bacteria in freshwater sediment. *Environmental Microbiology* 5: 798-803.

Amer R., Díez B. & El-Shehawy R. (2009). Diversity of hepatotoxic cyanobacteria in the Nile Delta, Egypt. *Journal of Environmental Monitoring* 11: 126-133.

Anderson-Glenna M. J., Bakkestuen V. & Clipson N. J. W. (2008). Spatial and temporal variability in epilithic biofilm bacterial communities along an upland river gradient. *FEMS Microbiology Ecology* 64: 407-418.

Bent S. J. & Forney L. J. (2008). The tragedy of the uncommon: Understanding limitations in the analysis of microbial diversity. *ISME Journal* 2: 689-695.

Berg K. A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P. & Rapala J. (2008). High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME Journal* 3: 314-325.

Bittencourt-Oliveira M. D. C., De Oliveira M. C. & Bolch C. J. S. (2001). Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (cpcBA). *Journal of Phycology* 37: 810-818.

Bond N., Lake P. & Arthington A. (2008). The impacts of drought on freshwater ecosystems: an Australian perspective. *Hydrobiologia* 600: 3-16.

Boucher D., Jardillier L. & Debroas D. (2006). Succession of bacterial community composition over two consecutive years in two aquatic systems: A natural lake and a lake-reservoir. *FEMS Microbiology Ecology* 55: 79-97.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Brümmer I. H. M., Felske A. & Wagner-Döbler I. (2003). Diversity and Seasonal Variability of {beta}-Proteobacteria in Biofilms of Polluted Rivers: Analysis by Temperature Gradient Gel Electrophoresis and Cloning. *Appl. Environ. Microbiol.* 69: 4463-4473.

Bruno L., Billi D., Albertano P. & Urzi C. (2006). Genetic characterization of epilithic cyanobacteria and their associated bacteria. *Geomicrobiology Journal* 23: 293-299.

Burrows W. D. & Renner S. E. (1999). Biological warfare agents as threats to potable water. *Environ Health Perspectives* 107: 975-984.

Cadel-Six S., Peyraud-Thomas C., Brient L., De Marsac N. T., Rippka R. & Me?jean A. (2007). Different genotypes of anatoxin-producing cyanobacteria coexist in the Tarn River, France. *Applied and Environmental Microbiology* 73: 7605-7614.

Caetano S., Miguel R., Mendes P., Galvão H. & Barbosa A. (2001). Cyanobacteria blooms and cyanotoxin occurrence in the Guadiana (SE Portugal) - preliminary results. *Ecotoxicology and Environmental Restoration* 4: 53-59.

Charron D. F., Thomas M. K., Waltner-Toews D., Aramini J. J., Edge T., Kent R. A., Maarouf A. R. & Wilson J. (2004). Vulnerability of waterborne diseases to climate change in Canada: A review. *Journal of Toxicology and Environmental Health - Part A* 67: 1667-1677.

Chorus I. & Fastner J., 2001. Recreational Exposure to Cyanotoxins. In: I. Chorus, (ed.), Cyanotoxins - occurrence, causes, consequences. Springer, Berlin, Germany: 190-199.

Christiansen G., Molitor C., Philmus B. & Kurmayer R. (2008). Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. *Molecular Biology and Evolution* 25: 1695-1704.

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Codd G. A., Azevedo S. M. F. O., Bagchi S. N., Burch M. D., Carmichael W. W., Harding W. R., Kaya K. & Utkilen H. C., CYANONET - A Global Network for Cyanobacterial Bloom and Toxin Risk Management - Initial Situation Assessment and Recommendations. IHP-VI Technical Documents in Hydrology, Vol. 76. UNESCO, Paris, 2005a.

Codd G. A., Edwards C., Beattie K. A., Lawton L. A., Campbell D. L. & Bell S. G., 1995. Toxins from cyanobacteria (blue-green algae). In: W. Wiessner, E. Schnepf & R. C. Starr, (eds.), Algae, Environment and Human Affairs. Biopress Ltd., Bristol, England: 1-17.

Codd G. A., Metcalf J. S. & Beattie K. A. (1999). Retention of *Microcystis aeruginosa* and microcystin by salad lettuce (*Lactuca sativa*) after spray irrigation with water containing cyanobacteria. *Toxicon* 37: 1181-1185.

Codd G. A., Morrison L. F. & Metcalf J. S. (2005b). Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology* 203: 264-272.

Cottrell M. T., Waidner L. A., Yu L. & Kirchman D. L. (2005). Bacterial diversity of metagenomic and PCR libraries from the Delaware River. *Environmental Microbiology* 7: 1883-1895.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

Das B. & Singh P. K. (1977). Isolation, growth and nitrogen fixation of a gas vacuole-less mutant of the blue green alga *Anabaena aphanizomenoides*. *Cytobios* 17: 151-158.

de Figueiredo D. R., Antunes S. C., Pereira M. J. & Gonçalves F. (2004a). Chronic effects of *Aphanizomenon flos-aquae* on the survival and reproduction of daphnids. *Fresenius Environmental Bulletin* 13: 665-670.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004b). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Azeiteiro U. M., Gonçalves F. & Pereira M. J. (2004c). *Aphanizomenon flos-aquae* grown under different nutrient concentrations and the effects of its exudates on growth of two green algae. *Fresenius Environmental Bulletin* 13: 657-664.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Degerholm J., Gundersen K., Bergman B. & Söderbäck E. (2006). Phosphorus-limited growth dynamics in two Baltic Sea cyanobacteria, *Nodularia* sp. and *Aphanizomenon* sp. *FEMS Microbiology Ecology* 58: 323-332.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Dorigo U., Volatier L. & Humbert J. F. (2005). Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Research* 39: 2207-2218.

Dow C. S. & Swoboda U. K., 2000. Cyanotoxins. In: B. A. Whitton & M. Potts, (eds.), The Ecology of Cyanobacteria. Kluwer Academic Publishers, The Netherlands: 613-632.

Dyble J., Paerl H. W. & Neilan B. A. (2002). Genetic characterization of *Cylindrospermopsis raciborskii* (cyanobacteria) isolates from diverse geographic origins based on nifH and cpcBA-IGS nucleotide sequence analysis. *Applied and Environmental Microbiology* 68: 2567-2571.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Falconer I. R. & Humpage A. R. (2006). Cyanobacterial (blue-green algal) toxins in water supplies: Cylindrospermopsins. *Environmental Toxicology* 21: 299-304.

Figueredo C. C., Giani A. & Bird D. F. (2007). Does allelopathy contribute to *Cylindrospermopsis raciborskii* (cyanobacteria) bloom occurrence and geographic expansion? *Journal of Phycology* 43: 256-265.

Fitzgerald D. J., 2001. Cyanotoxins and Human Health - Overview. In: I. Chorus, (ed.), Cyanotoxins - occurrence, causes, consequences. Springer, Berlin, Germany: 179-190.

Flores E. & Herrero A. (2010). Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Micro* 8: 39-50.

Fromin N., Hamelin J., Tarnawski S., Roesti D., Jourdain-Miserez K., Forestier N., Teyssier-Cuvelle S., Gillet F., Aragno M. & Rossi P. (2002). Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environmental Microbiology* 4: 634-643.

Galvão H. M., Reis M. P., Valério E., Domingues R. B., Costa C., Lourenço D., Condinho S., Miguel R., Barbosa A., Gago C., Faria N., Paulino S. & Pereira P. (2008). Cyanobacterial blooms in natural waters in southern Portugal: A water management perspective. *Aquatic Microbial Ecology* 53: 129-140.

Gehringer M. M., Govender S., Shah M. & Downing T. G. (2003). An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. *Environmental Toxicology* 18: 142-148.

Haande S., Rohrlack T., Ballot A., RÃ¸berg K., Skulberg R., Beck M. & Wiedner C. (2008). Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) isolates from Africa and Europe. *Harmful Algae* 7: 692-701.

Hindák F. (2000). Morphological variation of four planktic nostocalean cyanophytes - Members of the genus *Aphanizomenon* or *Anabaena*? *Hydrobiologia* 438: 107-116.

Innok S., Matsumura M., Boonkerd N. & Teaumroong N. (2005). Detection of *Microcystis* in lake sediment using molecular genetic techniques. *World Journal of Microbiology and Biotechnology* 21: 1559-1568.

Jacoby J. M., Collier D. C., Welch E. B., Hardy F. J. & Crayton M. (2000). Environmental factors associated with a toxic bloom of Microcystis aeruginosa. *Canadian Journal of Fisheries and Aquatic Sciences* 57: 231-240.

Janson S., Matveyev A. & Bergman B. (1998). The presence and expression of *hetR* in the non-heterocystous cyanobacterium *Symploca* PCC 8002. *FEMS Microbiology Letters* 168: 173-179.

Jeeji-Bai N. (1976). Morphological variation of certain blue-green algae in culture: *Scytonema stuposum* (Kütz.) Born. *Schweizerische Zeitschrift für Hydrologie* 38: 55-62.

Kaebernick M. & Neilan B. A. (2001). Ecological and molecular investigations of cyanotoxin production. *FEMS Microbiology Ecology* 35: 1-9.

Kardinaal W. E. A., Tonk L., Janse I., Hol S., Slot P., Huisman J. & Visser P. M. (2007). Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. *Applied and Environmental Microbiology* 73: 2939-2946.

Kaštovský J., Hauer T., Mareš J., Krautová M., Bešta T., Komárek J., Desortová B., Heteša J., Hindáková A., Houk V., Janeček E., Kopp R., Marvan P., Pumann P., Skácelová O. & Zapomělová E. (2010). A review of the alien and expansive species of freshwater cyanobacteria and algae in the Czech Republic. *Biological Invasions* DOI 10.1007/s10530-010-9754-3.

Kearns K. & Hunter M. (2001). Toxin-producing *Anabaena flos-aquae* induces settling of *Chlamydomonas reinhardtii*, a competing motile alga. *Microbial Ecology* 42: 80-86.

Komárek J. (1991). A review of water-bloom forming *Microcystis* species, with regard to populations from Japan. *Archives of Hydrobiology* Suppl 92: 115-127.

Komárek J. (2006). Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. *Algae* 21: 349-375.

Komárek J. (2010a). Modern taxonomic revision of planktic nostocacean cyanobacteria: a short review of genera. *Hydrobiologia* 639: 231-243.

Komárek J. (2010b). Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). *Hydrobiologia* 639: 245-259.

Komárek J. & Anagnostidis K., 1999. Cyanoprokariota - 1. Teil Chroococcales. In: H. Ettl, G. Gärtner, H. Heynig & D. Mollenhauer, (eds.), Sübwasserflora von Mitteleuropa, Band 19/1. G. Fischer Verlag, Jena, Stuttgart, Lübeck, Ulm.

Komárek J. & Kaštovský J. (2003). Coincidences of structural and molecular characters in evolutionary lines of cyanobacteria. *Algological Studies (Cyanobacterial Research 4)* 109: 305-325.

Komárek J. & Komárková J. (2006). Diversity of *Aphanizomenon*-like cyanobacteria. *Czech Phycology* 6: 1-32.

Lee H.-W., Lee S.-Y., Lee J.-W., Park J.-B., Choi E.-S. & Park Y. K. (2002). Molecular characterization of microbial community in nitrate-removing activated sludge. *FEMS Microbiology Ecology* 41: 85-94.

Lehtimäki J., Lyra C., Suomalainen S., Sundman P., Rouhiainen L., Paulin L., Salkinoja-Salonen M. & Sivonen K. (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *International Journal of Systematic and Evolutionary Microbiology* 50: 1043-1053.

Lehtimaki J., Moisander P., Sivonen K. & Kononen K. (1997). Growth, Nitrogen Fixation, and Nodularin Production by Two Baltic Sea Cyanobacteria. *Applied and Environmental Microbiology* 63: 1647-1656.

Li R., Carmichael W. W. & Pereira P. (2003). Morphological and 16s rRNA gene evidence for reclassification of the paralytic shellfish toxin producing *Aphanizomenon flos-aquae* LMECYA 31 as *Aphanizomenon issatschenkoi* (Cyanophyceae). *Journal of Phycology* 39: 814-818.

Li R., Watanabe M. & Watanabe M. M. (2000). Taxonomic studies of planktic species of *Anabaena* based on morphological characteristics in cultured strains. *Hydrobiologia* 438: 117-138.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Lodders N., Stackebrandt E. & Nübel U. (2005). Frequent genetic recombination in natural populations of the marine cyanobacterium *Microcoleus chthonoplastes*. *Environmental Microbiology* 7: 434-442.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Markensten H., Moore K. & Persson I. (2010). Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate. *Ecological Applications* 20: 752-767.

Marzorati M., Wittebolle L., Boon N., Daffonchio D. & Verstraete W. (2008). How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental Microbiology* 10: 1571-1581.

Mazel D., Houmard J., Castets A. M. & Tandeau de Marsac N. (1990). Highly repetitive DNA sequences in cyanobacterial genomes. *Journal of Bacteriology* 172: 2755-2761.

Mazur-Marzec H. (2006). Characterization of phycotoxins produced by cyanobacteria. *Oceanological and Hydrobiological Studies* 35: 85-109.

Mischke U. (2003). Cyanobacteria associations in shallow polytrophic lakes: influence of environmental factors. *Acta Oecologica* 24.

Mitraki C., Crisman T. L. & Zalidis G. (2004). Lake Koronia, Greece: Shift from autotrophy to heterotrophy with cultural eutrophication and progressive water-level reduction. *Limnologica - Ecology and Management of Inland Waters* 34: 110-116.

Muylaert K., Van der Gucht K., Vloemans N., De Meester L., Gillis M. & Vyverman W. (2002). Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. *Applied and Environmental Microbiology* 68: 4740-4750.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G. & Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 73: 127-141.

Neilan B. A., Saker M. L., Fastner J., Törökné A. & Burns B. P. (2003). Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. *Molecular Ecology* 12: 133-140.

Newton R. J., Jones S. E., Helmus M. R. & McMahon K. D. (2007). Phylogenetic ecology of the freshwater Actinobacteria acI lineage. *Applied and Environmental Microbiology* 73: 7169-7176.

Nixdorf B., Mischke U. & Rücker J. (2003). Phytoplankton assemblages and steady state in deep and shallow eutrophic lakes - An approach to differentiate the habitat properties of Oscillatoriales. *Hydrobiologia* 502: 111-121.

Nübel U., Garcia-Pichel F. & Muyzer G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology* 63: 3327-3332.

Oliver R. L. & Ganf G. G., 2000. Freshwater blooms. In: B. A. Whitton & M. Potts, (eds.), The Ecology of Cyanobacteria. Kluwer Academic Publishers: 149-194.

Palinska K. A., Liesack W., Rhiel E. & Krumbein W. E. (1996). Phenotype variability of identical genotypes: The need for a combined approach in cyanobacterial taxonomy demonstrated on *Merismopedia*-like isolates. *Archives of Microbiology* 166: 224-233.

Pearce D. A., van der Gast C. J., Woodward K. & Newsham K. K. (2005). Significant changes in the bacterioplankton community structure of a maritime Antartic freshwater lake following nutrient enrichment. *Microbiology* 151: 3237-3248.

Pope P. B. & Patel B. K. C. (2008). Metagenomic analysis of a freshwater toxic cyanobacteria bloom. *FEMS Microbiology Ecology* 64: 9-27.

Possemiers S., Verthé K., Uyttendaele S. & Verstraete W. (2004). PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. *FEMS Microbiology Ecology* 49: 495-507.

Pouria S., De Andrade A., Barbosa J., Cavalcanti R. L., Barreto V. T. S., Ward C. J., Preiser W., Poon G. K., Neild G. H. & Codd G. A. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 352: 21-26.

Preußel K., Stüken A., Wiedner C., Chorus I. & Fastner J. (2006). First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. *Toxicon* 47: 156-162.

Rajaniemi P., Hrouzek P., Kastovska K., Willame R., Rantala A., Hoffmann L., Komarek J. & Sivonen K. (2005). Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *International Journal of Systematic and Evolutionary Microbiology* 55: 11-26.

Ramette A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiology Ecology* 62: 142-160.

Rapala J., Niemela M., Berg K. A., Lepisto L. & Lahti K., Removal of cyanobacteria, cyanotoxins, heterotrophic bacteria and endotoxins at an operating surface water treatment plant. Water Science and Technology, Vol. 54, 2006, pp. 23-28.

Rapala J., Sivonen K., Lyra C. & NiemelÃ¤ S. I. (1997). Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Applied and Environmental Microbiology* 63: 2206-2212.

Rasmussen U. & Svenning M. M. (1998). Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. *Applied and Environmental Microbiology* 64: 265-272.

Riemann L. & Winding A. (2001). Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microbial Ecology* 42: 274-285.

Saadoun I. M. K., Schrader K. K. & Blevins W. T. (2001). Environmental and nutritional factors affecting geosmin synthesis by Anabaena SP. *Water Research* 35: 1209-1218.

Saker M. L., Fastner J., Dittmann E., Christiansen G. & Vasconcelos V. M. (2005a). Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. *Journal of Applied Microbiology* 99: 749-757.

Saker M. L., Jungblut A. D., Neilan B. A., Rawn D. F. K. & Vasconcelos V. M. (2005b). Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium *Aphanizomenon flos-aquae*. *Toxicon* 46: 555-562.

Saker M. L., Nogueira I. C. G., Vasconcelos V. M., Neilan B. A., Eaglesham G. K. & Pereira P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55: 243-250.

Schembri M. A., Neilan B. A. & Saint C. P. (2001). Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermosis raciborskii*. *Environmental Toxicology* 16: 413-421.

Schiefer W., Schütz K., Hachtel W. & Happe T. (2002). Molecular cloning and characterization of hetR genes from filamentous cyanobacteria. *Biochimica et Biophysica Acta - Gene Structure and Expression* 1577: 139-143.

Šimek K., Horňák K., Jezbera J., Nedoma J., Znachor P., Hejzlar J. & Sed'a J. (2008). Spatio-temporal patterns of bacterioplankton production and community composition related to phytoplankton composition and protistan bacterivory in a dam reservoir. *Aquatic Microbial Ecology* 51: 249-262.

Singh P. K. (1976). Growth and nitrogen fixation of mutants defective in heterocyst differentiation in the blue green alga *Cylindrospermum* sp. *Zeitschrift fur Allgemeine Mikrobiologie* 16: 453-463.

Smith J. K., Parry J. D., Day J. G. & Smith R. J. (1998). A PCR technique based on the Hip1 interspersed repetitive sequence distinguishes cyanobacterial species and strains. *Microbiology* 144: 2791-2801.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

Stefaniak K. & Kokociński M. (2005). Occurence of invasive *Cyanobacteria* species in polimictic lakes of the Wielkopolska region (western Poland). *Ocean. Hydrob. Studies* 34: 137-148.

Steffensen D. A. (2008). Economic cost of cyanobacterial blooms. *Advances in experimental medicine and biology* 619: 855-865.

Stewart I., Webb P., Schluter P. & Shaw G. (2006). Recreational and occupational field exposure to freshwater cyanobacteria – a review of anecdotal and case reports, epidemiological studies and the challenges for epidemiologic assessment. *Environmental Health: A Global Access Science Source* 5: 6.

Stüken A., Rücker J., Endrulat T., Preussel K., Hemm M., Nixdorf B., Karsten U. & Wiedner C. (2006). Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. *Phycologia* 45: 696-703.

Tian C., Tan J., Wu X., Ye W., Liu X., Li D. & Yang H. (2009). Spatiotemporal transition of bacterioplankton diversity in a large shallow hypertrophic freshwater lake, as determined by denaturing gradient gel electrophoresis. *J. Plankton Res.* 31: 885-897.

Tooming-Klunderud A., Mikalsen B., Kristensen T. & Jakobsen K. S. (2008). The mosaic structure of the *mcyABC* operon in *Microcystis*. *Microbiology* 154: 1886-1899.

Ueno Y., Nagata S., Tsutsumi T., Hasegawa A., Watanabe M. F., Park H.-D., Chen G.-C., Chen G. & Yu S.-Z. (1996). Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* 17: 1317-1321.

Valdor R. & Aboal M. (2007). Effects of living cyanobacteria, cyanobacterial extracts and pure microcystins on growth and ultrastructure of microalgae and bacteria. *Toxicon* 49: 769-779.

Valério E., Faria N., Paulino S. & Pereira P. (2008). Seasonal variation of phytoplankton and cyanobacteria composition and associated microcystins in six Portuguese freshwater reservoirs. *Annales de Limnologie* 44: 189-196.

Valério E., Pereira P., Saker M. L., Franca S. & Tenreiro R. (2005). Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. *Harmful Algae* 4: 1044-1052.

van Apeldoorn M. E., van Egmond H. P., Speijers G. J. A. & Bakker G. J. I. (2007). Toxins of cyanobacteria. *Molecular Nutrition & Food Research* 51: 7-60.

Van Der Gucht K., Cottenie K., Muylaert K., Vloemans N., Cousin S., Declerck S., Jeppesen E., Conde-Porcuna J. M., Schwenk K., Zwart G., Degans H., Vyverman W. & De Meester L. (2007). The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20404-20409.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Vasconcelos V., 2001. Freshwater cyanobacteria and their toxins in Portugal. In: I. Chorus, (ed.), Cyanotoxins - Occurrence, Causes, Consequences. Springer, Berlin: 62-67.

Vasconcelos V. M. (1999). Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research* 32: 249-254.

Vasconcelos V. M., Campos T., Amorim A. & Soares A. M. V. M. (1993). Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 193-201.

Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 30: 2377-2384.

Wei C., Bao S., Zhu X. & Huang X. (2008). Spatio-temporal variations of the bacterioplankton community composition in Chaohu Lake, China. *Progress in Natural Science* 18: 1115-1122.

WHO, 2003. Algae and cyanobacteria in fresh water. Guidelines for safe recreational water environments. Vol. 1: Coastal and fresh waters. World Health Organization, Geneva, Switzerland: 136-158.

Wiedner C., Rücker J., Brüggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.

Wiegand C. & Pflugmacher S. (2005). Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicology and Applied Pharmacology* 203: 201-218.

Wilson A. E., Sarnelle O., Neilan B. A., Salmon T. P., Gehringer M. M. & Hay M. E. (2005). Genetic Variation of the Bloom-Forming Cyanobacterium *Microcystis aeruginosa* within and among Lakes: Implications for Harmful Algal Blooms. *Applied and Environmental Microbiology* 71: 6126-6133.

Wood S. A., Rasmussen J. P., Holland P. T., Campbell R. & Crowe A. L. M. (2007). First report of the cyanotoxin anatoxin-a from *Aphanizomenon issatschenkoi* (cyanobacteria). *Journal of Phycology* 43: 356-365.

Wu X., Xi W., Ye W. & Yang H. (2007). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Xi W., Wu X., Ye W. & Yang H. (2007). Changes in bacterial community structure during preceding and degraded period of cyanobacterial bloom in a bay of the Taihu Lake. *Chinese Journal of Applied and Environmental Biology* 13: 97-103.

Xing P. & Kong F. (2007). Intra-habitat heterogeneity of environmental factors regulating bacterioplankton community composition in Lake Taihu, China. *Aquatic Microbial Ecology* 48: 113-122.

Yannarell A. C. & Triplett E. W. (2005). Geographic and Environmental Sources of Variation in Lake Bacterial Community Composition. *Appl. Environ. Microbiol.* 71: 227-239.

Yoshida M., Yoshida T., Satomi M., Takashima Y., Hosoda N. & Hiroishi S. (2008). Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. *Journal of Applied Microbiology* 105: 407-415.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Zapomělová E. (2006). Current taxonomic issues with planktonic representatives of the genus *Anabaena* (Cyanobacteria) with special reference to their morphological features; literary review. *Czech Phycology* 6: 33-47.

Zapomelová E., Jezberová J., Hrouzek P., Hisem D., Reháková K. & Komárková J. (2009). Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (Cyanobacteria) and their reclassification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana*). *Journal of Phycology* 45: 1363-1373.

Zehr J. P. & McReynolds L. A. (1989). Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Applied and Environmental Microbiology* 55: 2522-2526.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology* 25: 763-772.

Zhang C. C., Laurent S., Sakr S., Peng L. & Bédu S. (2006). Heterocyst differentiation and pattern formation in cyanobacteria: A chorus of signals. *Molecular Microbiology* 59: 367-375.

Zhou L., Yu H. & Chen K. (2002). Relationship between microcystin in drinking water and colorectal cancer. *Biomedical and Environmental Sciences* 15: 166-171.

Zwart G., Crump B. C., Kamst-van Agterveld M. P., Hagen F. & Han S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* 28: 141-155.

Chapter I

Characterization of bacterioplankton community in lentic freshwater bodies with eutrophic potential

Water temperature, nutrients and pH as major seasonal modulators of bacterioplankton community at the temperate eutrophic shallow Fermentelos Lake (Central Portugal)

In: de Figueiredo D. R., Pereira M. J. & Correia A. (2010). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26: 1067-1077.

Abstract

At the present time, there is still a lack of information about environmental parameters modulating variations on bacterial diversity in temperate lakes, particularly from Portugal. Fermentelos Lake (Central Portugal) is a shallow water body that sustains an important wetland area. The strong nutrient inputs from agriculture and industrial runoffs have led to its current eutrophic status. The present work aimed to understand which factors modulate the seasonal bacterioplankton diversity at this lake using *16S rRNA* PCR-DGGE (denaturing gradient gel electrophoresis) and multivariate analysis. Environmental data demonstrated eutrophic features throughout all samples with nitrate concentrations reaching 12.0 mg N (NO_3^-) L^{-1} in March 2006, while the highest conductivity (609 µS cm^{-1}), soluble reactive phosphorus (0.37 mg L^{-1}), total suspended solids (87.2 mg L^{-1}) and chlorophyll *a* (286.6 µg L^{-1}) levels were recorded in August 2007. Over the past two decades there was a general increase in nitrate, pH and conductivity levels at this lake, suggesting the eutrophication process is still in progress. Multivariate analysis showed that summer *vs.* winter DGGE patterns could be established for bacterial assemblages and were mainly defined by water temperature and chlorophyll *a*. *Actinobacteria* were dominant throughout the study period although a general preference for higher temperature, pH, total suspended solids, conductivity, soluble reactive phosphorus (SRP) and chlorophyll *a* was observed. The highest nitrogen sources were related to *Bacteroidetes* and phototrophic eukaryote (cryptophycean) dominance. The expansion of *Betaproteobacteria, Alphaproteobacteria* and *Cyanobacteria* phylotypes was generally associated to high temperature, pH, conductivity and SRP values.

Keywords

eutrophication, Fermentelos Lake, bacterioplankton diversity, 16SrDNA-DGGE, CCA.

Introduction

Eutrophication of lentic freshwater systems is being enhanced by climatic changes with drought episodes increasing water retention times, particularly in shallow water bodies, and leading to important changes in physical and chemical characteristics as well as in the composition of the living communities (Mitraki et al., 2004). The bacterial community composition (BCC) is altered with the development enhancement of some groups such as *Cyanobacteria* and *Actinobacteria* (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007) which may put at risk the water quality (Zaitlin & Watson, 2006; Smith et al., 2008). The massive growth of *Cyanobacteria*, in particular, can lead to toxic outcomes and endanger human health safety (Codd, 2000; de Figueiredo et al., 2004b). Therefore, the establishment of water management strategies requires previous knowledge on the ecological dynamics of target communities in the water body. Culture-independent molecular methodologies may play an important role in monitoring management plans since techniques such as PCR-DGGE (polymerase chain reaction denaturing gradient gel electrophoresis) have proven over the last years to be suitable for a general and rapid characterization of water bacterial assemblages allowing to compare temporal or/and spatial patterns (Muyzer et al., 1993; Muyzer & Smalla, 1998; Lyautey et al., 2005; Tian et al., 2009) since it considers also the diversity of microorganisms not cultivable at the present time (Bent & Forney, 2008). As for most Portuguese water bodies, the information on the shifts of BCC dynamics at Fermentelos Lake is inexistent or very scarce (de Figueiredo et al., 2007) which restricts the development of effective water management planning. Thus, it is urgent to obtain data and make public studies on the seasonal bacterial diversity as the present one.

Fermentelos Lake is a eutrophic shallow water body located in Western Central Portugal and which sustains an important wetland area in this region (Gil, 1988). Since long time, it has recurrently been suffering from a strong nutrient and pesticides input from agriculture runoffs but also domestic effluents and heavy metal contamination from industry (Rino & Gil, 1987; Gil, 1988; Pinho et al., 1988; Calado et al., 1991; Cerqueira et al., 2005). Considering the eutrophic status of the lake for the last twenty five years, the present work aimed to study, for the first time, the seasonal shifts in bacterioplankton diversity at Fermentelos Lake using *16S rRNA* PCR-DGGE band profiles and relate them to variation of environmental parameters through multivariate analysis.

Materials and methods

Sampling and environmental parameters

Fermentelos Lake is a fishing and recreational water body located in Fermentelos, Western Central Portugal (40° 34' N and 8° 30' W) which has been considered eutrophic to hypereutrophic (Calado & Craveiro, 1995; de Figueiredo et al., 2007). Cértima River is its main tributary [with

high nutrient and organic charge (Rino & Gil, 1987; Cerqueira et al., 2005)] along with Ribeira do Pano as well as runoffs from surrounding fields. The lake's area and depth ranges are 3-5 Km^2 and 1-5 m, respectively, depending on seasonality (Gil, 1988; Calado & Craveiro, 1995). The high nutrient levels and the massive development of macrophytes in the lake have been previously described (Gil, 1988; Calado et al., 1991; Almeida et al., 2006). The sampling was performed in winters and summers 2005-07, springs 2006-07 and autumn 2006 (see Table 1). Water samples were taken sub-superficially using sterile bottles at about 1 m from the shore. Samples were placed at 4 ºC under dark conditions until subsequent treatment within 12 hours. Water temperature was measured *in situ* and pH and conductivity were measured in laboratory at 20 ºC using specific electrodes. Precipitation data for 7 days (Precip7d) and 1 month (Precip1m) before sampling date were obtained from Portuguese Water Institute data series (http://snirh.inag.pt/). Parameters such as total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium, nitrate and nitrite concentrations were determined according to standard procedures (APHA, 1992; Rodier, 1996).

DNA extraction and PCR amplification of bacterial 16S rDNA fragments

Total DNA from environmental water samples was extracted as follows: 100 mL of the water samples were filtered through 0.22 µm polycarbonate sterile filters, resuspended in 2 mL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0] and centrifugated; lysozyme was added after resuspension in 200 µL of TE and incubation was performed at 37ºC for 1h; the following DNA extraction and purification were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania); DNA was finally suspended in TE buffer and stored at -20 ºC. PCR amplification of bacterial *16S rRNA* gene fragments was performed with the primers 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, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 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 30s at 92 °C, 30s at 55 °C and 30s at 72 °C, and a final extension step at 72 °C for 30 min. Negative control reactions without any template DNA were performed simultaneously and the size of amplicons was verified by electrophoresis in a 1.5% agarose gel using GeneRuler™ 1 kb DNA ladder as molecular weight marker and ethidium bromide staining.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1mm 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, California, USA) using 0.5x 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%).Image digitalization was performed using a Molecular Imager FX™ system (Bio-Rad Laboratories, Hercules, California, USA).

DGGE bands excision, cloning and sequencing

The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL of sterile milli-Q-purified water from which 5 µL of the eluted DNA was used in PCR amplification with the original primer pair. The purity of the DNA band was verified through DGGE and, if necessary, the extraction procedure was repeated. When necessary, the isolated bands were cloned using the TOPO TA cloning kit with the pCR 2.1-TOPO vector (Invitrogen, Portugal). Prior to cloning, an A tail was added to PCR products according to manufacturers' instructions. In order to discard false positive clones, amplicons were obtained from each clone using the vector primers M13R/T7F and its size checked by electrophoresis in 1.5% agarose gels as described above. The migration point of each cloned band with the targeted size was verified through DGGE after a nested PCR amplification with the primer pair 338F-GC and 518R. However, sequencing was made with PCR amplicons using the vector primers M13R/T7, optimizing the sequence length obtained. Each amplicon was purified with the concertTM rapid PCR purification system (Gibco BRL, Eggenstein, Germany) before it was commercially sequenced (STABVida, Portugal). Randomly, the identity of co-migrating bands from different lanes was confirmed (for instance, band 40 – see Fig. 2 and Table 3).

Nucleotide sequence accession numbers and phylogenetic analysis

The bacterial 16S rDNA partial sequences determined in the present study were deposited in the GenBank database under the accession numbers GQ253058-GQ253072 and GQ266143. A BLAST search was used to determine the similarity of the sequences obtained against sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov). Alignment for phylogenetic analyses of the partial 16S rDNA sequences was carried out using the CLUSTAL X software version 1.83 (Thompson et al., 1997). A phylogenetic tree was built using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 replicates. TreeView version 1.6.6 (Page, 1996) was used to display the trees.

Statistical analysis

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 the subsequent analyses. The presence or absence of co-migration points was converted to a binary matrix (0/1) and cluster analysis was performed using the unweighted pair group method with mathematical averages (UPGMA) based on the Bray-Curtis

similarity coefficient with PRIMER 6 software (Clarke & Gorley, 2006). The distribution of samples according to environmental parameters was assessed through principal component analysis (PCA) after data standardization (subtracting the mean from each observation and dividing by the corresponding standard deviation) (ter Braak, 1995). The UPGMA was also used to compare the samples on the basis of environmental parameters, but the dendrogram was created with the similarities calculated using the Pearson correlation coefficient. Pearson correlation coefficient was also used to assess significant correlations between environmental parameters. Canonical correspondence analysis (CCA) (ter Braak, 1986) was performed to reveal relationships between the distribution of the dominant DGGE phylotypes (using a matrix built with band relative intensities) and environmental variables (see also ter Braak, 1995). CANOCO 4.5 (Scientia Software) software was used. CCA requires a unimodal species-environment relationship (ter Braak & Verdonschot, 1995; ter Braak, 1995) which can be assumed in cases where the length of gradient of the first detrended correspondence analysis axis run on species data is >2 (Wu et al., 2006). This requirement was fulfilled by our data set (length of gradient = 2.959). The environmental parameters which better described the distribution of the species data were *a priori* identified by forward selection (Magnan et al., 1994; ter Braak & Verdonschot, 1995). Explanatory variables were added to the analysis until the model's explanatory power was not significantly improved by the addition of further variables, as assessed by a Monte Carlo permutation test (499 unrestricted permutations; ALFA = 0.10, following Magnan et al., 1994). Downweighting of rare species (i.e. phylotypes) was also performed. The statistical significance of the relationship, in the final model (including only selected environmental variables), was assessed by a Monte Carlo (unrestricted) permutation test.

Results

Environmental parameters and PCA

Environmental parameters are summarized in Table 1. Maxima and minima values are highlighted in bold. Water temperature, pH, TSS, conductivity, SRP and Chl *a* levels were generally higher during summer months while precipitation and nitrate concentration showed higher values during winter. Negative correlations (n = 9; $P < 0.05$) could be detected between nitrate concentrations and water temperature (r = -0.90), conductivity (r = -0.80), pH (r = -0.74) and TSS (r = -0.77); TSS and nitrite (r = -0.76), precip7d (r = -0.76) and precip1m (-0.71). Significant positive correlations were recorded between TSS and SRP (r = 0.84), water temperature (r = 0.73), conductivity (r = 0.68) and Chl *a* (r = 0.68); Chl *a* and SRP (r = 0.71), pH and water temperature (r = 0.75); precip7d and precip1m (r = 0.68).

Table 1. Characterization of the Fermentelos Lake samples and environmental data recorded during the study period (2005-2007).

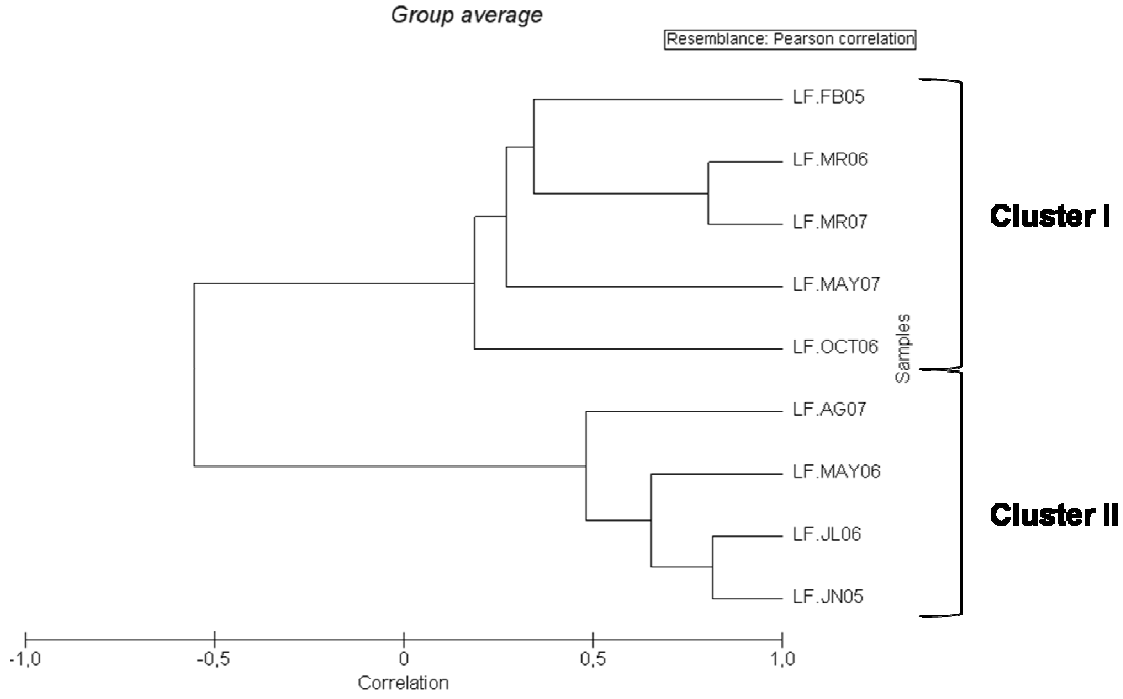
		Average daily precipitation* (mm)											
									$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	SRP	
Sampling date	Code	7 days before	1 month before	Seasonal (1932-1998)	Water temp (ºC)	pH	TSS (mg L^{-1})	Chl *a* (µg L^{-1})	(mg L^{-1})				Conductivity (µS cm^{-1})
23rd February 2005	LF.FB05	2.0	0.6	3.6	**10**	7.58	26.0	22.1	0.12	6.6	0.08	0.12	578
14th June 2005	LF.JN05	0.3	0.6	1.9	22	8.16	82.0	17.1	< 0.01	< 0.1	< 0.01	0.24	566
9th March 2006	LF.MR06	2.4	2.4	3.6	10	**7.36**	**10.4**	15.8	0.14	**12.0**	0.14	0.08	457
23rd May 2006	LF.MAY06	0.4	**0.2**	1.4	23	**8.51**	55.7	163.4	0.08	2.0	0.05	0.15	524
27th July 2006	LF.JL06	**0.1**	0.4	1.9	**24**	8.12	76.6	139.6	< 0.01	< 0.1	< 0.01	0.12	**610**
3rd October 2006	LF.OCT06	**5.5**	4.0	4.9	21	7.72	17.9	47.9	0.08	1.5	0.14	0.03	567
5th March 2007	LF.MR07	1.5	**4.2**	3.6	13	7.74	20.7	**10.6**	0.10	9.2	0.15	0.05	**414**
7th May 2007	LF.MAY07	3.8	1.9	1.4	15	8.22	14.5	39.7	**0.26**	4.8	0.03	0.01	487
29th August 2007	LF.AG07	0.7	0.5	1.9	20	8.24	**87.2**	**286.6**	0.05	0.2	**0.22**	**0.37**	609

* The average precipitation levels were obtained from Portuguese Water Institute (http://snirh.inag.pt/).

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 (%)
FB05	**3** (LF1_cl85)	GQ253058	*Bacteroidetes*	Uncultured ***Bacteroidetes*** bacterium clone PRD18H08 (AY948070)	Parker River, USA	99
FB05	**11** (LF2_cl82)	GQ253059	*Bacteroidetes*	Uncultured **Flavobacteriales** bacterium clone LiUU-9-259 (AY509340)	Limmaren Lake, Sweden	99
MR07	**15** (LF17_cl104)	GQ253060	*Betaproteobacteria*	**Betaproteobacterium** TEGF004 (AB426583)	Teganuma Lake, Japan	100
OCT06	**25** (LF16_cl103)	GQ253061	*Alphaproteobacteria*	Uncultured **alphaproteobacterium** clone TH_h31 (EU980280)	Taihu Lake, China	100
MR06	**28** (LF3_cl1)	GQ253062	*Cyanobacteria*	***Synechococcus*** sp. KORDI-78 (FJ497748)	East China Sea	100
MR07	**29** (LF18_clD6)	GQ253063	*Eukaryota* (choroplast)	Uncultured **phototrophic eukaryote** clone TH_a83 (EU273242) *Cryptomonas curvata* ccac 0006 (AM709636)	Taihu Lake, China Freshwater, England	100 100
MAY06	**30** (LF12)	GQ266143	*Actinobacteria*	Uncultured **actinobacterium** clone TH1-97 (AM690889)	Taihu Lake, China	100
MR07	**31** (LF19_clD3)	GQ253064	*Bacteroidetes*	Uncultured ***Flavobacteria*** bacterium clone LiUU-22-12 (EF061027)	Limmaren Lake, Sweden	98
MR06	**32** (LF7_cl118)	GQ253065	*Bacteroidetes*	Uncultured ***Bacteroidetes*** bacterium clone TH_d91 (EU373118)	Taihu Lake, China	99
OCT06	**36** (LF20_clD11)	GQ253066	*Actinobacteria*	Uncultured bacterium clone MFBC5E01 (EU592620)	Gatun Lake, Panama	100
JL06	**38** (LF15)	GQ253067	*Cyanobacteria*	Uncultured ***Synechococcus*** sp. clone Kanui-2 (EF638720)	Kanui Lake, New Zealand	100
MR06	**40** (LF8_clII6)	GQ253068	*Actinobacteria*	Uncultured **actinobacterium** clone TW1F11 (EU117985)	Twin Valley Lake, USA	100
MAY07	**40** (LF21_clD13)	GQ253069	*Actinobacteria*	Uncultured **actinobacterium** clone TW1F11 (EU117985)	Twin Valley Lake, USA	100
MAY06	**41** (LF14_cl77)	GQ253070	*Betaproteobacteria*	Uncultured **betaproteobacterium** clone TH_e45 (EU980140)	Taihu Lake, China	99
MR07	**42** (LF23_clII12)	GQ253071	*Betaproteobacteria*	Uncultured **betaproteobacterium** clone TH_e45 (EU980140) ***Polynucleobacter*** sp. USHIF012 (AB470466)	Taihu Lake, China Lake Ushikunuma, Japan	100 100
OCT06	**45** (LF22_clII22)	GQ253072	*Actinobacteria*	Uncultured **actinobacterium** clone TH_f7 (EU980163)	Taihu Lake, China	100

(a)



(b)

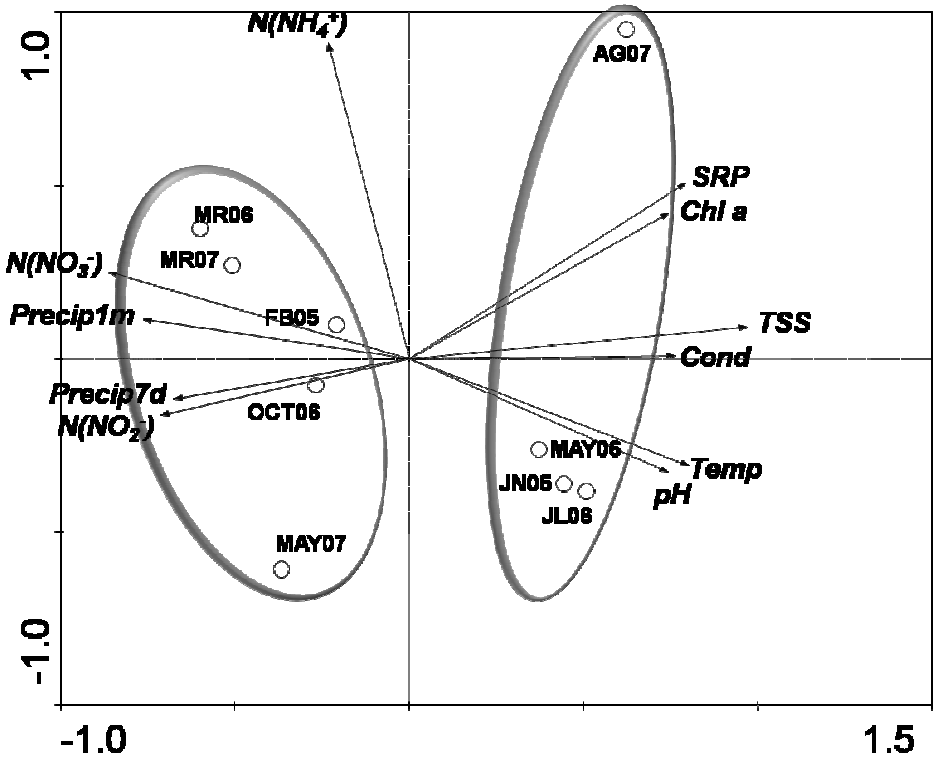


Fig. 1. (a) cluster analysis dendrogram and (b) PCA ordination biplot of Fermentelos Lake samples according to environmental parameters recorded from 2005 to 2007 (see sample codes in table 1); the first two axes accounted for 71.0% of the total variance of samples' distribution.

The cluster analysis dendrogram evidences two main seasonal clusters: cluster I grouping winter samples (but including also the autumn and spring samples OCT06 and MAY07, respectively) and cluster II aggregating summer samples (including also the spring sample MAY06). PCA showed a general seasonal gradient with the first axis mainly defined by winter samples on the negative side and summer samples on the positive side (as shown in Fig. 1b); sample AG07 defined the positive side of the second axis and MAY07 defined its negative side. The first axis was mainly related to inorganic nutrient sources such as SRP and nitrate levels, precipitation, TSS, water temperature, conductivity, pH and Chl *a* while the second axis was mostly defined by ammonium concentration. These first two axes could explain 71.0% of the total variance of samples' distribution.

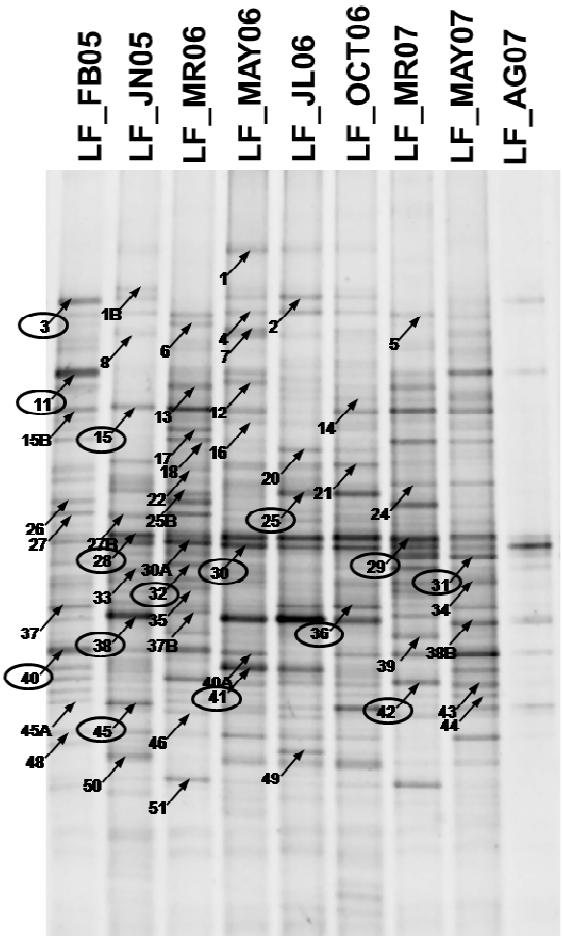


Fig. 2. DGGE profiles containing the bacterial 16S rDNA fragments from Fermentelos Lake samples taken from 2005 to 2007. The code above each lane refers to each sample (see table 1) and the bands numbering corresponds to the different migration positions considered for the analyses; the sequenced bands (see table 3) are encircled.

Analysis of DGGE band profiles

The 16S rDNA-DGGE band profiles obtained for Fermentelos Lake are presented in Fig. 2. A total of 184 bands were recorded corresponding to 55 different band migration positions and the number of bands *per* sample was in average of 20 ± 5 (n = 9). Sixteen DGGE bands were successfully isolated and sequenced (Table 2 and Fig. 3). The sequences obtained affiliated with members of *Cyanobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria* and *Bacteroidetes* groups as well as with a phototrophic eukaryote. Cluster analysis using the DGGE profiles (Fig. 4a) revealed a clear seasonal clustering of samples. Cluster I included winter samples and was distinctly separated from cluster II which included the spring and summer samples. Nevertheless, summer of 2007 showed to be different from the other summer samples as previously observed for cluster analysis based on environmental parameters.

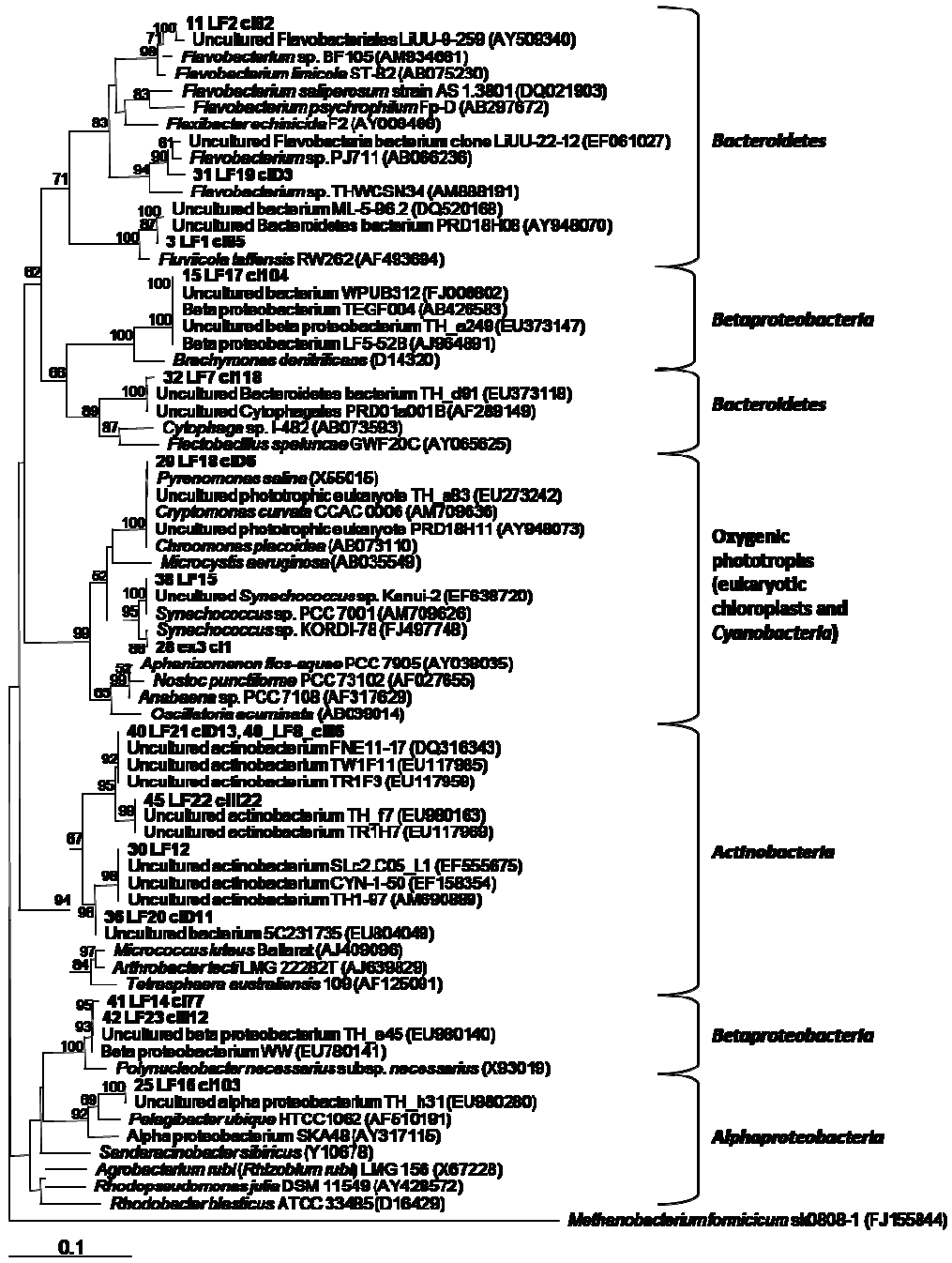


Fig. 3. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel (Fig. 3). The archaeal sequence from *Methanobacterium formicicum* strain sk0808-1 was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

Canonical Correspondence Analysis (CCA)

The CCA analysis (Fig.4b) based on DGGE band relative intensities and the environmental parameters selected by forward selection (temperature and chlorophyll *a*) could explain 48.1% of the BCC variation (31.1% for the first axis and 17.0% for the second axis). Water temperature was strongly correlated with the first axis (-0.96) and chlorophyll *a* with the second axis (0.88). Two main clusters could be observed along the seasonal (temperature) gradient in the first axis: samples

collected at warmer temperatures (and DGGE bands such as 7, 16 and 49) on the negative side and winter samples (and DGGE bands such as 17, 25B and 29) on the positive side. Sample AG07 (and DGGE bands 3 and 38B) defined the positive side of the second axis while the negative side was defined by the typical winter samples MR06 and MR07 (and band 29).

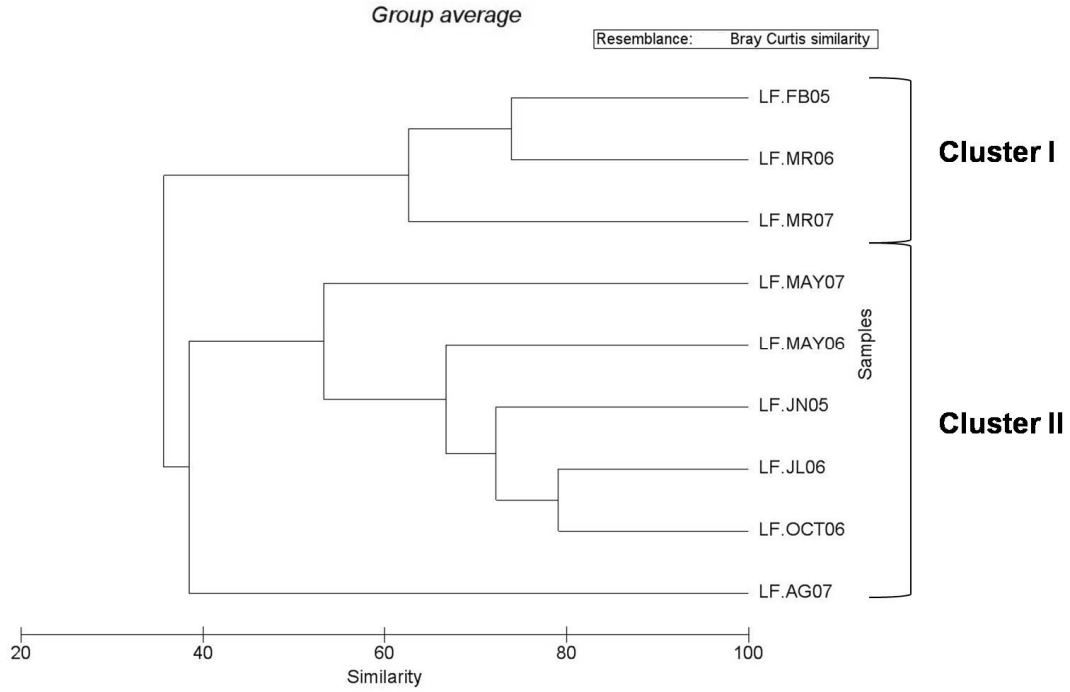
Discussion

During the study period, Fermentelos Lake showed eutrophic to hypereutrophic features as reported over the past two decades (Gil, 1988; Calado et al., 1991; Calado & Craveiro, 1995; Pereira, 1999; de Figueiredo et al., 2007). A general trend for the increase of pH and conductivity as well as nitrate levels has been recorded by comparing to previous data (Calado et al., 1991; Pereira, 1999). Our results suggest that the environmental seasonality at the lake is mainly associated with TSS, inorganic nutrient sources, precipitation, water temperature, conductivity, pH and Chl *a*. Typical winter samples (MR06 and MR07) were mainly related to lower temperatures and higher levels of nitrate and average daily precipitation while characteristic summer samples (JN05 and JL06) were mostly related to increased values for temperature, TSS, conductivity, SRP, Chl *a* and pH. However, AG07 differed from typical summers by having the highest ammonium and SRP levels recorded, favouring phytoplankton development, as confirmed by the highest Chl *a* concentration. Moreover, FB05 did not show significant relationships with winters 2006 and 2007, indicative of a different environmental context. In fact, winter 2005 showed some of the lowest precipitation levels recorded over the last fifty years (de Figueiredo et al., 2007).

The variation of the BCC is known to depend on parameters such as water temperature and pH (Crump & Hobbie, 2005; Lindström et al., 2005; Yannarell & Triplett, 2005; Wu et al., 2007b) as well as the water retention time or water flow rate (Crump & Hobbie, 2005; Lindström et al., 2005) and the levels of nitrogen (Crump & Hobbie, 2005; Wu et al., 2007b; Wei et al., 2008; Zeng et al., 2009), phosphorus (Boucher et al., 2006; Xing & Kong, 2007) and chlorophyll *a* (Xing & Kong, 2007; Tian et al., 2009). In general, our results showed typical winter *vs.* summer DGGE profiles (mainly related to temperature and chlorophyll *a*) suggesting some predictability for inter-annual summer and winter BCC at Fermentelos Lake, in contrast with a high variability in autumn and spring BCC as observed for other water bodies (Crump & Hobbie, 2005).

Interestingly, the BCC from winter 2005 and winters 2006 and 2007 showed to be rather similar, in spite of the environmental differences found between these samples. These results suggest that, under a drought stress, typical winter BCC at Fermentelos Lake may resist; however, the fact that the precipitation levels during the week before sampling (in 23rd February 2005) were 3 fold higher than the month average may have attenuated potential drought effects over the bacterioplankton assemblage dynamics. Moreover, the BCC for summer 2007 showed to differ

(a)



(b)

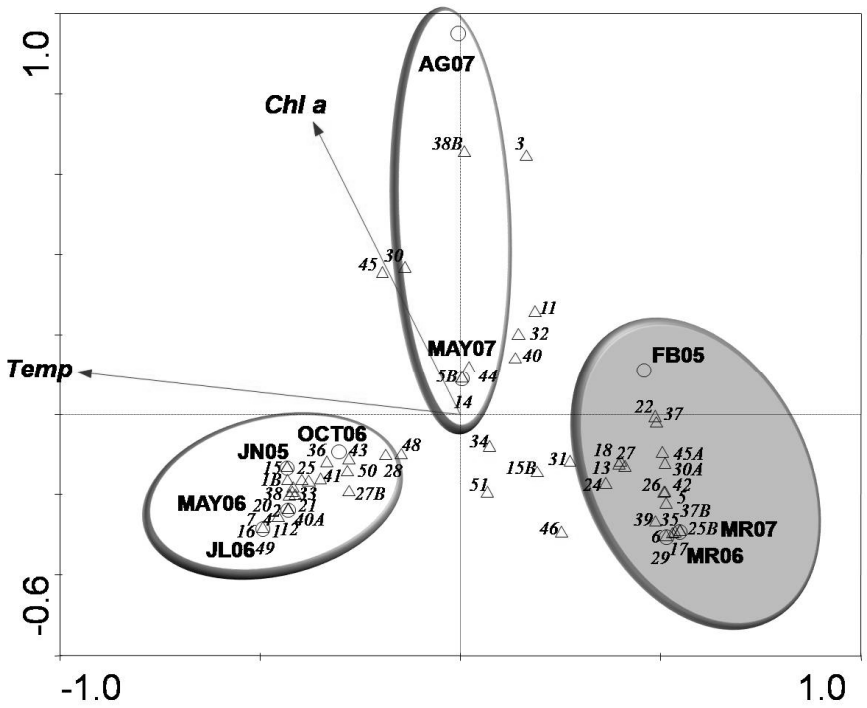


Fig. 4. (a) cluster analysis dendrogram of Fermentelos Lake samples according to DGGE band patterns and (b) CCA ordination triplot of Fermentelos Lake DGGE band patterns according to the selected environmental parameters recorded during the study period (2005-2007). DGGE bands numbering and samples coding are described in figure 2 and table 1, respectively.

from the other summer samples (as observed in PCA analysis) and it was mainly characterized by mild water temperatures, with nutrient availability and high Chl *a* levels, indicative of "spring-like" conditions.

The low temperatures and high concentration of nitrogen sources were related to the dominance of *Bacteroidetes* as observed in other eutrophic water bodies (Brümmer et al., 2000; Zwart et al., 2002; Wu et al., 2007b; Xi et al., 2007), namely at Fermentelos Lake (de Figueiredo et al., 2007). The Cytophagales sequence was similar to those detected in the Chinese Taihu Lake and at the Parker River, USA, during winter (Zwart et al., 2002; Crump & Hobbie, 2005) while the Flavobacteriales sequences were similar to those from eutrophic shallow lakes (Eiler & Bertilsson, 2004; Wu et al., 2007b) and their dominance was related to high ammonium, SRP and Chl *a* levels. The dominance of the cryptophycean phylotype was highly correlated with the lowest conductivity, pH and Chl *a* levels but a very high nitrate concentration as observed during *Cryptomonas* spp. dominance in other shallow eutrophic lakes (Lindström, 2000; Ye et al., 2007). In fact, the presence of *Cryptomonas* and *Chroomonas*-like species has been reported in Fermentelos Lake, namely during winter season (Calado et al., 1991; Pereira, 1999) as in the present study. The lowest Chl *a* concentration suggests an opportunistic behaviour of this microalga to develop when there are no phytoplankton competitors in high densities, as previously reported (Tolotti et al., 2003). The dominance of cryptophycean algae has also shown to affect the BCC (Lindström, 2000) and our results suggest a co-domination with *Flavobacteria* sp. (*Bacteroidetes*) and *Polynucleobacter* sp. (Burkholderiaceae, *Betaproteobacteria*). The *Alphaproteobacteria* and *Betaproteobacteria* sequences from this study were most similar to those found in Asian shallow eutrophic lakes including Taihu Lake (Wu et al., 2007b; Xi et al., 2007). The alphaproteobacterial phylotype was more related to summer-like conditions mainly defined by high temperatures, as observed by Wu et al. (2007b). One of the *Betaproteobacteria* phylotypes occurred in summer season and other in winter season; this fact may indicate that this group has a broader diversity of environmental requirements although a preference for low TN/TP has been noticed as recorded before (Wu et al., 2007b). However, the phylotype affiliated with the Comamonadaceae family (Burkholderiales, *Betaproteobacteria*) was recorded only during the dry summer of 2005, under high temperature, SRP and pH levels and the lowest nitrogen sources concentrations. The *Actinobacteria* sequences had total match with sequences from meso- to eutrophic lakes in Northern Europe (Allgaier & Grossart, 2006a), USA (Newton et al., 2007; Navarro et al., 2009) and Australia (Pope & Patel, 2008). In general, the *Actinobacteria* phylotypes were associated with mild water temperatures and higher levels of ammonium, SRP and chlorophyll *a* levels although this group was well represented throughout seasons as previously observed for lentic eutrophic water bodies (De Wever et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006b; Newton et al., 2007; Wu et al., 2007b; Xi et al., 2007). The cyanobacterial phylotype showed similarity with sequences of marine

and freshwater *Synechococcus* spp.; their tolerance to high salinity ranges, by special mechanisms (Ladas & Papageorgiou, 2000; Takeyama et al., 2000), may explain the ubiquity of similar sequences throughout freshwater, brackish and marine systems. At Fermentelos Lake, the development of cyanobacteria was enhanced in summer and related to high temperature, pH and conductivity values, as previously observed for this (Calado et al., 1991; Pereira, 1999) and other lakes (Dokulil & Teubner, 2000; De Wever et al., 2005; Van Der Gucht et al., 2005; de Figueiredo et al., 2006; Ke et al., 2008).

The recorded dominance of phylotypes belonging to *Bacteroidetes*, *Betaproteobacteria, Alphaproteobacteria, Cyanobacteria* and *Actinobacteria* may be the result of the strong eutrophication of the lake as recorded for other eutrophic water bodies (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007; Wu et al., 2007b) and water quality problems may increase in a near future (Zaitlin & Watson, 2006; Smith et al., 2008). The co-dominance of *Actinobacteria*, *Alphaproteobacteria*, *Cyanobacteria* as well as *Betaproteobacteria* phylotypes has been recorded before (Xi et al., 2007; Pope & Patel, 2008) as well as the desynchronizing dominance of *Cyanobacteria* and *Alphaproteobacteria* in summer and *Bacteroidetes* in winter (Xi et al., 2007). Interestingly, sequences from this study matched sequences retrieved from the shallow carbon-rich Parker River (USA), whose BCC dynamics has shown to be related to temperature and nutrient sources (Zwart et al., 2002; Crump & Hobbie, 2005), and mostly from the shallow eutrophic Taihu Lake (China) where temperature and chlorophyll *a* play also an important role for BCC variation (Wu et al., 2007a; Xing & Kong, 2007) as suggested by our data.. Interestingly, Taihu Lake in spite of being much larger (Liu & Qiu, 2007) has many similarities with Fermentelos Lake such as the average water depth, the discharge of industrial and urban effluents that reach the lake, the strong presence of macrophytes in some areas (Wu et al., 2007a) and the frequent dominance of phytoplankton by Cryptophyta (Peng et al., 2007; Ye et al., 2007) but also the annual range for environmental parameters including suspended solids, chlorophyll *a* and nutrients concentrations, conductivity and pH (Gao et al., 2007; Ke et al., 2008). This may be very useful since many studies for the recovery of Taihu Lake have already been conducted (Chang et al., 2006; Liu & Qiu, 2007; Ye et al., 2007; Yu et al., 2007; Jin et al., 2008; Li et al., 2008; Mao et al., 2008) and may help defining management strategies for eutrophication control at Fermentelos Lake.

As a conclusion, the results from the present work suggest that the eutrophication process is still being enhanced at Fermentelos Lake with increased levels of nitrate, conductivity and pH. Characteristic summer *vs.* winter bacterial assemblages could be defined during the study period and showed to be mainly related with factors such as water temperature and chlorophyll *a* levels. *Actinobacteria* phylotypes were present during the study period but their dominance was related to high temperature and chlorophyll *a* values along with high levels of pH, TSS, conductivity and

SRP. The development of *Betaproteobacteria, Alphaproteobacteria* and *Cyanobacteria* phylotypes was generally also associated to high temperature, pH, conductivity and SRP values. The highest nitrogen sources were associated to *Bacteroidetes* and phototrophic eukaryote (cryptophycean) dominance. These results add important information to the ecology of BCC in temperate freshwater lakes, particularly in Portuguese territory, which may give important ecological background data for the development of lake management plans.

References

Allgaier M. & Grossart H. P. (2006a). Diversity and seasonal dynamics of *Actinobacteria* populations in four lakes in Northeastern Germany. *Applied and Environmental Microbiology* 72: 3489-3497.

Allgaier M. & Grossart H. P. (2006b). Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

Almeida A. S., Gonçalves A. M. M., Pereira J. L. & Gonçalves F. (2006). The impact of *Eichhornia crassipes* on green algae and cladocerans. *Fresenius Environmental Bulletin* 15: 1531-1538.

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Bent S. J. & Forney L. J. (2008). The tragedy of the uncommon: Understanding limitations in the analysis of microbial diversity. *ISME Journal* 2: 689-695.

Boucher D., Jardillier L. & Debroas D. (2006). Succession of bacterial community composition over two consecutive years in two aquatic systems: A natural lake and a lake-reservoir. *FEMS Microbiology Ecology* 55: 79-97.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Calado A. J. & Craveiro S. C. (1995). Notes on the ecology of Synurophycean algae found in Portugal. *Nord J Bot* 15: 641-654.

Calado A. J., Freitas A. M. & Veloso V. M. (1991). Algas da Pateira de Fermentelos numa situação de Inverno. *Revista de Biologia da Universidade de Aveiro* 4: 55-71.

Cerqueira M. A., Vieira F. N., Ferreira R. V. & Silva J. F. (2005). The water quality of the Cértima River Basin (Central Portugal). *Environmental Monitoring and Assessment* 111: 297-306.

Chang H. Q., Yang X. E., Fang Y. Y., Pu P. M., Li Z. K. & Rengel Z. (2006). In-situ nitrogen removal from the eutrophic water by microbial-plant integrated system. *Journal of Zhejiang University. Science. B.* 7: 521-531.

Clarke K. R. & Gorley R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Gao G., Qin B., Sommaruga R. & Psenner R. (2007). The bacterioplankton of Lake Taihu, China: Abundance, biomass, and production. *Hydrobiologia* 581: 177-188.

Gil M. C., Estudo ecológico das Diatomáceas dos rios Águeda, Agadão e Alfusqueiro. Department of Biology, Vol. PhD. University of Aveiro, Aveiro, 1988, pp. 389.

Jin X., Lu S., Hu X., Jiang X. & Wu F. (2008). Control concept and countermeasures for shallow lakes' eutrophication in China. *Frontiers of Environmental Science and Engineering in China* 2: 257-266.

Ke Z., Xie P. & Guo L. (2008). Controlling factors of spring-summer phytoplankton succession in Lake Taihu (Meiliang Bay, China). *Hydrobiologia* 607: 41-49.

Ladas N. P. & Papageorgiou G. C. (2000). The salinity tolerance of freshwater cyanobacterium *Synechococcus* sp. PCC 7942 is determined by its ability for osmotic adjustment and presence of osmolyte sucrose. *Photosynthetica* 38: 343-348.

Li L., Li Y., Biswas D. K., Nian Y. & Jiang G. (2008). Potential of constructed wetlands in treating the eutrophic water: Evidence from Taihu Lake of China. *Bioresource Technology* 99: 1656-1663.

Lindström E. S. (2000). Bacterioplankton community composition in five lakes differing in trophic status and humic content. *Microbial Ecology* 40: 104-113.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Liu W. & Qiu R. (2007). Water eutrophication in China and the combating strategies. *Journal of Chemical Technology and Biotechnology* 82: 781-786.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Magnan P., Rodríguez M. A., Legendre P. & Lacasse S. (1994). Dietary variation in a freshwater fish species: relative contributions of biotic interactions, abiotic factors, and spatial structure. *Canadian Journal of Fisheries and Aquatic Sciences* 51: 2856-2865.

Mao J., Chen Q. & Chen Y. (2008). Three-dimensional eutrophication model and application to Taihu Lake, China. *Journal of Environmental Sciences* 20: 278-284.

Mitraki C., Crisman T. L. & Zalidis G. (2004). Lake Koronia, Greece: Shift from autotrophy to heterotrophy with cultural eutrophication and progressive water-level reduction. *Limnologica - Ecology and Management of Inland Waters* 34: 110-116.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G. & Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 73: 127-141.

Navarro J., Moser D., Flores A., Ross C., Rosen M., Dong H., Zhang G. & Hedlund B. (2009). Bacterial Succession within an Ephemeral Hypereutrophic Mojave Desert Playa Lake. *Microbial Ecology* 57: 307-320.

Newton R. J., Jones S. E., Helmus M. R. & McMahon K. D. (2007). Phylogenetic ecology of the freshwater Actinobacteria acI lineage. *Applied and Environmental Microbiology* 73: 7169-7176.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Peng X., Fanxiang K., Huansheng C. & Min Z. (2007). Relationship between bacterioplankton and phytoplankton community dynamics during late spring and early summer in Lake Taihu, China. *Acta Ecologica Sinica* 27: 1696-1702.

Pereira M. J., Utilização de algas na avaliação de contaminação de águas doces por metais. Department of Biology, Vol. PhD. University of Aveiro, Aveiro, 1999, pp. 611.

Pinho P., Praça F. & Torres A., Estudo para o ordenamento da área envolvente da Pateira de Fermentelos. In: R. e. C. d. N. Serviço Nacional de Parques, (Ed.). Departamento de Ambiente e Ordenamento da Universidade de Aveiro, Aveiro, 1988.

Pope P. B. & Patel B. K. C. (2008). Metagenomic analysis of a freshwater toxic cyanobacteria bloom. *FEMS Microbiology Ecology* 64: 9-27.

Rino J. M. A. & Gil M. C. P. (1987). Diatomáceas do rio Cértima - Estudo preliminar. *Revista de Biologia da Universidade de Aveiro* 1: 53 - 103.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Saitou N. & Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

Takeyama H., Nakayama H. & Matsunaga T. (2000). Salinity-regulated replication of the endogenous plasmid pSY10 from the marine cyanobacterium *Synechococcus* sp. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology* 84-86: 447-453.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

ter Braak C. J. F. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology* 67: 1167-1179.

ter Braak C. J. F., 1995. Ordination. In: R. H. G. Jongman, C. J. F. ter Braak & O. F. R. Tongeren, (eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, UK: 91-173.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. & Higgins D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Tian C., Tan J., Wu X., Ye W., Liu X., Li D. & Yang H. (2009). Spatiotemporal transition of bacterioplankton diversity in a large shallow hypertrophic freshwater lake, as determined by denaturing gradient gel electrophoresis. *J. Plankton Res.* 31: 885-897.

Tolotti M., Thies H., Cantonati M., Hansen C. M. E. & Thaler B. (2003). Flagellate algae (Chrysophyceae, Dinophyceae, Cryptophyceae) in 48 high mountain lakes of the Northern and Southern slope of the Eastern Alps: Biodiversity, taxa distribution and their driving variables. *Hydrobiologia* 502: 331-348.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Wei C., Bao S., Zhu X. & Huang X. (2008). Spatio-temporal variations of the bacterioplankton community composition in Chaohu Lake, China. *Progress in Natural Science* 18: 1115-1122.

Wiedner C., Rücker J., Brüggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.

Wu Q. L., Zwart G., Schauer M., Kamst-van Agterveld M. P. & Hahn M. W. (2006). Bacterioplankton Community Composition along a Salinity Gradient of Sixteen High-Mountain Lakes Located on the Tibetan Plateau, China. *Appl. Environ. Microbiol.* 72: 5478-5485.

Wu Q. L., Zwart G., Wu J., Kamst-Van Agterveld M. P., Liu S. & Hahn M. W. (2007a). Submersed macrophytes play a key role in structuring bacterioplankton community composition in the large, shallow, subtropical Taihu Lake, China. *Environmental Microbiology* 9: 2765-2774.

Wu X., Xi W., Ye W. & Yang H. (2007b). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Xi W., Wu X., Ye W. & Yang H. (2007). Changes in bacterial community structure during preceding and degraded period of cyanobacterial bloom in a bay of the Taihu Lake. *Chinese Journal of Applied and Environmental Biology* 13: 97-103.

Xing P. & Kong F. (2007). Intra-habitat heterogeneity of environmental factors regulating bacterioplankton community composition in Lake Taihu, China. *Aquatic Microbial Ecology* 48: 113-122.

Yannarell A. C. & Triplett E. W. (2005). Geographic and Environmental Sources of Variation in Lake Bacterial Community Composition. *Appl. Environ. Microbiol.* 71: 227-239.

Ye C., Xu Q., Kong H., Shen Z. & Yan C. (2007). Eutrophication conditions and ecological status in typical bays of Lake Taihu in China. *Environmental Monitoring and Assessment* 135: 217-225.

Yu X., Nipper J. & Yan N. (2007). Recommendations of eutrophication control of Taihu Lake from an international view. *Acta Geographica Sinica* 62: 899-906.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology* 25: 763-772.

Zwart G., Crump B. C., Kamst-van Agterveld M. P., Hagen F. & Han S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* 28: 141-155.

Seasonal diversity of bacterioplankton community at the eutrophic shallow Vela Lake (Central Western Portugal)

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Abstract

The occurrence of cyanobacterial blooms is increasing worldwide enhanced by climatic changes. Vela Lake (Central Western Portugal) is a temperate eutrophic shallow water body where blooms of cyanobacteria are frequent during spring and summer months. However, there are no studies contextualizing their occurrence in the whole bacterioplankton community. The present work aimed to investigate the seasonal bacterioplankton diversity at Vela Lake, using *16S rRNA* PCR-DGGE (denaturing gradient gel electrophoresis). Environmental data demonstrated eutrophic to hypereutrophic features throughout all samples and cluster analysis and PCA showed a clear seasonal segregation of samples mainly defined by factors such as water temperature, precipitation, inorganic nitrogen sources, pH and Chl *a* levels. Dominant phylotypes showed to affiliate with groups such as *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria* and *Betaproteobacteria* and the seaasonal shifts on the bacterial assemblage showed to be mostly modulated by factors such as water temperature and conductivity. Nevertheless, *Cyanobacteria* showed to dominate the bacterioplankton assemblage across seasons following a dominance succession of phylotypes corresponding to sequences of *Aphanizomenon aphanizomenoides*, *Aph. gracile* and *Synechococcus* spp..

Keywords

eutrophication, Vela Lake, bacterioplankton diversity, 16SrDNA-DGGE.

Introduction

Climatic changes lead to an enhancement of eutrophication in shallow lakes, as these are very vulnerable to drought conditions which cause a rapid decrease in the water level and lead to important changes in water physical and chemical characteristics as well as in the composition of the aquatic communities (Mitraki et al., 2004). The bacterial community composition (BCC), in particular, can rapidly change its composition and the development of groups such as *Cyanobacteria* and *Actinobacteria* (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007) is enhanced, which may put at risk the water quality (Zaitlin & Watson, 2006; Smith et al., 2008). *Cyanobacteria* can develop into blooms that can lead to toxic outcomes and endanger human health safety (Codd, 2000; de Figueiredo et al., 2004b). More, pathogenic bacteria including *Aeromonas*, *Vibrio*, *Acinetobacter* and *Pseudomonas* have been also related to the development of cyanobacterial blooms which increases even further the human health risk (Berg et al., 2008).

Cyanobacterial blooms are frequent at Vela Lake (Figueira da Foz, Central Western Portugal), with the dominance of potentially microcystin-producing cyanobacteria such as *Microcystis* spp., *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* and *Cylindrospermopsis raciborskii* (Vasconcelos et al., 1993a; Saker et al., 2003; de Figueiredo et al., 2006). However, as for most Portuguese water bodies, the information on the shifts of the whole bacterial community composition (BCC) dynamics at Vela Lake is inexistent or very scarce (de Figueiredo et al., 2007), which limits the development of effective water management planning.

In the present work, a PCR-DGGE (Denaturing Gradient Gel Electrophoresis) approach, using primers that target regions of the *16S rRNA* gene directly from environmental samples, was used to investigate the seasonal variation in bacterioplankton diversity at Vela Lake. Environmental parameters were also determined in order to find the most important factors behind the BCC seasonality through multivariate analysis.

Materials and methods

Study area, sampling and environmental parameters

The shallow eutrophic Vela Lake is located at Quiaios (Figueira da Foz, Central Portugal) and it has a depth range of 0.9-2.4 m with an area of approximately 70 ha surrounded by forest of *Pinus* spp. and acacias on its West side and agriculture fields, livestock farms and urban areas on the East side (Abrantes et al., 2009b). This lake is mainly used for recreational and agricultural purposes. The agriculture, in particular, provides high amounts of fertilizers and pesticides that are lixiviated into the lake water with rainfall (Abrantes et al., 2009b). Sampling was performed at (40°16'23"N, 8°47'34"W) from 2005 to 2007 in the dates presented in Table 1. Samples were taken sub-superficially at about 1 m from the shore in each sampling site using 2L sterile bottles and were immediately placed at 4 ºC in

Table 1. Characterization of the Vela Lake samples and environmental data recorded during the study period (2005-2007).

Sampling date	Code	Average daily precipitation* (mm)		Water temp (ºC)	pH	TSS (mg L^{-1})	Chl *a* (µg L^{-1})	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	SRP	Conductivity (µS cm^{-1})
		7 days before	1 month before					(mg L^{-1})				
31st January 2005	VL.JA05	**0.0**	0.4	**8.7**	7.87	31.7	**15.4**	0.02	0.6	**0.6**	<0.1	505
7th April 2005	VL.AP05	0.1	0.5	12.0	8.06	36.1	64.1	< 0.01	0.5	<0.1	<0.1	**475**
23rd June 2005	VL.JN05	**0.0**	**0.1**	25.8	8.80	42.4	67.3	< 0.01	0.1	<0.1	<0.1	516
19th July 2005	VL.JL05	0.1	**0.1**	25.7	8.77	12.1	84.4	< 0.01	0.1	<0.1	<0.1	584
17th August 2005	VL.AG05	0.0	0.5	**26.8**	8.72	41.1	**159.9**	< 0.01	0.1	0.1	<0.1	647
29th September 2005	LV.ST05	0.2	0.7	26.0	8.61	**68.4**	88.7	< 0.01	0.1	<0.1	<0.1	**828**
7th November 2005	LV.NV05	**5.2**	**5.2**	17.0	8.69	74.0	123.2	< 0.01	0.1	0.1	<0.1	622
9th March 2006	LV.MR06	2.4	2.8	13.1	**7.82**	25.8	70.3	**0.15**	1.1	0.6	<0.1	629
22nd May 2006	LV.MAY06	0.3	1.1	21.4	7.94	17.6	40.0	< 0.01	<0.1	<0.1	<0.1	610
25th June 2006	LV.JN06	**0.0**	1.0	22.1	8.49	**10.8**	72.7	< 0.01	0.1	<0.1	<0.1	647
19th July 2006	LV.JL06	0.6	0.2	25.0	8.48	13.7	77.6	< 0.01	0.1	0.2	<0.1	614
1st March 2007	LV.MR07	2.1	4.6	16.0	8.55	12.5	36.3	0.04	**1.5**	0.5	<0.1	510
21st August 2007	LV.AG07	1.4	0.5	20.2	**9.00**	52.7	158.3	< 0.01	0.1	0.1	<0.1	488

* The average precipitation levels were obtained from Portuguese Water Institute (http://snirh.inag.pt/).

the dark until further treatment in the laboratory (within 4 hours) for DNA extraction and determination of environmental parameters. Water temperature, conductivity, pH and dissolved oxygen were determined *in situ* using portable water testing meters (WTW LF 330 conductivity meter, WTW 340-A pH meter and WTW OXI 320 oxygen meter). In the laboratory, the total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$) and nitrite ($N\text{-}NO_2$) concentrations were determined in laboratory according to standard procedures (APHA, 1992; Rodier, 1996).

DNA extraction and PCR amplifications

The total DNA from environmental water samples was extracted after filtering 100 mL of the sampled water through 0.22 µm polycarbonate sterile filters (more than one if the first filter clogs). Collected cells were resuspended in 2 mL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0] and centrifuged. After resuspension in 200 µL of TE, lysis was carried out by adding lysozyme (1 $mg.mL^{-1}$) and incubating at 37 ºC for 1h. DNA extraction and purification was performed using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and DNA was resuspended in TE buffer and stored at -20 ºC.

Amplifications by PCR was performed with the primers 338F-GC/518R (Muyzer et al., 1993), universal for Bacteria. The primer pair QmetF/ QmetR was used for targeting the *mcyA* region of the microcystin synthetase operon (Wilson et al., 2005) with a 40 bp GC-rich sequence added to the 5' end of the forward primer. All primers were commercially synthesized by STABVida (Oeiras, Portugal). PCRs were carried out in a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 15 pmol of each primer, 1 U of *Taq* DNA polymerase and 50-200 ng template DNA. The PCR programs for the primer set targeting the *16S rRNA* gene fragments followed the original procedures (Muyzer et al., 1993; Wilson et al., 2005). A final extension step at 72 °C for 30 min was included for both programs. PCR and negative control reactions were checked by electrophoresis and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder) in a 1.5% agarose gel stained with ethidium bromide. Visualization was performed on a UV transmilluminator.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analysed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1mm 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, California, USA) using 0.5x 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 gels were then stained for 5 min in an ethidium bromide solution (5%) and then gently destained with agitation in distilled water for 10 min before image digitalization

in a Molecular Imager FX™ system (Bio-Rad Laboratories, Hercules, California, USA). The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL 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 verified through DGGE and the extraction procedure was repeated until the targeted band isolation was obtained. More, if necessary, the isolated bands were cloned using the TA cloning kit from Invitrogen. Prior to cloning an A tailing was performed for PCR products 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

Sequencing of PCR amplicons was made using the vector primers M13R / T7. Each amplicon was purified with the concertTM rapid PCR purification system (Gibco BRL, Eggenstein, Germany) before it was commercially sequenced (STABVida, Portugal). The bacterial 16S rDNA partial sequences determined in the present study were submitted to the GenBank database. A BLAST search (http://www.ncbi.nlm.nih.gov) was used to explore similarity of the sequences obtained in this study against sequences deposited in the GenBank database. The alignment of the partial 16S rDNA sequences was carried out using the CLUSTAL X software version 1.8 (Thompson et al., 1994). A phylogenetic tree was built using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 replicates. TreeView version 1.6.6 (Page, 1996) was used to display the trees.

Statistical 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 & Gorley, 2006). Pearson's correlation coefficient was also used to assess relationships between environmental parameters and against bands (considering their occurrence and relative 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. Redundancy analysis (RDA) was performed to reveal relationships between the

distribution of the dominant DGGE phylotypes and environmental variables using CANOCO 4.5 (Scientia Software). Forward selection was applied to choose the significant environmental parameters ($P < 0.05$) for the RDA using a Monte Carlo permutation test (499 unrestricted permutations) (ter Braak & Verdonschot, 1995).

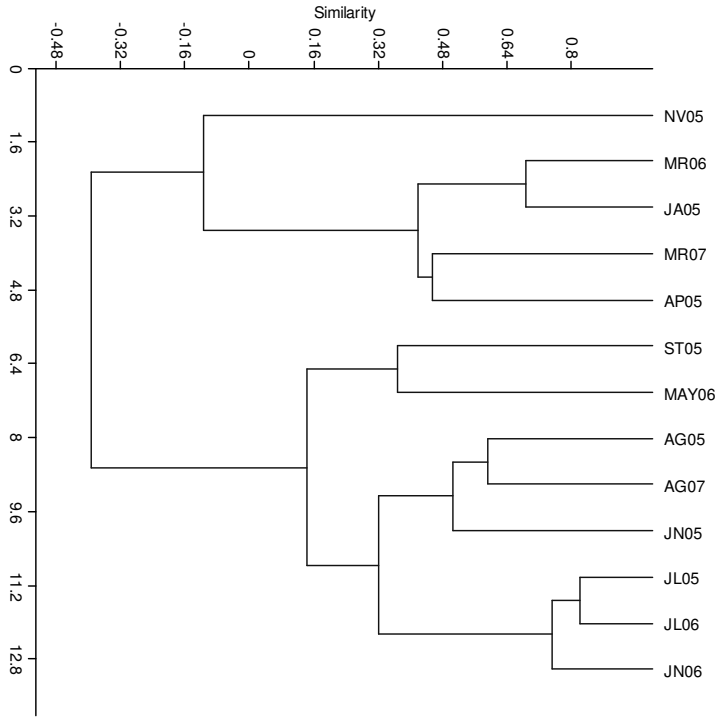
Results

Environmental parameters and PCA

Environmental parameters are summarized in Table 1. Maxima and minima values are highlighted in bold. Water temperature, pH, conductivity and Chl *a* levels were generally higher during summer months while the nitrogen sources concentrations showed higher values during winter. The average daily precipitation levels 7 days and 1 month before sampling dates both showed the lowest levels in summer samples and maxima of 5.2 mm, for both parameters, in NV05. Water temperature ranged between 8.7 and 26.8 ºC, in JA05 and AG05, respectively. The values for pH ranged between 7.82 in MR06 and 9.00 in AG07. The lowest conductivity level was recorded in AP05 (475 µS cm^{-1}) while a maximum of 828 µS cm^{-1} was recorded ST05. TSS concentration ranged from 10.8 to 68.4 $mg.L^{-1}$ in JN06 and ST05, respectively. Chl *a* concentration ranged between 15.4 and 159.9 µg L^{-1} (in JA05 and AG05, respectively). Nitrate concentrations were generally low (0.1 mg N (NO_3^-) L^{-1}) during summer months and with a maximum of 1.5 mg N (NO_3^-) L^{-1} in MR07; nitrite levels were undetectable during summer and maximum of 0.15 mg N (NO_2^-) L^{-1}) in MR06. Ammonia was also generally undetectable in spring and summer samples and maxima concentrations were recorded in JA05 and MR06 with 0.6 mg N (NH_4^+) L^{-1}. SRP concentration was undetected throughout all samples. Pearson correlation coefficient revealed significant correlations between environmental parameters. Positive correlations were found between: pH and water temperature ($r = 0.74$) or Chl *a* ($r = 0.58$); ammonium and nitrite ($r = 0.70$) or nitrate ($r = 0.77$); nitrate and nitrite ($r = 0.68$); and between Precip7d and SRP ($r = 0.68$) or Precip1m ($r = 0.77$). Negative correlations ($n = 13$; $P < 0.05$) could be detected between water temperature and ammonium ($r = -0.66$) and nitrate concentrations ($r = -0.60$).

The cluster analysis dendrogram clearly evidenced two main seasonal clusters: one grouping summer samples (including also the spring sample MAY06 and early autumn ST05) and another cluster aggregating winter samples (but including also the spring samples AP05 and the autumn sample NV05). PCA showed also a clear seasonal gradient with the first axis mainly defined by summer samples on the negative side and winter samples on the positive side (as shown in Fig. 1b); sample NV05 defined the positive side of the second axis and JA05 defined its negative side. The first axis was mainly related to water temperature and inorganic nitrogen sources while the second axis was mostly defined by precipitation levels. These first two axes could explain 62.4% of the total variance of samples' distribution.

(a)



(b)

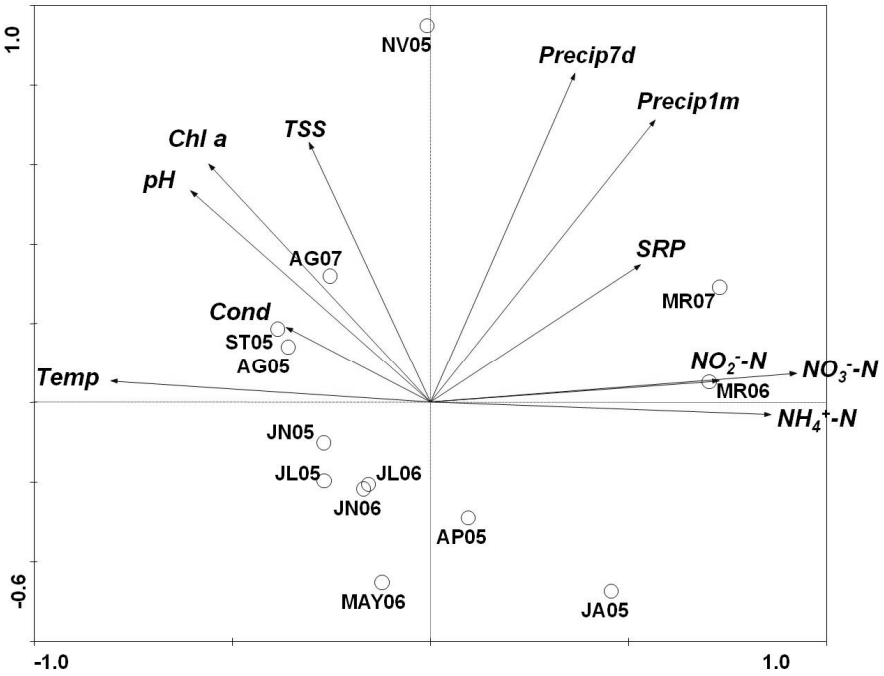


Fig. 1. (a) cluster analysis dendrogram and (b) PCA ordination biplot of Vela Lake samples according to environmental parameters recorded from 2005 to 2007 (see sample codes in table 1).

Analysis of DGGE band profiles and sequenced bands

The DGGE band profiles obtained for Vela Lake samples are presented in Fig. 2. A total of 194 bands were recorded and corresponded to 45 different migration positions; the number of bands *per* sample was in average 15 ± 7 (n = 13). Cluster analysis of DGGE profiles showed two main groups: one including samples from summers 2006 and 2007, and the other group included sample MR07 separated from the remaining samples. Interestingly, inside this last group, sample from winter 2005 clustered together with spring and summer samples 2005 rather than with the other winter samples. RDA analysis showed a gradient mainly related with water temperature (0.68) across the first axis and conductivity (0.90) along the second axis. The first axis was defined by JN05 (and bands 31 and 36) in the positive side whereas the negative side was defined by winter samples MR06 and JA05 (and bands 37 and 23). Sample ST05 and band 23 defined the positive side of the second axis and AP05 (and bands 12, 14 and 28) defined its negative side. Both these first two axes could explain 28.5 % of the total BCC variation.

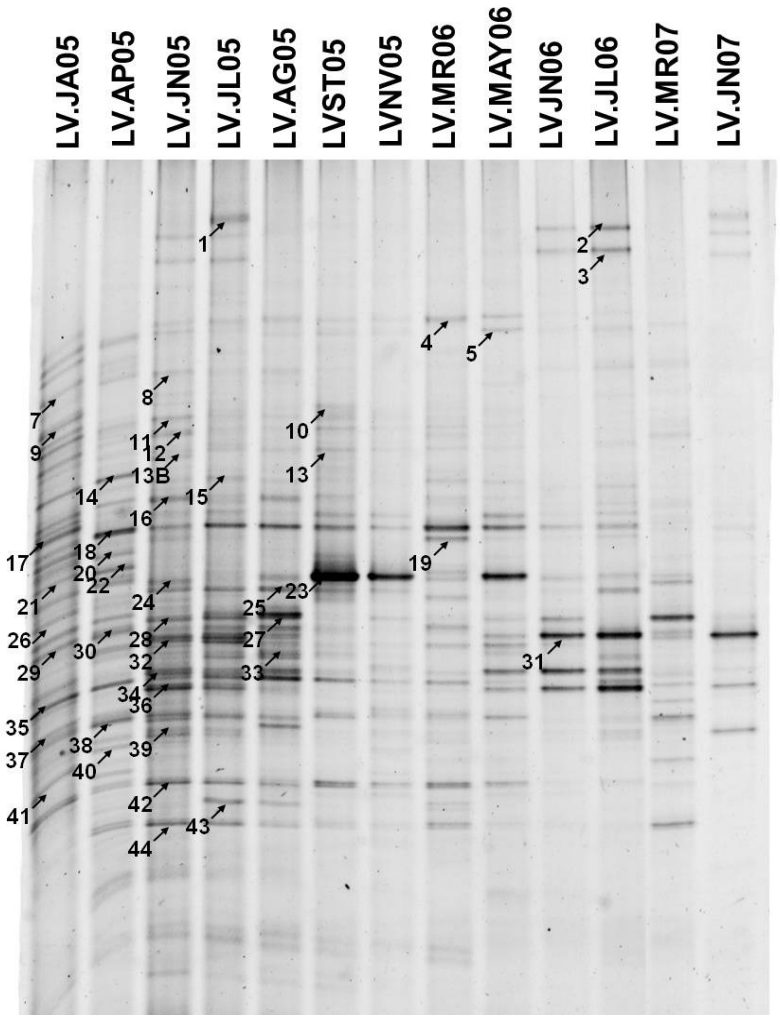
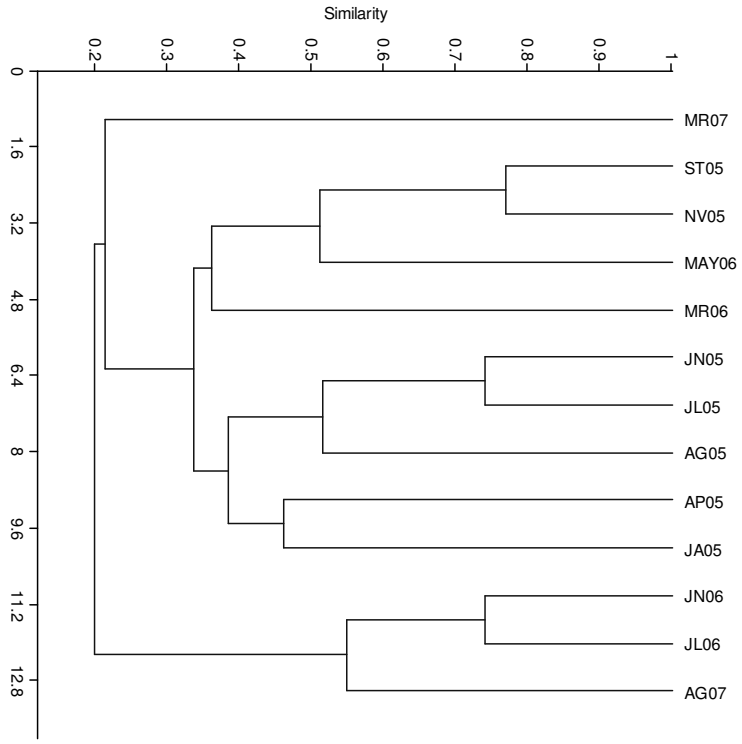


Fig. 2. DGGE profiles containing the bacterial 16S rDNA fragments from Vela Lake samples taken from 2005 to 2007. The code above each lane refers to each sample (see table 1) and the bands numbering corresponds to the different migration positions considered for the analyses.

The phylogenetic affiliation of the sequenced DGGE bands (most intense) is presented in Table 2. Most intense 16S rDNA partial sequences showed to affiliate with groups such as *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria* and *Betaproteobacteria*. The persistence of some

(a)



(b)

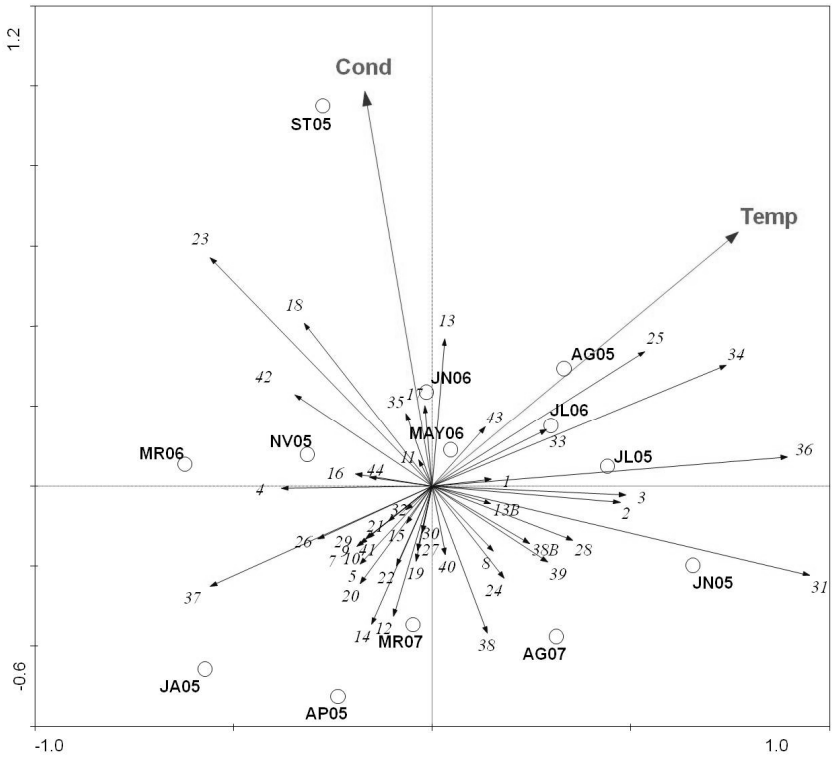


Fig. 3. (a) cluster dendrogram of Crestuma reservoir samples according to DGGE band patterns and (b) RDA ordination triplot of Vela Lake DGGE band patterns according to the environmental parameters recorded during the study period (2005-2007). DGGE bands numbering and samples coding are described in figure 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/cloned bands.

Migration position	Samples	NCBI Accession Nº	Phylogenetic affiliation	Closest relatives (accession Nº)	Percentage similarity (%)
17_A	**JA05**	DQ900047 (de Figueiredo et al., 2007)	*Cyanobacteria*	*Cyanobacterium* sp. MS-M-47 (FJ460126)	99
19_L	**JN07**	submitted	*Alphaproteobacteria*	Uncultured alpha proteobacterium clone WA0.2-0d-24 (HM153614)	100
23_B	**ST05, NV05, MAY06**	submitted	*Cyanobacteria*	*Synechococcus* sp. KORDI-78 (FJ497748)	100
31_C	**JN05, JN06**	submitted	*Cyanobacteria*	*Aphanizomenon aphanizomenoides* UADFA13 (FJ895123)	100
34_I	**MAY06**	submitted	*Cyanobacteria*	*Synechococcus* sp. 2LT05S01 (FM177502)	100
35_E	**JA05, AG05**	submitted	*Betaproteobacteria*	Uncultured *Polynucleobacter* sp. clone SR14 (HM208443)	
36_F	**JN05, JN06, JL06**	submitted	*Cyanobacteria*	*Aphanizomenon gracile* UADFA12 (FJ895127)	100
37_Y	**MR07**	submitted	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Jab PL1W2D10 (HM486215)	100
38B_N	**JN07**	submitted	*Cyanobacteria*	Leptolyngbya foveolarum PMC302.07 (GQ859653)	100
42_G	**MAY06**	submitted	*Actinobacteria*	Uncultured actinomycete clone CR-FL3 16S (AF141389)	100
44_HJ	**JA05, JN05, MR07**	submitted	*Actinobacteria*	Uncultured Actinomycetales bacterium LW9m-5-32 (EU640399)	100

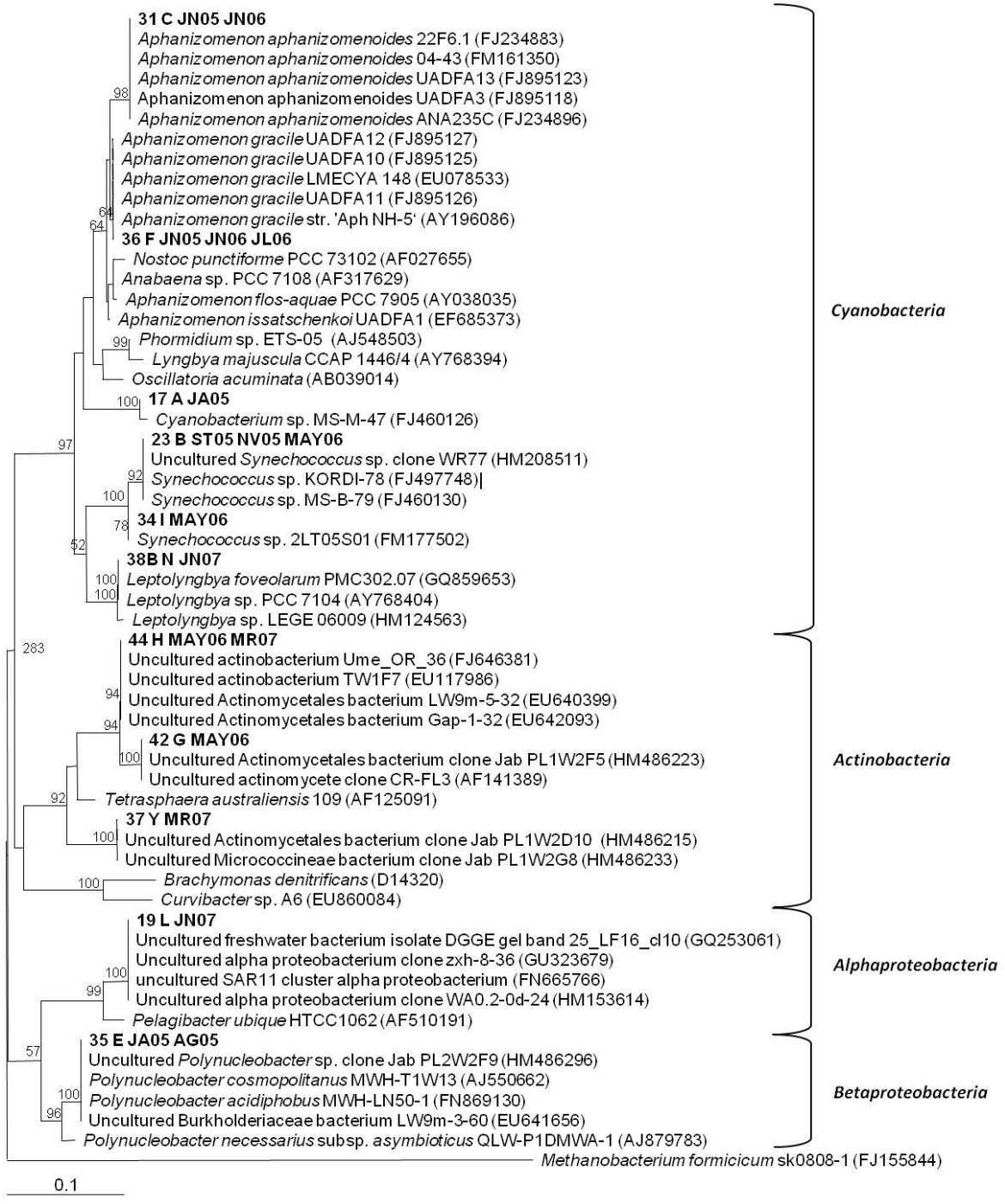


Fig. 4. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel (Fig. 3). The archaeal sequence from *Methanobacterium formicicum* strain sk0808-1 was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

phylotypes across almost all samples could be recorded (e.g. bands 35, 42 and 44) and others were characteristic from samples with similar seasonal conditions (e.g. bands 23, 31 and 36). *Cyanobacteria* showed to dominate the bacterioplankton assemblage across seasons. Interestingly, the cyanobacterial phylotypes corresponding to the migration points 31 and 36 affiliated with

sequences belonging to strains of *Aphanizomenon aphanizomenoides* and *Aph. gracile*, correspondingly, isolated from Vela Lake (de Figueiredo et al., 2010a). Other cyanobacterial dominant phylotypes were similar to sequences of the genera *Synechococcus* and *Leptolyngbya*.

Discussion

The nitrite and phosphate concentrations at Vela Lake are usually low or undetectable during summer months and increase during winter (de Figueiredo et al., 2006) as recorded in the present study. During summer 2005, Vela Lake showed increased values for pH, water temperature, chl *a* and conductivity levels, by comparing to previous studies (Barros, 1994; de Figueiredo et al., 2006). This points out to a deterioration of the trophic status of the lake, as recorded over the past twenty years. In 1989, Vela lake was considered oligo- to β-mesosaprobic (Calado, 1990) but in 1993 its eutrophic condition was already reported based on nutrient levels, phytoplankton densities and cyanobacterial massive development (Barros et al., 1993). In fact, 2005 was considered to have one of the most intense droughts over the last half century for the Portuguese territory, according to the Water Resources Information National System, with eutrophication enhancement effects on water bodies recorded already in winter (de Figueiredo et al., 2007).

In general, our results showed typical winter *vs.* summer DGGE profiles suggesting some predictability for inter-annual summer and winter BCC at Vela Lake, in contrast with a high variability in autumn and spring BCC, as observed for other water bodies (Crump & Hobbie, 2005). The dominant 16S rDNA partial sequences retrived from DGGE profiles showed to affiliate with groups such as *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria* and *Betaproteobacteria* which may indicate the strong eutrophication of the lake as recorded for other eutrophic water bodies (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007; Wu et al., 2007b) and water quality problems may increase in a near future (Zaitlin & Watson, 2006; Smith et al., 2008). The co-dominance of *Actinobacteria*, *Alphaproteobacteria*, *Cyanobacteria* as well as *Betaproteobacteria* phylotypes has been recorded before (Xi et al., 2007; Pope & Patel, 2008).

In the present study, conductivity and water temperature were the parameters most related to the BCC variation across samples. Parameters such as water temperature and pH (Crump & Hobbie, 2005; Lindström et al., 2005; Yannarell & Triplett, 2005; Wu et al., 2007b) as well as the water retention time or water flow rate (Crump & Hobbie, 2005; Lindström et al., 2005) and the levels of nitrogen (Crump & Hobbie, 2005; Wu et al., 2007b; Wei et al., 2008; Zeng et al., 2009), phosphorus (Boucher et al., 2006; Xing & Kong, 2007) and chlorophyll *a* (Xing & Kong, 2007; Tian et al., 2009) are known to be major modulators of bacterial community composition . In this study, the persistence of some phylotypes through samples could be recorded under similar seasonal conditions. Cyanobacteria showed to dominate the bacterioplankton assemblage across seasons. For example, the

phylotype corresponding to *Aphanizomenon aphanizomenoides* (as well as *Aphanizomenon gracile*) was always dominant in June and July samples whereas *Synechococcus* sp. was dominant in autumn and spring. Interestingly, these *Aphanizomenon* phylotypes showed total match with sequences of *Aph. aphanizomenoides* and *Aph. gracile* strains isolated from Vela Lake in 2004 and 2001 (de Figueiredo et al., 2010a) indicating its persistence at this lake over the past years. It was dominant during all summer with co-dominance of Burkholderiaceae (*Betaproteobacteria*) and Actinomycetales (*Actinobacteria*) phylotypes, and also the cyanobacterium *Microcystis viridis* (in June - see chapter III of the present thesis). Actinomycetales and Burkholderiales bacteria have proven to enhance the growth of cyanobacteria belonging to cyanobacteria from the genera *Microcystis* and *Anabaena* (Berg et al., 2008). The dominance of cyanobacteria during late spring and summer months is recurrent at Vela Lake, but there is usually a succession in the bloom-forming species (Barros et al., 1993; de Figueiredo et al., 2006). The development of *Aphanizomenon* spp. blooms is associated with very low concentrations or absence of nitrogen sources (nitrate, nitrite and ammonium) due to N-fixing capability, but under phosphate availability (Teubner *et al.*, 1999; Kahru *et al.*, 2000; de Figueiredo *et al.*, 2004). *M. aeruginosa* usually dominates cyanobacterial blooms during late summer at Vela Lake (de Figueiredo et al., 2006). In general, the development of cyanobacteria was enhanced in summer and related to high temperature, pH and conductivity values, as previously observed for other lakes (Dokulil & Teubner, 2000; De Wever et al., 2005; Van Der Gucht et al., 2005; Ke et al., 2008; de Figueiredo et al., 2010b). The development of the *Leptolyngbya* phylotype was related to higher chlorophyll *a* levels whereas the dominance of *Synechococcus*-like phylotypes showed to be significantly positively related with total suspended solids levels but also with water temperature and conductivity, depending on the phylotype. *Betaproteobacteria* are frequently dominant in eutrophic lakes (Altmann et al., 2003; Boucher et al., 2006) as recorded in the present study. The *Alphaproteobacteria* phylotype was found to be identical to a sequence retrieved from Fermentelos Lake, also a eutrophic lake located in Central Portugal (de Figueiredo et al., 2007; de Figueiredo et al., 2010b) which suggests a possible transversal persistence of some organisms across different water bodies with similar characteristics, as previously recorded (Van Der Gucht et al., 2007). In general, *Actinobacteria* are recorded across seasons in lentic eutrophic water bodies (De Wever et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006b; Newton et al., 2007; Wu et al., 2007b; Xi et al., 2007). However, the occurrence of two Actinomycetales phylotypes (bands 37 and 44) showed to be favoured by higher levels of SRP, nitrate and precipitation. The Actinomycetes group is known to have members that produce compounds that can give bad odour and taste to the water (Zaitlin & Watson, 2006); thus, its dominance in a recreational lake may degrade the water quality for the lake's users.

References

Abrantes N., Pereira R., de Figueiredo D. R., Marques C. R., Pereira M. J. & Gonçalves F. (2009). A whole sample toxicity assessment to evaluate the sub-lethal toxicity of water and sediment elutriates from a lake exposed to diffuse pollution. *Environmental Toxicology* 24: 259-270.

Allgaier M. & Grossart H. P. (2006). Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

Altmann D., Stief P., Amann R., Beer D. d. & Schramm A. (2003). *In situ* distribution and activity of nitrifying bacteria in freshwater sediment. *Environmental Microbiology* 5: 798-803.

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Barros P., Implicações ecotoxicológicas de cianobactérias em cladóceros. Faculty of Sciences and Technology, Vol. MSc. University of Coimbra, Coimbra, 1994.

Barros P., Silveira S., Ribeiro R., Gonçalves F., Vasconcelos V. & Soares A. M. V. M. (1993). Estrutura populacional fitoplanctónica nas lagoas das Braças, Vela e Mira (região centro-litoral). Resultados preliminares. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 1-8.

Berg K. A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P. & Rapala J. (2008). High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME Journal* 3: 314-325.

Boucher D., Jardillier L. & Debroas D. (2006). Succession of bacterial community composition over two consecutive years in two aquatic systems: A natural lake and a lake-reservoir. *FEMS Microbiology Ecology* 55: 79-97.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Calado A. J., Fundamentação da aplicação do sistema de saprobia às algas de água doce do Centro de Portugal. Department of Biology. University of Aveiro, Aveiro, 1990.

Clarke K. R. & Gorley R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

de Figueiredo D., Pereira M. & Correia A. (2010a). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26: 1067-1077.

de Figueiredo D. R., Alves A., Pereira M. J. & Correia A. (2010b). Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). *Journal of Plankton Research* 32: 239-252.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Ke Z., Xie P. & Guo L. (2008). Controlling factors of spring-summer phytoplankton succession in Lake Taihu (Meiliang Bay, China). *Hydrobiologia* 607: 41-49.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Mitraki C., Crisman T. L. & Zalidis G. (2004). Lake Koronia, Greece: Shift from autotrophy to heterotrophy with cultural eutrophication and progressive water-level reduction. *Limnologica - Ecology and Management of Inland Waters* 34: 110-116.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Newton R. J., Jones S. E., Helmus M. R. & McMahon K. D. (2007). Phylogenetic ecology of the freshwater Actinobacteria acI lineage. *Applied and Environmental Microbiology* 73: 7169-7176.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Pope P. B. & Patel B. K. C. (2008). Metagenomic analysis of a freshwater toxic cyanobacteria bloom. *FEMS Microbiology Ecology* 64: 9-27.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Saitou N. & Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Saker M. L., Nogueira I. C. G., Vasconcelos V. M., Neilan B. A., Eaglesham G. K. & Pereira P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55: 243-250.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

Thompson J. D., Higgins D. G. & Gibson T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

Tian C., Tan J., Wu X., Ye W., Liu X., Li D. & Yang H. (2009). Spatiotemporal transition of bacterioplankton diversity in a large shallow hypertrophic freshwater lake, as determined by denaturing gradient gel electrophoresis. *J. Plankton Res.* 31: 885-897.

Van Der Gucht K., Cottenie K., Muylaert K., Vloemans N., Cousin S., Declerck S., Jeppesen E., Conde-Porcuna J. M., Schwenk K., Zwart G., Degans H., Vyverman W. & De Meester L. (2007). The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20404-20409.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Vasconcelos V. M., Campos T., Amorim A. & Soares A. M. V. M. (1993). Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 193-201.

Wei C., Bao S., Zhu X. & Huang X. (2008). Spatio-temporal variations of the bacterioplankton community composition in Chaohu Lake, China. *Progress in Natural Science* 18: 1115-1122.

Wiedner C., Rücker J., Brüggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.

Wilson A. E., Sarnelle O., Neilan B. A., Salmon T. P., Gehringer M. M. & Hay M. E. (2005). Genetic Variation of the Bloom-Forming Cyanobacterium *Microcystis aeruginosa* within and among Lakes: Implications for Harmful Algal Blooms. *Applied and Environmental Microbiology* 71: 6126-6133.

Wu X., Xi W., Ye W. & Yang H. (2007). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Xi W., Wu X., Ye W. & Yang H. (2007). Changes in bacterial community structure during preceding and degraded period of cyanobacterial bloom in a bay of the Taihu Lake. *Chinese Journal of Applied and Environmental Biology* 13: 97-103.

Xing P. & Kong F. (2007). Intra-habitat heterogeneity of environmental factors regulating bacterioplankton community composition in Lake Taihu, China. *Aquatic Microbial Ecology* 48: 113-122.

Yannarell A. C. & Triplett E. W. (2005). Geographic and Environmental Sources of Variation in Lake Bacterial Community Composition. *Appl. Environ. Microbiol.* 71: 227-239.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology* 25: 763-772.

Zwart G., Crump B. C., Kamst-van Agterveld M. P., Hagen F. & Han S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* 28: 141-155.

Seasonal variation of bacterioplankton assemblage at Crestuma reservoir (downstream Douro River, North Western Portugal)

In: de Figueiredo D. R., Silva R. A., Castro B. B., Pereira M. J. & Correia A. (in preparation for submission). Seasonal variation of bacterioplankton assemblage at Crestuma reservoir (downstream Douro River, North Western Portugal).

Abstract

Crestuma reservoir is an important water body for the Northern Portugal; it isused for drinking water supply of Porto city suburbs. The present work aimed to investigate seasonal shifts in bacterioplankton community composition (BCC) at this reservoir (using *16S rRNA* PCR-DGGE) and relate them with environmental variables through multivariate analysis. Parameters such as water temperature, pH, conductivity and concentrations of total suspended solids (TSS), nutrients, precipitation and chlorophyll *a* levels were considered for the analyses. Environmental data suggested meso- to eutrophic features for this water body. Variables such as precipitation, nitrite, TSS, pH and conductivity levels showed to have major impact on characterization of samples. However, the seasonal BCC variation showed to depend mainly on water temperature and conductivity, as observed for many other water bodies. Dominant phylotypes showed to belong to members of *Betaproteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Actinobacteria* groups.

Keywords

Crestuma reservoir, bacterioplankton diversity, 16SrDNA-DGGE, RDA analysis.

Introduction

The increasing level of eutrophication in freshwater systems is becoming a concerning issue, particularly in water bodies used for drinking water supply. Climatic changes lead to increased water retention times during summer months and water quality is degraded with the risk of waterborne diseases outbreaks (Charron et al., 2004; Bond et al., 2008a). For instance, the development of bacterial groups such as *Cyanobacteria* and *Actinobacteria* can lead to water quality degradation (Zaitlin & Watson, 2006; Smith et al., 2008). More, the occurrence of cyanobacterial blooms in drinking water supplies may also put at risk the human health safety due to the production of cyanotoxins (Codd, 2000; de Figueiredo et al., 2004b). Although the development and toxicity of cyanobacterial blooms have been relatively studied at Crestuma reservoir over the last decade (Vasconcelos et al., 1996; Ferreira et al., 2001; Teles et al., 2006) there are almost no studies concerning the total bacterioplankton community composition (BCC) (de Figueiredo et al., 2007). Culture-independent molecular methodologies such as PCR-DGGE (polymerase chain reaction - denaturing gradient gel electrophoresis) can be used for the characterization of BCC through time by comparing the resulting band patterns (Muyzer et al., 1993; Muyzer & Smalla, 1998; Lyautey et al., 2005).

Crestuma reservoir is mainly used for hydroelectric power generation but also for drinking water supply of Porto city and periphery suburbs (Cabecinha et al., 2009a). Eutrophic conditions have been recently recorded at this water body (de Figueiredo et al., 2007; Cabecinha et al., 2009a); pollution sources comprise domestic wastewater (including from transnational large population agglomerates upstream Douro River) but also runoff from agriculture and industrial effluents (Vasconcelos et al., 1993b; Cabecinha et al., 2009b). The development of toxic cyanobacteria at Crestuma reservoir has been reported (Vasconcelos et al., 1993b; Ferreira et al., 2001). Unfortunately, in spite of the research made over the last years the removal of cyanobacterial cells along with cyanotoxins from water is still difficult and expensive which leads to its ineffective elimination from drinking waters worldwide (de Figueiredo et al., 2004b). This can be dangerous to human health since chronic poisoning by low doses of tumour promoter microcystins, in particular, has been related to the development of liver and colorectal cancer (Ueno et al., 1996; de Figueiredo et al., 2004b; Hernández et al., 2009).

The present study aimed to screen the bacterioplankton diversity at Crestuma reservoir using *16S rRNA* PCR-DGGE. The seasonal variation of BBC was investigated by relating shifts in DGGE band patterns and environmental parameters through multivariate analysis.

Materials and methods

Sampling and environmental parameters

Crestuma reservoir is located downstream the transnational Douro River (Cabecinha et al., 2009b) just before Porto city and 22 Km from the river mouth and it comprises an area of 1298 ha (through a length of 44 Km), a maximum water volume storing of about 110 million m^3 and an average depth of 13 m (Ferreira et al., 2001; Teles et al., 2006; Cabecinha et al., 2009b). It is used for hydroelectric power generation, for drinking water supply of Porto city and periphery suburbs and also for recreational purposes. In spite of the water residence time is considered very low at Crestuma reservoir (2.24 days), the eutrophication which has been recorded has been related to domestic wastewater (including from large Spanish population agglomerates upstream Douro River) but also runoff from agriculture and industry (Vasconcelos et al., 1993b; Cabecinha et al., 2009b). The sampling was performed in winters 2005-07, springs 2006-07, summers 2005-06 and autumn 2006 (see Table 1). Water samples were taken sub-superficially using sterile bottles at about 1 m from the shore. Samples were placed at 4 ºC under dark conditions until subsequent treatment within 12 hours. Water temperature was measured *in situ* but pH and conductivity were measured in laboratory at 20 ºC by using specific electrodes. Parameters such as total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium, nitrate and nitrite concentrations were determined in laboratory according to standard procedures (APHA, 1992; Rodier, 1996).

DNA extraction

Total DNA from environmental water samples was extracted by filtering 150 mL of the water samples through 0.22 µm polycarbonate sterile filters, resuspension in 2 mL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0] and centrifugation. Lysozyme was added after resuspension in 200 µL of TE and incubation was performed at 37ºC for 1h. The following DNA extraction and purification were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). DNA was finally suspended in TE buffer and stored at -20 ºC.

PCR amplification of bacterial 16S rDNA fragments

PCR amplification of bacterial *16S rRNA* gene fragments was performed with the primers 338F-GC/518R (Muyzer et al., 1993), universal for bacteria. Primers were synthesized by STABVida (Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 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 30s at 92 °C, 30s at 55 °C and 30s at 72 °C, and a final extension step at 72 °C for 30 min. Negative control reactions without any template DNA were performed simultaneously. The PCR amplicons were electrophoresed in a 1.5% agarose gel and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder). The gel was stained with ethidium bromide and visualized on a UV transmilluminator.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1mm 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, California, USA) using 0.5x 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, California, USA).

DGGE bands excision, cloning and sequencing

The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL of sterile milli-Q-purified water from which 5 µL of the eluted DNA was used in PCR amplification with the original primer pair. The isolation and identity of each DNA band was verified through DGGE and, if necessary, the extraction procedure was repeated until the targeted band isolation was obtained. The isolated bands were then cloned using the TOPO TA cloning kit with the pCR 2.1-TOPO vector (Invitrogen, Portugal). Prior to cloning, an A tailing was performed for PCR products according to manufacturers' instructions. In order to screen for false positive clones, the size of the amplicons from PCR using the vector primers M13R/T7F was checked in 1.5% agarose gels as described above using the molecular weight marker GeneRuler™ 1 kb DNA ladder. The migration point of each cloned sequence with the targeted size was verified through DGGE after a nested PCR amplification with the primer pair 338F-GC and 518R. However, sequencing was made with PCR amplicons using the vector primers M13R/T7, optimizing the sequence length information. Each amplicon was purified with the concertTM rapid PCR purification system (Gibco BRL, Eggenstein, Germany) before it was commercially sequenced (STABVida, Portugal).

Statistical analysis

Image analysis of DGGE profiles was performed using the the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories, Hercules, CA, USA) and a densitometric profile was established for each sample to determine the relative contribution of each band to the total signal in the lane sample (bands with a relative contribution of less than 0.5 % in each lane were not considered for the subsequent analyses). The presence or absence of co-migration points was converted to a binary matrix (0/1) and cluster analysis was performed using the unweighted pair group method with mathematical averages (UPGMA) based on the Bray-Curtis similarity coefficient with PRIMER 6 software (Clarke & Gorley, 2006). Principal component analysis (PCA) was used to assess the distribution of samples according to environmental parameters after data standardization

(subtracting the mean from each observation and dividing by the corresponding standard deviation) (ter Braak, 1995). Cluster analysis of samples according to environmental parameters was also executed using the UPGMA. The dendrogram was created with the similarities calculated using the Pearson correlation coefficient (95% probability) which was also used to assess significant correlations within environmental parameters and with bands considering their occurrence and relative intensity. Redundancy analysis (RDA) was performed to reveal relationships between the distribution of the dominant DGGE phylotypes (using a matrix built with band relative intensities after log transformation) and environmental variables using CANOCO 4.5 (Scientia Software). The environmental parameters which better described the distribution of the species data were *a priori* identified by forward selection (Magnan et al., 1994; ter Braak & Verdonschot, 1995) using a Monte Carlo permutation test (499 unrestricted permutations; ALPHA = 0.10).

Results

Environmental parameters

The code and environmental parameters recorded for Crestuma reservoir samples are summarised in Table 1. Water temperature ranged between 13 and 26 ºC, in FB05 and AG05, respectively. The values for pH showed to be higher during spring months with a maximum of 8.60 in MR07 and a minimum of 7.60 in MAY07. Samples MR06 and MR07 showed the lowest conductivity levels (215 and 214 µS cm^{-1}, respectively) while a maximum of 385 µS cm^{-1} was recorded in MR05. Nitrate concentration ranged from 2.9 to 7.0 mg N (NO_3^-) L^{-1} in AG05 and MR05, respectively, while nitrite levels were lowest (0.01 mg N (NO_2^-) L^{-1}) in MR05 and OCT06, and highest in AG05 with 0.26 mg N (NO_2^-) L^{-1}. Ammonia was undetectable in 2005 and 2006 samples (except for OCT06) and reached a maximum of 0.09 mg N (NH_4^+) L^{-1} in MR07. SRP concentration was undetectable in MAY06 and JL06 and reached a maximum of 0.15 mg L^{-1} in AG05. TSS levels ranged between 3.08 and 12.10 mg.L^{-1} (in MR05 and AG05, respectively). Chl *a* concentration ranged from 2.7 to 17.5 µg L^{-1} in OCT06 and MAY06, in that order. The average daily precipitation levels 7 days and 1 month before sampling date showed be different with ranges from 0.0 (MR05, AG05 and JL06) to 10.9 mm (OCT06) and from 0.0 (AG05) to 4.8 mm (OCT06), respectively. With the exception of OCT06, all samples showed precipitation levels way below the seasonal averages from the past century, since 1913.

The Pearson correlation coefficient was calculated to assess relationships between the recorded environmental parameters (n = 8; $P < 0.05$). Positive significant correlations were found between ammonium and pH (r = 0.76), nitrite and TSS (r = 0.89) and precip7days and precip1month (r = 0.74); nitrate concentration showed a significant negative correlation with water temperature (r = -0.83). According to environmental parameters, Pearson correlation coefficient among samples (n =

Table 1. Characterization of Crestuma reservoir samples and environmental data recorded during the study period (2005-2007).

		Average daily precipitation* (mm)			Water temp (ºC)	pH	TSS (mg L^{-1})	Chl *a* (µg L^{-1})	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	SRP	Conductivity (µS cm^{-1})
Sampling date	Code	7 days before	1 month before	Seasonal (1913-2004)					(mg L^{-1})				
13th March 2005	BCR_MR05	**0.0**	1.0	5.0	**13**	7.65	3.08	8.1	0.01	7.0	< 0.01	0.13	385
15th August 2005	BCR_AG05	**0.0**	**0.0**	1.0	26	7.79	12.10	9.1	0.14	2.9	< 0.01	0.15	318
12th March 2006	BCR_MR06	1.2	4.4	5.0	12	7.74	4.23	3.4	0.02	5.5	< 0.01	0.05	215
30rd May 2006	BCR_MAY06	0.4	0.3	2.5	17	8.08	7.48	17.5	0.04	6.0	< 0.01	< 0.01	233
11th July 2006	BCR_JL06	**0.0**	0.3	1.0	**25**	7.95	5.32	16.0	0.05	3.3	< 0.01	< 0.01	313
27th September 2006	BCR_SP06	**10.9**	**4.8**	4.8	25	7.81	3.25	2.7	0.01	3.0	0.03	0.08	288
1st March 2007	BCR_MR07	0.1	0.4	5.0	14	8.60	7.09	3.8	0.02	4.2	0.09	0.06	216
9th May 2007	BCR_MAY07	1.8	2.3	2.5	19	7.60	5.02	7.7	0.03	5.1	0.01	0.03	299

* The average precipitation levels were obtained from Portuguese Water Institute (http://snirh.pt/) for the nearest available meteorological monitoring point.

Table 2. Accession number and phylogenetic affiliation for the sequenced bacterial DGGE bands shown in Fig. 2.

Band	NCBI Accession Nº (reference)	Phylogenetic affiliation	Closest relatives (accession Nº)	Origin	Percentage similarity (%)
BCR10_cl101	Submitted (this study)	*Betaproteobacteria*	Uncultured beta proteobacterium clone WA0.2-0d-59 (HM153631)	Taihu Lake, China	99
BCR12_clA8	Submitted (this study)	*Bacteroidetes*	Uncultured **Flavobacteriaceae** LW18m-2-67 (EU642364)	Lake Michigan, Wisconsin, USA	99
BCR13_cl104	Submitted (this study)	*Cyanobacteria*	Uncultured *Synechococcus* sp. clone WR77 (HM208511)	Freshwater China	100
BCR16B_cl97	Submitted (this study)	*Bacteroidetes*	Uncultured Bacteroidetes bacterium clone ly4 (GQ203644)	anaerobic sludge in ABR reactor	99
BCR16_cl96	Submitted (this study)	*Firmicutes*	Uncultured Firmicutes clone E_RAMPD3502RT35 (GQ242585)	tidal freshwater sediment	98
BCR17_cl50	Submitted (this study)	*Actinobacteria*	Uncultured Micrococcineae bacterium Jab PL2W2G4 (HM486300)	wetlands	100
BCR18_clA7	Submitted (this study)	*Bacteroidetes*	Uncultured **Sphingobacteria** bacterium LW18m-3-23 (EU640250)	Lake Michigan, Wisconsin, USA	100
BCR24	DQ900029 (de Figueiredo et al., 2007)	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Ac70F5 (GU472674)	Lake Pavin (France)	100

11; $P < 0.05$) only indicated significant negative correlations for FB06 and JL06 (r = -0.70) and MAY06 and SP06 (r = -0.66). Cluster analysis (Fig. 1a) showed two main clusters: one including typical winter samples MR05 and FB06 and another with summer samples AG05 and JL06. However, this last cluster also included spring sample MAY06 and winter sample MR07 suggesting that winter in 2007 had special features more similar to spring and summer conditions. PCA (Fig. 1b) showed a general seasonal gradient with the first axis generally defined by winter and autumn samples (FB06 and SP06, respectively) on the negative side and summer samples on the positive side (AG05 and JL06) while the winter sample MR07 defined the positive side of the second axis and autumn sample SP06 defined its negative side. The first axis was mainly related to precipitation, TSS and nitrite levels while the second axis was mostly defined by pH and conductivity. These first two axes could explain 54.7% of the total variance of samples distribution.

DGGE band patterns analysis

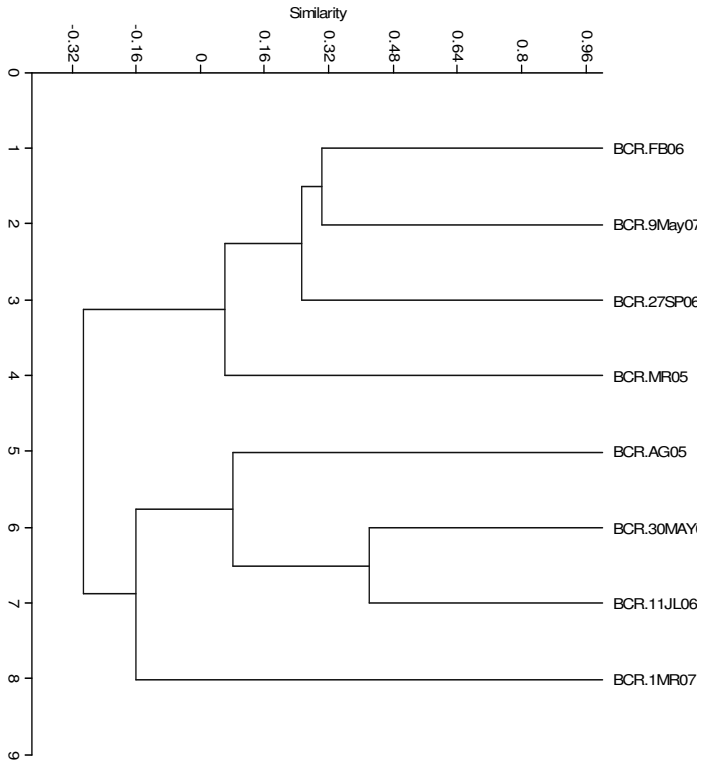
The DGGE profiles obtained for Crestuma reservoir are presented in Fig. 2. A total of 253 bands (with a relative intensity of more than 0.5% of the total sum of the bands intensity in the corresponding line) were recorded and corresponded to 65 different band migration positions. The number of bands *per* sample showed an average of 34 ± 6 (n = 8). Ubiquitous phylotypes could be detected throughout the samples corresponding to bands 2, 6, 9, 10, 12, 16, 18, 21, 24, 25, 28 and 28B. Cluster analysis (Fig. 3a) suggests a seasonal clustering of samples based on DGGE band profiles. Two major clusters were observed: one including summer and early autumn samples and the other including winter and spring samples. The RDA suggests a seasonal gradient along the first axis mainly based on water temperature (-0.97); summer samples and bands 11 and 13 define the axis' negative side and winter samples and bands 17, 9C and 16B define its positive side. However, conductivity (0.94) showed to be also an important modulator for BCC variation along the second axis, with sample MR05 (and bands 9B, 27B and 20) defining its positive side and MR07 along with MR06 (and bands 8 and 19) defined the negative side. The first two RDA axes could explain 46.2% of the total variance.

The affiliation of the successfully sequenced DGGE bands is presented in Table 2. The sequences showed the highest similarities with members of major phylogenetic groups such as *Betaproteobacteria*, *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria* but also the *Firmicutes* group (Fig. 4). In general, all sequenced phylotypes had the highest similarities with sequences retrieved from freshwater environments, namely lakes.

Discussion

Crestuma reservoir has been considered to have a low ecological status (eutrophic to hyper-eutrophic) based on parameters such as nutrient levels and secchi disk depth (de Figueiredo et al., 2007; Cabecinha et al., 2009a) although Chl *a* levels may vary seasonally giving an oligotrophic to

(a)



(b)

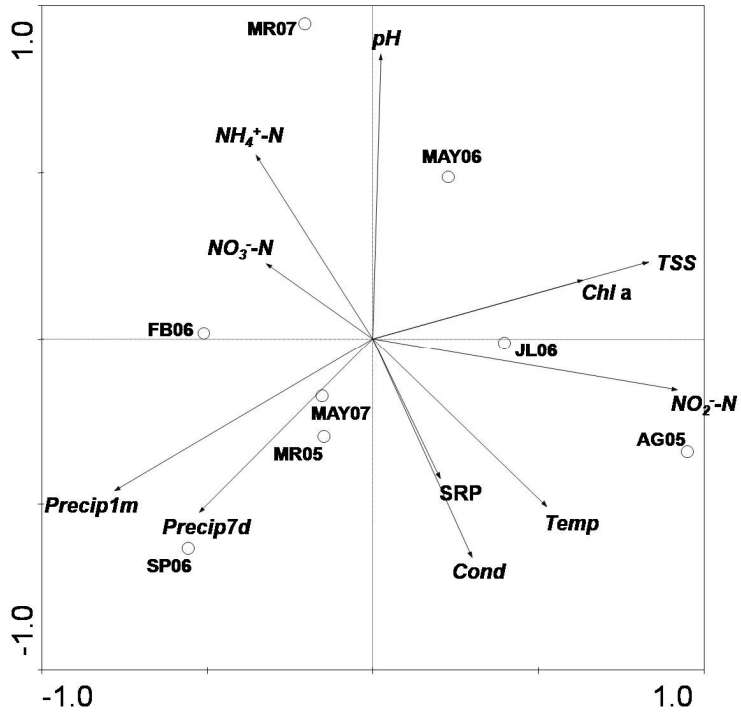


Fig. 1. (a) cluster dendrogram and (b) PCA ordination biplot of Crestum reservoir samples according to environmental parameters recorded from 2005 to 2007 (see sample codes in table 1).

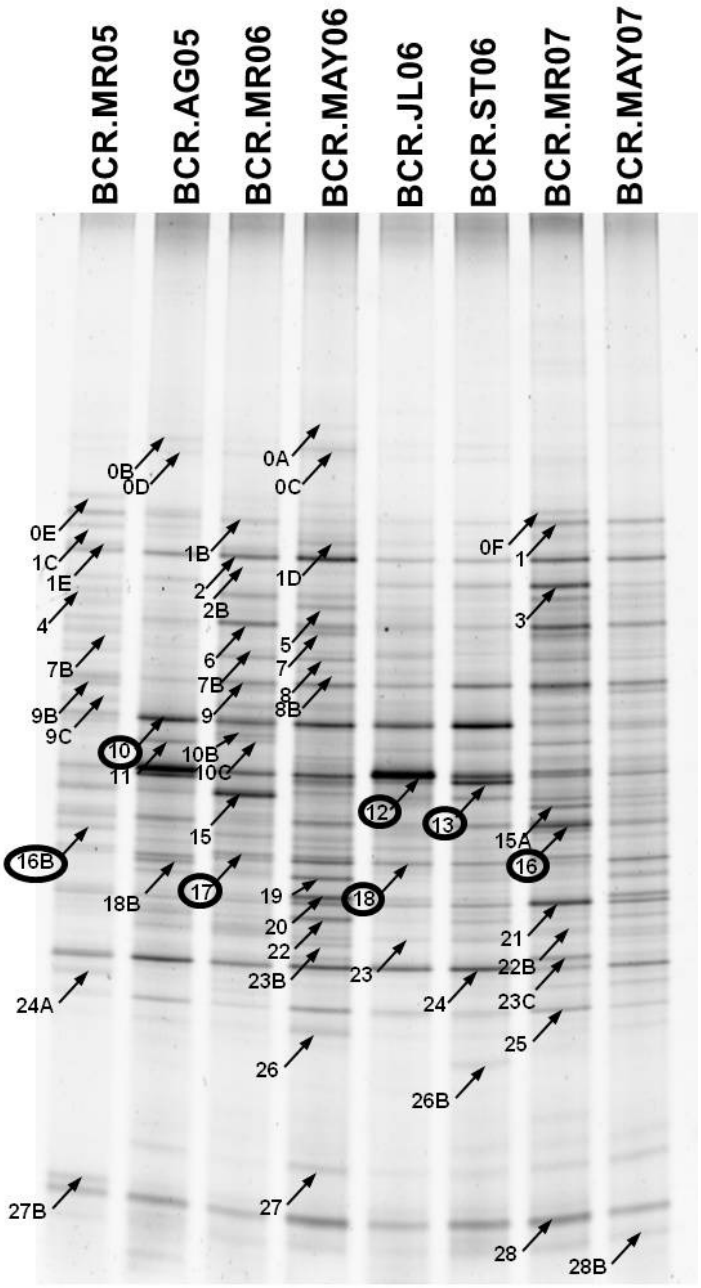
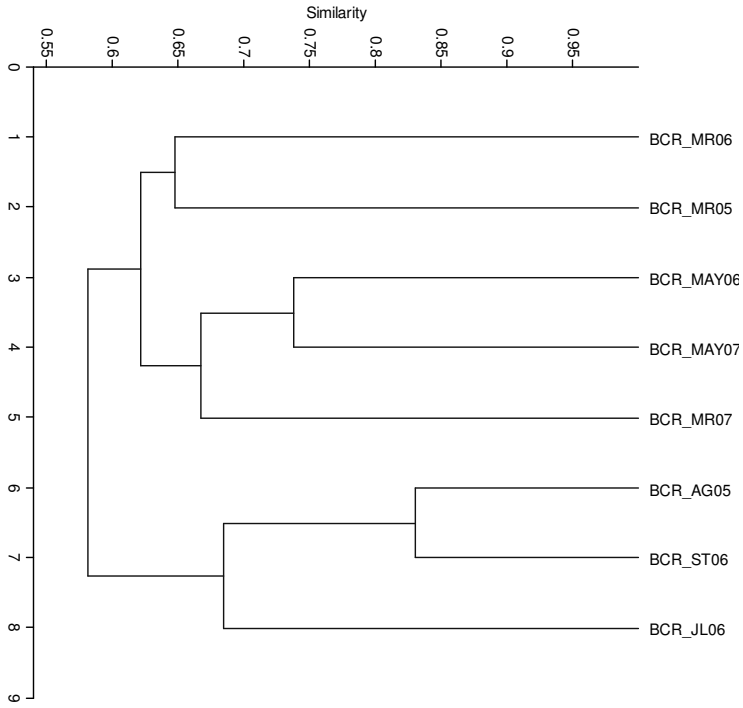


Fig. 2. DGGE profiles containing the bacterial 16S rDNA fragments from Crestuma reservoir samples taken from 2005 to 2007. The code above each lane refers to each sample (see table 1) and the bands numbering corresponds to the different migration positions considered for the analyses.

eutrophic status to the reservoir as also shown in the present study (Nürnberg, 1996). However, in general, samples showed meso- to eutrophic features (Harper, 1992). The recorded levels for environmental parameters showed to be within the average values recorded over the last two decades, according to time series data (http://snirh.pt) from Portuguese Water Institute (INAG), except for conductivity (higher in all samples, particularly in winter 2005) with Chl *a* which had higher values in winter 2005 and spring and summer 2006. Cluster analysis and PCA suggested a seasonal clustering of samples although the cluster with typical winter samples MR05 and FB06 also included spring and autumn samples MAY07 and SP06, respectively, related to the high precipitation levels recorded. More, the cluster with summer samples and spring sample MAY06 also included the winter sample MR07 mainly due to the low precipitation values but also to the high pH levels recorded. Thus, temperature showed not to be the expected main seasonal variable but precipitation did. This indicates that climatic changes are leading to a new perception of seasonality based on new factors such as precipitation levels (which are leading to more extreme rain or drought episodes (Charron et al., 2004; Bond et al., 2008a)) rather than typical variables such as temperature. Interestingly, the analyses based on the DGGE band profiles showed a more seasonal-consistent clustering of samples, mainly influenced by a temperature gradient. The cluster analysis based on DGGE band patterns suggested proximity between summer and early autumn and between winter and spring samples. Actually, although summer *vs.* winter BCC can be usually

(a)



(b)

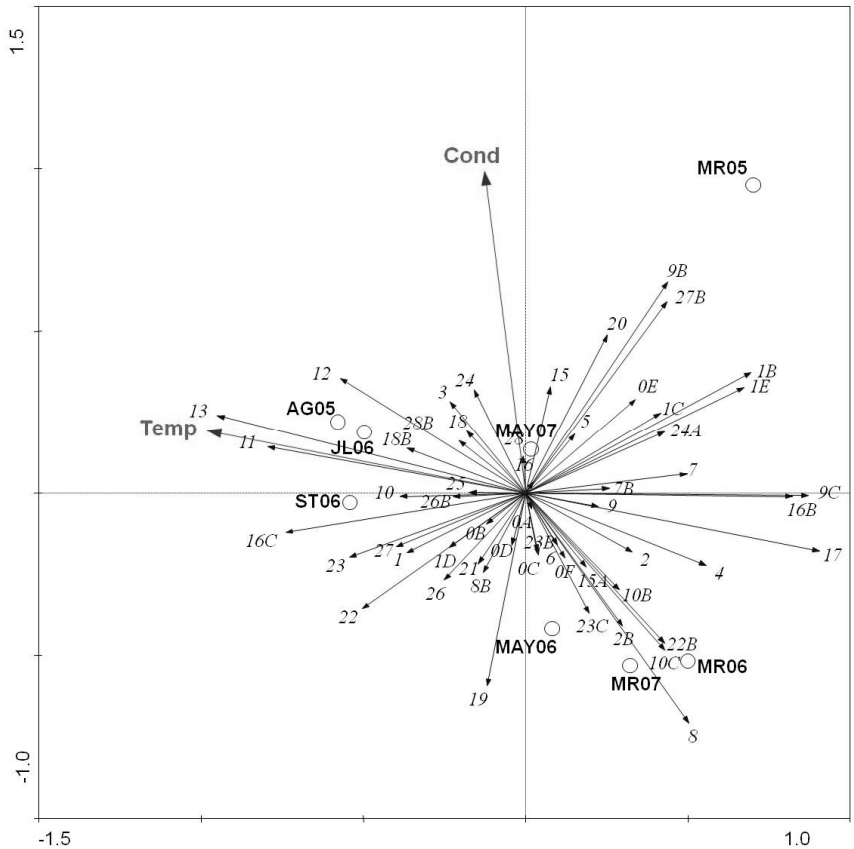


Fig. 3. (a) cluster dendrogram of Crestuma reservoir samples according to DGGE band patterns and (b) RDA ordination triplot of Crestuma reservoir DGGE band patterns according to the environmental parameters recorded during the study period (2005-2007). DGGE bands numbering and samples coding are described in figure 2 and table 1, respectively.

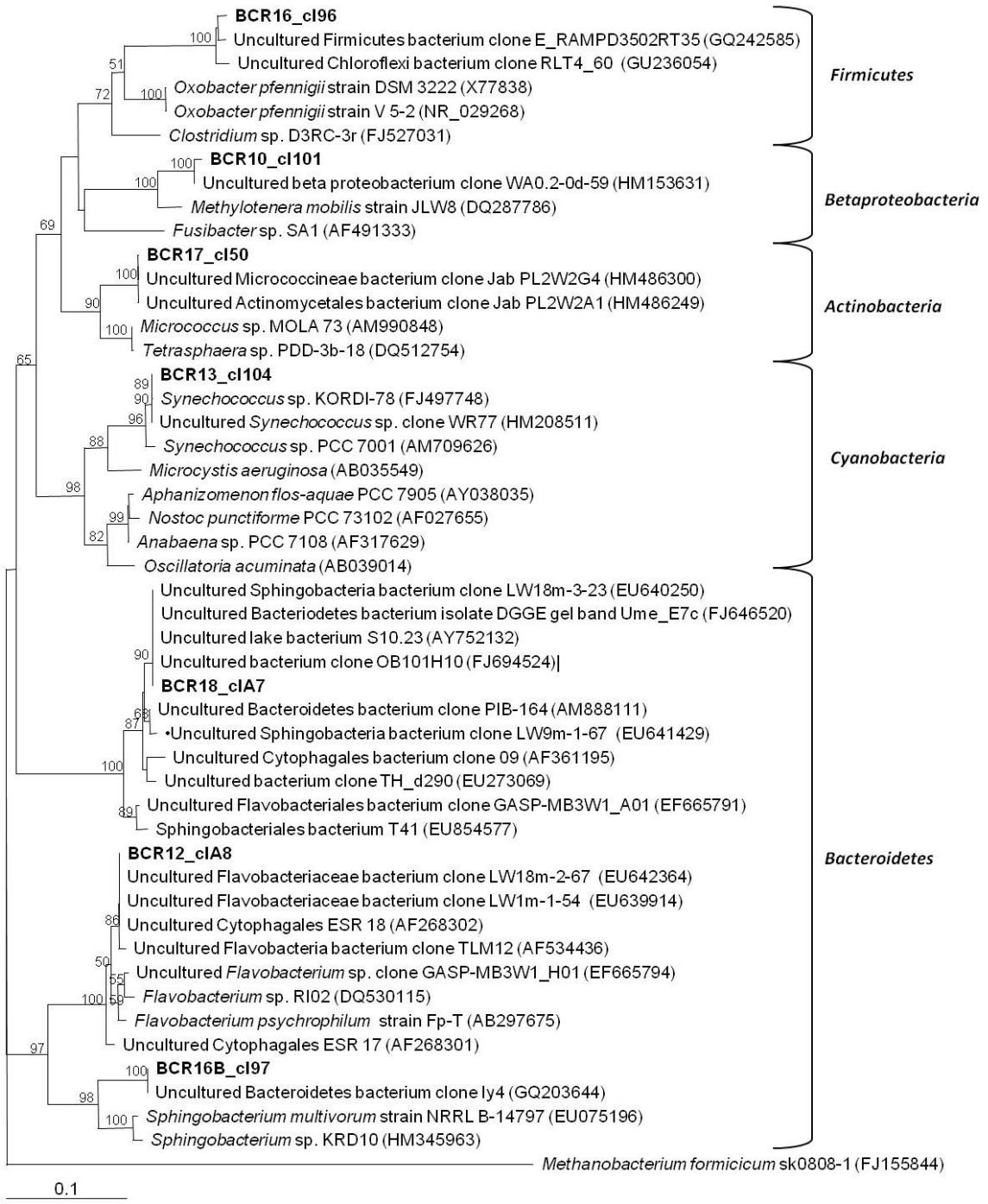


Fig. 4. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel (Fig. 3). The archaeal sequence from *Methanobacterium formicicum* strain sk0808-1 was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

predictable in freshwater bodies, a high variability in autumn and spring samples has been recorded (Crump & Hobbie, 2005). In fact, water temperature is known to be a major modulator for BCC variation in freshwater bodies (Crump & Hobbie, 2005; Lindström et al., 2005; de Figueiredo et al., 2010b). However, conductivity is not usually presented a major BCC modulator, contrary to results in the present study. Multivariate analysis showed that BCC in winter 2005 differed from the other winter samples and it was mainly defined by high conductivity levels. This must be due to the fact that winter 2005 was considered one of the driest over the past century (de Figueiredo et al., 2007). This suggests that the intensification of drought episodes enhanced by climatic changes may lead to increased conductivity levels and cause major impacts on BCC.

In shallow eutrophic and hypereutrophic lakes a dominance of the *Alpha-* and *Beta-proteobacteria*, *Bacteroidetes* and *Actinobacteria* groups can be found (Eiler & Bertilsson, 2004; Van der Gucht *et al.*, 2005; Allgaier & Grossart, 2006). *Bacteroidetes*, particularly Sphingobactereaceae and Flavobacteriaceae bacteria, were found among the dominant DGGE bands at Crestuma reservoir samples. *Flavobacterium* dominance is frequent in Northern Europe eutrophic rivers (Brümmer *et al.*, 2000). At Crestuma reservoir, the cyanobacterial phylotype recorded was most similar to *Synechococcus* spp. and showed to dominate in late summer which has been observed for other lentic water bodies, namely Vela Lake (see previous sub-chapter of this thesis). Nevertheless, during summer months, the development of toxic cyanobacteria such as neurotoxic *Aphanizomenon flos-aquae* (Ferreira et al., 2001) and hepatotoxic strains of *Microcystis aeruginosa* (mostly MC-LR producers) had already been reported as representing potential health risks for human consumers (Vasconcelos et al., 1993b; Vasconcelos et al., 1996; de Figueiredo et al., 2004b).

References

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Bond N., Lake P. & Arthington A. (2008). The impacts of drought on freshwater ecosystems: an Australian perspective. *Hydrobiologia* 600: 3-16.

Cabecinha E., Cortes R., Cabral J. A., Ferreira T., Lourenço M. & Pardal M. A. (2009a). Multi-scale approach using phytoplankton as a first step towards the definition of the ecological status of reservoirs. *Ecological Indicators* 9: 240-255.

Cabecinha E., Cortes R., Pardal M. A. & Cabral J. A. (2009b). A Stochastic Dynamic Methodology (StDM) for reservoir's water quality management: Validation of a multi-scale approach in a south European basin (Douro, Portugal). *Ecological Indicators* 9: 329-345.

Charron D. F., Thomas M. K., Waltner-Toews D., Aramini J. J., Edge T., Kent R. A., Maarouf A. R. & Wilson J. (2004). Vulnerability of waterborne diseases to climate change in Canada: A review. *Journal of Toxicology and Environmental Health - Part A* 67: 1667-1677.

Clarke K. R. & Gorley R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

de Figueiredo D., Pereira M. & Correia A. (2009). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* doi:10.1007/s11274-009-0272-3.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

Ferreira F. M. B., Soler J. M. F., Fidalgo M. L. & Ferna?ndez-Vila P. (2001). PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal). *Toxicon* 39: 757-761.

Harper D., 1992. Eutrophication of freshwaters: principles, problems and restoration. Chapman et Hall, London.

Hernández J. M., López-Rodas V. & Costas E. (2009). Microcystins from tap water could be a risk factor for liver and colorectal cancer: A risk intensified by global change. *Medical Hypotheses* 72: 539-540.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Magnan P., Rodríguez M. A., Legendre P. & Lacasse S. (1994). Dietary variation in a freshwater fish species: relative contributions of biotic interactions, abiotic factors, and spatial structure. *Canadian Journal of Fisheries and Aquatic Sciences* 51: 2856-2865.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G. & Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 73: 127-141.

Nürnberg G. K. (1996). Trophic State of Clear and Colored, Soft- and Hardwater Lakes with Special Consideration of Nutrients, Anoxia, Phytoplankton and Fish. *Lake and Reservoir Management* 12: 432 - 447.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

Teles L. O., Vasconcelos V., Pereira E. & Saker M. (2006). Time series forecasting of cyanobacteria blooms in the Crestuma Reservoir (Douro River, Portugal) using artificial neural networks. *Environmental Management* 38: 227-237.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

ter Braak C. J. F., 1995. Ordination. In: R. H. G. Jongman, C. J. F. ter Braak & O. F. R. Tongeren, (eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, UK: 91-173.

Ueno Y., Nagata S., Tsutsumi T., Hasegawa A., Watanabe M. F., Park H.-D., Chen G.-C., Chen G. & Yu S.-Z. (1996). Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* 17: 1317-1321.

Vasconcelos V. M., Evans W. R., Carmichael W. W. & Namikoshi M. (1993). Isolation of microcystin-LR from a Microcystis (cyanobacteria) waterbloom collected in the drinking water reservoir for Porto, Portugal. *Journal of Environmental Science and Health - Part A Environmental Science and Engineering* 28: 2081-2094.

Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 30: 2377-2384.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Chapter II

Characterization of bacterioplankton community in riverine water bodies with eutrophic potential

Seasonal dominance of *Bacteroidetes* and *Betaproteobacteria* downstream the nutrient-rich Sousa and Antuã rivers (North Western Portugal)

In: de Figueiredo D. R, Pereira M. J. & Correia A. (in preparation for submission). Seasonal dominance of *Bacteroidetes* and *Betaproteobacteria* downstream the nutrient-rich Sousa and Antuã rivers (North Western Portugal).

Abstract

Sousa River and Antuã River (North Western Portugal) are known for their pollution associated with high nutrient levels. Both these rivers are affluents of estuaries which makes them important contributors to the nutrient levels in each estuary. The present study aimed to screen the seasonality in the bacterioplankton community composition (BCC) downstream both rivers using the culture-independent molecular methodology *16S rRNA* PCR-DGGE. The seasonal shifts in DGGE band patterns were also related to environmental parameters through multivariate analysis to evaluate potential modulators for the BCC variation in both rivers. Parameters such as water temperature, pH, conductivity and concentrations of total suspended solids (TSS), nutrients, precipitation and chlorophyll *a* levels were considered for the analyses. Environmental data suggested eutrophic to hypereutrophic features for these river sections. Variables such as precipitation, pH, temperature, conductivity, SRP, nitrate and Chl *a* levels showed to have major impact on characterization of samples from the two rivers. *Bacteroidetes* and *Betaproteobacteria* phylotypes showed to dominate both rivers BCC. For Sousa River, the seasonality of bacterial assemblage showed to be mainly related with on water temperature and ammonium concentration whereas ammonium concentration and precipitation levels were the main seasonal BCC modulators at Antuã River.

Keywords

Sousa River, Antuã River, bacterioplankton diversity, 16SrDNA-DGGE, RDA analysis.

Introduction

Global warming and water pollution have major impacts on the bacterioplankton community (Paerl et al., 2003; Hall & Cotner, 2007; Zeng et al., 2009). However, there is still a lack of information concerning the ecology of riverine bacterioplankton assemblages, namely in Portugal. The study of the bacterial community composition (BCC) in rivers is not very explored by comparing to lakes since its variation is not as predictable as in lentic systems due to the high oscillations in the water flow and contribution of allochthonous bacteria from soil, groundwater and wastewater (Brümmer et al., 2000; Crump & Hobbie, 2005). However, predictable seasonal changes for the BCC within a river but also among similar rivers have been reported (Crump & Hobbie, 2005).

In north-western Portugal, Sousa River has been considered eutrophic due to the high nutrient concentrations recorded (de Figueiredo et al., 2007). In general, in spite of its nutrient pollution problems (as reported in the present work) and the potential impact of untreated tailings and wells from abandoned gold mines (Ferreira da Silva et al., 2004), there are not many studies concerning this freshwater system. Antuã River is also known to have serious pollution problems (still associated with untreated effluents from industry and a dense population pressure) leading to high nutrient levels (Silva & Oliveira, 2007; Cerqueira et al., 2008). In spite of secondary affluents, both these rivers have a direct impact on the water quality of two important estuaries from Northern Portugal. Sousa River is an affluent of Douro River in the beginning of Douro estuary and Antuã River is an affluent of Ria de Aveiro estuary. Thus, the study of these riverine water bodies, particularly downstream, is of major importance.

The present study aimed to screen, for the first time, the seasonality in the bacterioplankton diversity downstream Sousa River and Antuã River, using the culture-independent molecular methodology *16S rRNA* PCR-DGGE (polymerase chain reaction - denaturing gradient gel electrophoresis) (Muyzer et al., 1993; Muyzer & Smalla, 1998; Lyautey et al., 2005). The seasonal shifts in DGGE band patterns were also related to environmental parameters through multivariate analysis to evaluate potential modulators for the BCC variation in both rivers.

Materials and methods

Sampling and environmental parameters

Sousa River is a tributary of the Douro River (northern Portugal) and its river basin has an area of about 555 Km^2. Antuã River is about 38 Km long and its basin reaches approximately 149 Km^2 (Cerqueira et al., 2008). This river affluxes directly into the Ria de Aveiro estuary. The sampling sites were located downstream the rivers: at Foz do Sousa, Gondomar (41°5'37"N and 8°30'9"W), and at Estarreja (40°45'5"N and 8°34'3"W) for Sousa River and Antuã River,

Table 1. Characterization of samples collected at Sousa River (RS) and environmental data recorded during the corresponding study period.

Sampling date	Code	Average daily precipitation* (mm)			Water temp (ºC)	pH	TSS (mg L^{-1})	Chl *a* (µg L^{-1})	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	SRP	Conductivity (µS cm^{-1})
		7 days before	1 month before	Seasonal (1913-2004)					(mg L^{-1})				
13th March 2005	RS.MR05	**0.0**	1.0	5.0	**11**	7.04	8.4	**0.6**	**< 0.01**	12.6	**< 0.01**	0.24	214
15th August 2005	RS.AG05	**0.0**	**0.0**	1.0	24	7.29	**45.0**	**5.5**	0.15	**30.8**	0.03	**1.32**	**396**
12th March 2006	RS.MR06	1.2	4.4	5.0	12	7.22	13.5	2.2	0.16	16.0	0.09	0.15	165
30rd May 2006	RS.MAY06	0.4	0.3	2.5	20	7.08	11.6	2.7	**0.21**	28.0	0.02	0.72	204
11th July 2006	RS.JL06	**0.0**	0.3	1.0	**25**	7.47	**3.5**	2.6	0.15	16.8	0.06	0.50	272
27th September 2006	RS.SP06	**10.9**	**4.8**	4.8	20	**7.90**	12.6	1.6	0.11	**7.0**	0.11	0.43	200
1st March 2007	RS.MR07	0.1	0.4	5.0	14	7.50	11.1	0.8	0.06	18.9	**0.12**	**0.09**	**152**
9th May 2007	RS.MAY07	1.8	2.3	2.5	19	**6.87**	16.8	2.4	0.20	16.8	0.07	0.32	185

* The average precipitation levels were obtained from Portuguese Water Institute (http://snirh.pt/) for the nearest available meteorological monitoring point.

Table 2. Characterization of samples collected at Antuã River (RAN) and environmental data recorded during the corresponding study period.

Sampling date	Code	Average daily precipitation* (mm)			Water temp (ºC)	pH	TSS (mg L^{-1})	Chl *a* (µg L^{-1})	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	SRP	Conductivity (µS cm^{-1})
		7 days before	1 month before	Seasonal (1976-2005)					(mg L^{-1})				
30rd May 2006	RAN.MAY06	**0.0**	**0.1**	3.2	18	7.07	**4.2**	1.9	0.29	**26.5**	**0.05**	0.87	234
11th July 2006	RAN.JL06	0.0	0.8	**1.2**	**24**	**7.61**	5.3	3.8	**1.89**	12.0	0.21	**1.55**	**318**
30th September 2006	RAN.SP06	**4.3**	2.7	**5.4**	20	7.34	**35.1**	2.7	0.12	**11.2**	**0.33**	0.27	171
1st March 2007	RAN.MR07	3.0	**5.9**	4.6	**14**	7.50	21.4	**0.4**	**0.09**	19.6	0.30	**0.15**	**163**
9th May 2007	RAN.MAY07	0.5	1.8	3.2	18	**7.05**	14.8	2.1	0.88	22.2	0.20	0.77	220
31st August 2007	RAN.AG07	0.3	**0.1**	**1.2**	**24**	7.40	5.1	**4.8**	0.33	17.8	0.06	1.06	289

* The average precipitation levels were obtained from Portuguese Water Institute (http://snirh.pt/) for the nearest available meteorological monitoring point.

respectively. For Sousa River, the sampling was performed in winters 2005-07, springs 2006-07, summers 2005-06 and autumn 2006 (see tables 1 and 2). For Antuã River, sampling was carried out seasonally from spring 2006 to summer 2007. Water samples were taken sub-superficially using sterile bottles at about 1 m from the shore. Samples were placed at 4 ºC under dark conditions until subsequent treatment within 12 hours. Water temperature was measured *in situ* but pH and conductivity were measured in laboratory at 20 ºC by using specific electrodes. Parameters such as total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium, nitrate and nitrite concentrations were determined in laboratory according to standard procedures (APHA, 1992; Rodier, 1996).

DNA extraction

Total DNA from environmental water samples was extracted by filtering 150 mL of the water samples through 0.22 µm polycarbonate sterile filters, resuspension in 2 mL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0] and centrifugation. Lysozyme was added after resuspension in 200 µL of TE and incubation was performed at 37ºC for 1h. The following DNA extraction and purification were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). DNA was finally suspended in TE buffer and stored at -20 ºC.

PCR amplification of bacterial 16S rDNA fragments

PCR amplification of bacterial *16S rRNA* gene fragments was performed with the primers 338F-GC/518R (Muyzer et al., 1993), universal for bacteria. Primers were synthesized by STABVida (Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 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 30s at 92 °C, 30s at 55 °C and 30s at 72 °C, and a final extension step at 72 °C for 30 min. Negative control reactions without any template DNA were performed simultaneously. The PCR amplicons were electrophoresed in a 1.5% agarose gel and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder). The gel was stained with ethidium bromide and visualized on a UV transmilluminator.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1mm 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, California, USA) using 0.5x 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, California, USA).

DGGE bands excision, cloning and sequencing

The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL of sterile milli-Q-purified water from which 5 µL of the eluted DNA was used in PCR amplification with the original primer pair. The isolation and identity of each DNA band was verified through DGGE and, if necessary, the extraction procedure was repeated until the targeted band isolation was obtained. The isolated bands were then cloned using the TOPO TA cloning kit with the pCR 2.1-TOPO vector (Invitrogen, Portugal). Prior to cloning, an A tailing was performed for PCR products according to manufacturers' instructions. In order to screen for false positive clones, the size of the amplicons from PCR using the vector primers M13R/T7F was checked in 1.5% agarose gels as described above using the molecular weight marker GeneRuler™ 1 kb DNA ladder. The migration point of each cloned sequence with the targeted size was verified through DGGE after a nested PCR amplification with the primer pair 338F-GC and 518R. However, sequencing was made with PCR amplicons using the vector primers M13R/T7, optimizing the sequence length information. Each amplicon was purified with the concertTM rapid PCR purification system (Gibco BRL, Eggenstein, Germany) before it was commercially sequenced (STABVida, Portugal).

Phylogenetic analysis and nucleotide sequence accession numbers

The bacterial 16S rDNA partial sequences determined in the present study were submitted to the GenBank database. A BLAST search was used to explore similarity of these sequences against sequences deposited in the GenBank database. The sequences alignment for phylogenetic analyses was carried out using the CLUSTAL X software version 1.83 (Thompson et al., 1997). A phylogenetic tree was built using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 replicates. TreeView version 1.6.6 (Page, 1996) was used to display the trees.

Statistical analysis

Image analysis of DGGE profiles was performed using the the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories, Hercules, CA, USA) and a densitometric profile was established for each sample to determine the relative contribution of each band to the total signal in the lane sample (bands with a relative contribution of less than 0.5 % in each lane were not considered for the subsequent analyses). The presence or absence of co-migration points was converted to a binary matrix (0/1) and cluster analysis was performed using the unweighted pair group method with mathematical averages (UPGMA) based on the Bray-Curtis similarity coefficient with PRIMER 6 software (Clarke & Gorley, 2006). Principal component analysis

(PCA) was used to assess the distribution of samples according to environmental parameters after data standardization (subtracting the mean from each observation and dividing by the corresponding standard deviation) (ter Braak, 1995). Cluster analysis of samples according to environmental parameters was also executed using the UPGMA. The dendrogram was created with the similarities calculated using the Pearson correlation coefficient (95% probability) which was also used to assess significant correlations within environmental parameters and with bands (considering their occurrence and relative intensity). Redundancy analysis (RDA) was performed to reveal relationships between the distribution of the dominant DGGE phylotypes (using a matrix built with band relative intensities after log transformation) and environmental variables using CANOCO 4.5 (Scientia Software). The environmental parameters which better described the distribution of the species data were *a priori* identified by forward selection (Magnan et al., 1994; ter Braak & Verdonschot, 1995) using a Monte Carlo permutation test (499 unrestricted permutations; ALPHA = 0.12).

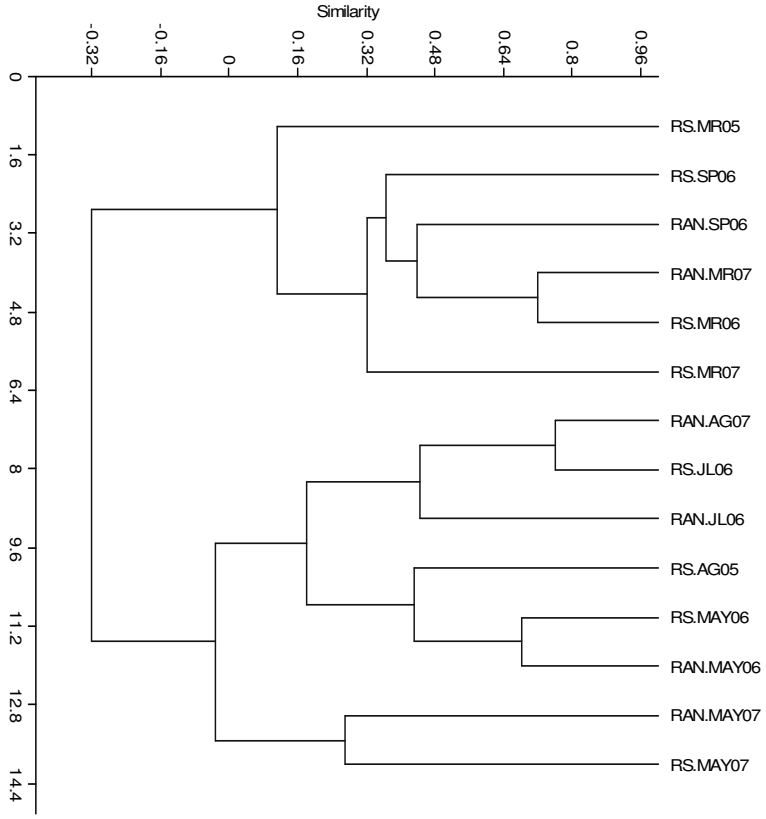
Results

Environmental parameters

The environmental parameters determined for Sousa River samples are summarised in Table 1 (minima and maxima values are highlighted in bold). The average daily precipitation levels 7 days and 1 month before sampling dates both showed no precipitation in summers 2005 and 2006, and maxima of 10.9 and 5.9 mm, respectively, in RS.SP06 and RAN.MR07. With the exception of autumn 2006 and RAN.AG07, all samples showed precipitation levels way below the seasonal averages from the past century (time series data from INAG). Water temperature ranged between 11 and 25 ºC, at RS.MR05 and RS.JL06, respectively. The values for pH ranged between 6.87 in MAY07 and 7.90 in SP06 for RS. The lowest conductivity level was recorded at RS.MR07 (152 µS cm^{-1}) while a maximum of 396 µS cm^{-1} was recorded at RS.AG05. TSS concentration ranged from 3.5 to 45.0 $mg.L^{-1}$ at RS.JL06 and RS.AG05, in that order. Chl *a* concentration ranged between 0.4 and 5.5 µg L^{-1} (at RAN.SP06 and RS.MAY06, respectively). Nitrate concentrations were very high for both rivers, ranging from 7.0 to 30.8 mg N (NO_3^-) L^{-1} at RS.SP06 and RS.AG05, respectively; nitrite levels were undetectable at RS.MR05 and highest (1.89 mg N (NO_2^-) L^{-1}) at RAN.JL06. Ammonia was also undetectable at RS.MR05 and reached a maximum of 0.33 mg N (NH_4^+) L^{-1} at RAN.MR07. SRP concentration ranged from 0.09 to 1.55 mg L^{-1} at RS.MR07 and RAN.AG05, respectively.

The Pearson correlation coefficient showed relationships between the recorded environmental parameters ($P < 0.05$; $n = 14$). Conductivity showed positive correlations with water temperature ($r = 0.72$) and Chl *a* ($r = 0.82$) and SRP ($r = 0.88$) concentrations. Chl *a* concentration

(a)



(b)

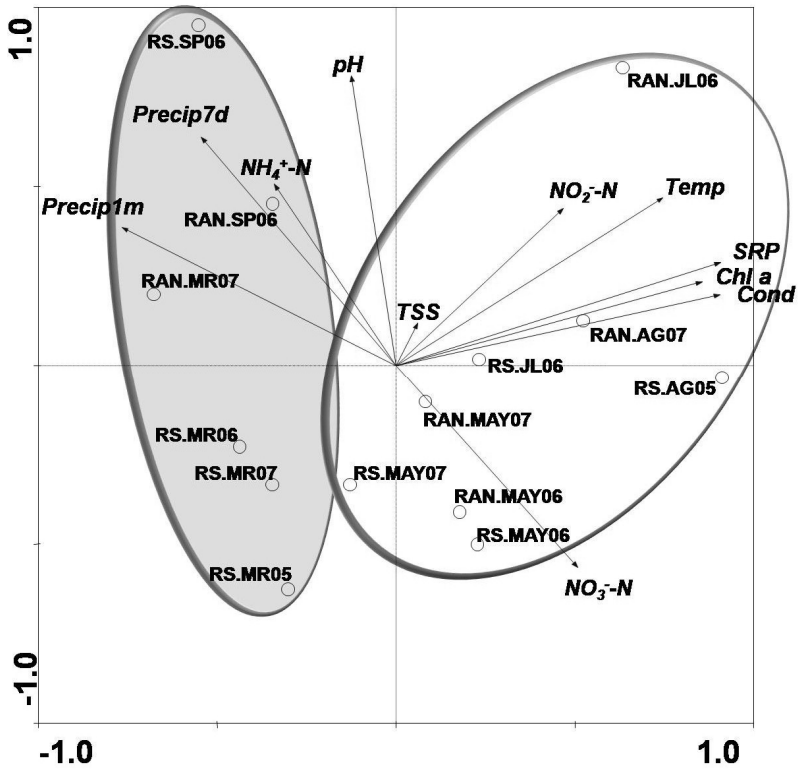


Fig. 1. (a) cluster dendrogram and (b) PCA ordination biplot of Sousa and Antuã rivers samples according to environmental parameters recorded from 2005 to 2007 (see sample codes in table 1).

also correlated with SRP and water temperature (both with $r = 0.79$). SRP was found to have also positive significant correlations with water temperature ($r = 0.72$), nitrite concentration ($r = 0.68$). Precipitation 7d was positively correlated with precipitation 1m ($r = 0.68$) and pH ($r = 0.56$) but negatively correlated with nitrate ($r = -0.57$). Precipitation 1m also had significant negative correlations with SRP ($r = -0.55$) and conductivity ($r = -0.56$).

In general, cluster analysis (Fig. 1a) evidenced two main groups: the first cluster including winter samples but also autumn 2006 samples; the second cluster included summer and spring samples. PCA (Fig. 1b) showed a general seasonal gradient along the first axis defined by winter and autumn samples on the negative side and summer samples and spring samples on the positive side. The second axis was defined by samples RS.SP06 and RAN.JL06 on the positive side while sample RS.MR05 defined its negative side, followed by spring 2006 samples. The first axis was mainly related to conductivity, SRP, Chl *a* levels while the second axis was mostly defined by Precip7d, pH and nitrate concentration. These first two axes could explain 62.0% of the total variance of samples distribution.

DGGE band patterns analysis and sequencing of dominant bands

The analysis of DGGE profiles obtained for Sousa River (Fig. 2) showed a total of 121 bands corresponding to 31 different band migration positions. The number of bands *per* sample showed an average of 15 ± 3 ($n = 8$). Cluster analysis (Fig. 3a) based on DGGE band profiles evidenced a seasonal clustering of samples. Two major clusters were observed: one including winter samples and the other including spring, summer and autumn samples. The RDA suggested a seasonal gradient along the first axis mainly based on water temperature (-0.97) and defined by summer samples (and bands 4, 17, 3 and 12) on the negative side and winter samples (and band 3B) on the negative side. However, conductivity (0.74) showed to be also an important BCC modulator along the second axis, defined by sample RS_AG05 (and band 14) on the positive side and RS_MAY07 (and bands 10 and 11) on the negative side. The first two RDA axes could explain 63.6% of the total variance.

A total of 77 bands (corresponding to 25 different band migration positions) were considered for the statistical analyses using DGGE profiles from Antuã River (Fig. 2). The number of bands *per* sample showed an average of 13 ± 3 ($n = 6$). Cluster analysis (Fig. 4a) showed the segregation of sample JL06 from two other groups: one including autumn and winter samples and the other including spring samples and AG07. The RDA showed a gradient based on ammonium concentration (-0.91) along the first axis which was defined by MR07 (and bands 26 and 34) on the negative part and AG07 (and band 23) on the positive side. The second axis was defined by sample JL06 (and band 22) on the negative side and ST06 (and bands 6 and 34) on the positive side; this axis was mostly related to precipitation 7 days before sampling date (0.77). This RDA showed that the first axes could explain 57.5% of the total variance.

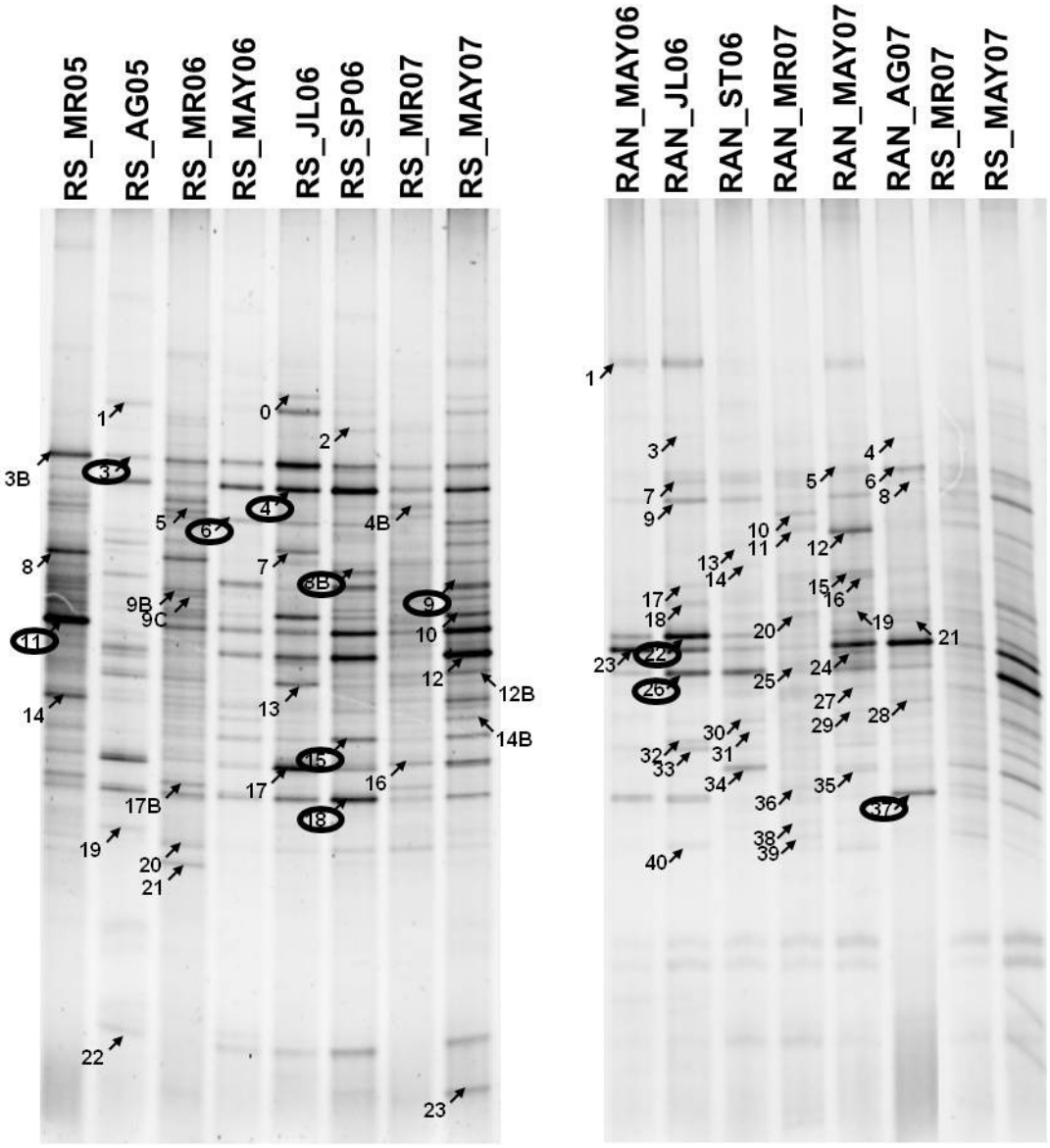


Fig. 2. DGGE profiles containing the bacterial 16S rDNA fragments of samples collected from Sousa River (from 2005 to 2007) and Antuã River (from 2006 to 2007). The code above each lane refers to each sample (see tables 1 and 2) and the bands numbering corresponds to the different migration positions considered for the analyses. Samples RS.MR07 and RS.MAY07 were used as reference to compare between gels.

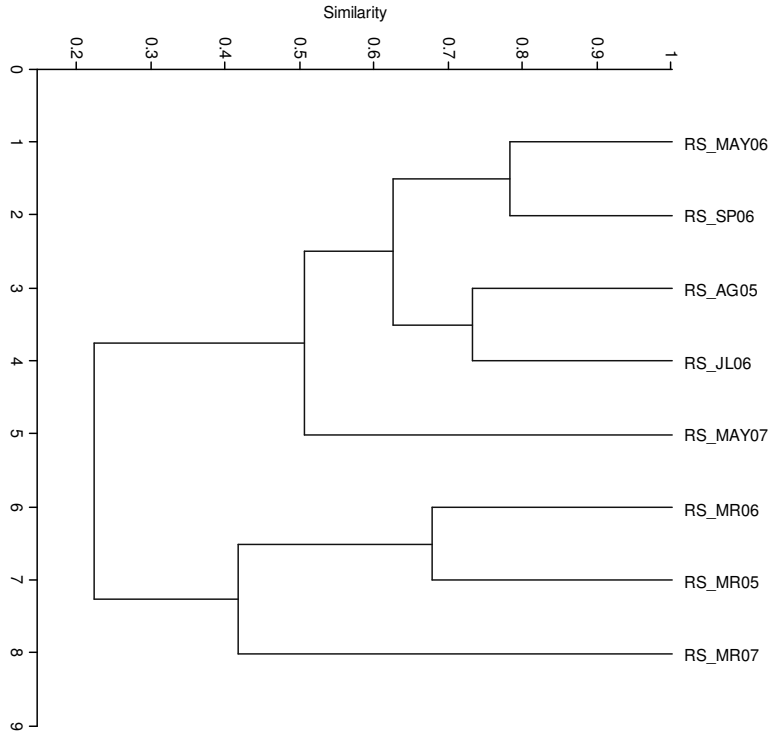
The phylogenetic affiliation of the sequenced bands after a BLAST search corresponded to the groups *Bacteroidetes*, *Betaproteobacteria*, *Actinobacteria* and *Alphaproteobacteria*. The sequencing results are shown in Table 3 and Fig. 5. *Bacteroidetes* and *Betaproteobacteria* phylotypes showed to dominate the bacterial assemblage in both Sousa and Antuã rivers.

Discussion

Environmental parameters

All samples showed nutrient levels characteristic from eutrophic to hypereutrophic water bodies (Nürnberg, 1999). According to time series data from Portuguese Water Institute (INAG),

(a)



(b)

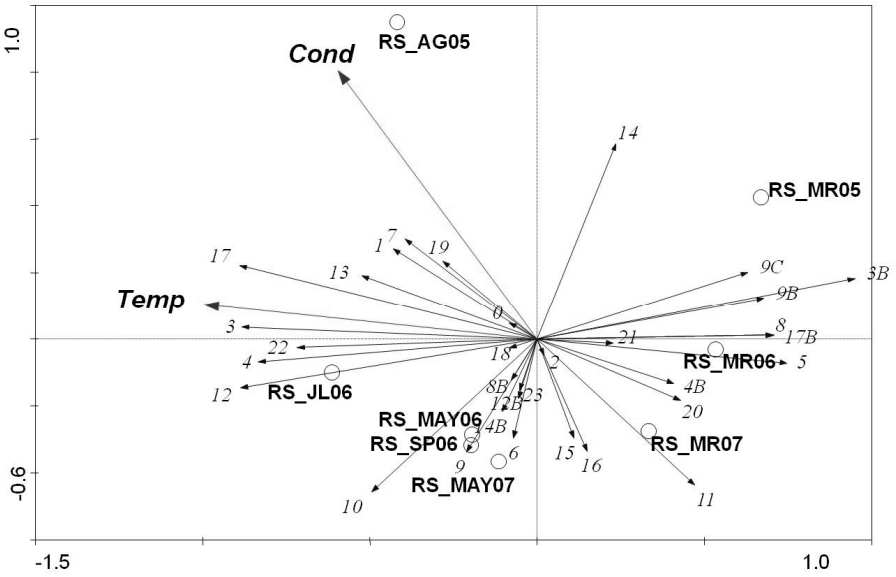
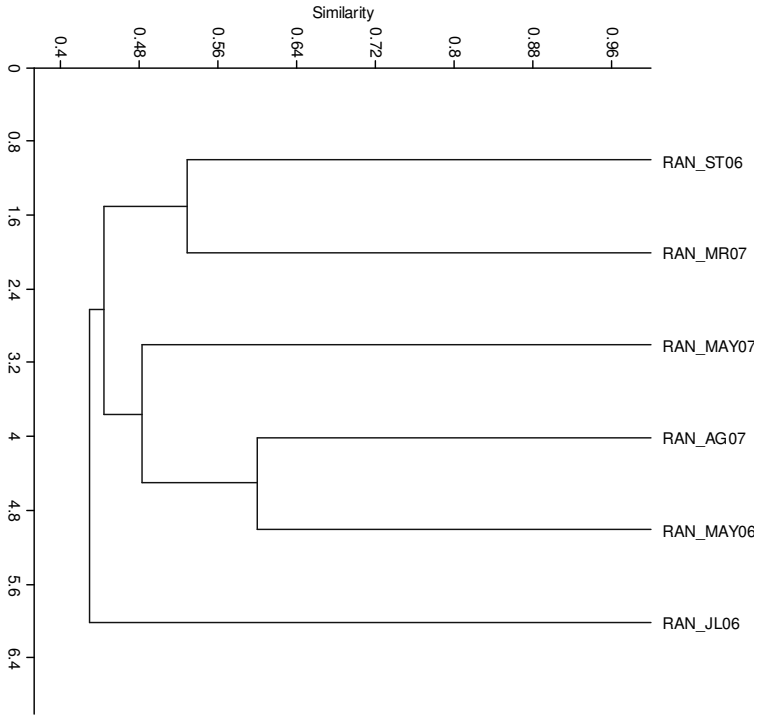


Fig. 3. (a) cluster dendrogram of Sousa River samples according to DGGE band patterns and (b) RDA ordination triplot of Sousa River DGGE band patterns according to the environmental parameters recorded during the study period (2005-2007). DGGE bands numbering and samples coding are described in figure 2 and table 1, respectively.

(a)



(b)

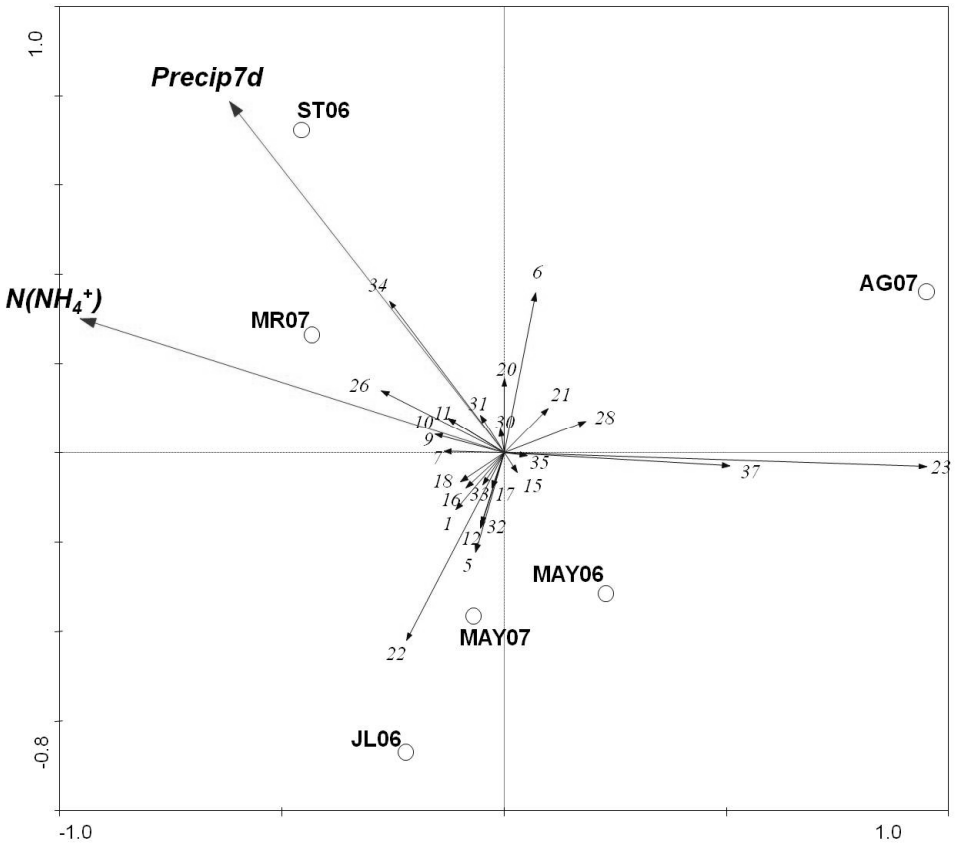


Fig. 4. (a) cluster dendrogram of Antuã River samples according to DGGE band patterns and (b) RDA ordination triplot of Antuã River DGGE band patterns according to the environmental parameters recorded during the study period (2005-2007). DGGE bands numbering and samples coding are described in figure 2 and table 1, respectively.

Table 3 – Sample, accession number, closest relative (after a BLAST search) and corresponding percentage similarity for the 16S rDNA bacterial partial sequences from excised bands.

Band	NCBI Accession Nº	Phylogenetic affiliation	Closest relatives (accession Nº)	Percentage similarity (%)
RS3_ cl60	submitted	*Bacteroidetes*	Uncultured Flavobacteriaceae bacterium clone LW18m-1-69 (EU642285)	100
RS4_ cl46	submitted	*Betaproteobacteria*	Uncultured *Curvibacter* sp. clone FL_8 (HQ008565) Uncultured freshwater bacterium isolate DGGE gel band 15_LF17_cl10	100
RS6_ cl53	submitted	*Betaproteobacteria*	Uncultured Comamonadaceae bacterium clone Gap-2-96 (EU642206)	100
RS8B_ cl31	submitted	*Bacteroidetes*	Uncultured bacterium partial 16S rRNA gene, clone OctG7_5 (FN296583)	99
RS9_ cl45	submitted	*Bacteroidetes*	Uncultured *Arcicella* sp. clone Jab PL2W2H5 (HM486311)	100
RS11_ cl23	submitted	*Bacteroidetes*	*Flavobacterium* sp. 3AR3-35 (GU295965) Uncultured bacterium isolate DGGE gel band C28_cl38 (GU908482)	98 98
RS15_ cl39	submitted	*Betaproteobacteria*	Polynucleobacter necessarius subsp. asymbioticus strain MWH-P1-05-14 (FN556008)	100
RS18_ cl32	submitted	*Actinobacteria*	Uncultured actinobacterium partial 16S rRNA gene, clone ZS-2-33 (FN668214)	99
RAN26 _cl20	submitted	*Betaproteobacteria*	Uncultured *Curvibacter* sp. clone FL_8 (HQ008565)	98
RAN22 _cl21	submitted	*Bacteroidetes*	*Flavobacterium* sp. ARSA-103 (GU295971)	100
RAN37 _cl2	submitted	*Alphaproteobacteria*	*Rhodobacter* sp. Cr4-37 (GU441681)	99

the values for nitrate recorded in the present work were way above the average from the last decade at Sousa River; SRP and conductivity values were also higher than the average but temperature, nitrite, TSS and pH levels were within the range previously recorded. Antuã River showed conductivity, pH, phosphate and nitrate values within the range reported in other studies (Silva & Oliveira, 2007; Cerqueira et al., 2008), although TSS achieved high levels associated to the higher precipitation that occurred in autumn 2006. Along the Sousa River, the agriculture has an important representation, particularly cultures of corn (Ferreira da Silva et al., 2004) which is related to high amounts of inorganic nutrients from agriculture runoff. The high nitrate and phosphate levels recorded at Antuã River are not surprising as well, as they have been reported before (Silva & Oliveira, 2007; Cerqueira et al., 2008) and also associated with agriculture along with domestic and industrial effluents (Cerqueira et al., 2008). Cluster analysis and PCA suggested a clear seasonal clustering of samples (winter and autumn *vs.* summer and spring samples) mainly associated with factors such as precipitation, conductivity, pH, SRP, nitrate and Chl *a* levels. Nevertheless, sample RS.MR05 showed to differ from the other winter samples and samples from spring 2007 also

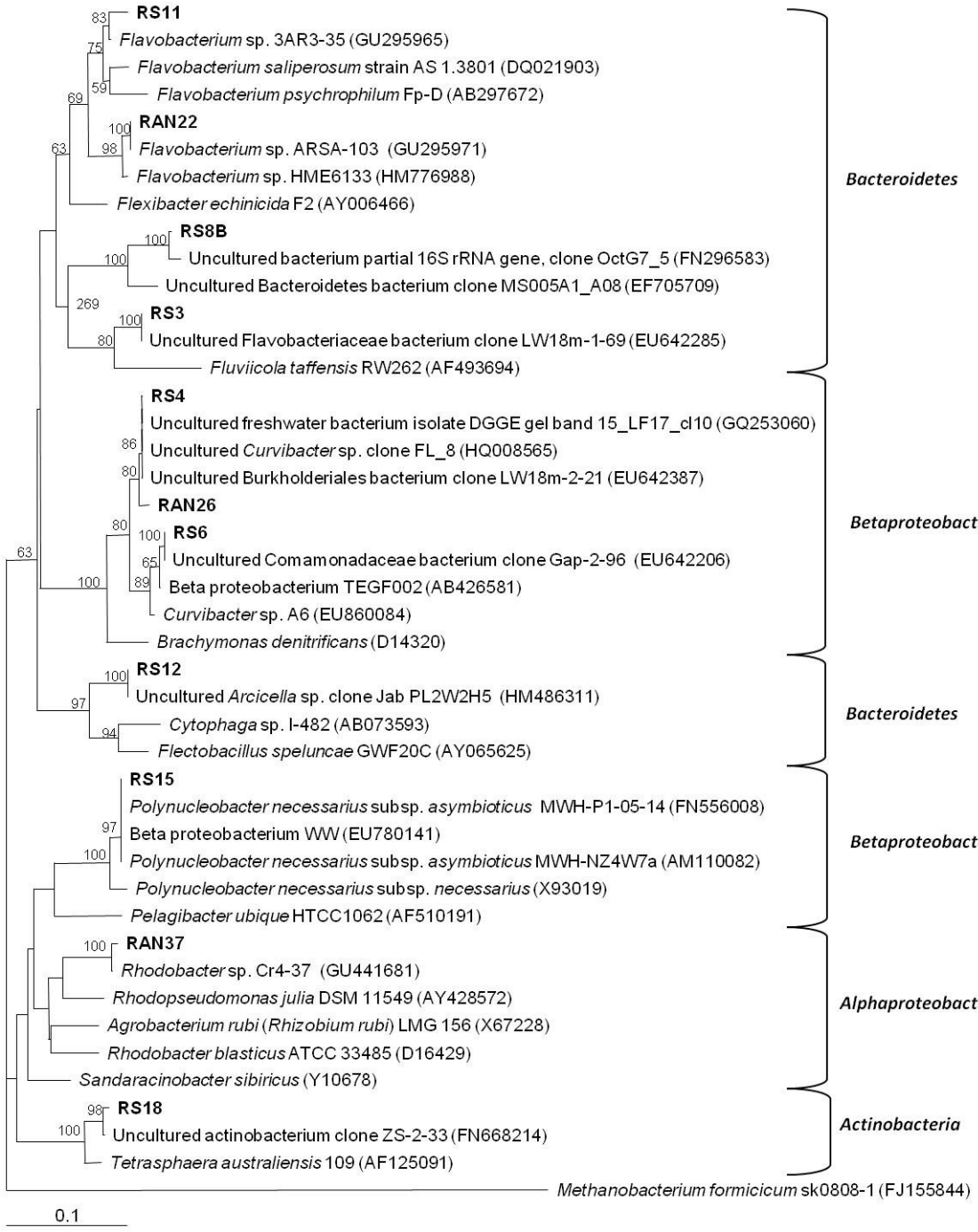


Fig. 5. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel (Fig. 2). The archaeal sequence from *Methanobacterium formicicum* strain sk0808-1 was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

differed from spring 2006 samples (which were more similar to summer samples). In fact, the winter sample RS.MR05 was related to precipitation levels lower than the average values for winter season according to time series data from INAG.

Bacterial diversity according to environmental parameters

The cluster analysis based on DGGE profiles from Sousa River suggested a seasonal clustering of samples. The BCC of winter samples was well distinct from the summer samples, as recorded for other studies concerning temperate rivers (Crump & Hobbie, 2005). In spite of the extreme conditions felt during the drought of 2005, the winter and summer BCC at the Sousa River showed to maintain a typical diversity, suggesting that the bacterioplankton assemblage can resist and remain relatively stable under severe climatic conditions. This may be due to the fact that this is a lotic system, since in lentic water bodies this drought in 2005 has shown to have impact on the BCC diversity (e.g. Crestuma reservoir, North Western Portugal – unpublished data). However, spring samples were not as predictable, as previously reported (Crump & Hobbie, 2005), although a similarity with summer samples was evidenced. The RDA of Sousa River suggested a seasonal gradient mainly based on water temperature, as vastly reported for many freshwater bodies (Crump & Hobbie, 2005; Lindström et al., 2005; de Figueiredo et al., 2010b) but also on conductivity. For Antuã River, ammonium and precipitation levels were the most important factors for BCC variation, which is not very frequently reported. In fact, the study of the bacterial community composition (BCC) in rivers is less explored than in lakes because its variation is not as predictable as in lentic system. There are high oscillations in the water flow and the contribution of allochthonous bacteria from soil, groundwater and wastewater must be considered (Brümmer et al., 2000; Crump & Hobbie, 2005). Nevertheless, predictable seasonal changes for the BCC within a river but also among similar rivers have been reported (Crump & Hobbie, 2005) but still with the river flow rate along with temperature as major modulators.

Significant correlations could be retrieved among the dominance of some phylotypes and environmental parameters for both Sousa ($P < 0.05$; $n = 8$) and Antuã ($P < 0.05$; $n = 6$) rivers. *Bacteroidetes* took over the bacterial assemblage at Sousa River, as previously recorded (de Figueiredo et al., 2007). The dominance of *Bacteroidetes*, particularly *Flavobacterium* spp., is frequent in eutrophic rivers from Northern Europe (Brümmer *et al.*, 2000) and has been related to high nutrient levels (de Figueiredo et al., 2007) as they are nitrate-reducing bacteria (Nijburg & Laanbroek, 1997). At Sousa River, dominant phylotypes showed high sequence similarities with Flavobacteriales bacteria from lakes and rivers. More, one phylotype showed to match a sequence retrieved from the Portuguese Cértima River, which may raise the hypothesis of phylotypes' sharing among water bodies with similar characteristics; this phylotype dominance was negatively correlated with temperature and chlorophyll *a*. However, the other Flavobacteriales phylotype was positively correlated with water temperature. For Antuã River, a phylotype similar to *Flavobacterium* sp. was found to dominate during summer 2006 but not 2007 (which showed "springuish" conditions by clustering with spring samples rather than with summer 2006). *Betaproteobacteria* showed to have also an important representation in Sousa River and a negative

correlation with conductivity levels. *Betaproteobacteria* dominance in Sousa River showed a positive correlation with water temperature and SRP levels whereas at Antuã River its dominance was positively correlated with ammonium concentration. In contrast, ammonium concentration was negatively correlated with the dominance of an *Alphaproteobacteria* phylotype at Antuã River. An actinobacterium phylotype was found dominant in autumn 2006 at Sousa River, although its presence occurred throughout all samples; nevertheless, it showed a positive correlation with pH. *Actinobacteria* dominance is common in eutrophic water bodies (De Wever et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006b; Newton et al., 2007; Wu et al., 2007b; Xi et al., 2007). Interesting, this phylotype showed total match with sequences retrieved from other Portuguese water bodies, both lentic and lotic (de Figueiredo et al., 2007), suggesting a transversal persistance for its occurrence. Further investigation should be made to assess the spatial variation of bacterioplankton assemblages between the different riverine tributaries and the corresponding Douro and Ria de Aveiro estuaries.

References

Allgaier M. & Grossart H. P. (2006). Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Cerqueira M. A., Silva J. F., Magalhães F. P., Soares F. M. & Pato J. J. (2008). Assessment of water pollution in the Antuã River basin (Northwestern Portugal). *Environmental Monitoring and Assessment* 142: 325-335.

Clarke K. R. & Gorley R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

de Figueiredo D., Pereira M. & Correia A. (2010) Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26:1067-1077.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Ferreira da Silva E., Serrano Pinto L., Patinha C. & Cardoso Fonseca E. (2004). Site-Specific Characterization of Castromil Brownfield Area Related to Gold Mining Activities. *Environmental Geochemistry and Health* 26: 13-25.

Hall E. K. & Cotner J. B. (2007). Interactive effect of temperature and resources on carbon cycling by freshwater bacterioplankton communities. *Aquatic Microbial Ecology* 49: 35-45.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G. & Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 73: 127-141.

Newton R. J., Jones S. E., Helmus M. R. & McMahon K. D. (2007). Phylogenetic ecology of the freshwater Actinobacteria acI lineage. *Applied and Environmental Microbiology* 73: 7169-7176.

Nürnberg G. K. (1999). Determining trophic state in experimental lakes. *Limnology and Oceanography* 44: 1176-1179.

Paerl H. W., Dyble J., Moisander P. H., Noble R. T., Piehler M. F., Pinckney J. L., Steppe T. F., Twomey L. & Valdes L. M. (2003). Microbial indicators of aquatic ecosystem change: Current applications to eutrophication studies. *FEMS Microbiology Ecology* 46: 233-246.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Saitou N. & Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Silva J. F. & Oliveira F. (2007). Eutrophication in the River Vouga basin - Impacts on the quality of water for public supply. *IAHS-AISH Publication*: 139-147.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

ter Braak C. J. F., 1995. Ordination. In: R. H. G. Jongman, C. J. F. ter Braak & O. F. R. Tongeren, (eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, UK: 91-173.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. & Higgins D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Wu X., Xi W., Ye W. & Yang H. (2007). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Xi W., Wu X., Ye W. & Yang H. (2007). Changes in bacterial community structure during preceding and degraded period of cyanobacterial bloom in a bay of the Taihu Lake. *Chinese Journal of Applied and Environmental Biology* 13: 97-103.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology*: 1-10.

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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Abstract

The information on bacterial community composition (BCC) in Portuguese water bodies is very scarce. Cértima River (Western Central 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 16SrDNA-DGGE (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*, TSS, TOC, 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 SRP, 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 (*Eh*) and high temperature, pH, TSS and Chl *a* levels while another one showed a negative correlation with Chl *a* values.

Keywords

riverine bacterioplankton diversity, 16SrDNA-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 & Cotner, 2007; Zeng et al., 2009) which may endanger the quality and safety of water used for human purposes (de Figueiredo et al., 2004b; Zaitlin & 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 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 & 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 twenty years (Rino & 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; de Figueiredo et al., 2010b).

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 16SrDNA PCR - DGGE (Denaturing Gradient Gel Electrophoresis) (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 & 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

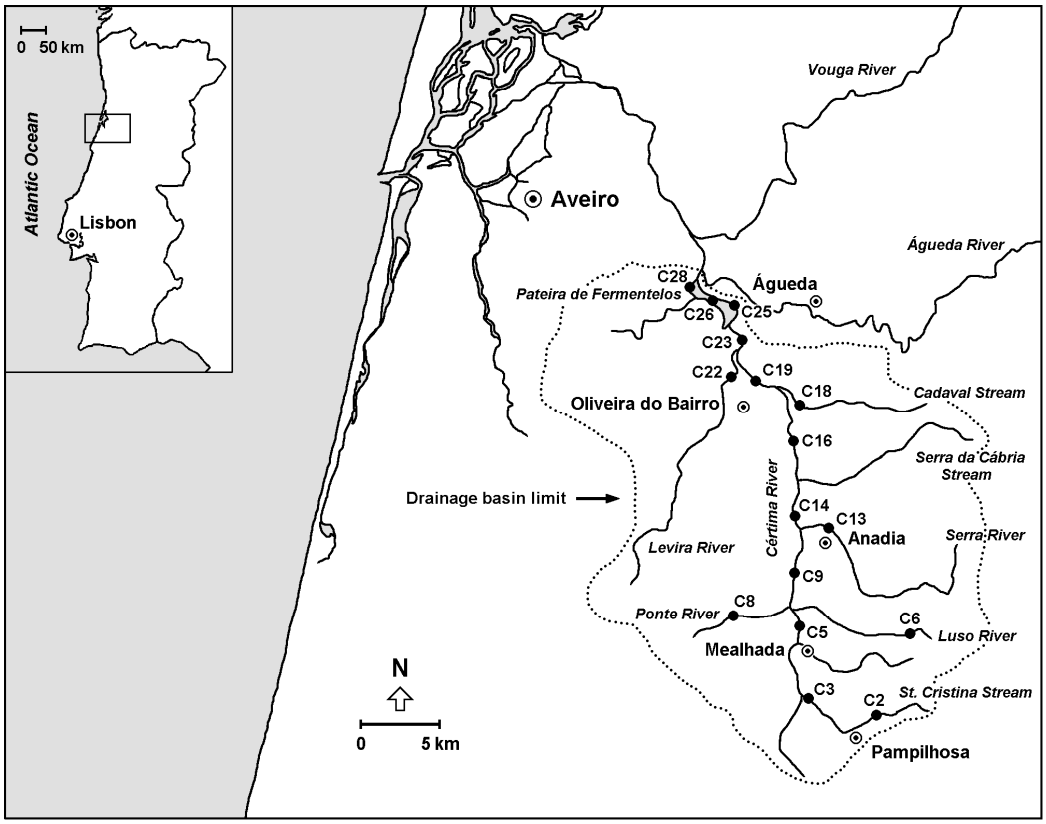


Fig. 1. Location of sampling sites along the Cértima River Basin (see sample codes in table 1).

late May 2007, during 3 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 4ºC 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 of TE, lysozyme was added and incubation was performed at 37ºC for 1h. 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 ºC. 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, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5%

dimethylsulfoxide (DMSO), 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 30s at 92 °C, 30s 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 transmilluminator.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized 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, California, USA) using 0.5x 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, California, USA). The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL 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 neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 replicates. **Tree**View version 1.6.6 (Page, 1996) was used to display the **tree**s.

Multivariate analysis

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 (ºC)	Cond (µS cm^{-1})	*Eh*	Chl *a* (µg L^{-1})	O_2 sat. (%)	Diss. O_2	TOC	BOD_5	TSS	SO_4^{2-}	HCO_3^-	NO_2^--N	NO_3^--N	NH_4^+-N	Kjeld-N	TN	Org-N	SRP
									(mg L^{-1})												
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

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 & 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 & 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. 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.

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, TOC, 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.

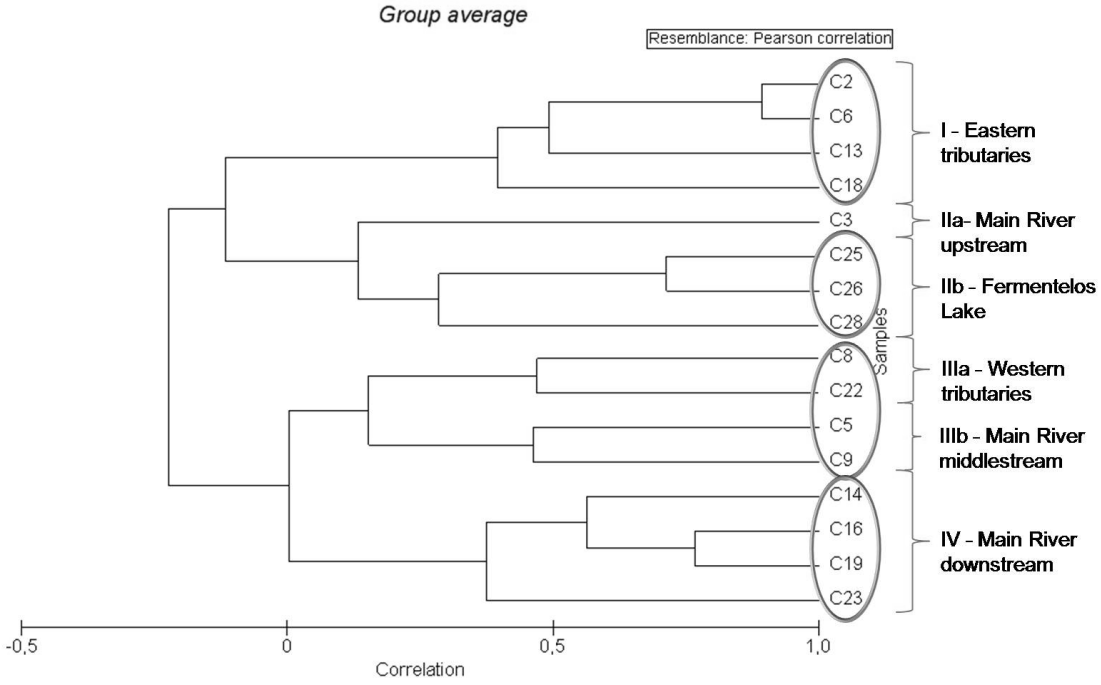
The PCA biplot puts in evidence this gradient (Fig. 2b): samples C2, C6 and C13 (with the highest water quality) appear on the negative side of the first axis and sample C5 (characterized by a strong organic charge) appears on the positive side. This axis showed to be mainly defined by organic pollutants, 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 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.

(a)



(b)

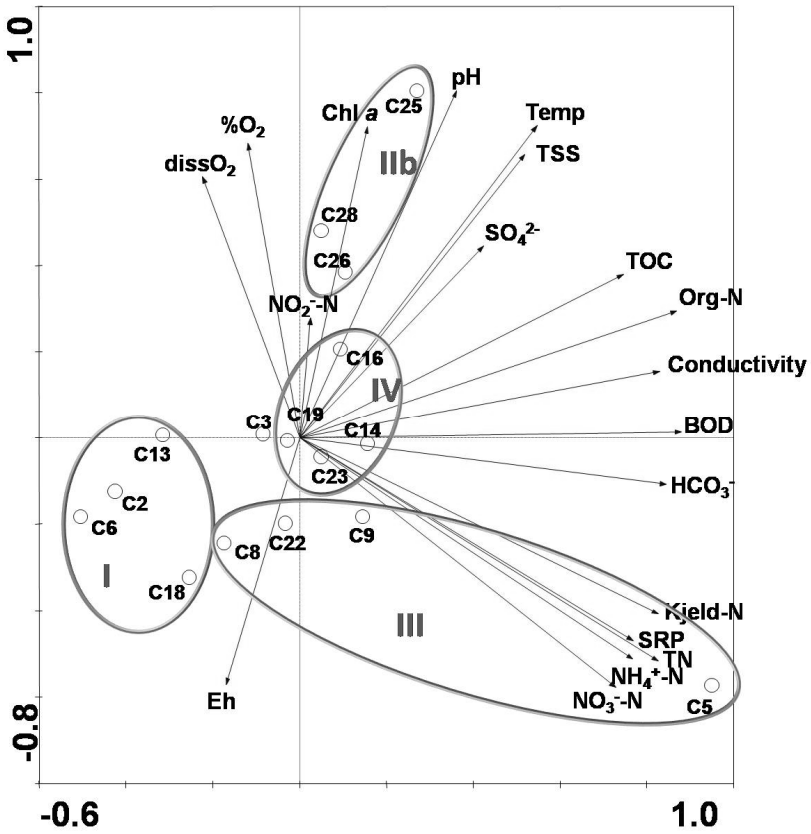


Fig. 2. (a) cluster dendrogram and (b) PCA ordination biplot of Cértima River Basin samples according to environmental parameters recorded in May 2007 (see sample codes in table 1).

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*	Gamma proteobacterium MH154 (EU052746) Gamma proteobacterium IMCC1704 (DQ664237)	Microalgae culture association Freshwater pond, Republic of Korea	96
C28	**14_ cl51**	GU908477	*Betaproteobacteria*	Uncultured bacterium clone 3C002569 (EU801309) Beta proteobacterium MWH-C5 (AJ938026)	Chesapeake Bay, MD, USA Freshwater Lake Mondsee, Austria	99 98
C16	**15_cl24**	GU908478	*Betaproteobacteria*	Uncultured beta proteobacterium 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) Uncultured candidate division TM7 bacterium clone Skagenf60 (DQ640706)	Wastewater, Palestine Activated sludge, Denmark	99
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 beta proteobacterium clone GC1m-1-96	Lake Michigan, Wisconsin, USA	100
C5	**32_cl56**	GU908486	*Bacteria (Bacteroidetes)*	Uncultured bacterium clone D5-43 (DQ113698)	Dog duodenum	98

Sequencing and phylogenetic affiliation of dominant phylotypes in DGGE profiles

A total of twenty-five 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

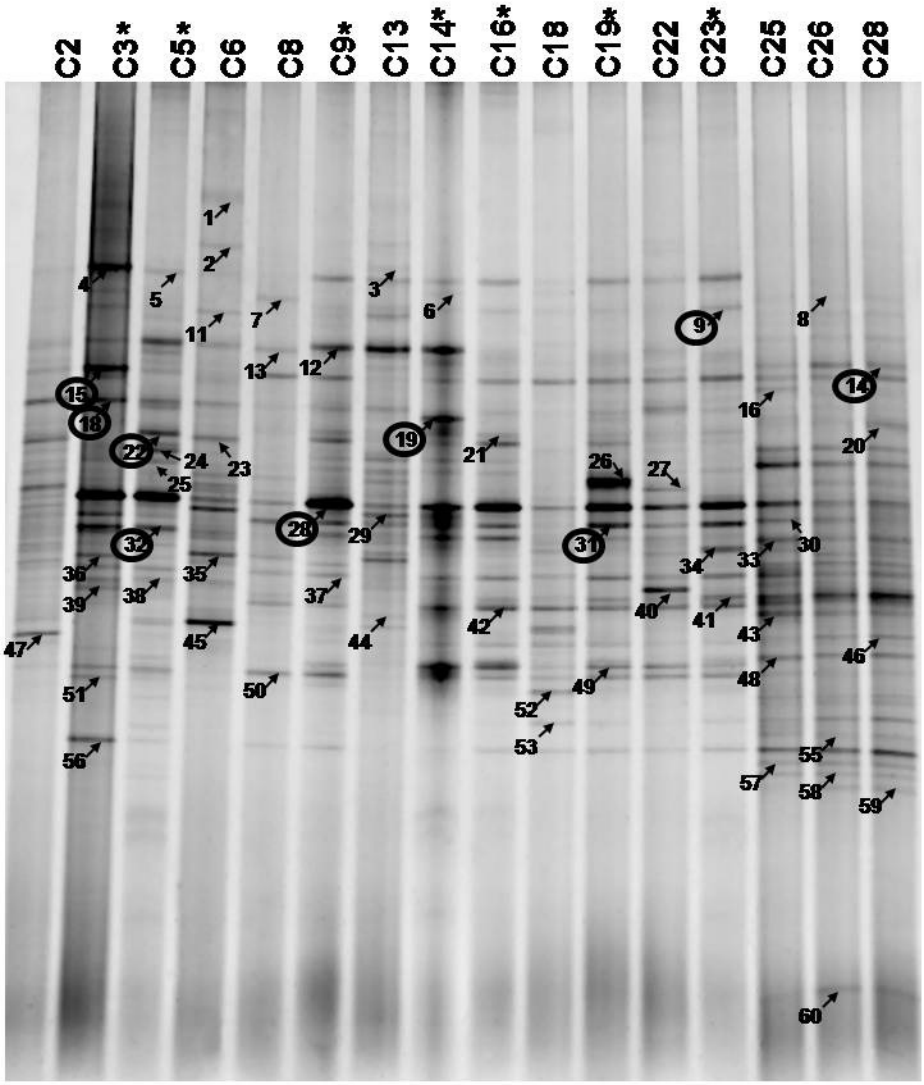
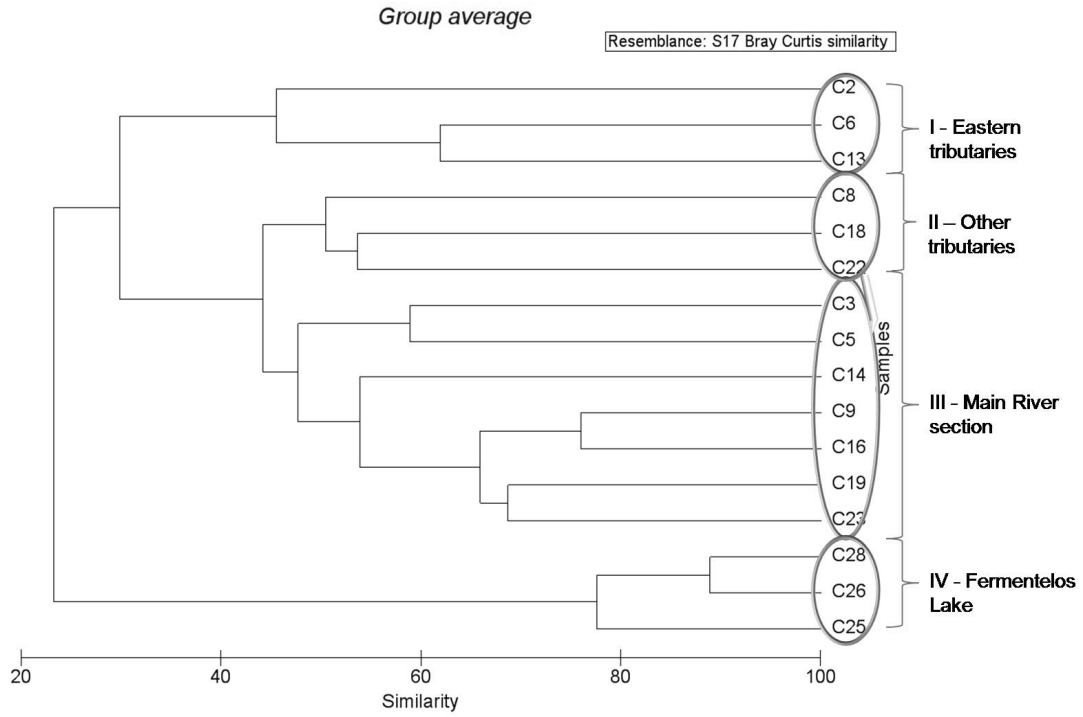


Fig. 3. DGGE 16S rDNA band profiles for samples obtained along Cértima River Basin in May 2007. The code above each lane refers to each sample (see table 1) and the bands numbering corresponds to the different migration positions considered for the analyses.

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).

(a)



(b)

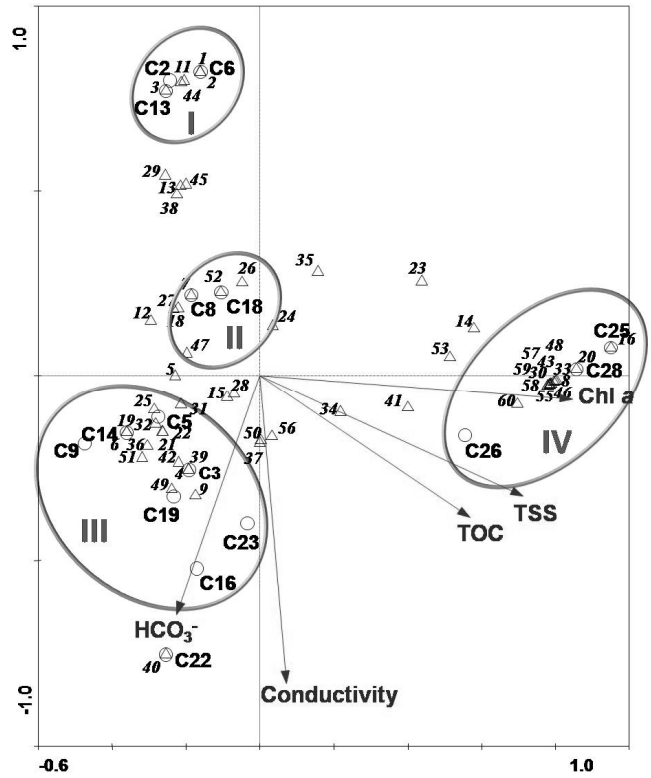


Fig. 4. (a) dendrogram and (b) CCA ordination triplot of Cértima River DGGE band patterns according to the environmental parameters recorded during May 2007. DGGE bands numbering and samples coding are described in figure 2 and table 1, respectively.

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 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 & 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 & 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 & 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, SRP and ammonium levels were much higher than previous records (Rino & 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 & 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 agriculture (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 & 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 & 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

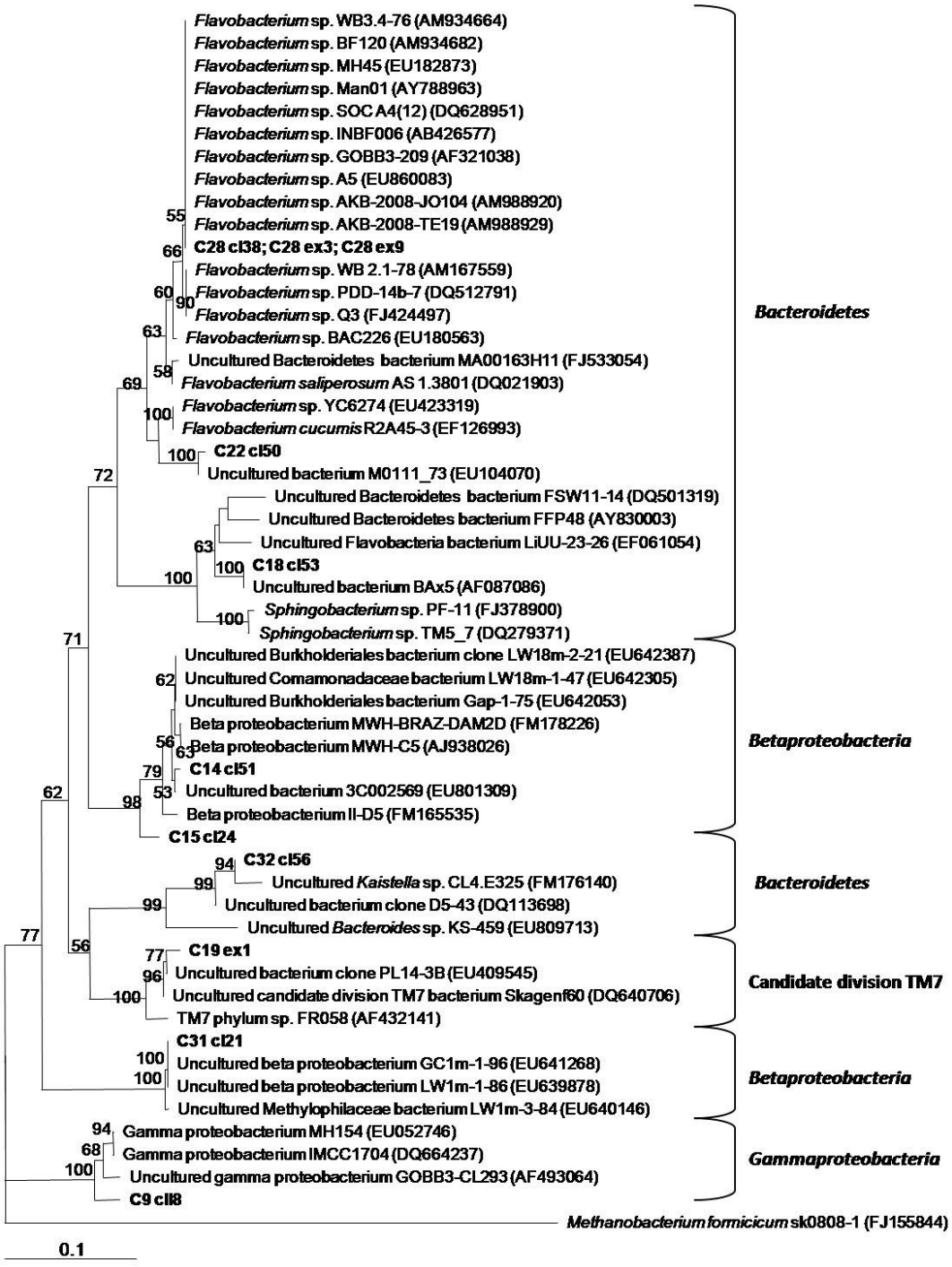


Fig. 5. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel of Cértima River Basin samples (Fig. 3). The archaeal sequence of *Methanobacterium formicicum* strain sk0808-1 (FJ155844) was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

usually discharged just upstream this spot (Rino & 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; de Figueiredo et al., 2010b).

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 & Grossart, 2006b).

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 (*Eh*) 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 & Cotner, 2007; Berggren et al., 2009), along with the water flow and retention time (Crump & Hobbie, 2005; Lindström et al., 2005), Chl *a* (Muylaert et al., 2002; Allgaier & Grossart, 2006b; Šimek et al., 2008) and sulphate 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 amongst 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 & 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; Xi et al., 2007; de Figueiredo et al., 2010b). 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 & 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 & Grossart, 2006b) 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 & Grossart, 2006b). Bands 14 and 15 were affiliated with the family Comamonadaceae (Burkholderiales; *Betaproteobacteria*) from lakes and rivers (Crump & Hobbie, 2005; Mueller-Spitz et al., 2009). In fact, members of this family have been recorded at Fermentelos Lake (de Figueiredo et al., 2010b) and are abundant in rivers with organic pollution levels such as Cértima River (Brümmer et al., 2003). Band 14 correlated with low *Eh* 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 *Eh* 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.

References

Allgaier M. & Grossart H. P. (2006). Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

Almeida S. F. P. (2001). Use of diatoms for freshwater quality evaluation in Portugal. *Limnetica* 20: 205-213.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Appelo C. A. J. & Postma D., 2005. Geochemistry, groundwater and pollution. A.A.Balkema Publishers, Amsterdam, the Netherlands.

Awadallah R. M., Soltan M. E., Shabeb M. S. A. & Moalla S. M. N. (1998). Bacterial removal of nitrate, nitrite and sulphate in wastewater. *Water Research* 32: 3080-3084.

Bacelar-Nicolau P., Nicolau L. B., Marques J. C., Morgado F., Pastorinho R. & Azeiteiro U. M. (2003). Bacterioplankton dynamics in the Mondego estuary (Portugal). *Acta Oecologica* 24: S67-S75.

Berg K. A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P. & Rapala J. (2008). High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME Journal* 3: 314-325.

Berggren M., Laudon H. & Jansson M. (2009). Hydrological control of organic carbon support for bacterial growth in boreal headwater streams. *Microbial Ecology* 57: 170-178.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Brümmer I. H. M., Felske A. & Wagner-Döbler I. (2003). Diversity and Seasonal Variability of {beta}-Proteobacteria in Biofilms of Polluted Rivers: Analysis by Temperature Gradient Gel Electrophoresis and Cloning. *Appl. Environ. Microbiol.* 69: 4463-4473.

Calado A. J., Fundamentação da aplicação do sistema de saprobia às algas de água doce do Centro de Portugal. Department of Biology. University of Aveiro, Aveiro, 1990.

Calado A. J., Almeida S. F. P. & Craveiro S. C. (2005). Phycological surveys and monitoring in Portugal - a summary. *ALGAS - Boletín de la Sociedad Española de Ficologia* Septiembre 2005: 36-41.

Calado A. J., Freitas A. M. & Veloso V. M. (1991). Algas da Pateira de Fermentelos numa situação de Inverno. *Revista de Biologia da Universidade de Aveiro* 4: 55-71.

Cerqueira M. A., Vieira F. N., Ferreira R. V. & Silva J. F. (2005). The water quality of the Cértima River Basin (Central Portugal). *Environmental Monitoring and Assessment* 111: 297-306.

Clarke K. R. & Gorley R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Cortecci G., Dinelli E., Bencini A., Adorni-Braccesi A. & La Ruffa G. (2002). Natural and anthropogenic SO4 sources in the Arno river catchment, northern Tuscany, Italy: a chemical and isotopic reconnaissance. *Applied Geochemistry* 17: 79-92.

Cottrell M. T., Waidner L. A., Yu L. & Kirchman D. L. (2005). Bacterial diversity of metagenomic and PCR libraries from the Delaware River. *Environmental Microbiology* 7: 1883-1895.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

de Figueiredo D., Pereira M. & Correia A. (2010). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26: 1067-1077.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

Ducharne A., Baubion C., Beaudoin N., Benoit M., Billen G., Brisson N., Garnier J., Kieken H., Lebonvallet S., Ledoux E., Mary B., Mignolet C., Poux X., Sauboua E., Schott C., Théry S. & Viennot P. (2007). Long term prospective of the Seine River system: Confronting climatic and direct anthropogenic changes. *Science of the Total Environment* 375: 292-311.

Ferreira R. S. V., Avaliação hidroquímica e da qualidade da água na bacia do rio Cértima (Portugal). Department of Environment and Planning, Vol. MSc. University of Aveiro, Aveiro, 2007.

Gil M. C., Estudo ecológico das Diatomáceas dos rios Águeda, Agadão e Alfusqueiro. Department of Biology, Vol. PhD. University of Aveiro, Aveiro, 1988, pp. 389.

Hall E. K. & Cotner J. B. (2007). Interactive effect of temperature and resources on carbon cycling by freshwater bacterioplankton communities. *Aquatic Microbial Ecology* 49: 35-45.

Haukka K., Kolmonen E., Hyder R., Hietala J., Vakkilainen K., Kairesalo T., Haario H. & Sivonen K. (2006). Effect of Nutrient Loading on Bacterioplankton Community Composition in Lake Mesocosms. *Microbial Ecology* 51: 137-146.

Kong Y., Xia Y., Nielsen J. L. & Nielsen P. H. (2007). Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. *Microbiology* 153: 4061-4073.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Mueller-Spitz S. R., Goetz G. W. & McLellan S. L. (2009). Temporal and spatial variability in nearshore bacterioplankton communities of Lake Michigan. *FEMS Microbiology Ecology* 67: 511-522.

Muylaert K., Van der Gucht K., Vloemans N., De Meester L., Gillis M. & Vyverman W. (2002). Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. *Applied and Environmental Microbiology* 68: 4740-4750.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Nürnberg G. K. (1996). Trophic State of Clear and Colored, Soft- and Hardwater Lakes with Special Consideration of Nutrients, Anoxia, Phytoplankton and Fish. *Lake and Reservoir Management* 12: 432 - 447.

Paerl H. W., Dyble J., Moisander P. H., Noble R. T., Piehler M. F., Pinckney J. L., Steppe T. F., Twomey L. & Valdes L. M. (2003). Microbial indicators of aquatic ecosystem change: Current applications to eutrophication studies. *FEMS Microbiology Ecology* 46: 233-246.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Pereira M. J., Utilização de algas na avaliação de contaminação de águas doces por metais. Department of Biology, Vol. PhD. University of Aveiro, Aveiro, 1999, pp. 611.

Pinho P., Praça F. & Torres A., Estudo para o ordenamento da área envolvente da Pateira de Fermentelos. In: R. e. C. d. N. Serviço Nacional de Parques, (Ed.). Departamento de Ambiente e Ordenamento da Universidade de Aveiro, Aveiro, 1988.

Riemann L. & Winding A. (2001). Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microbial Ecology* 42: 274-285.

Rino J. M. A. & Gil M. C. P. (1987). Diatomáceas do rio Cértima - Estudo preliminar. *Revista de Biologia da Universidade de Aveiro* 1: 53 - 103.

Rooney-Varga J. N., Giewat M. W., Savin M. C., Sood S., Legresley M. & Martin J. L. (2005). Links between phytoplankton and bacterial community dynamics in a coastal marine environment. *Microbial Ecology* 49: 163-175.

Saitou N. & Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Saksena D. N., Garg R. K. & Rao R. J. (2008). Water quality and pollution status of Chambal river in National Chambal sanctuary, Madhya Pradesh. *Journal of Environmental Biology* 29: 701-710.

Šimek K., Horňák K., Jezbera J., Nedoma J., Znachor P., Hejzlar J. & Sed'a J. (2008). Spatio-temporal patterns of bacterioplankton production and community composition related to phytoplankton composition and protistan bacterivory in a dam reservoir. *Aquatic Microbial Ecology* 51: 249-262.

Teles M., Pacheco M. & Santos M. A. (2007). Endocrine and metabolic responses of *Anguilla anguilla* L. caged in a freshwater-wetland (Pateira de Fermentelos-Portugal). *Science of the Total Environment* 372: 562-570.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

ter Braak C. J. F., 1995. Ordination. In: R. H. G. Jongman, C. J. F. ter Braak & O. F. R. Tongeren, (eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, UK: 91-173.

Thompson J. D., Higgins D. G. & Gibson T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

Tong S. T. Y., Liu A. J. & Goodrich J. A. (2007). Climate change impacts on nutrient and sediment loads in a midwestern agricultural watershed. *Journal of Environmental Informatics* 9: 18-28.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Xi W., Wu X., Ye W. & Yang H. (2007). Changes in bacterial community structure during preceding and degraded period of cyanobacterial bloom in a bay of the Taihu Lake. *Chinese Journal of Applied and Environmental Biology* 13: 97-103.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology*: 1-10.

Zwart G., Crump B. C., Kamst-van Agterveld M. P., Hagen F. & Han S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* 28: 141-155.

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Chapter III

Characterization of bacterioplankton community from Portuguese freshwater bodies under severe drought conditions – increased risks associated with cyanobacterial blooms

Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies

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Abstract

In order to investigate the bacterial diversity in a number of rivers, reservoirs and lakes in northern and central Portugal during the winter of 2004/5 (atypically dry), we applied molecular methodologies, namely denaturing gradient gel electrophoresis with primers targeting fractions of the bacterial 16S rRNA gene. Environmental parameters such as pH, conductivity, inorganic nutrients, total suspended solids and chlorophyll a were determined in order to characterize the trophic status of the studied water bodies. We found water bodies with oligotrophic to hypereutrophic characteristics. Organisms belonging to the *Bacteroidetes* and *Alphaproteobacteria* were found at the highest pH environment. *Bacteroidetes* were also related to high nutrient concentrations. *Verrucomicrobia* were associated with the most oligotrophic reservoir and low pH values. *Actinobacteria* were present in all samples from lakes and reservoirs, indicating its preference for lentic water bodies. *Cyanobacteria* dominance was related to high pH and conductivity levels. In general the conductivity values recorded in winter 2005 were the highest over recent years and chlorophyll a also reached very high levels. The data emphasize an enhanced risk of eutrophication for the studied water bodies, especially in the subsequent months when the temperature rises.

Keywords

eutrophication, bacterial diversity, DGGE analysis, 16S rRNA gene.

Introduction

Superficial freshwaters are increasingly suffering from eutrophication owing to factors either of natural or anthropogenic origin, posing great concern given that the quality of drinking water supplies is affected (Codd, 2000; Craun et al., 2002; Zaitlin & Watson, 2006). Waterborne pathogens still cause disease outbreaks in both developing and developed countries (Craun et al., 2002). Thus, monitoring strategies and in particular those directed to determining the composition of microbial assemblages are becoming more and more necessary for the management of these water systems.

The Portuguese winter of 2004/5 was considered to be one of the driest over the past decades, posing even greater risks of eutrophication of inland waters in the subsequent months. Eutrophication enhances phenomena such as the occurrence of cyanobacterial blooms, which may be toxic (Codd, 2000; Dokulil & Teubner, 2000; de Figueiredo et al., 2004b; Mazur-Marzec, 2006). Although this type of event has been reported for several Portuguese rivers, lakes and reservoirs during summer months (Vasconcelos, 2001) the freshwater bacterioplankton community as a whole has not been subject to intensive investigation.

In the last decade, culture-independent DNA approaches have led to faster and more detailed determinations of microbial diversity in natural aquatic environments. Denaturing gradient gel electrophoresis (DGGE), in particular, is a method that takes advantage of PCR amplification of highly conserved domains within the 16S rRNA gene and has been widely applied to assessment of microbial diversity (Muyzer et al., 1993; Muyzer & Smalla, 1998; Muylaert et al., 2002; Lyautey et al., 2005).

This study was designed to assess the bacterial diversity in several Portuguese rivers, reservoirs and lakes in winter 2004/5, which was considered one of the driest over the last century according to environmental time series data from the Portuguese Water Institute (INAG; http://snirh.inag.pt/). This study also aimed to gather ecological information from environmental parameters with data from modern molecular approaches. As there are no internationally published studies concerning the bacterial composition in these water bodies, a comparison with other similar European water systems was performed.

Materials and methods

Sampling

The river sections chosen for this study were in accordance with monitoring stations established by the INAG for water quality reports and real-time monitoring of abiotic parameters (conductivity, temperature, pH and dissolved oxygen). The reservoirs sampled receive waters from different origins and present distinct physical and chemical characteristics, as reported also by the INAG. All the aquatic systems are located in northern and central Portugal (Table 1 and Fig. 1). Sampling was performed during late February and March using 2-L sterile bottles. Samples were taken

subsuperficially at about 1m from the shore to ensure that sediment was not collected. Samples were immediately placed at 4 1C in the dark until further treatment for no longer than 12 h. From each sample DNA was extracted (see below) and environmental parameters were determined as well as chlorophyll a (Chl *a*).

Environmental parameters and chlorophyll a

The water conductivity and pH were measured in the laboratory, at 20 1C, using specific electrodes. Owing to the wide variations in temperature and dissolved oxygen values during the day (according to real-time data from the INAG), these values were not considered for the present analysis as the samples were not all taken at the same time of the day. Other environmental parameters, such as the total suspended solids (TSS), Chl *a*, soluble reactive phosphorus (SRP), ammonium, nitrate and nitrite concentrations were determined in the laboratory according to standard procedures (APHA, 1995; Rodier, 1996).

DNA extraction

The total DNA from environmental water samples was extracted after filtering 300mL of the sampled water through 0.22-mm polycarbonate sterile filters (more than one if the first filter clogged). This procedure was used for all samples. As an additional analysis for some samples, a successive filtration through 5- and 0.22-mm polycarbonate filters was performed in order to compare the attached and free-living bacterial communities, respectively. Collected cells were resuspended in 2mL of TE buffer [10mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. After resuspension in 200 mL TE, lysis was carried out by adding 1mgmL_1 lysozyme and incubating at 37 1C for 1 h as described by Henriques et al. (2004). DNA extraction and purification were performed using the genomic DNA purification kit (MBI Fermentas) and DNA was resuspended in TE buffer and stored at _20 1C.

PCR amplification of the bacterial 16S rRNA gene fragments

Amplification by PCR was performed with the primer pair 518R (50-ATTACCGCGGCTGCTGG-30) and 338F-GC (50-GACTCCTACGGGAGGCAGCAG-30 with a GC clamp attached) (Muyzer et al., 1993). The primers were synthesized by MWG-Biotech AG. PCR reactions were carried out with Taq polymerase, nucleotides and buffers from MBI Fermentas, in a Bio-Rad iCycler Thermal Cycler. PCRs were performed in 50-mL reaction mixtures each containing 3mM MgCl2, 200 mM of each nucleotide, 1_PCR buffer with (NH4)2SO4%, 5% dimethylsulfoxide (DMSO), 15 pmol of each primer, 1U of Taq DNA polymerase and 50–200 ng template DNA. For each PCR reaction an initial denaturation step at 94 1C for 5 min was carried out; following this, 30 cycles of 30 s at 92 1C (denaturation), 30 s at 55 1C (annealing) and 30 s at 72 1C (extension) were completed. A final extension step at 72 1C for 7 min was included. Negative control reactions without any template DNA were carried out simultaneously. PCR products (c. 200 bp long) were checked by electrophoresis in 1.5% agarose gels at 80 V for 1.5 h in Tris-acetate-EDTA buffer. A GeneRulerTM

Table 1 Environmental data recorded in winter season of 2005 at the studied rivers, lakes and reservoirs.

Hydrographical basin	Sampling station	Code	Average daily precipitation	Average winter precipitation* (mm)		Average precipitation on sampling month* (mm)		Chl *a*	TSS	pH	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	DIN	SRP	Conductivity
			7 days before sampling* (mm)	2004/5	From 1940/41 to 1997/98	2004/5	From 1940/41 to 1997/98	($\mu g.L^{-1}$)	($mg.L^{-1}$)		($mg.L^{-1}$)					($\mu S.cm^{-1}$)
Cávado River	Cávado River (Bico bridge)	RCV1	10.3	88	188	187.3	154	1.9	8.08	6.51	0.02	4.7	<0.1	4.7	<0.1	39
Cávado River	Cávado River (after Bico)	RCV2	10.3	88	188	187.3	154	0.6	3.05	6.03	0.11	6.2	<0.1	6.3	<0.1	103
Douro River	Azibo Reservoir	BAZ	3.7	18	60	32.2	52	0.5	3.08	6.76	0.01	0.2	<0.1	0.2	<0.1	76
Douro River	Crestuma Reservoir	BCR	0.0	34	142	59.4	117	8.1	4.08	7.65	0.01	7.0	<0.1	7.0	0.1	385
Douro River	Douro River (after Crestuma)	RD1	0.0	34	142	59.4	117	9.1	8.36	7.74	0.03	2.8	<0.1	2.9	0.1	296
Douro River	Sousa River	RS	0.0	34	142	59.4	117	0.6	12.10	7.04	<0.01	12.6	<0.1	12.6	0.2	214
Mondego River	Arouce River (Gevim)	GV	5.2	20	149	40.3	155	<0.3	0.10	6.53	0.01	0.8	<0.1	0.8	<0.1	42
Mondego River	Alva River (Mucela bridge)	RAL	5.2	20	149	40.3	155	1.5	0.19	6.75	0.01	2.0	<0.1	2.0	<0.1	59
Mondego River	Caldeirão Reservoir	BCL	9.5	51	205	93.3	161	3.5	2.35	6.60	0.01	0.4	<0.1	0.4	<0.1	28
Mondego River	Fagilde Reservoir	BFG	2.0	20	149	18.1	123	12.2	11.46	8.72	0.11	2.3	<0.1	2.4	<0.1	80
Mondego River	Aguieira Reservoir	BAG	5.2	20	149	40.3	155	3.4	2.63	6.91	0.02	3.9	<0.1	4.0	<0.1	97
Vouga River	Águeda River (Águeda bridge)	RAG	0.1	37	222	23.2	233	0.9	1.70	6.81	0.01	2.7	<0.1	2.7	<0.1	60
Vouga River	Fermentelos Lake	LF	0.1	37	222	23.2	233	22.1	25.97	7.58	0.12	6.6	0.1	6.8	0.1	578
-	Vela Lake	LV	0.1	34	107	11.7	125	15.4	31.70	7.87	0.02	0.6	0.6	1.2	<0.1	505
-	Mira Lake	LM	3.7	34	107	47.3	70	76.9	13.50	8.19	0.12	5.0	<0.1	5.0	<0.1	450

* These data were obtained from Portuguese Water Institute (http://snirh.inag.pt/).

DNA Ladder Mix (MBI Fermentas) was run on both sides of each gel. The gels were stained with ethidium bromide, and visualized on a UV transilluminator.

DGGE

The DGGE was performed to separate the PCR-amplified 16S rRNA gene fragments (with identical length but potentially different sequence) from bacteria using a 35–60% denaturing gradient (100% denaturing gradient is 7M urea and 40% deionized formamide) in 1mm vertical polyacrylamide gels [8% w/v acrylamide in 0.5_ TAE buffer (20mM Tris, 10mM acetic acid and 0.5mM EDTA)].Electrophoresis was performed in a DCodeTM universal mutation detection system (Bio-Rad Laboratories) using 0.5_TAE buffer for 5.5 h at 200V. An initial 15-min step at 20V was also performed. The gel was then stained for 5 min in an ethidium bromide solution (5%) and then destained (with gentle agitation) in distilled water for 20 min before image digitalization in a molecular imager FXTM system (Bio-Rad Laboratories).

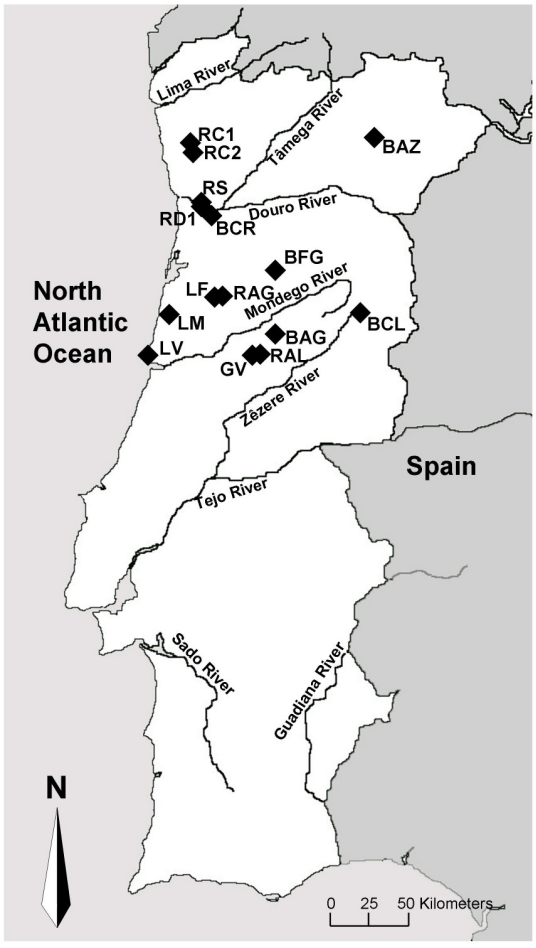


Fig. 1. Location of the sampling stations. Each sampled water body station has a corresponding code given in Table 1.

Statistical analysis

Principal component analysis (PCA) was performed to observe the distribution of the sampled water bodies according to their environmental parameters. Before running the analysis, the environmental data were standardized by subtractingthe mean from each observation and dividing by the corresponding SD. Cluster analysis of the samples according to the environmental parameters was performed using the unweighted pair group method with mathematical averages (UPGMA). Correlations were calculated using the Pearson coefficient of similarity (95% probability) and a relatedness tree was built by the PRIMER 5 software (Clarke & Gorley, 2001). In order to evaluate the correlation between the environmental parameters the Pearson correlation coefficient was applied.

DNA sequencing of excised DGGE bands

Bands from DGGE profiles elected for sequencing were excised from the gel using a sterilized blade. After addition of 10 mL of sterile milli-Q-purified water, PCR reamplification was performed immediately in order to avoid the overnight incubation that could lead to a higher

contamination of the dominant DNA sequence. The primer pair used for reamplification were the same as those used in the original PCR. DGGE was performed to confirm the identity of the eluted DNA bands. To perform sequence analysis, the amplicons were purified with the concertTM rapid PCR purification system (Gibco BRL) and then used as templates for the sequencing reactions with the ABI PRISMs bigdye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) using the primer 518R. Sequencing reaction products were analysed in an automatic DNA sequencer (ABI PRISMs 310 genetic analyser, PE Applied Biosystems). Similarity to sequences deposited in the GenBank database was searched by BLAST (http://www.ncbi.nlm.nih.gov).

Nucleotide sequence accession numbers

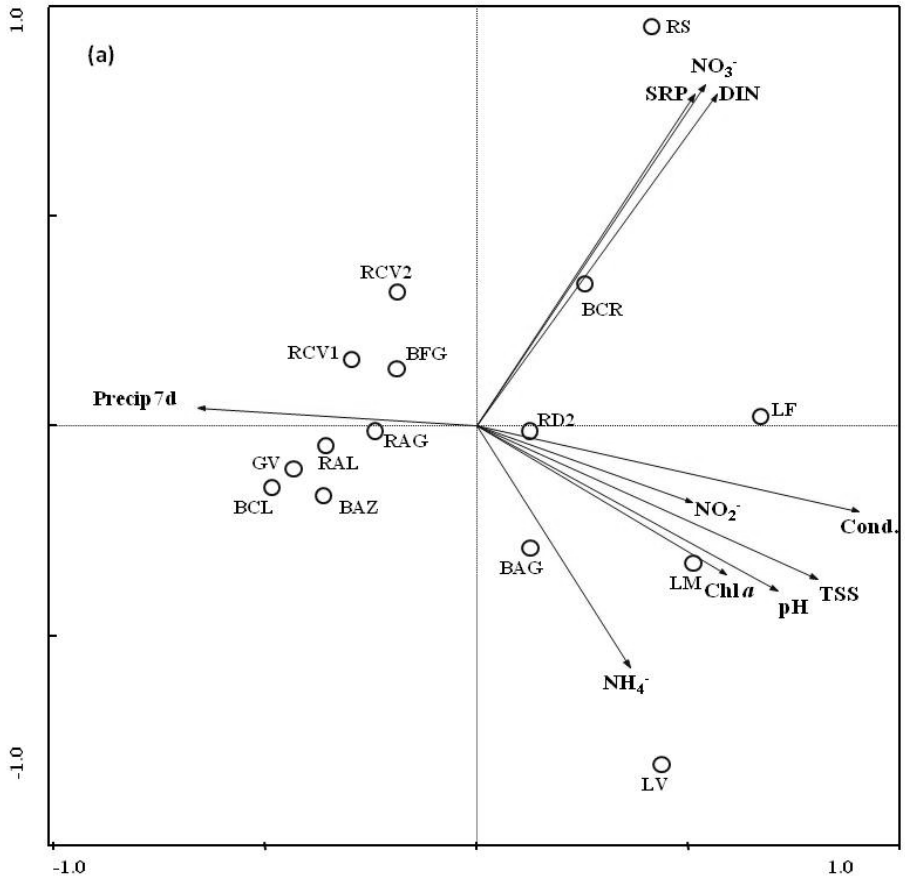
Bacterial partial sequences from excised bands were deposited in the GenBank database under accession nos. DQ900024–DQ900050.

Results

Environmental parameters and PCA

The environmental parameters considered are summarized in Table 1. The pH ranged from 6.03 (at Cávado River) to 8.72 (at Fagilde reservoir). Conductivity was highest in lake water (ranging from 450 at Mira Lake to 578 mS cm_1 at Fermentelos Lake) followed by Crestuma reservoir and Douro River (385 and 296 mS cm_1, respectively). The lowest conductivity value was recorded at Caldeirão reservoir (28 mS cm_1). With regard to nutrient concentrations, SRP and dissolved inorganic nitrogen (DIN) attained the highest values at Sousa River, with 0.23 and 12.6 mg L_1, respectively. The lowest DIN concentration was observed for Azibo reservoir (0.02 mg L_1) and SRP was undetectable at Arouce, Alva and Águeda rivers, Vela and Mira lakes and Caldeirão reservoir, and only 0.01 mg L_1 at Azibo reservoir. In deep lakes or reservoirs such as Crestuma and Aguieira reservoirs, with high oxygen levels, inorganic nitrogen is usually available as nitrate whereas in shallow lakes with low oxygen levels such as Vela Lake, nitrogen is found as ammonia. Accordingly, the highest ammonium levels were present in the shallow Fermentelos and Vela lakes (0.1 and 0.6 mg L_1, respectively).

The average daily precipitation 7 days before the sampling date in the vicinity of each station was higher at Cávado River and Caldeirão reservoir (10.3 and 9.5 mm, respectively) and undetectable for the Vouga basin stations. Precipitation levels for winter 2004/5 were below the average for winter seasons between 1940/41 and 1997/98 (Table 1) at all sampling stations, with the exception of Cávado River where heavy rainfall in March raised the monthly average to 187.3 mm. TSS was highest at the lakes (ranging from 13.50 to 31.70 mg L_1 in Mira and Vela lakes, respectively) followed by Sousa River (12.10 mg L_1) and Fagilde reservoir (11.46 mg L_1). The lowest TSS values were recorded for the Arouce and Alva Rivers, 0.10 and 0.19 mg L_1, respectively. The highest Chl *a* concentrations were observed in the lakes (ranging from 15.4 to



(b)

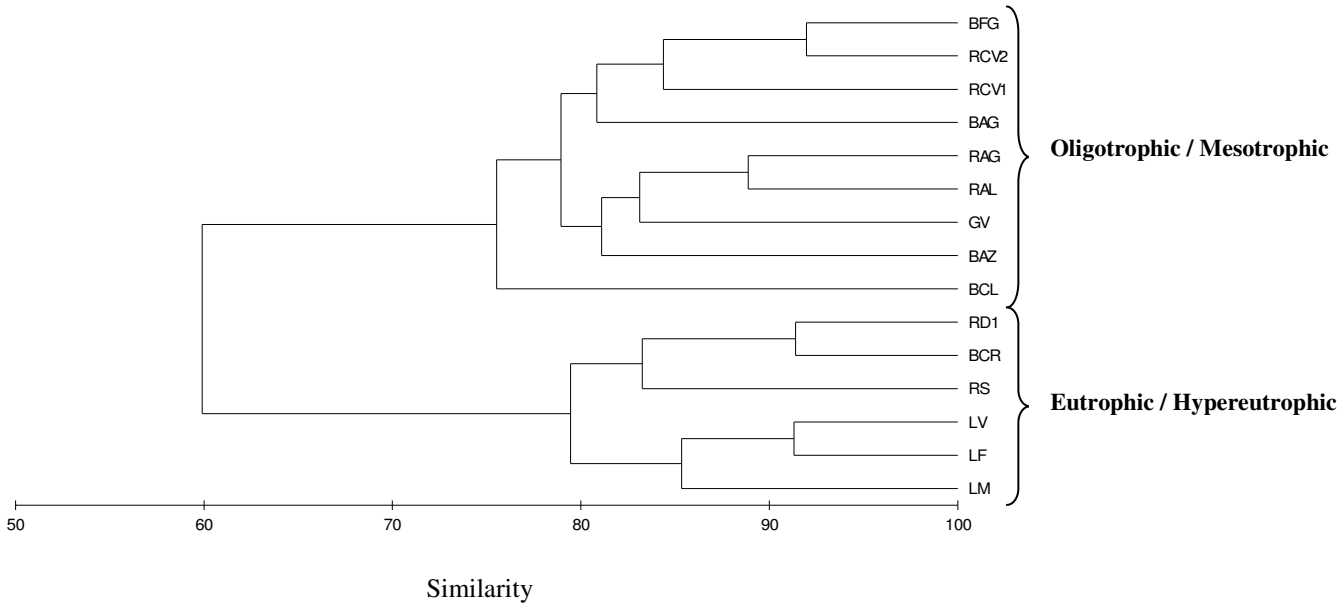


Fig. 2. (a) PCA ordination biplot of sampling stations and environmental parameters; (b) dendrogram of sampling stations clustered according to the environmental parameters.

76.9 mg L_1) followed by Fagilde reservoir (12.2 mg L_1), Douro River (9.1 mg L_1) and Crestuma reservoir (8.1 mg L_1). Chl *a* was undetectable at Arouce River and had the lowest values at Azibo Reservoir (0.5 mg L_1) and at Cávado and Sousa Rivers (both stations with 0.6 mg L_1).Correlation between environmental parameters was calculated for Po0.05 and n = 15. pH was significantly positively correlated with conductivity (r = 0.60), Chl *a* (r = 0.59) and SST (r = 0.57) but negatively correlated with precipitation (r = 0.64). Conductivity was shown to have a highly significant positive correlation with SST (r = 0.81) and negative correlation with precipitation (r =_0.60). SRP had a highly significant positive correlation with nitrate and DIN (r = 0.90 for both). Nitrite showed a significant positive correlation with Chl *a* (r = 0.62). Ammonium was highly significantly positively correlated with SST (r = 0.74), and DIN was totally positively correlated with nitrate (r = 1.00). Ordination of samples by PCA according to environmental parameters revealed a distribution of sampling sites along the first axis mainly related to conductivity and precipitation, whereas nutrient concentrations (SRP and nitrate) were mostly correlated to the second axis. PC1 accounted for 40.3% and PC2 for 27.3% of the total variance among the sampling sites. The spread of data (Fig. 2a) indicates a general progression from the high water quality river and reservoir scores in the negative left quadrant towards the eutrophic lakes and Sousa River scores in the right side of the graph. On the negative side, the first axis is defined by the Arouce River and Caldeirão and Azibo reservoirs while in the extreme positive side are Fermentelos and Mira lakes. The second axis positive area is defined by the Sousa River station and the negative extreme is defined by the Vela Lake station, which is strongly defined by high ammonium levels. The dendrogram constructed (Fig. 2b) shows also the clustering of meso-/oligotrophic versus eutrophic/hypereutrophic water bodies. From Table 1 it can be seen that this separation is highly correlated with conductivity as suggested by the PCA analysis.

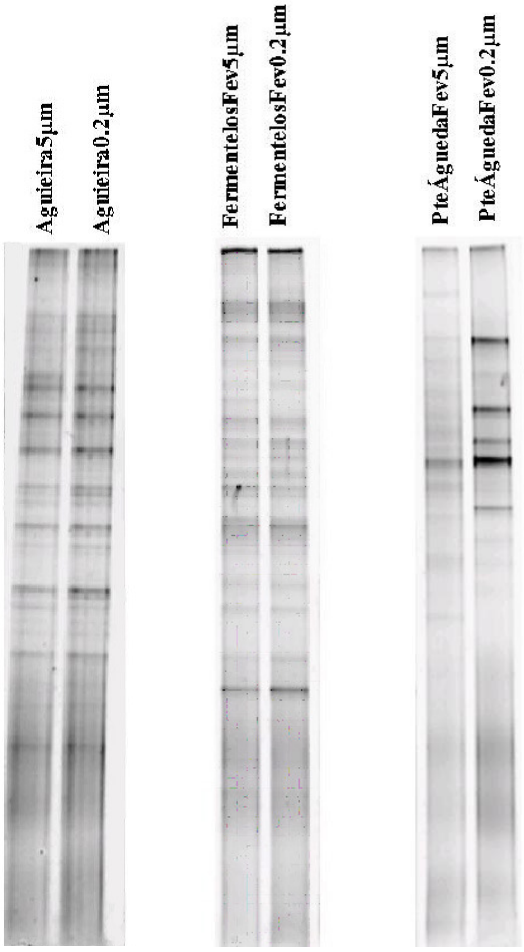


Fig. 3. Differences in DGGE profiles of attached (5 mm) and free-living (0.22 mm) bacterial communities for some of the water bodies sampled in winter 2004/5.

Analysis of DGGE profiles

The methodology used to extract DNA did not discriminate between free-living and particle-attached bacteria present in the samples (see Fig. 4). However, a previous careful analysis of selected samples allowed us to conclude that free-living bacterial groups/organisms were in general also present in the attached bacterial assemblage (see Fig. 3). For Aguieira reservoir and Fermentelos Lake these free-living and attached communities showed very similar DGGE profiles, whereas remarkable differences among these two communities were found for Águeda River. From the DGGE profiles shown in Fig. 4 it is clear that there was a high variability between the bacterial assemblages, although some bands are common to several water bodies (e.g. band 38). The phylogenetic affiliation of each sequenced DGGE band is presented in Table 2 and shows that they belong to members of the main bacterial groups found in freshwaters: *Betaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia* , *Actinobacteria*, *Cyanobacteria* and *Alphaproteobacteria*. The majority of the excised bands corresponded to a single DNA sequence; some bands, however, were revealed to be a mixture of two DNA sequences and were excluded from the sequencing results. DGGE has some limitations (Muyzer & Smalla, 1998) such as the possible co-migration of bands with different sequences. Thus, in order to confirm that matching bands corresponded to identical phylotypes, more than one band with equal position in the gel was excised and sequenced. The same nucleotide sequence was obtained for each band position (e.g. band 38).

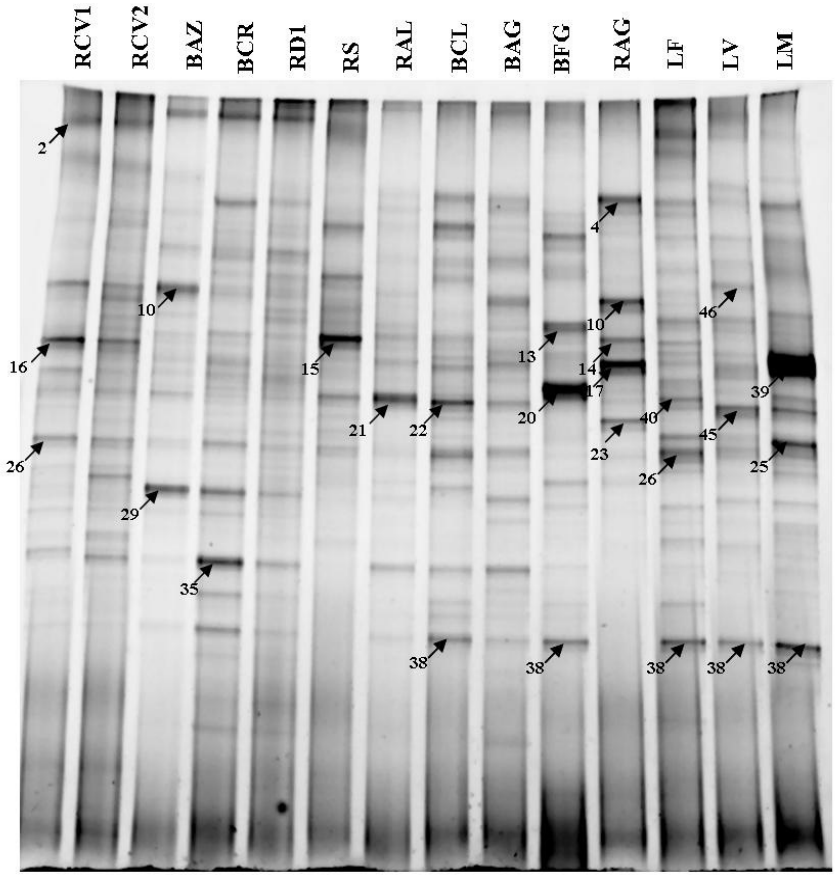


Fig. 4. DGGE gel containing the bacterial 16S rRNA gene fragments to assess the bacterial diversity in several Portuguese rivers, reservoirs and lakes in winter 2004/5. The code above each lane refers to each sampled water body (see Table 1).

Cávado River was dominated by *Betaproteobacteria* although *Actinobacteria* were also well represented. Different organisms of *Actinobacteria* were dominant at Azibo and Crestuma

reservoirs while *Bacteroidetes* were dominant in the bacterial assemblage at Sousa River. Alva River was dominated by an autotrophic eukaryote and at Caldeirão reservoir *Verrucomicrobia* dominated in spite of a strong representation of *Actinobacteria*. Aguieira reservoir did not have a dominant group but instead bands with similar low intensity (isolation of these bands was extremely difficult, excluding their deposition in the NCBI database). The Fagilde reservoir bacterial assemblage was almost completely dominated by *Alphaproteobacteria*, followed by *Bacteroidetes* and *Actinobacteria*. Águeda River was showed to have *Bacteroidetes* dominating the bacterial community along with different *Betaproteobacteria*. At Fermentelos Lake the *Actinobacteria* and *Bacteroidetes* were among the dominant bacterial groups. Following the assumption that each different DGGE band corresponds to a different organism, the richest sample in species was Fermentelos Lake and the poorest was Alva River. Vela Lake showed dominant bacteria belonging to the *Betaproteobacteria* and *Actinobacteria*. The relatively strong presence of a eukaryotic chloroplast was also found at Vela Lake. Mira Lake presented a bloom of *Cyanobacteria* codominating with *Actinobacteria*. Members of the *Actinobacteria* group proved to be dominant at the majority of the samples, with the exception of Alva, Águeda and Sousa rivers (lotic water systems). In addition, all the lentic water systems including most reservoirs (except Azibo reservoir) and all lakes showed an intense presence of the same actinobacterium (band 38), as proven after sequencing several bands with this same migration point (see Table 2).

Discussion

PCA and trophic status of the water bodies

From the PCA results for the sampling stations, a gradient was distinguishable that coincides with the trophic status of the water bodies. The spread of data suggests a general progression from the high water quality Arouce River and Caldeirão and Azibo reservoirs towards the eutrophic Sousa River and the hypereutrophic Mira and Fermentelos lakes. This clustering of the sampled water bodies by trophic status is also presented in the dendrogram of Fig. 2b. The distribution of the samples was mainly related to conductivity and nutrient concentrations. The water bodies with the best water quality had the lowest conductivities and low nutrient concentrations, while the eutrophic and hypereutrophic water bodies had higher values for these parameters. Thus, an increase in these parameters may suggest a deterioration of the trophic status of a water body. The INAG reported that for winter 2004/5 precipitation levels were far below the average data for winter seasons between 1940/41 and 1997/ 98 at all sampling stations. Data from the recent years also made available by INAG indicated trophic characteristics from the sampled water bodies similar to our data, although in 2005 the conductivity levels were generally higher than average (see further below), suggesting there may have been a deterioration of the trophic status of the studied water bodies.

Oligotrophic water bodies

The water bodies which had conditions within the range for oligotrophic status (clear water, low algal growth and productivity, and low nutrient levels; Harper, 1992) included Caldeirão and Azibo reservoirs, both located upstream in the basins of the rivers Mondego and Douro, respectively, and Arouce River, also located upstream in the Mondego River Basin. Caldeirão reservoir (mainly used for hydroelectricity generation) showed environmental parameters in winter 2004/5 that were all within the values registered for winters 2000/1, 2001/2 and 2002/3. Despite the fact that TSS and nutrient values were very low and fitted with oligotrophic conditions, Chl *a* concentration in winter 2004/5 was characteristic of mesotrophic waters with a moderate productivity. Gevim station (on the Arouce River) had the lowest TSS value and Chl *a* was undetectable (although between 2002 and 2003 this ranged from 4.04 to 6.66 mg L_1 in the winter season). Nutrient concentration, conductivity and pH were also lower than in previous years. For Azibo reservoir (which is used for irrigation and as a supply of drinking water) the values recorded for the environmental parameters in winter 2004/5 were also within the range recorded since 1994 although conductivity was higher than the winter average.

Mesotrophic water bodies

The mesotrophic water bodies (with moderate nutrient levels and productivity) (Harper, 1992) included the lotic sections of Cávado, Águeda and Alva rivers. Cávado River (Bico bridge station) had pH values considered to be normal for this water system but nitrate levels higher than the average and that fall within those for hypertrophic conditions (Harper, 1992), indicating there is the risk of enhanced eutrophication if phosphate levels become slightly higher. Conductivity in winter 2004/5 was the highest over the past 10 years and Chl *a* concentration was also high in relation to the winter average, also suggesting an increase in biological productivity and potential deterioration of its trophic status. Station RCV2 (downstream from RCV1) in this river showed an increase in nutrient levels and in conductivity but decreased Chl *a*, SST and pH. For Águeda River, the recorded environmental parameter values were found to be normal, although conductivity was generally higher in 2005 than in the winter seasons from previous years (since 1998). Alva River also showed normal values for these environmental variables, although in 2005 the lowest SST values were recorded and conductivity was higher than the average for the past 15 years.

Eutrophic and hypereutrophic water bodies

Some of the studied water bodies that were considered to be eutrophic (green water, high nutrient levels and high productivity) (Harper, 1992) included Vela Lake, Sousa River and Crestuma, Aguieira and Fagilde reservoirs. According to the INAG, the sampled water from Aguieira reservoir (used as a drinking water supply, irrigation source and also for hydroelectric purposes and recreation) had values within the normal range for the environmental parameters TSS, pH, conductivity and nutrient concentration. Nevertheless, for winter 2004/5 relatively high values for

Table 2 Accession number and phylogenetic affiliation for the selected sequenced bacterial DGGE bands shown in Fig. 2. Closest relatives found in the database after a BLAST search are also indicated.

DGGE band	NCBI Accession Nº	Phylogenetic affiliation	Closest relative (accession Nº)	Percentage similarity (%)
2RCV	DQ900024	*Beta-proteobacteria*	*Beta-proteobacterium* HTCC540 (AY584577)	98
16RCV	DQ900025	*Beta-proteobacteria*	Uncultured Green Bay ferromanganous micronodule bacterium MNA1 (AF293004)	100
26RCV	DQ900026	*Actinobacteria*	Uncultured *actinobacterium* clone PRD18G08 (AY948060)	100
10BAZ	DQ900027	*Actinobacteria*	Uncultured *actinobacterium* clone Sta2-15 (AY562323)	100
29BAZ	DQ900028	*Actinobacteria*	Uncultured *actinomycete* clone CR-FL3 (AF141389)	100
35BCR	DQ900029	*Actinobacteria*	Uncultured *actinobacterium* clone TW11-1 (DQ316387)	100
15RS	DQ900030	*Bacteroidetes*	*Flavobacterium* sp. GOBB3-209 (AF321038)	100
21RAL	DQ900031	Eukaryota (chloroplast)	Uncultured phototrophic eukaryote clone PRD18H02; chloroplast (AY948066)	99
22BCL	DQ900032	*Verrucomicrobia*	Uncultured *Verrucomicrobia les* clone CR-FL1 (AF141387)	99
38BCL	DQ900033	*Actinobacteria*	Uncultured *actinobacterium* clone PRD18H10 (AY948072)	100
13BFG	DQ900034	*Bacteroidetes*	Uncultured *Cytophagales* ESR 2 (AF268286)	100
20BFG	DQ900035	*Alpha-proteobacteria*	*Sphingomonas* sp. GOBB3-C201 (AF321036)	100
38BFG	DQ900036	*Actinobacteria*	Uncultured actinobacterium clone PRD18H10 (AY948072)	100
4 RAG	DQ900037	*Beta-proteobacteria*	*Beta-proteobacterium* HTCC540 (AY584577)	98
10RAG	DQ900038	*Beta-proteobacteria*	Uncultured *beta-proteobacterium* clone PRD18F02 (AY948045)	99
14RAG	DQ900039	*Bacteroidetes*	*Flavobacterium* sp. GOBB3-209 (AF321038)	99
17RAG	DQ900040	*Bacteroidetes*	Uncultured *Cytophagales* bacterium clone 30 (AF361200)	100
23RAG	DQ900041	*Beta-proteobacteria*	*Beta-proteobacterium* LI2-55 (AJ964892)	99
26LF	DQ900042	*Actinobacteria*	Uncultured *actinobacterium* clone PRD18G08 (AY948060)	100
38LF	DQ900043	*Actinobacteria*	Uncultured *actinobacterium* clone PRD18H10 (AY948072)	100
40LF	DQ900044	*Bacteroidetes*	*Flavobacterium* sp. Rud06 (AY788977)	100
38LV	DQ900045	*Actinobacteria*	Uncultured *actinobacterium* clone PRD18H10 (AY948072)	100
45LV	DQ900046	*Beta-proteobacteria*	Uncultured *beta-proteobacterium* clone DH-9 (DQ076174)	100
46LV	DQ900047	Eukaryota (chloroplast)	Uncultured phytoplankton ESR 1 (AF268285)	100
25LM	DQ900048	*Actinobacteria*	Uncultured *actinomycete* clone CRO-FL14 (AF141592)	99
38LM	DQ900049	*Actinobacteria*	Uncultured *actinobacterium* clone PRD18H10 (AY948072)	100
39LM	DQ900050	*Cyanobacteria*	*Synechococcus* sp. MW73B4 (AY151250)	100

pH, conductivity and Chl *a* were found (indicating an intensification in productivity). Over the last 15 years there has been a tendency for conductivity to increase at this reservoir and Chl *a* has also shown increasingly high values since the winter of 2001/2. The high nitrate concentrations (characteristic of eutrophic waters) found in this reservoir may pose risks to its trophic status maintenance. Since 1989, at Crestuma reservoir (used for hydroelectric exploration) high nutrient concentrations have been recorded (reaching 0.38 mg L_1 for ammonium, 0.33 mg L_1 for nitrite,

18.2 mg L_1 for nitrate and 1.05 mg L_1 for SRP) that fit within the range of values found in hypertrophic water bodies (Harper, 1992). Yet, Chl *a* values are usually within the range reported for mesotrophic conditions. In winter 2004/5, nutrient levels were lower but Chl *a* concentration was higher than the average, suggesting a higher algal productivity in the reservoir under the drought conditions felt in 2005. Additionally, highest conductivity (385 mS cm_1) over the last 15 years was attained at Crestuma reservoir in winter 2004/5 as well as high concentrations of Chl *a*, both indicating the eutrophic status of this water body. The Douro River station just downstream of Crestuma reservoir showed that Chl *a*, SRP and nitrate concentrations were higher than in 2002 and 2003. It should be borne in mind that this station is subject to tidal influence, although sampling was made during low tide in order to minimize its influence. The Sousa River flows into the Douro River downstream of Crestuma reservoir and it is a nutrient-enriched water body as verified from INAG time-series data since 1991 (SRP ranging from 0.02 to 0.42 mg L_1, nitrite concentration reaching 0.32 mg L_1 and nitrate concentration ranging from 4.19 to 20.00 mg L_1); 2005 was no exception, with high nutrient values that fit with the hypereutrophic conditions in spite of the low algal productivity as represented by levels of Chl *a*. In winter 2004/5 the values recorded for TSS in the Sousa River were lower than the average although conductivity attained the highest value (214 mS cm_1). In recent years, Fagilde reservoir (used for municipal water supply) has shown to have problems in terms of water quality, with high nutrient concentrations, particularly nitrate (with a maximum of 7.70 mg L_1 in February 2001). Since 2001, the highest values for pH (8.72), conductivity (80 mS cm_1) and TSS (11.46 mg L_1) were recorded in the drought conditions of winter 2004/5. Chl *a* peaks during winter were observed in 2002 and 2005 (13.44 and 12.2 mg L_1, respectively), suggesting a high productivity characteristic of eutrophic waters in Fagilde reservoir. Vela Lake showed considerably increased TSS and conductivity values as compared with those for winter 2001 (2–15 mg L_1 and 327–419 mS cm_1, respectively) (de Figueiredo et al., 2006). Chl *a*, pH and nutrient concentrations were generally within the range of values recorded during the winters of 1993 (Barros, 1994) and 2001 (de Figueiredo et al., 2006).

The water bodies with conditions that suggest a hypereutrophic status (Harper, 1992) with very high nutrient concentrations and very biologically productive (supporting large numbers of plants, fish and other animals) include Mira and Fermentelos lakes. Mira Lake showed high nitrate concentrations and Chl *a* levels which indicate that there is a high productivity that may increase further if SRP concentration increases. Fermentelos Lake is known to be hypereutrophic as a result of its high nutrient levels, which have as their main source the C´ertima River which transports effluents from agriculture (fertilizers and pesticides) along with domestic and industrial wastewater discharge (Cerqueira et al., 2008).

Trophic status vs. bacterial diversity

Both particle-attached and free-living bacteria were considered together here as other studies have considered them as interactive assemblages (Riemann & Winding, 2001). Yet, in the few samples where these two communities were considered separately and when DGGE profiles were compared it was evident that many bands were common to both profiles, possibly suggesting the above-mentioned interaction and a rapid exchange between both bacterial fractions as suggested before (Hollibaugh et al., 2000). By looking at Fig. 4 and following the assumption that each different DGGE band corresponds to different bacteria, the richest sample in terms of species was Fermentelos Lake and the sample with fewest species was Alva River (dominated by phytoplankton). These water samples had very distinct environmental characteristics, indicating there may be a relationship between the trophic level of a water body and its bacterial species richness, as verified by Van der Gucht et al. (2005). We detected *Cyanobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Verrucomicrobia* and *Actinobacteria*, which are typical worldwide freshwater bacteria (Zwart et al., 2002; Cottrell et al., 2005; Lindström et al., 2005; Van Der Gucht et al., 2005).

Oligotrophic water bodies

The *Verrucomicrobia* group dominated the bacterial assemblage at Caldeirão reservoir. In fact, this bacterial group has been associated with oligotrophic conditions (Urbach et al., 2001; Zwart et al., 2002; Phung et al., 2004) and with low pH values and long THRT (theoretical hydrological retention time) (Lindström et al., 2005), as verified for this reservoir. Azibo reservoir had two dominant *Actinobacteria*. For one of these, the closest relative after the BLAST search was from mesotrophic riverine freshwaters (Cottrell et al., 2005). The other *Actinobacteria* belonged to the Actinomycetes group, whose dominance may pose some risk to the drinking water quality as there are some Actinomycetes able to produce terpenoids (such as geosmine) and pyrazines that give a bad taste and odour to the water (Zaitlin & Watson, 2006). Thus, in spite of the fact that Azibo reservoir has been characterized as an oligotrophic water body, attention should be given to its bacterial dominance in winter.

Mesotrophic water bodies

Alva River was dominated by phytoplankton, with the closest relative found in the NCBI database from a temperate river (Crump & Hobbie, 2005). Diatoms were found as dominant in qualitative determinations under an optical microscope at the time of sampling. The dominance of diatoms is usual in winter for several Iberian freshwater bodies (Sabater & Munoz, 1990; de Figueiredo et al., 2006) when nutrient availability is greater and temperatures are lower. Nevertheless, Chl *a* concentration was low, indicating there was not a significant phytoplanktonic bloom occurring at that time in spite of the high nitrate levels (that were compensated for by the undetectable SRP).

As already reported for other water bodies, the dominance of *Betaproteobacteria* at Cávado and Águeda rivers shows that it may be associated with relatively low pH values and short THRT (Lindström et al., 2005) but high nitrogen levels (Brümmer et al., 2000; Lee et al., 2002). Members of the *Betaproteobacteria* can be found in acidic water bodies (López-Archilla et al., 2004) and as part of denitrifying populations in environments with high nitrate concentrations (Lee et al., 2002). Besides *Betaproteobacteria*, Águeda River had a strong dominance of *Bacteroidetes* , particularly a Cytophagales bacterium characteristic of a mesoeutrophic reservoir (Šimek et al., 2001). However, these bacteria also codominated this bacterial community along with another *Betaproteobacteria* and a different *Bacteroidetes* (similar to a *Flavobacterium* sp. able to catabolize riverine dissolved organic matter; Kisand et al., 2002). In Cávado River, an actinobacterium was shown to be the same as that in the hypereutrophic Fermentelos Lake, suggesting there are conditions shared among these two water bodies. The high nitrate concentration is common to both water systems and may explain in part the presence of this Actinobacterium.

Eutrophic and hypereutrophic water bodies

The considered eutrophic water bodies were Vela Lake, Sousa River and Crestuma, Aguieira and Fagilde reservoirs. In shallow eutrophic and hypereutrophic lakes a dominance of the *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes* and *Actinobacteria* groups can be found (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006a) as was the case for these eutrophic water bodies. An *Actinobacteria*l clone associated with a eutrophic Germanlake (Allgaier & Grossart, 2006a) had the highest similarity with the dominant bacterial organism at Crestuma reservoir, showing it contains bacteria characteristic of its trophic status. *Bacteroidetes* , particularly a *Flavobacterium* sp. able to catabolize riverine dissolved organic matter (Kisand et al., 2002), had 100% similarity with the bacterium that dominated the bacterial assemblage at Sousa River where nutrient levels were very high. The dominance of *Flavobacterium* species is frequent in northern Europe eutrophic rivers (Brümmer et al., 2000). Integrons were also recorded in this Sousa River sample (unpublished data), indicating the risk for transfer of genetic determinants of resistance to antimicrobials and intensifying the water quality problem at this river. Aguieira reservoir did not present a dominant group but instead it had some bands that have similar low intensity (the isolation of these bands was extremely difficult, and thus they were not deposited in the NCBI database). The Fagilde reservoir bacterial assemblage was almost completely dominated by *Alphaproteobacteria*, particularly a *Sphingomonas* sp. associated with a bacterial community able to catabolize riverine dissolved organic matter (Kisand et al., 2002). This dominance may be associated with long THRT (Lindström et al., 2005) and high pH values as well as high nutrient and TSS concentrations, as reported for other eutrophic freshwaters (Zwart et al., 2002). The *Bacteroidetes* group was also highly represented along with *Actinobacteria*. The *Bacteroidetes* organism was found to be similar to a Cytophagales bacterium associated with a phytoplankton

bloom in a mesotrophic lake during late winter (Riemann & Winding, 2001). In fact, Cytophaga – *Flavobacterium* organisms are known to occur in high densities in European eutrophic reservoirs (Mašín et al., 2003). At Vela Lake *Betaproteobacteria* were dominant but *Actinobacteria* were also well represented. *Betaproteobacteria* are known to be nitrifying bacteria (performing ammonia oxidization) (Altmann et al., 2003), which may explain their dominance in Vela Lake where relatively high levels of ammonium were recorded along with very low nitrate levels. The dominance of *Actinobacteria* during autumn and winter is also observed for other lakes (Burkert et al., 2003; Allgaier & Grossart, 2006a). A eukaryotic chloroplast was also detected among the DNA partial sequences with moderate intensity and according to a qualitative phytoplankton screening of samples it should belong to a green alga.

For the hypereutrophic lakes we found that at Fermentelos Lake the *Actinobacteria* and *Bacteroidetes* were among the dominant bacterial groups. The *Bacteroidetes* group is known to appear in eutrophic lakes with high conductivities (Van Der Gucht et al., 2005). The *Bacteroidetes* organism was a *Flavobacterium* sp. again related to high nitrate levels but also related to SRP availability. Mira Lake presented a bloom of *Cyanobacteria* (*Synechococcus* sp.) co-dominated with two different clones of *Actinobacteria*. The group of *Actinobacteria* dominant in both Fermentelos and Mira lakes is known to have high densities in lakes during winter (Burkert et al., 2003; Allgaier & Grossart, 2006a). The dominance of *Cyanobacteria* in lakes is also very frequent worldwide and is usually associated with characteristics such as elevated temperatures and low total N/total P ratios in turbid waters with high pH and conductivity (Codd, 2000; Dokulil & Teubner, 2000; De Wever et al., 2005; Van Der Gucht et al., 2005; de Figueiredo et al., 2006). Yet, in similar shallow Portuguese lakes (Barros, 1994; de Figueiredo et al., 2006) this dominance occurs usually in late spring and summer months, which suggests there may have existed an effect on the bacterial dynamics owing to the drought felt during winter 2004/5. In addition, the eutrophication process is known to lead to dominance of *Cyanobacteria* even in almost oligotrophic conditions (Pearce et al., 2005). A dominance of *Cyanobacteria* is concerning due to the production of cyanotoxins, and *Synechococcus* spp. have proven to have toxic potential (Mazur-Marzec, 2006).

Concluding remarks

Data from our study suggest that pH along with nutrient levels are in fact important parameters in determining differences in the bacterial communities among the studied water bodies. *Verrucomicrobia* were associated with the oligotrophic Caldeirão reservoir with low pH values and long THRT as in other oligotrophic water bodies (Urbach et al., 2001; Zwart et al., 2002; Phung et al., 2004; Lindström et al., 2005). The *Alphaproteobacteria* group dominated the bacterial community at the eutrophic Fagilde reservoir, related to long THRT, high pH values as well as high nutrient and TSS concentrations as previously reported (Zwart et al., 2002; Lindström

et al., 2005). *Betaproteobacteria* were dominant from the lotic mesotrophic Cávado and Águeda rivers to the eutrophic Vela Lake. It is a ubiquitous group that may dominate the bacterial assemblages from eutrophic to oligotrophic freshwater systems (Böckelmanna et al., 2000; Brümmer et al., 2000; O'Sullivan et al., 2002; Mašín et al., 2003; Lindström et al., 2005) and sediment (Stein et al., 2001; Altmann et al., 2003; Hullar et al., 2006). The *Bacteroidetes* group is known to occur at mesotrophic and eutrophic water bodies (Riemann & Winding, 2001; Van Der Gucht et al., 2005). In this study, *Bacteroidetes* were found at the most extreme high pH conditions at the eutrophic Fagilde reservoir and extremely high nutrient concentrations at the eutrophic Sousa River and hypereutrophic Fermentelos Lake, although they were also dominant at the mesotrophic Águeda River. In fact, in European eutrophic waters Cytophaga–*Flavobacterium* organisms are known to occur in high densities (Brümmer et al., 2000; Mašín et al., 2003) and the high nitrate levels have been shown to be related to the dominance of *Flavobacterium* sp. given that they are nitrate-reducing bacteria (Nijburg & Laanbroek, 1997). At Sousa and Águeda rivers the *Flavobacterium* clones were very similar and associated with extremely low Chl *a* levels, which may suggest a preferable noncoexistence with phytoplankton; interactions between phytoplankton and bacteria (particularly the attached bacteria community) have been reported by Rooney-Varga et al. (2005). *Cyanobacteria* almost completely dominated the bacterial community at the hypereutrophic Mira Lake where pH and conductivity were very high, as reported for other eutrophic lakes (De Wever et al., 2005; Van Der Gucht et al., 2005; de Figueiredo et al., 2006). A specific *Actinobacteria*l species proved to be associated with most reservoirs (except Azibo reservoir) and all lakes. This dominance of *Actinobacteria* in lentic water systems has been described previously (Burkert et al., 2003; De Wever et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006a).

In general, the environmental parameters indicate that the trophic status of the studied water bodies during the winter 2004/5 deteriorated in relation to previous years, according to time-series data from the INAG, suggesting that the drought felt in 2005 had an influence on the normal characteristics of the water. Conductivity was higher, and as this significantly positively correlates with pH, it may also have had an influence on the bacterial assemblage of these water bodies given that pH has been shown to be an important environmental parameter for the determination of bacterial assemblages (Lindstr¨om et al., 2005). A small alteration in pH may thus induce determinant changes in the composition of the autochthonous bacterial community leading to the dominance of groups such as *Bacteroidetes, Alphaproteobacteria* and *Cyanobacteria*. Mesocosm experiments such as that described by Torsvik et al. (1998) could provide important information to verify these changes. Historical data concerning bacterial assemblages in the studied water bodies would also be essential to determine whether the winter 2004/5 drought (with an accentuated pH increase in some water bodies) indeed caused major changes in the dominant bacterial groups. The

lack of such historical studies also makes this study an important contribution to the construction of a phylogenetic database with relevant sequence information obtained from the extracted environmental DNA of the studied water bodies for future comparative studies.

References

Allgaier M. & Grossart H. P. (2006). Diversity and seasonal dynamics of *Actinobacteria* populations in four lakes in Northeastern Germany. *Applied and Environmental Microbiology* 72: 3489-3497.

Altmann D., Stief P., Amann R., Beer D. d. & Schramm A. (2003). *In situ* distribution and activity of nitrifying bacteria in freshwater sediment. *Environmental Microbiology* 5: 798-803.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Barros P., Implicações ecotoxicológicas de cianobactérias em cladóceros. Faculty of Sciences and Technology, Vol. MSc. University of Coimbra, Coimbra, 1994.

Böckelmanna U., Manz W., Neu T. R. & Szewzyk U. (2000). Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe River of Germany by cultivation and molecular methods. *FEMS Microbiology Ecology* 33: 157-170.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Burkert U., Warnecke F., Babenzien D., Zwirnmann E. & Pernthaler J. (2003). Members of a readily enriched b-proteobacterial clade are common in surface waters of a humic lake. *Applied and Environmental Microbiology* 69: 6550-6559.

Cerqueira M. A., Silva J. F., Magalhães F. P., Soares F. M. & Pato J. J. (2008). Assessment of water pollution in the Antuã River basin (Northwestern Portugal). *Environmental Monitoring and Assessment* 142: 325-335.

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Cottrell M. T., Waidner L. A., Yu L. & Kirchman D. L. (2005). Bacterial diversity of metagenomic and PCR libraries from the Delaware River. *Environmental Microbiology* 7: 1883-1895.

Craun G. F., Nwachuku N., Calderon R. L. & Craun M. F. (2002). Outbreaks in drinking-water systems, 1991-1998. *Journal of Environmental Health* 65: 16-23.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Harper D., 1992. Eutrophication of freshwaters: principles, problems and restoration. Chapman et Hall, London.

Hollibaugh J. T., Wong P. S. & Murrell M. C. (2000). Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California. *Aquatic Microbial Ecology* 21: 103-114.

Hullar M. A. J., Kaplan L. A. & Stahl D. A. (2006). Recurring seasonal dynamics of microbial communities in stream habitats. *Applied and Environmental Microbiology* 72: 713-722.

Kisand V., Cuadros R. & Wikner J. (2002). Phylogeny of Culturable Estuarine Bacteria Catabolizing Riverine Organic Matter in the Northern Baltic Sea. *Appl. Environ. Microbiol.* 68: 379-388.

Lee H.-W., Lee S.-Y., Lee J.-W., Park J.-B., Choi E.-S. & Park Y. K. (2002). Molecular characterization of microbial community in nitrate-removing activated sludge. *FEMS Microbiology Ecology* 41: 85-94.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

López-Archilla A. I., Gérard E., Moreira D. & López-García P. (2004). Macrofilamentous microbial communities in the metal-rich and acidic River Tinto, Spain. *FEMS Microbiology Letters* 235: 221-228.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Mašín M., Jezbera J., Nedoma J., Straškrabová V., Hejzlar J. & Šimek K. (2003). Changes in bacterial community composition and microbial activities along the longitudinal axis of two canyon-shaped reservoirs with different inflow loading. *Hydrobiologia* 504: 99-113.

Mazur-Marzec H. (2006). Characterization of phycotoxins produced by cyanobacteria. *Oceanological and Hydrobiological Studies* 35: 85-109.

Muylaert K., Van der Gucht K., Vloemans N., De Meester L., Gillis M. & Vyverman W. (2002). Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. *Applied and Environmental Microbiology* 68: 4740-4750.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G. & Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 73: 127-141.

Nijburg J. W. & Laanbroek H. J. (1997). The influence of *Glyceria maxima* and nitrate input on the composition and nitrate metabolism of the dissimilatory nitrate-reducing bacterial community. *FEMS Microbiology Ecology* 22: 57-63.

O'Sullivan L. A., Weightman A. J. & Fry J. C. (2002). New degenerate *Cytophaga-Flexibacter-Bacteroides*-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in river taff epilithon. *Applied and Environmental Microbiology* 68: 201-210.

Pearce D. A., van der Gast C. J., Woodward K. & Newsham K. K. (2005). Significant changes in the bacterioplankton community structure of a maritime Antartic freshwater lake following nutrient enrichment. *Microbiology* 151: 3237-3248.

Phung N. T., Lee J., Kang K. H., Chang I. S., Gadd G. M. & Kim B. H. (2004). Analysis of microbial diversity in oligotrophic microbial fuel cells using 16S rDNA sequences. *FEMS Microbiology Letters* 233: 77-82.

Riemann L. & Winding A. (2001). Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microbial Ecology* 42: 274-285.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Rooney-Varga J. N., Giewat M. W., Savin M. C., Sood S., Legresley M. & Martin J. L. (2005). Links between phytoplankton and bacterial community dynamics in a coastal marine environment. *Microbial Ecology* 49: 163-175.

Sabater S. & Munoz I. (1990). Successional dynamics of the phytoplankton in the lower part of the River Ebro. *Journal of Plankton Research* 12: 573-592.

Šimek K., Pernthaler J., Weinbauer M. G., Horňák K., Dolan J. R., Nedoma J., Mašín M. & Amann R. (2001). Changes in Bacterial Community Composition and Dynamics and Viral Mortality Rates Associated with Enhanced Flagellate Grazing in a Mesoeutrophic Reservoir. *Applied and Environmental Microbiology* 67: 2723-2733.

Stein L. Y., Duc M. T. L., Grundl T. J. & Nealson K. H. (2001). Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environmental Microbiology* 3: 10-18.

Urbach E., Vergin K., Young L. & Morse A. (2001). Unusual bacterioplankton community structure in ultra-oligotrophic Crater Lake. *Limnology and Oceanography* 46: 557-572.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Vasconcelos V., 2001. Freshwater cyanobacteria and their toxins in Portugal. In: I. Chorus, (ed.), Cyanotoxins - Occurrence, Causes, Consequences. Springer, Berlin: 62-67.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Zwart G., Crump B. C., Kamst-van Agterveld M. P., Hagen F. & Han S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* 28: 141-155.

Dominant bacterioplankton phylotypes in Portuguese water bodies under a severe summer drought scenario – an overview of the future?

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Abstract

During the year of 2005 one of the strongest droughts over the last decades was observed for the Portuguese territory. In the region of Alentejo (southern Portugal) those effects were more intense, with several reservoirs becoming dry. A set of 20 Portuguese water bodies were analysed through denaturing gradient gel electrophoresis (16SrDNA-DGGE) to assess its bacterioplankton diversity under these particular conditions. The trophic status of the water bodies was evaluated though determination of parameters such as pH, conductivity, total suspended solids and chlorophyll *a*, soluble reactive phosphorus, nitrite, nitrate and ammonium concentrations. Most of the water bodies fell into the meso- to eutrophic and hypereutrophic state, except for Caldeirão reservoir which showed oligotrophic conditions. In general, the levels for conductivity, pH and total suspended solids at the studied water bodies were higher than the average data from previous years. Generally, dominant phylotypes belonged to *Cyanobacteria*, *Actinobacteria* *Alphaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes* groups. *Bacteroidetes* dominance was observed at Sousa River and related to high nutrient levels while *Cyanobacteria* (Nostocales and Oscillatoriales) dominated in Alentejo reservoirs and higher conductivity values. However, *Actinobacteria* showed ubiquitous phylotypes throughout several samples suggesting its persistence over geographically distant water bodies. Further investigation should be conducted to assess the real impact of the drought on bacterioplankton communities at these water bodies by comparing to different climatic conditions on subsequent summers.

Keywords

Portuguese water bodies, drought, bacterial diversity, 16SrDNA-DGGE analysis.

Introduction

The concern with the global climatic changes is increasing worldwide since episodes such as extreme precipitation or drought are becoming more frequent and the maintenance of water quality in freshwater bodies may be endangered with potential increase of waterborne disease outbreaks (Charron et al., 2004; Masciopinto et al., 2007; Greer et al., 2008) which makes the establishment of water management strategies indispensable (Bond et al., 2008b). Bacterial communities are greatly affected by these environmental oscillations and can rapidly change their composition (Van der Gucht et al., 2001; Muylaert et al., 2002; Jardillier et al., 2005). The eutrophication of freshwater systems is also enhanced by drought conditions, particularly in shallow water bodies with high water retention time, leading to the development of bacterial groups such as *Alphaproteobacteria*, *Cyanobacteria* and *Actinobacteria* (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007) that may put at risk the water quality (Izaguirre & Taylor, 1995; Zaitlin & Watson, 2006; Smith et al., 2008) and the human health safety (Pouria et al., 1998; de Figueiredo et al., 2004b; Mazur-Marzec, 2006). Interestingly, at a large spatial scale, the composition of bacterial assemblages seems to be mainly explained by differences in environmental conditions rather than biogeography (Van Der Gucht et al., 2007) which suggests there may be a transversal global pattern for similar freshwater bodies under common environmental contexts. This could help in the development of management strategies to mitigate drought outcomes.

In 2005, Portugal suffered one of the most intense droughts over the last fifty years, according to time series data made available by the Water Resources Information National System (http://snirh.pt/) with a trend for water bodies eutrophication (de Figueiredo et al., 2007). In Alentejo region drought conditions are recurrent (Caetano et al., 2001) but the summer of 2005 brought severe consequences with some reservoirs becoming dry as reported by the National Commission for Drought in 2005 (http://www.inag.pt/inag2004/port/divulga/actualidades/seca/relatorios/RelatorioParlamento.pdf). For Portuguese freshwater bodies there is a lack of information concerning their bacterioplankton communities although several reports on *Cyanobacteria* occurrence have been published over the last decade (Vasconcelos et al., 1996; Vasconcelos, 1999; Vasconcelos, 2001; Saker et al., 2003; Valério et al., 2005; de Figueiredo et al., 2006).

The present study aimed to explore if there can be a general biogeographical pattern for Portuguese bacterioplankton assemblages under the severe climatic conditions of drought occurring in 2005. The diversity of dominant bacterioplankton phylotypes in 20 freshwater bodies from Northern and Southern Portugal was analysed using denaturing gradient gel electrophoresis (16SrDNA-DGGE), a culture-independent molecular methodology (Muyzer et al., 1993; Lyautey et al., 2005).

Materials and Methods

Sampling and environmental parameters

A set of 20 samples was taken from river sections, reservoirs and lakes belonging to Douro, Vouga, Mondego, Sado and Guadiana River Basins (Table 1 and Fig. 1). Samples are spread throughout the NorthCentral and Southern regions of Portugal in a range of 379 Km. The reservoirs are of great importance for the water supply of surrounding populations, particularly at the Alentejo region. During the end of July and beginning of August 2005, water samples were taken sub-superficially using sterile bottles at about 1 m from the shore avoiding sediment collection. Samples were placed at 4ºC under dark conditions until subsequent treatment within hours. The pH and conductivity were measured at 25ºC by using specific electrodes. Since temperature and oxygen levels can have major oscillations during the day they were not considered in the present comparative study. Parameters such as the total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium (N-NH_4), nitrate (N-NO_3) and nitrite (N-NO_2) concentrations were determined in laboratory according to standard procedures (APHA, 1992; Rodier, 1996).

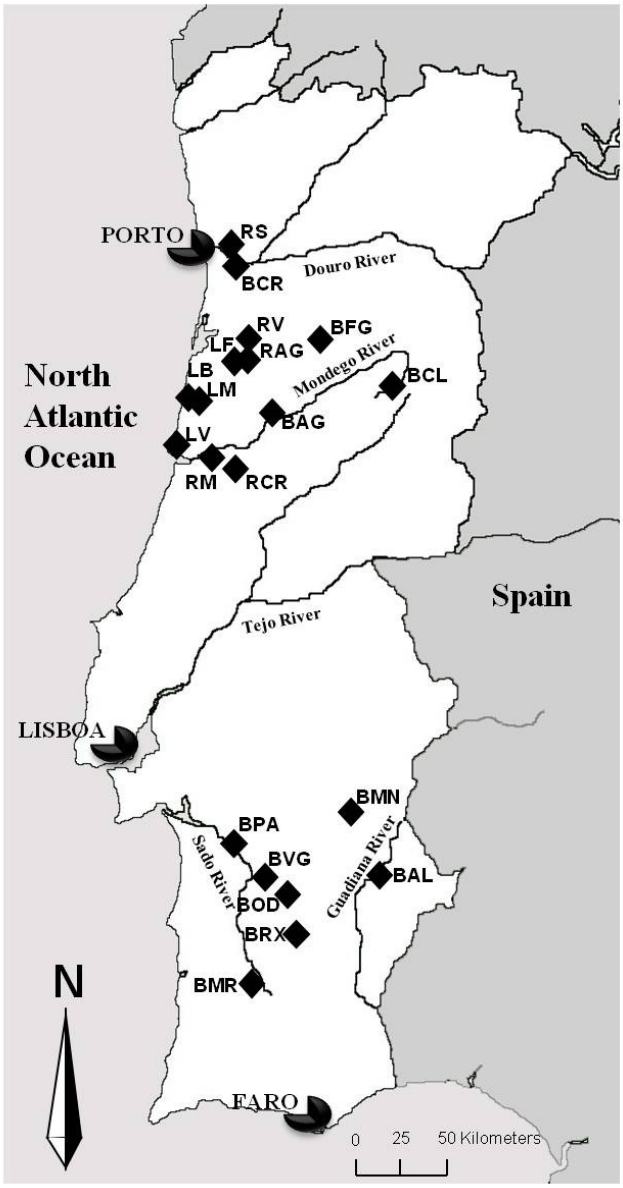


Fig. 1. Geographical location of the sampled water bodies (the corresponding codes are presented in Table 1).

DNA extraction

Total DNA from environmental water samples was extracted by filtering 150 mL of the water samples through 0.22 µm polycarbonate sterile filters, resuspension in 2 mL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0] and centrifugation. Lysozyme was added after resuspension in 200 µL of TE and incubation was performed at 37ºC for 1h. The following

DNA extraction and purification were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). DNA was finally suspended in TE buffer and stored at -20 ºC.

PCR amplification of bacterial 16S rDNA fragments

PCR amplification of bacterial *16S rRNA* gene fragments was performed with the primers 338F-GC/518R (Muyzer et al., 1993), universal for bacteria. Primers were synthesized by STABVida (Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 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 30s at 92 °C, 30s at 55 °C and 30s at 72 °C, and a final extension step at 72 °C for 30 min. Negative control reactions without any template DNA were performed simultaneously. The PCR amplicons were electrophoresed in a 1.5% agarose gel and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder). The gel was stained with ethidium bromide and visualized on a UV transmilluminator.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analyzed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1mm 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, California, USA) using 0.5x 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, California, USA).

DGGE bands excision, cloning and sequencing

The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL of sterile milli-Q-purified water from which 5 µL of the eluted DNA was used in PCR amplification with the original primer pair. The isolation and identity of each DNA band was verified through DGGE and, if necessary, the extraction procedure was repeated until the targeted band isolation was obtained. The isolated bands were then cloned using the TOPO TA cloning kit with the pCR 2.1-TOPO vector (Invitrogen, Portugal). Prior to cloning, an A tailing was performed for PCR products according to manufacturers' instructions. In order to screen for false positive clones, the size of the amplicons from PCR using the vector primers M13R/T7F was checked in 1.5% agarose gels as described above using the molecular weight marker GeneRuler™ 1 kb DNA ladder. The migration point of each cloned sequence with

Table 1. Characterization of the studied water bodies and environmental data recorded in summer 2005.

Hydrographical River Basin	Location	Original water body	Reservoir/Lake	Code	Month average water retention levels * (dam^3) Summer 2005	Before 2005	Chl *a* ($\mu g\ L^{-1}$)	TSS ($mg\ L^{-1}$)	pH
Vouga	Macinhata do Vouga, Águeda	Vouga River	-	RV	-	-	18.7	7.46	7.
Vouga	Águeda, Águeda	Águeda River	-	RAG	-	-	3.6	3.72	7.
Vouga	Fermentelos, Águeda	Cértima River	Fermentelos Lake	LF	-	-	17.1	**82.00**	8.
Vouga	Mira, Mira	-	Mira Lake	LM	-	-	38.8	19.13	9.
Vouga	Mira, Mira	-	Barrinha de Mira Lake	LB	-	-	112.4	25.80	**9.**
Douro	Crestuma, Porto	Douro river	Crestuma reservoir	BCR	100028	103101 (1990-2004)	9.1	3.91	7.
Douro	Foz do Sousa, Porto	Sousa River	-	RS	-	-	5.5	9.62	7.
Mondego	Quiaios, Figueira da Foz	-	Vela Lake	LV	-	-	**159.9**	41.13	8.
Mondego	S. Martinho do Bispo, Coimbra	Mondego River	-	RM	-	-	2.3	**2.22**	7.
Mondego	Ceira, Coimbra	Ceira River	-	RCR	-	-	110.2	16.35	7.
Mondego	Aguieira, Penacova	Mondego River	Aguieira reservoir	BAG	328343	338255 (1990-2004)	49.1	11.88	9.
Mondego	Caldeirão, Guarda	Ribeira do Caldeirão	Caldeirão reservoir	BCL	3918	3717 (1994-2004)	**1.4**	2.37	7.
Mondego	Fagilde, Viseu	Dão River	Fagilde reservoir	BFG	1175	1916 (2006-2008)	7.9	4.83	7.
Guadiana	Évora, Évora	Degebe River	Monte Novo reservoir	BMN	6910	11829 (1990-2004)	11.2	3.77	8.
Guadiana	Moura, Beja	Guadiana River	Alqueva reservoir	BAL	-	-	3.6	2.76	8.
Sado	Aljustrel, Beja	Ribeira do Roxo	Roxo reservoir	BRX	16527	32455 (1967-2004)	12.8	31.50	8.
Sado	Alcácer do Sal, Setúbal	Rio Xarrama	Vale do Gaio reservoir	BVG	6112	33266 (1990-2004)	29.4	12.84	8.
Sado	Ferreira do Alentejo, Beja	Ribeira de Odivelas	Odivelas reservoir	BDV	36714	52498 (1972-2004)	8.5	30.20	8.
Sado	Alcácer do Sal, Setúbal	Ribeira das Alcáçovas	Pego do Altar reservoir	BPA	21907	51168 (1990-2004)	2.9	5.62	8.
Sado	Ourique, Beja	Sado River	Monte da Rocha reservoir	BMR	30276	58010 (1990-2004)	2.1	25.26	8.

* The average water retention levels were obtained from Portuguese Water Institute (http://snirh.pt/).

the targeted size was verified through DGGE after a nested PCR amplification with the primer pair 338F-GC and 518R. However, sequencing was made with PCR amplicons using the vector primers M13R/T7, optimizing the sequence length information. Each amplicon was purified with the concertTM rapid PCR purification system (Gibco BRL, Eggenstein, Germany) before it was commercially sequenced (STABVida, Portugal).

Phylogenetic analysis

The bacterial 16S rDNA partial sequences determined in the present study were submitted to the GenBank database. A BLAST search (http://www.ncbi.nlm.nih.gov) was used to explore similarity of the sequences obtained in this study against sequences deposited in the GenBank database. The alignment of the partial 16S rDNA sequences was carried out using the CLUSTAL X software version 1.8 (Thompson et al., 1994). A phylogenetic tree was built using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 replicates. TreeView version 1.6.6 (Page, 1996) was used to display the trees.

Statistical analysis

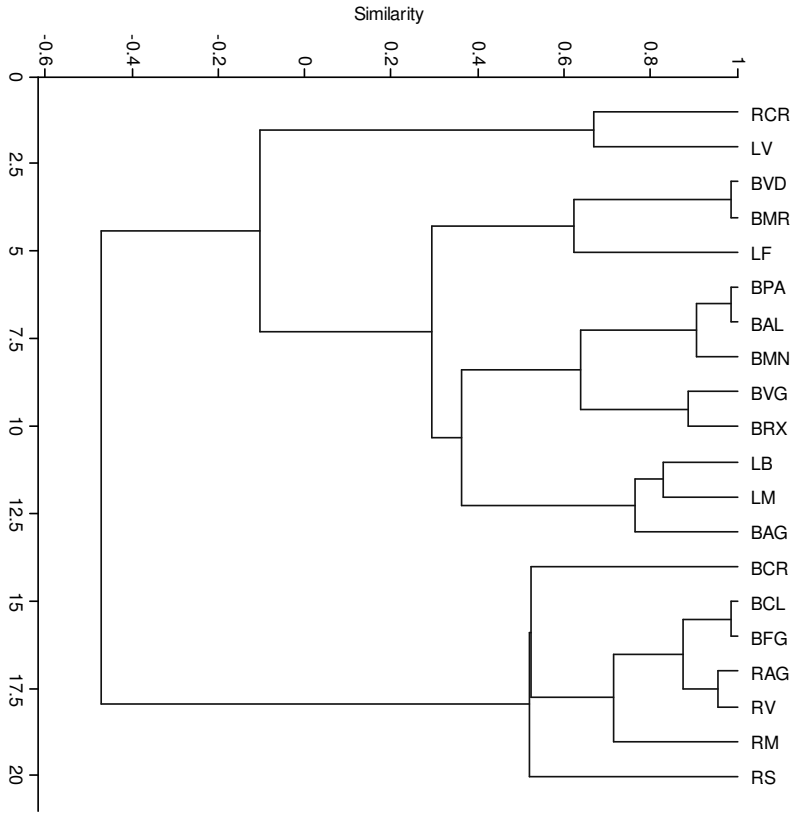
Principal component analysis (PCA) was used to assess the distribution of samples according to environmental parameters after data standardization (subtracting the mean from each observation and dividing by the corresponding standard deviation) (ter Braak, 1995). Cluster analysis of samples according to environmental parameters was also executed using the UPGMA. The dendrogram was created with the similarities calculated using the Pearson correlation coefficient (95% probability) which was also used to assess significant correlations within environmental parameters and between these and dominant bands in DGGE profiles.

Results

Environmental parameters

The sample codes used for the samples and the corresponding environmental parameters are summarised in Table 1. The values for pH varied between 7.24 and 9.47, at Raiva reservoir and Barrinha de Mira Lake, respectively. Ceira River showed the minimum conductivity level (6 µS cm^{-1}) recorded while Roxo reservoir showed the maximum value (1512 µS cm^{-1}). Sousa River attained the highest concentrations for nitrate [30.8 mg N (NO_3^-) L^{-1}] and soluble reactive phosphorus (1.32 mg L^{-1}) while Mondego River showed the highest records for the other nitrogen sources [0.47 mg N (NO_2^-) L^{-1} and 1.18 mg N (NH_4^+) L^{-1}]. Chlorophyll *a* concentration ranged between 1.4 and 159.9 µg L^{-1} at Caldeirão reservoir and Vela Lake, correspondingly. Total suspended solids had minimum values at Mondego River and Caldeirão reservoir (2.22-2.37 mg L^{-1}) and a maximum of 82.00 mg L^{-1} at Fermentelos Lake. INAG records also show that during summer 2005 the water retention levels in Portuguese reservoirs, particularly at Alentejo region,

(a)



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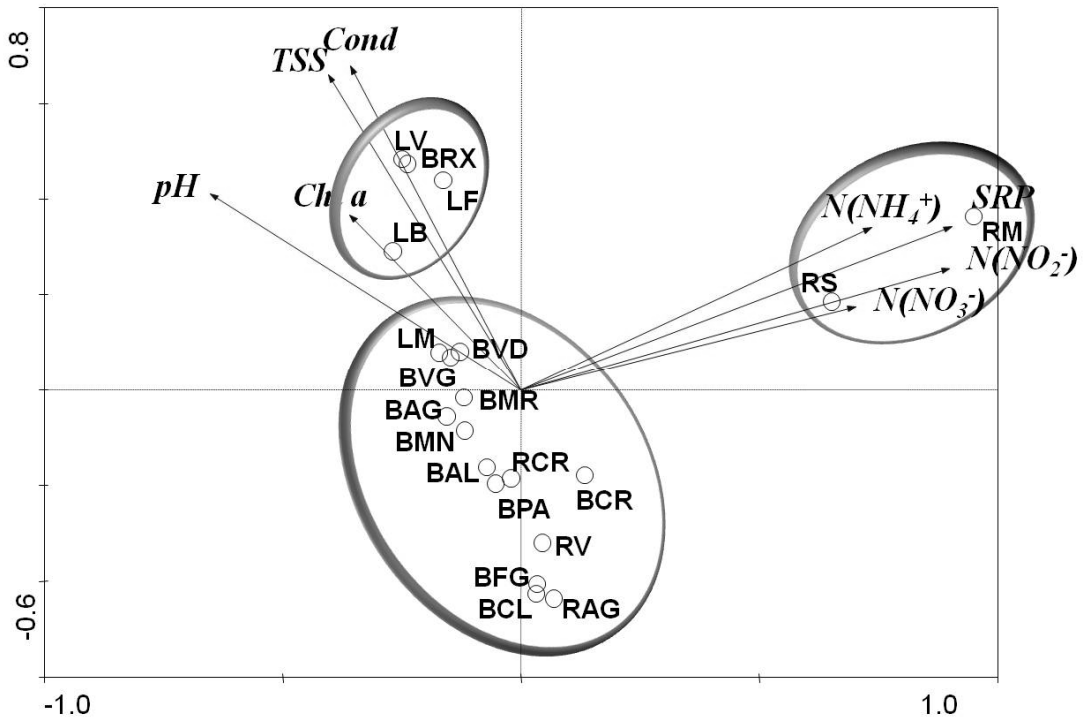

Fig. 2. (a) cluster dendrogram and (b) PCA ordination biplot of sampled water bodies according to environmental parameters recorded in summer 2005 (see sample codes in table 1).

were far below the average from the last half century (see Table 1). The Pearson correlation coefficient was calculated to assess significant relationships ($P < 0.05$; $n = 20$) between the recorded environmental parameters. Conductivity showed to be positively correlated with pH ($r = 0.49$) and total suspended solids ($r = 0.45$). Nitrite concentrations showed significant positive

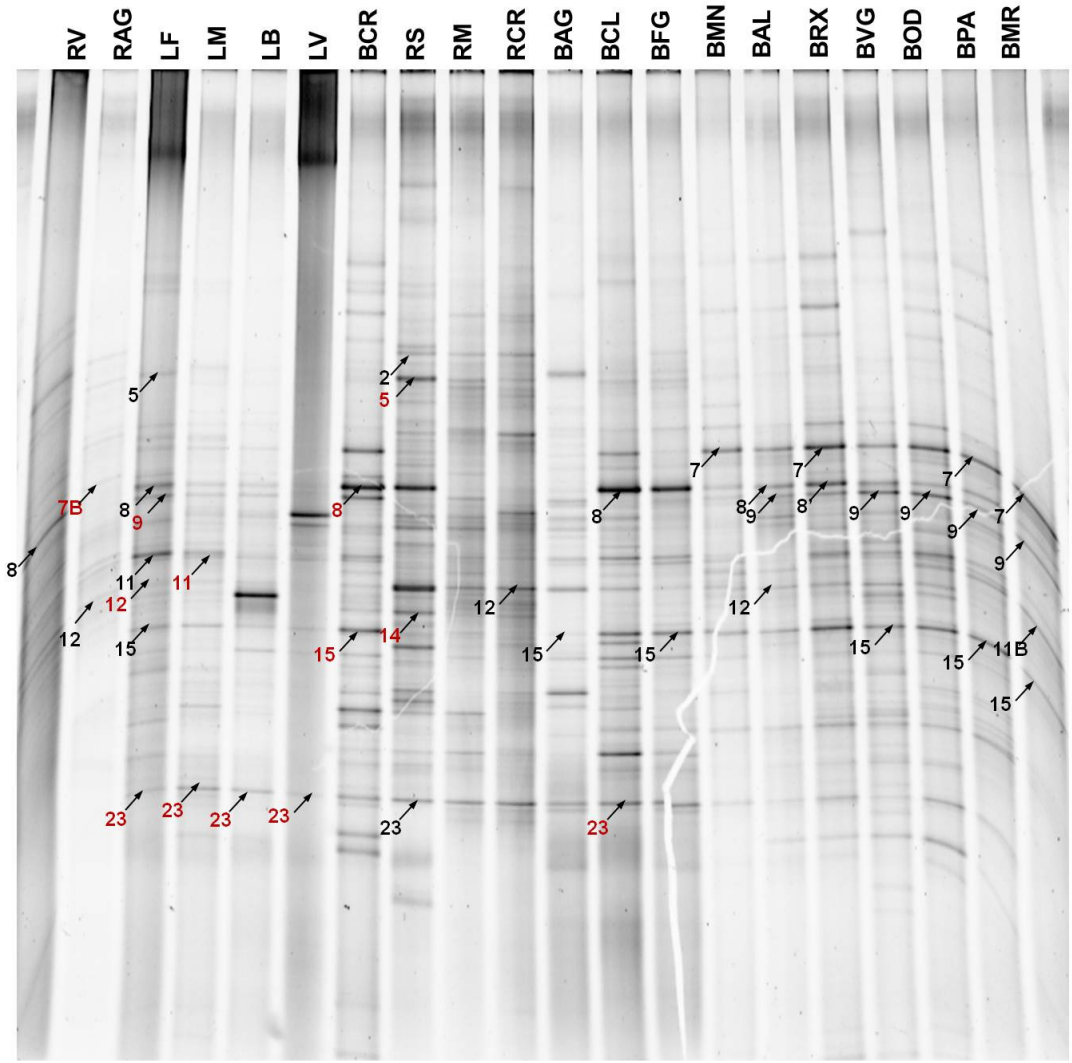

Fig. 3. DGGE profiles containing the bacterial 16S rDNA fragments from Portuguese water bodies sampled in summer 2005. The code above each lane refers to each sampled water body (see table 1); bands in black were excised and sequenced and bands in red correspond to migration points inferred from seasonal DGGE patterns from each sampling site (data not shown) and not direct sequencing.

correlations with ammonium ($r = 0.91$), soluble reactive phosphorus ($r = 0.83$) and nitrate levels ($r = 0.47$). Soluble reactive phosphorus was also shown to have highly significant correlations with nitrate ($r = 0.85$) and ammonium ($r = 0.66$). The dendrogram shown in Fig. 2a evidences the clustering of samples according to its trophic characteristics and PCA analysis (Fig. 2b) showed a gradient based on concentration of inorganic nutrient sources, with the evident segregation of the samples RM and RS (having the highest nutrient levels) in relation to the other samples. Another

gradient was observed, mostly related to conductivity levels and concentration of total suspended solids; this axis was defined by BRX and the hypereutrophic lakes LF and LV on the positive side whereas RAG and BCL defined its negative side. These first two axes could explain 63.0% of the total variance of samples distribution.

DGGE profiles and sequenced bands

Interestingly, according to Fig. 3, the DGGE profiles of the partial 16S rDNA sequences showed that in spite of the high variability among the bacterioplankton composition at the 20 water bodies there were bands common to several profiles from the studied reservoirs, suggesting the

Table 2. Accession number and phylogenetic affiliation for the sequenced bacterial DGGE bands.

band	Samples	Phylogenetic affiliation	Closest relatives (accession Nº)	Percentage similarity (%)
2	RS	*Bacteroidetes*	Uncultured **Flavobacteriaceae** bacterium clone LW18m-1-69 (EU642285)	100
5	LF, RS	*Betaproteobacteria*	Uncultured *Curvibacter* sp. clone FL_8 (HQ008565)	100
7	BRX, BPA, BMR, BMN	*Alphaproteobacteria*	Uncultured Sphingomonadales bacterium clone HS-S-207 (HM592611)	100
7B	RAG	*Alphaproteobacteria*	*Sphingomonas* sp. BF64A_I5 (HM141526)	100
8	BCR, BCL, LF, RV	*Cyanobacteria*	Uncultured *Synechococcus* sp. clone WR77 (HM208511)	100
8	BRX, BAL	*Cyanobacteria*	*Leptolyngbya fragilis* OL 03 (AM398794)	98
9	BPA, BMR, BDV, BAL, BVG, LF	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Jab PL1W2D10 (HM486215)	100
11	LF, LM	*Cyanobacteria*	Uncultured *Synechococcus* sp. clone 97 (FN860052)	100
11	BMR	*Cyanobacteria*	*Aphanizomenon gracile* LMECYA 148 (EU078533)	99
12	LF, RCR, RAG	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Jab PL2W2A1 (HM486249)	100
12	BAL	*Bacteroidetes*	*Flavobacterium* sp. ARSA-19 (GU295970)	100
14	RS18_cl32	*Actinobacteria*	Uncultured actinobacterium clone ZS-2-33 (FN668214)	99
15	BCR	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Ac70F5 (GU472674)	100
15	LF, BPA, BMR, BVG, BFG, BAG	*Actinobacteria*	Uncultured actinomycete clone CR-FL3 (AF141389)	100
23	LF, LM, LB, LV, RS, BCL	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Jab PL2W2F8 (HM486295)	100

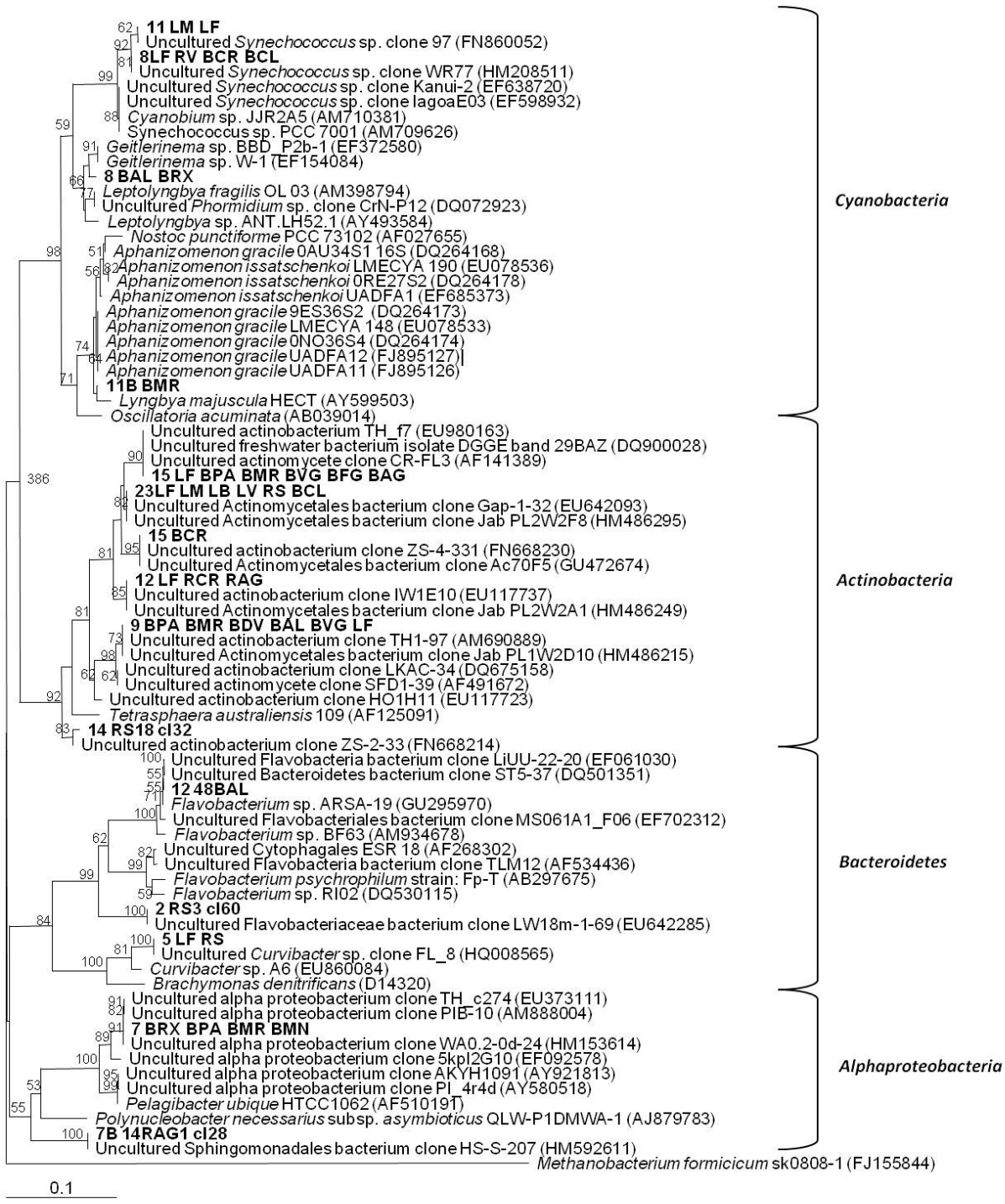

Fig. 4. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel (Fig. 3). The archaeal sequence from *Methanobacterium formicicum* strain sk0808-1 was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

prevalence of some phylotypes under these drought conditions. DGGE patterns from Alentejo reservoirs, in particular, showed to be rather similar. The phylogenetic affiliation of the sequenced DGGE bands (most intense) is presented in Table 2 and Fig. 4. Most intense 16S rDNA partial sequences belonged to the *Actinobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Betaproteobacteria* and *Alphaproteobacteria* groups. Interestingly, the sequence analysis gathered with information from other previous studies (data not shown) allowed evidencing that some migration points from geographically distant freshwater bodies corresponded, in fact, to a same phylotype. This was the case of migration points 9 (with southern and northern samples) and 23 (with samples from northern and central Portugal). However, in some cases (as in migration points 8, 11, 12 and 15), although *a priori* correspondence could be established among different sites, sequence results showed that there were more than one phylotype corresponding to a same migration point considered.

Discussion

Environmental parameters and trophic status *of the water bodies*

According to the clustering and PCA analyses a gradient could be observed regarding the trophic status of the studied water bodies. This gradient began with the oligotrophic Caldeirão reservoir along with the mesotrophic Águeda River and Fagilde reservoir and goes towards the eutrophic and nutrient-rich Sousa and Mondego Rivers and the hypereutrophic Fermentelos and Vela Lakes. The main factors behind this trophic gradient include conductivity, total suspended solids, chlorophyll *a* and nutrients concentration.

According to INAG time series data, the year of 2005 had precipitation levels far below average for the last 60 years which directly determined the low water retention levels recorded in summer 2005 at the Portuguese dams, especially at Alentejo region (Southern Portugal). Environmental parameters recorded in this study showed differences by comparing to time series data from previous years. In general, the environmental parameters showed to be typical of drought conditions with generally high conductivity values as well as high pH and total suspended solids records by comparing to historical average data from INAG. Conductivity values at Sousa River, Vouga River, Mondego River, Crestuma reservoir, Monte de Rocha reservoir, Odivelas reservoir, Vale do Gaio reservoir, Pego do Altar reservoir, Aguieira reservoir, Fermentelos Lake and Vela Lake were way above the summer average values previosuly recorded. Conductivity at Roxo reservoir reached extremely high levels (a new maximum since 1995, according to data from INAG) which must be still linked to acid mine drainage from mill tailings impoundments of abandoned pyrite extraction mines in Aljustrel (Monteiro et al., 1995; Bobos et al., 2006). However, pH was high at Roxo reservoir which has been observed for other similar water bodies where huge seasonal shifts in pH and toxicity are observed with acidic pH recorded only in raining

season (Antunes et al., 2007). The pH recorded at Sousa River, Águeda River, Vouga River, Monte da Rocha reservoir, Crestuma reservoir, Aguieira reservoir, Caldeirão reservoir and Fermentelos Lake was also above the average values recorded for summer months over the last years. Total suspended solids at Monte da Rocha reservoir, Roxo reservoir and Odivelas reservoir were way above the average summer values recorded before. At Odivelas reservoir and Vela Lake, the Chl *a* concentration was also higher than the average from the last years. Sousa and Mondego rivers showed very high nitrate and SRP concentrations which may lead to accelerated eutrophication and nuisance algal or cyanobacterial blooms and,ultimately, to fish kills (due to the increased turbidity and depletion of dissolved oxygen (Harper, 1992). Thus, under drought conditions, industrial and domestic eflluents as well as the runoff from nutrient enrichment of agriculture fields should be even more reduced due to the augmentation of their impact on the water dodies which water level is severely decreased.

Bacterial DGGE profiles and dominant phylotypes

Interestingly, the DGGE profile analysis of the partial 16S rDNA sequences showed common bacterial phylotypes among several water bodies (as confirmed through sequencing) which suggests that there may be phylotypes transversely spread over the Portuguese territory under the environmental conditions recorded in this study. This aspect should be further explored in future work on bacterioplakton from Portuguese water bodies. Nevertheless, it has been previously recorded that, at a large spatial scale, the bacterial community composition of similar habitats from distant geographic regions (up to several thousands of km) is mainly explained by regional differences in environmental conditions rather than biogeography, e.g. lakes with similar environmental characteristics (shallow and meso- to eutrophic) have similar bacterial assemblages despite of their geographic distance (Van Der Gucht et al., 2007). Thus, biogeographic patterns will be mainly observed in studies focusing on similar habitats no matter what spatial scale is being considered (Van Der Gucht et al., 2007). This is due to the fact that bacterial communities can rapidly change according to environmental variations (Van der Gucht et al., 2001; Muylaert et al., 2002; Jardillier et al., 2005). However, for other migration points the *a priori* correspondence established among different sites showed to be erroneous, matching different sequences; the co-migration of bands and shortness of fragments are recognized limitating factors in DGGE (Dorigo et al., 2005; Marzorati et al., 2008), that may lead to this kind of ambiguity, not allowing a higher discrimination in the DGGE profiles. This highlights the limitations of DGGE analysis using these primers to compare band patterns from different locations.

The recorded groups *Cyanobacteria, Actinobacteria*, *Bacteroidete, Betaproteobacteria* and *Alphaproteobacteria* are characteristic from shallow eutrophic freshwater bodies (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007). The dominance of *Cyanobacteria* in lakes is frequent worldwide and it is usually associated with characteristics such

as high temperatures and low TN/TP ratios in turbid waters along with high pH and conductivity values (Codd, 2000; Dokulil & Teubner, 2000; De Wever et al., 2005; Van Der Gucht et al., 2005; de Figueiredo et al., 2006) such as the Alentejo reservoirs and Vela Lake, for instance. In Alentejo, cyanobacteria are frequently found forming blooms during summer months with production of toxins (Vasconcelos et al., 1996; 2001; Caetano et al., 2001; Moreno et al., 2003).The partial 16S rDNA sequences which had the filamentous cyanobacteria oscillatoriacean *Geitlerinema* and *Leptolyngbya* was found to be dominant at Roxo and Alqueva reservoirs whereas at Monte da Rocha reservoir, an *Aphanizomenon gracile* phylotype (similar to others from Portuguese and Northern Europe freshwaters) was dominant. At the Guadiana River Basin, summer cyanobacterial blooms occur frequently with the dominance of species from genera such as *Microcystis*, *Anabaena*, *Gomphosphaeria*, *Coelosphaerium*, *Lyngbya, Aphanizomenon*, *Oscillatoria*, *Merismopedia*, *Nostoc* and *Synechococcus* genera (Caetano et al., 2001; Rocha et al., 2002; Moreno et al., 2004; Domingues et al., 2005; Moreno et al., 2005). For the Sado River Basin the published research on cyanobacteria is very scarce (Monteiro et al., 1995) but the presence of *Cylindrospermopsis raciborski* has been reported at the Odivelas reservoir (Odivelas River) (Saker et al., 2003; Valério et al., 2005). In spite of these parcelled studies focused on cyanobacterial occurrence in Guadiana and Sado River Basins, there is no published information concerning their bacterioplankton composition as a whole. After qualitative microscope observation of samples (data not shown) it was possible to detect at Alqueva reservoir the presence of several chroococcalean colonial cyanobacteria such as *Gomphosphaeria* spp. but also nostocalean cyanobacterial species belonging to *Anabaena* and *Aphanizomenon* genera. Domingues et al. (2005) registered that when N:P values become lower and temperature higher, cyanobacteria start to replace green algae favouring the development of nitrogen-fixing cyanobacteria such as *Anabaena* sp.. Thus, under the general N unavailability recorded at the studied reservoirs, heterocyst-forming cyanobacteria may still develop massively although phosphorus unavailability usually limits their growth and favour cyanobacteria able to storage P (Dokulil & Teubner, 2000). Through microscopic observation it was possible to detect at the studied Sado River Basin reservoirs the presence of cyanobacteria belonging to *Chroococcus*, *Oscillatoria* and *Merismopedia* genera at BMR and *Aphanizomenon flos-aquae* at BPA; members of the genera *Anabaenopsis*, *Aphanizomenon* and *Planktothrix* were observed at BRX, while *Anabaena* sp. and *Oscillatoria* sp. appeared at BVG and *Aphanizomenon gracile*, *Aph. aphanizomenoides*, *Aph. issatschenkoi* and *Anabaena* spp. at BVD. Thus, although the dominance of cyanobacteria in the bacterioplankton community was limited to a couple of most intense bands in the DGGE profiles, their presence was detected through microscopy indicating they may develop and form blooms in the subsequent weeks after the sampling was performed. Two phylotypes affiliated with *Synechococcus* sp. were found to be dominant at freshwater bodies from the northern region of Portugal. One was shared by

RV, LF, BCR and BCL and it showed a negative relation with chlorophyll *a* levels; the other was shared by LF and LM, two water bodies considered eutrophic (de Figueiredo et al., 2007; de Figueiredo et al., 2010b). Alentejo reservoirs showed to share the dominance of a Sphingomonadales phylotype which showed to be related with high pH and conductivity levels; usually, the *Alphaproteobacteria* dominance is linked to freshwater eutrophication (Eiler & Bertilsson, 2004; Van der Gucht et al., 2005; de Figueiredo et al., 2007). The *Flavobacteriales* (*Bacteroidetes* group) phylotypes showed an important representation at the Alqueva reservoir and Sousa River. The *Flavobacterium* dominance is frequent in European meso- and eutrophic rivers (Brümmer et al., 2000; de Figueiredo et al., 2007) and the *Bacteroidetes* group, in general, is also known to appear in eutrophic lakes with high conductivities (Van der Gucht et al., 2005). *Actinobacteria* are frequently dominant in lakes and reservoirs with high water retention time (De Wever et al., 2005; Van Der Gucht et al., 2005; Allgaier & Grossart, 2006a; de Figueiredo et al., 2007). Alentejo reservoirs, Aguieira reservoir and Fermentelos Lake shared a same dominant Actinomycetales phylotype which was related to high pH levels; this phylotype had already been recorded at other Portuguese water bodies, namely Azibo reservoir (de Figueiredo et al., 2007). Another phylotype affiliated with Actinomycetales bacteria showed to be ubiquitous to the majority of the studied water bodies. Most of the studied reservoirs are used as drinking water supply and the dominance of Actinomycetes, in particular, has been linked to problems such as odour and taste nuisance in drinking water (Zaitlin & Watson, 2006). Therefore, this fact along with the development of cyanobacteria as well, there is the potential for risks concerning the maintenance of water quality for human consumption.

Conclusion

In the summer of 2005, severe drought conditions were recorded with consequences on the water quality of the studied Portuguese water bodies, particularly at Alentejo reservois. In general, the values for conductivity were very high as well as pH, by comparing to time series data. The bacterioplankton phylotypes found to be dominant at the studied water bodies mainly belonged to the *Cyanobacteria*, *Actinobacteria*, *Alphaproteobacteria* and *Bacteroidetes* groups, as observed for other eutrophic water bodies. In spite of the cyanobacterial blooms recorded throughout the last decades, studies on the bacterioplankton dynamics of the studied freshwater systems are not known which restricts the historical analysis of our data and the assessment of the impact of drought conditions on the studied bacterial assemblages. Thus, the present work is a major contribution to the knowledge of the bacterioplankton community in Portuguese water bodies. Since many of these water bodies provide the population with supply for drinking water and sustain most of the agriculture irrigation needs of their respective region, it is essential to go further in their ecological characterization in order to develop effective management plans for drought episodes such as the

one described in this study. Interestingly, common bacterial phylotypes could be recorded among several water bodies, suggesting a transversal persistence of some phylotypes over the Portuguese territory, under severe drought conditions. Global warming is expected to increase the drought episodes over the South European countries, which makes crucial to conduct more studies such as the present one.

References

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Baptista M. S. & Vasconcelos M. T. (2006). Cyanobacteria metal interactions: requirements, toxicity and ecological implications. *Critical Reviews in Microbiology* 32: 127 - 137.

Bond N. R., Lake P. S. & Arthington A. H. (2008). The impacts of drought on freshwater ecosystems: An Australian perspective. *Hydrobiologia*: 1-14.

Caetano S., Miguel R., Mendes P., Galvão H. & Barbosa A. (2001). Cyanobacteria blooms and cyanotoxin occurrence in the Guadiana (SE Portugal) - preliminary results. *Ecotoxicology and Environmental Restoration* 4: 53-59.

Charron D. F., Thomas M. K., Waltner-Toews D., Aramini J. J., Edge T., Kent R. A., Maarouf A. R. & Wilson J. (2004). Vulnerability of waterborne diseases to climate change in Canada: A review. *Journal of Toxicology and Environmental Health - Part A* 67: 1667-1677.

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

de Figueiredo D. R., Pereira M. J. & Correia A. (2010). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26: 1067-1077.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Domingues R. B., Barbosa A. & Galvão H. (2005). Nutrients, light and phytoplankton succession in a temperate estuary (the Guadiana, south-western Iberia). *Estuarine, Coastal and Shelf Science* 64: 249-260.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Greer A., Ng V. & Fisman D. (2008). Climate change and infectious diseases in North America: The road ahead. *Canadian Medical Association Journal* 178: 715-722.

Harper D., 1992. Eutrophication of freshwaters: principles, problems and restoration. Chapman et Hall, London.

Izaguirre G. & Taylor W. D. (1995). Geosmin and 2-methylisoborneol production in a major aqueduct system. *Water Science and Technology* 31: 41-48.

Jardillier L., Boucher D., Personnic S., Jacquet S., Thénot A., Sargos D., Amblard C. & Debroas D. (2005). Relative importance of nutrients and mortality factors on prokaryotic community composition in two lakes of different trophic status: Microcosm experiments. *FEMS Microbiology Ecology* 53: 429-443.

Kothe E., Bergmann H. & Bu?chel G. (2005). Molecular mechanisms in bio-geo-interactions: From a case study to general mechanisms. *Chemie der Erde* 65: 7-27.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Masciopinto C., La Mantia R., Carducci A., Casini B., Calvario A. & Jatta E. (2007). Unsafe tap water in households supplied from groundwater in the Salento Region of Southern Italy. *Journal of Water and Health* 5: 129-148.

Mazur-Marzec H. (2006). Characterization of phycotoxins produced by cyanobacteria. *Oceanological and Hydrobiological Studies* 35: 85-109.

Monteiro M. T., Oliveira R. & Vale C. (1995). Metal stress on the plankton communities of Sado River (Portugal). *Water Research* 29: 695-701.

Moreno I., Repetto G., Carballal E., Gago A. & Cameán A. M. (2005). Cyanobacteria and microcystins occurrence in the Guadiana River (SW Spain). *International Journal of Environmental Analytical Chemistry* 85: 461-474.

Moreno I. M., Pereira P., Franca S. & Camea?n A. (2004). Toxic cyanobacteria strains isolated from blooms in the Guadiana River (southwestern Spain). *Biological Research* 37: 405-417.

Muylaert K., Van der Gucht K., Vloemans N., De Meester L., Gillis M. & Vyverman W. (2002). Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. *Applied and Environmental Microbiology* 68: 4740-4750.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Dorigo U., Volatier L. & Humbert J. F. (2005). Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Research* 39: 2207-2218.

Marzorati M., Wittebolle L., Boon N., Daffonchio D. & Verstraete W. (2008). How to get more out of molecular fingerprints: Practical tools for microbial ecology. *Environmental Microbiology* 10: 1571-1581.

Pouria S., De Andrade A., Barbosa J., Cavalcanti R. L., Barreto V. T. S., Ward C. J., Preiser W., Poon G. K., Neild G. H. & Codd G. A. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 352: 21-26.

Rocha C., Galvão H. & Barbosa A. (2002). Role of transient silicon limitation in the development of cyanobacteria blooms in the Guadiana estuary, south-western Iberia. *Marine Ecology Progress Series* 228: 35-45.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Saker M. L., Nogueira I. C. G., Vasconcelos V. M., Neilan B. A., Eaglesham G. K. & Pereira P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55: 243-250.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

ter Braak C. J. F., 1995. Ordination. In: R. H. G. Jongman, C. J. F. ter Braak & O. F. R. Tongeren, (eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, UK: 91-173.

Valério E., Pereira P., Saker M. L., Franca S. & Tenreiro R. (2005). Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. *Harmful Algae* 4: 1044-1052.

Van Der Gucht K., Cottenie K., Muylaert K., Vloemans N., Cousin S., Declerck S., Jeppesen E., Conde-Porcuna J. M., Schwenk K., Zwart G., Degans H., Vyverman W. & De Meester L. (2007). The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20404-20409.

Van der Gucht K., Sabbe K., De Meester L., Vloemans N., Zwart G., Gillis M. & Vyverman W. (2001). Contrasting bacterioplankton community composition and seasonal dynamics in two neighbouring hypertrophic freshwater lakes. *Environmental Microbiology* 3: 680-690.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Vasconcelos V., 2001. Freshwater cyanobacteria and their toxins in Portugal. In: I. Chorus, (ed.), Cyanotoxins - Occurrence, Causes, Consequences. Springer, Berlin: 62-67.

Vasconcelos V. M. (1999). Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research* 32: 249-254.

Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 30: 2377-2384.

Wiedner C., Rucker J., Bruggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Spatial bacterioplankton diversity at Vela Lake (Central Western Portugal) during cyanobacterial blooms and under severe drought conditions

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Abstract

The shallow Vela Lake (Centralwestern Portugal) suffers frequently from cyanobacterial blooms development during summer months. The present work aimed to study the spatial diversity of bacterioplankton assemblage during cyanobacterial blooms occurring under the severe summer drought of 2005. The diversity of bacterioplankton and microcystin potential producers was analysed through *16S rRNA*- and *mcyA*-DGGE (denaturing gradient gel electrophoresis), respectively. A set of recorded environmental parameters was used for multivariate analyses. PCA showed spatial differentiation among sites mainly related to the levels of oxygen, pH, temperature, ammonium and chlorophyll *a*. 16S rDNA-DGGE results showed that the bacterial community clearly differed among the East and Western sampling sites, particularly under drought intensification. RDA analysis suggested that differences between spatial bacterioplankton assemblages were mainly related to oxygen levels, conductivity and water temperature. Dominant phylotypes belonged to *Cyanobacteria, Betaproteobacteria* and *Actinobacteria* groups. The dominance of bloom-forming cyanobacteria such as *Aphanizomenon aphanizomenoides*, *Aphanizomenon gracile* and *Microcystis viridis* could be recorded. In August, the chlorophyll *a* levels on the west side of the lake (more used for recreational purposes) rose way above the guidelines established by WHO under cyanobacterial dominance. Furthermore, the potential for microcystin production was recorded in all samples, indicating health risks for lake users; *mcyA*-DGGE showed spatial differences in the dominant phylotypes, mainly related with factors such as chlorophyll *a* and total suspended solids.

Keywords

shallow lake, cyanobacterial blooms, spatial bacterial diversity, DGGE, 16S rDNA, *mcyA*, multivariate analysis.

Introduction

The occurrence of cyanobacterial blooms is being enhanced by the global warming conditions that we are already beginning to face nowadays (Wiedner et al., 2007; Paerl & Huisman, 2008). Drought episodes are becoming more recurrent all around the world and effective managing strategies for water quality maintenance are urgent. For the Portuguese territory, the year of 2005 was considered one of the driest over the last half century and a trend for eutrophication enhancement of Portuguese water bodies had already been observed during late winter 2005 (de Figueiredo et al., 2007).

Cyanobacterial blooms occur frequently in freshwater eutrophic shallow lakes and are usually enhanced by low N:P levels, water stability, reduced transparency and increased water temperature and pH (Jacoby et al., 2000; Oliver & Ganf, 2000; de Figueiredo et al., 2006) although different species may have distinct preferences (Mischke, 2003; Nixdorf et al., 2003). During the development of cyanobacterial blooms there are potential toxic outcomes involved, namely for human health (Codd et al., 2005b; Mazur-Marzec, 2006). Microcystins, in particular, are secondary metabolites produced non-ribosomally which have proven to be potent hepatotoxins with serious risks for humans (Ueno et al., 1996; Pouria et al., 1998; de Figueiredo et al., 2004b). More, pathogenic bacteria including *Aeromonas*, *Vibrio*, *Acinetobacter* and *Pseudomonas* have been also related to the development of cyanobacterial blooms which increases even further the human health risk (Berg et al., 2008).

At the freshwater shallow Vela Lake (Figueira da Foz, Centralwestern Portugal), the occurrence of cyanobacterial blooms is frequent during summer months (Barros et al., 1993; Vasconcelos et al., 1993a; Saker et al., 2003; de Figueiredo et al., 2006). The massive development of potentially microcystin-producing cyanobacteria such as *Microcystis* spp. is recurrent (Vasconcelos et al., 1993a; de Figueiredo et al., 2006). Nevertheless, other potentially toxic species such as *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* and *Cylindrospermopsis raciborskii* have also been recorded as bloom-formers at this lake (Vasconcelos et al., 1993a; Saker et al., 2003; de Figueiredo et al., 2006). However, no simultaneous studies concerning the whole bacterial assemblage during cyanobacterial blooms have been published for this lake or similar Portuguese shallow lakes. Nowadays, DNA methodologies such as DGGE (Denaturing Gradient Gel Electrophoresis) have been widely used to characterize the bacterial community structure of environmental samples (Muyzer et al., 1993; Muylaert et al., 2002; Lyautey et al., 2005) using primers that target regions of the *16S rRNA* gene directly from environmental samples DNA consequently avoiding labouring and time-consuming isolating and culturing procedures.

The present work aimed to assess which bacteria co-dominate with bloom-forming cyanobacteria at this lake and if there is a spatial diversity inside the lake under strong drought

conditions. The microcystin-production potential and spatial diversity was also assessed through PCR analysis targeting the *mcyA* region of the microcystin synthetase operon.

Materials and Methods

Study area, sampling and environmental parameters

The shallow eutrophic Vela Lake is located at Quiaios (Figueira da Foz, Central Portugal) and it has a depth range of 0.9-2.4 m with an area of approximately 70 ha surrounded by forest of *Pinus* spp. and acacias on its West side and agriculture fields, livestock farms and urban areas on the East side (Abrantes et al., 2009b). This lake is mainly used for recreational and agricultural purposes. The agriculture, in particular, provides high amounts of fertilizers and pesticides that are lixiviated into the lake water with rainfall (Abrantes et al., 2009b). Sampling was performed in June, July and August 2005 at five locations around the lake which have accessibility for lake users (Fig. 1): sites 1, 2 and 3 on the West side and sites 4 and 5 on the East side. In April and September 2005 samples were also collected to be used as pre and post summer references but were not used for multivariate analyses. Samples were taken sub-superficially at about 1 m from the shore in each sampling site using 2L sterile bottles and were immediately placed at 4 ºC in the dark until further treatment in the laboratory (within 4 hours) for DNA extraction and determination of environmental parameters. Water temperature, conductivity, pH and dissolved oxygen were determined *in situ* using portable water testing meters (WTW LF 330 conductivity meter, WTW 340-A pH meter and WTW OXI 320 oxygen meter). In the laboratory, the total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$) and nitrite ($N\text{-}NO_2$) concentrations were determined in laboratory according to standard procedures (APHA, 1992; Rodier, 1996).

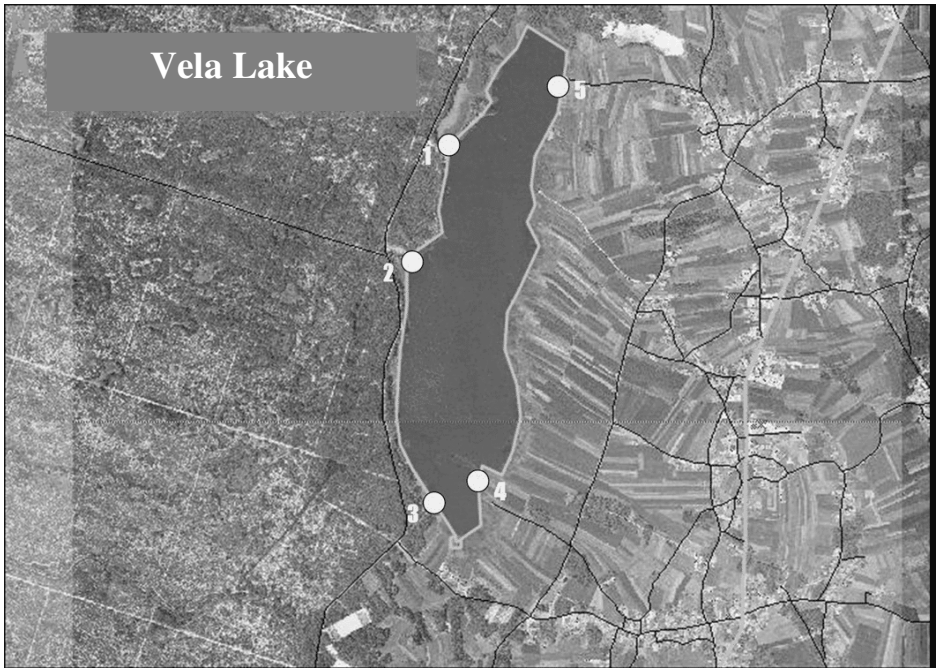


Fig. 1. Location of the sampling sites in Vela Lake (image from GoogleEarth application).

Table 1 Environmental data recorded in Vela Lake sampling sites during summer 2005 (samples LV4.AG05, LV3.SP05, LV4.SP05 and LV5.SP05 were omitted from the table because these sampling sites dried during the study period).

Sampling date	Sampling Site	Code	Temp. (ºC)	Dissolved oxygen (% sat. / mg.L^{-1})	Chl *a* (µg.L^{-1})	TSS (mg.L^{-1})	pH	N-NO_2	N-NO_3	N-NH_4	DIN	SRP	Conductivity (µS.cm^{-1})
										(mg.L^{-1})			
7th April 2005 (AP05)	LV1	AP1	19.1	-	69.4	36.13	8.06	<0.01	0.4	<0.01	0.4	<0.1	475
	LV2	AP2	19.4	-	64.8	45.20	8.34	<0.01	0.2	<0.01	0.2	<0.1	460
	LV3	AP3	19.6	-	67.1	42.07	8.38	<0.01	0.1	<0.01	0.1	<0.1	464
	LV4	AP4	20.1	-	64.8	46.27	8.44	<0.01	0.4	<0.01	0.4	<0.1	460
	LV5	AP5	19.9	-	64.8	33.70	8.49	<0.01	0.1	<0.01	0.1	<0.1	464
23rd June 2005 (JN05)	LV1	JN1	25.8	117.0 / 9.60	72.9	42.40	8.80	<0.01	<0.1	<0.01	<0.1	<0.1	516
	LV2	JN2	25.9	123.0 / 10.36	107.9	34.55	8.58	<0.01	<0.1	<0.01	<0.1	<0.1	516
	LV3	JN3	26.1	117.6 / 9.58	81.0	35.87	8.60	<0.01	<0.1	<0.01	<0.1	<0.1	510
	LV4	JN4	24.4	110.7 / 9.35	49.3	24.20	8.36	<0.01	<0.1	<0.01	<0.1	<0.1	519
	LV5	JN5	25	119.3 / 9.55	79.3	34.60	8.56	<0.01	<0.1	<0.01	<0.1	<0.1	521
19th July 2005 (JL05)	LV1	JL1	25.7	91.5 / 7.47	91.4	12.13	8.77	<0.01	<0.1	<0.01	<0.1	<0.1	584
	LV2	JL2	28.2	115.9 / 8.94	64.8	29.94	8.66	<0.01	<0.1	<0.01	<0.1	<0.1	516
	LV3	JL3	30.8	178.8 / 13.39	27.8	23.45	9.97	<0.01	<0.1	0.03	<0.1	<0.1	592
	LV4	JL4	30.9	116.7 / 8.72	24.7	52.35	9.97	<0.01	<0.1	<0.01	<0.1	<0.1	619
	LV5	JL5	31.2	188.3 / 14.02	46.3	52.80	9.77	<0.01	<0.1	0.01	<0.1	<0.1	575
17th August 2005 (AG05)	LV1	AG1	26.8	107.3 / 8.74	159.9	41.13	8.72	<0.01	<0.1	0.14	0.1	<0.1	647
	LV2	AG2	29.3	124.0 / 9.12	223.7	60.73	8.04	<0.01	<0.1	0.04	<0.1	<0.1	682
	LV3	AG3	31.9	373.0 / 27.90	53.6	26.47	10.50	<0.01	<0.1	0.01	<0.1	<0.1	773
	LV5	AG4	30.5	182.4 / 13.17	38.7	101.91	10.11	<0.01	<0.1	0.06	<0.1	<0.1	665
30th September 2005 (ST05)	LV1	ST1	22.4	-	88.7	68.40	8.61	<0.01	<0.1	<0.01	<0.1	<0.1	828
	LV2	ST2	22.6	-	63.1	63.00	8.87	<0.01	<0.1	<0.01	<0.1	<0.1	820

DNA extraction and PCR amplifications

The total DNA from environmental water samples was extracted after filtering 100 mL of the sampled water through 0.22 µm polycarbonate sterile filters (more than one if the first filter clogs). Collected cells were resuspended in 2 mL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0] and centrifuged. After resuspension in 200 µL of TE, lysis was carried out by adding lysozyme (1 $mg.mL^{-1}$) and incubating at 37 ºC for 1h. DNA extraction and purification was performed using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and DNA was resuspended in TE buffer and stored at -20 ºC.

Amplifications by PCR was performed with the primers 338F-GC/518R (Muyzer et al., 1993), universal for Bacteria. The primer pair QmetF/ QmetR was used for targeting the *mcyA* region of the microcystin synthetase operon (Wilson et al., 2005) with a 40 bp GC-rich sequence added to the 5' end of the forward primer. All primers were commercially synthesized by STABVida (Oeiras, Portugal). PCRs were carried out in a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 15 pmol of each primer, 1 U of *Taq* DNA polymerase and 50-200 ng template DNA. The PCR programs for the primer set targeting the *16S rRNA* gene fragments followed the original procedures (Muyzer et al., 1993; Wilson et al., 2005). A final extension step at 72 °C for 30 min was included for both programs. PCR and negative control reactions were checked by electrophoresis and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder) in a 1.5% agarose gel stained with ethidium bromide. Visualization was performed on a UV transmilluminator.

Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were analysed through DGGE using a 35-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1mm 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, California, USA) using 0.5x 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 gels were then stained for 5 min in an ethidium bromide solution (5%) and then gently destained with agitation in distilled water for 10 min before image digitalization in a Molecular Imager FX™ system (Bio-Rad Laboratories, Hercules, California, USA). The most intense bands from 16S rDNA-DGGE profiles were aseptically excised from the gel into 1.5mL Eppendorf tubes and washed in 10µL 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 verified through DGGE and the extraction procedure was repeated until the targeted band isolation was obtained. More, if necessary, the isolated bands were cloned using the TA cloning kit from Invitrogen. Prior to cloning an A tailing

was performed for PCR products 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

Sequencing of PCR amplicons was made using the vector primers M13R / T7. Each amplicon was purified with the concertTM rapid PCR purification system (Gibco BRL, Eggenstein, Germany) before it was commercially sequenced (STABVida, Portugal). The bacterial 16S rDNA partial sequences determined in the present study were submitted to the GenBank database. A BLAST search (http://www.ncbi.nlm.nih.gov) was used to explore similarity of the sequences obtained in this study against sequences deposited in the GenBank database. The alignment of the partial 16S rDNA sequences was carried out using the CLUSTAL X software version 1.8 (Thompson et al., 1994). A phylogenetic tree was built using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analyses were based on 1000 replicates. TreeView version 1.6.6 (Page, 1996) was used to display the trees.

Statistical 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 & Gorley, 2006). Pearson's correlation coefficient was also used to assess relationships between environmental parameters and against bands (considering their occurrence and relative 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. Redundancy analysis (RDA) was performed to reveal relationships between the distribution of the dominant DGGE phylotypes (using a matrix built with band relative intensities and subsequent logarithmic transformation) and environmental variables using CANOCO 4.5 (Scientia Software). Forward selection was applied to choose the significant (ALPHA = 0.10) environmental parameters for the RDA using a Monte Carlo permutation test (499 unrestricted permutations) (ter Braak & Verdonschot, 1995).

Results

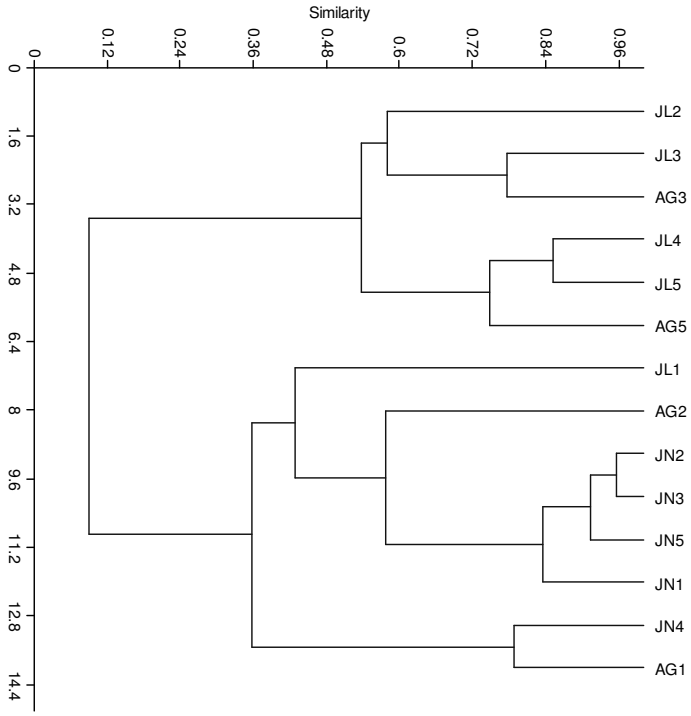
Environmental parameters

Environmental parameters determined for all samples are summarized in Table 1. For summer samples, water temperature and dissolved oxygen levels, in spite of their high dial variation, showed the highest values in July and August (up to 31.9 ºC and 27.9 $mg.L^{-1}$ at VL3.AG05). The water temperature was lower for June samples (between 24.4 and 26.1 ºC) while the lowest dissolved oxygen value was recorded in July at site 1 (7.47 $mg.L^{-1}$). The pH ranged from 8.04 (VL2.AG05) to 10.11 (VL5.AG05). Conductivity summer values varied between 510 (VL3.JN05) and 773 $\mu S.cm^{-1}$ (VL3.AG05). Total suspended solids showed no regular variability but a maximum of 101.91 $mg.L^{-1}$ was attained for VL5.AG05 and a minimum for VL1.JL05 (12.13 $mg.L^{-1}$). The maximum chlorophyll *a* concentration was recorded for VL2.AG05 (with 223.7 $\mu g.L^{-1}$) and the minimum for VL4.JL05 (24.7 $\mu g.L^{-1}$). Concerning summer nutrient concentrations for SRP, nitrate and nitrite, these were undetectable. The same was recorded for ammonium concentration, except in August when very low levels (0.1 $mgN(NH_4^-).L^{-1}$) were recorded. April samples showed lower values for temperature (as expected), pH, TSS and conductivity whereas nitrate concentration levels were higher. For early autumn samples, temperature was lower than summer samples but pH and TSS levels remained high and conductivity achieved new maxima values.

The Pearson correlation coefficient was calculated to assess relationships between the recorded environmental parameters. Water temperature showed to be highly positively correlated with pH ($r = 0.75$; $P < 0.001$; $n = 20$) followed by significant positive relationships with oxygen saturation and dissolved oxygen concentration ($r = 0.67$, and 0.60, respectively; $P < 0.05$; $n = 13$). Dissolved oxygen and oxygen saturation levels were highly correlated ($n = 13$) among themselves ($r = 1.00$; $P < 0.001$), as should be expected, but also with pH ($r = 0.69$ and 0.73, correspondingly; $P < 0.01$) and conductivity ($r = 0.65$ and 0.69, respectively; $P < 0.05$). Chl *a* levels were also correlated with ammonium concentration ($r = 0.44$; $P < 0.05$; $n = 20$). Significantly negative correlations ($P < 0.05$; $n = 20$) were detected between pH and Chl *a* ($r = -0.49$) and between conductivity and nitrate concentration ($r = -0.53$).

Pearson correlation coefficient using the environmental data also revealed highly significant correlations among summer samples ($n = 11$). JN1 was highly correlated with JN2, JN3 and JN5 (with $r = 0.76$, 0.89 and 0.85, respectively; $P < 0.01$). Interestingly, JN4 showed no significant correlations with the other June samples but rather with AG1 ($r = 0.79$; $P < 0.01$). JN2 showed to be correlated with JN3 and JN5 (with $r = 0.95$ and 0.90, respectively; $P < 0.001$) but also with AG2 ($r = 0.72$; $P < 0.05$) while JN3 was correlated with JN5 ($r = 0.92$; $P < 0.001$) and AG2 ($r = 0.64$; $P < 0.05$) as well. In spite of JL1 did not show to have significant correlations with the other JL samples, JL2 was correlated with JL3 and JL5 ($r = 0.67$ and 0.68, respectively; $P < 0.05$) while JL3

(a)



(b)

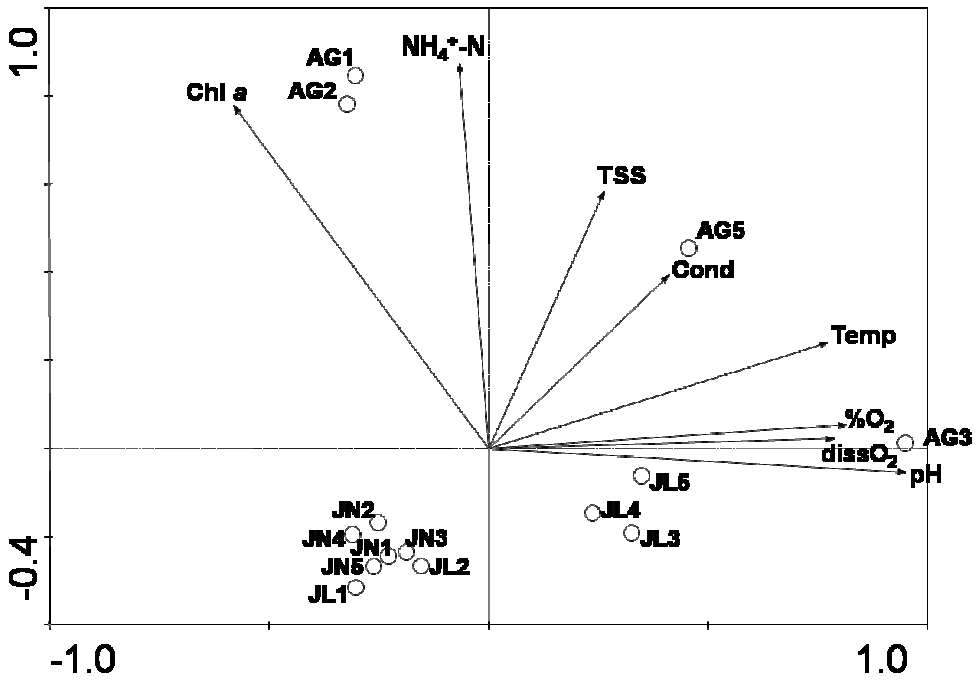


Fig. 2. a) dendrogram of sampling stations clustered according to the environmental parameters; b) PCA ordination biplot of sampling stations and environmental parameters.

correlated with JL4 (r = 0.73; P < 0.05), JL5 (r = 0.82; P < 0.01) and AG3 (r = 0.78; P < 0.01). JL4 was highly significantly correlated with JL5 (r = 0.85; P < 0.001) and with AG5 (r = 0.76; P < 0.01). JL5 showed significant correlations with AG4 (r = 0.71; P < 0.05) and AG5 (r = 0.74; P < 0.01) while only AG1 and AG2 showed to be correlated (r = 0.66; P < 0.05).

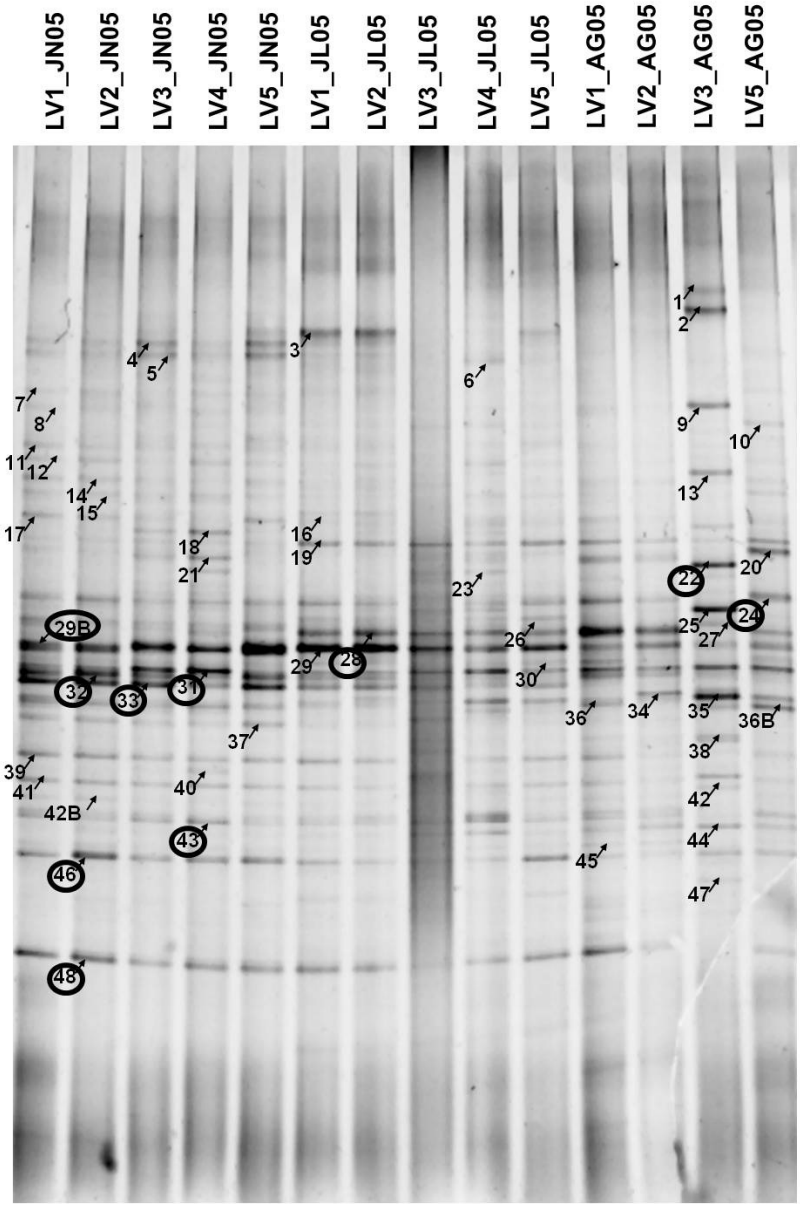


Fig. 3. Negative image of an ethidium bromide-stained DGGE pattern of the bacterioplankton assemblage at the five sampling sites in Vela Lake in June, July and August 2005. The codes at the top of the image refer to the sampling site and date. The circles refer to the excised and sequenced bands presented in Table 2.

The ordination of summer samples in the PCA analysis (Fig. 2b) shows that the sampling sites distribution along the first axis (defined by samples AG1 and AG2 on the positive side and JL1 on the negative side) was mainly related to oxygen availability, pH and temperature whereas Chl *a* and ammonium concentrations were mostly correlated to the second axis (defined by samples JN4 and JL1 on the negative side and AG3 on the positive side). Nitrite, nitrate and phosphate concentrations were excluded from the graphic because their contribution was null. PC1 accounted for 45.7% and PC2 for 26.1% (and both axes 71.8%) of the total variance among the sampling sites.

DGGE profiles, CCA and affiliation of sequenced bands

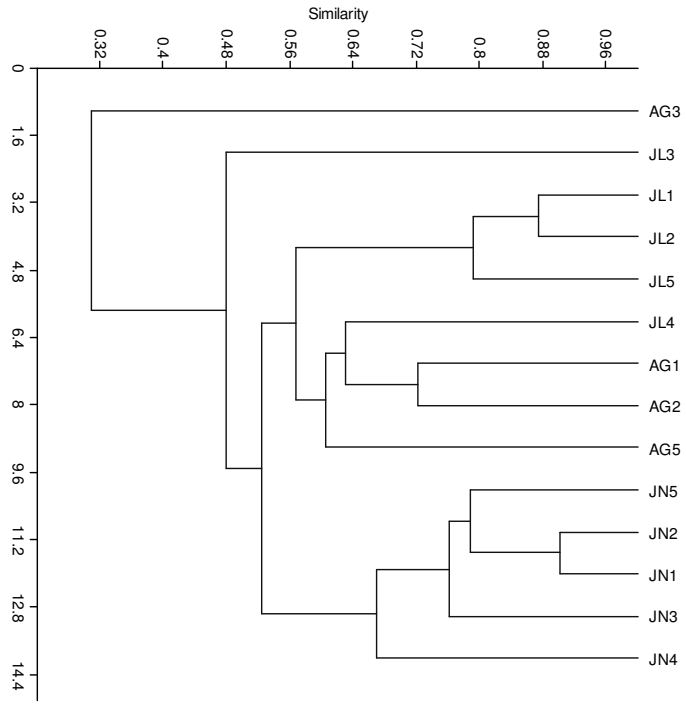
A total of 298 bands were analysed in the DGGE profiles obtained for Vela Lake bacterial spatial diversity in summer samples (Fig. 3) and corresponded to 48 different band migration points. The

number of bands *per* sample showed an average of 21 ± 3 (n = 14). Band 19, 29, 43 and 46 were present in all the lake's samples while bands 24, 31, 35, 39, 41, 44 and 48 were detected in more than 78% of samples. However, there were also recorded unique bands at JL4 (band 6), AG1 (band 36), AG3 sample (bands 1, 2, 9, 13, 42 and 47) and AG5 (band 10). Interestingly, August showed to have the highest variability among sampling locations probably due to the fact that some of these began to dry and the water depth was dramatically reduced sometimes to less than 20 cm. This was particularly severe for sites 3 and 5 (while site 4 was already dry in August) which showed distinct 16S rDNA DGGE profiles with unique phylotypes as described above. This is in accordance to the conclusions retrieved from the environmental parameters. Interestingly, cluster analysis based on band patterns from 16S rDNA DGGE showed a clear segregation of sample AG3 but the persistent grouping of sampling sites 1 and 2 (Fig. 4a) throughout the sampling period. RDA analysis (Fig. 4b) showed that the first two axes could explain up to 42.5 % of the total spatial and summer variation of the dominant bacterioplankton assemblage. The first axis was defined by August samples (except AG3) and bands 28, 38 and 31 on the positive side while the negative side was defined by June samples and bands 5 and 33. This axis was mostly related with conductivity (0.86) and water temperature (0.76). Sample AG3 (and bands 2 and 22) defined the positive side of the second axis and band 28 defined its negative side with dissolved oxygen (0.78) as the most related variable.

The phylogenetic affiliation of the sequenced DGGE bands (most intense) is presented in Table 2 and Fig. 5. Most intense 16S rDNA partial sequences showed to affiliate with cyanobacteria such as *Aphanizomenon aphanizomenoides*, *Aphanizomenon gracile*, *Microcystis viridis* and *Synechococcus* sp., actinobacteria from the order Actinomycetales, betaproteobacteria from the Burkholderiaceae family and *Verrucomicrobia*.

The DGGE based on the *mcyA* fragment showed a total of 165 bands (Fig. 6) corresponding to 27 different band migration points and an average of 12 ± 3 (n = 14) bands *per* sample. Bands 9 and 10 were present in all profiles. Cluster analysis (Fig. 7a) showed a clear segregation of sample AG5 from the other samples and two clusters were also evidenced: one including June and July (JL1, JL2 and JL5) samples and other including JL3 and JL4 the remaining August samples. RDA analysis (Fig. 4b) showed a clear clustering of *mcyA* fragments according to sampling date, evidencing a temporal succession of potential microcystin-producing phylotypes. The first two axes could explain 63.9% of the *mcyA* phylotypes' distribution. The first axis was mainly related to 16S rDNA fragments corresponding to bands 37 (0.90), 5 (0.90) and 15 (0.87) and it was defined by June samples (and *mcyA* bands 17 and 15) on the positive side whereas samples AG2 and AG3 (and *mcyA* bands 2, 3 and 25) defined its negative side. The positive side of the second axis was defined by samples JL1 and JL2 (and *mcyA* bands 18 and 21) and its positive side was defined by

(a)



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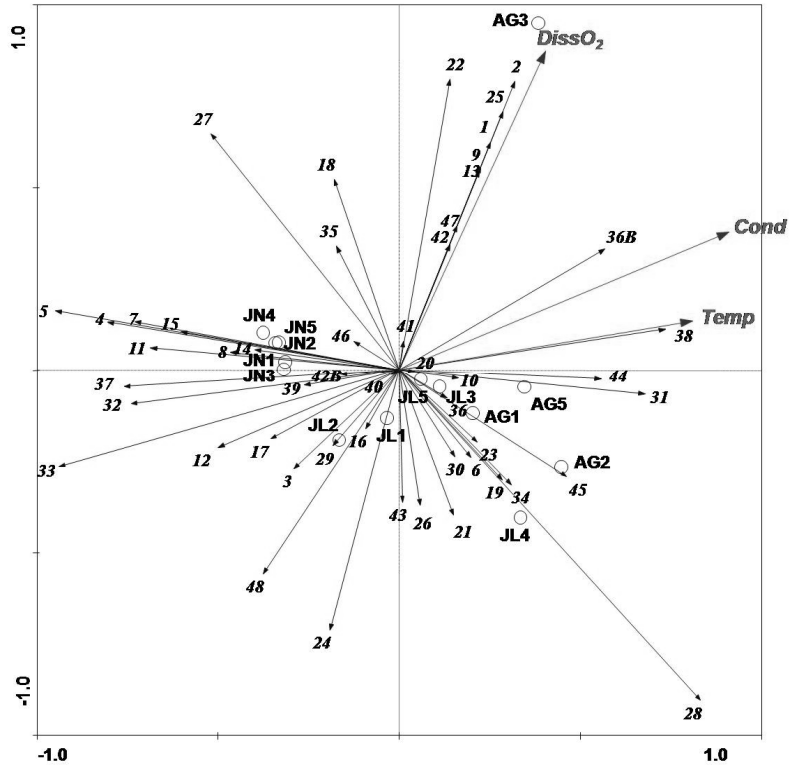


Fig. 4. (a) dendrogram of sampled water bodies according to 16Sr DNA-DGGE band patterns and (b) RDA ordination triplot of samples, 16S rDNA-DGGE bands and environmental data recorded in summer 2005 (see sample codes in table 1).

Table 2. Accession number and phylogenetic affiliation for the sequenced bacterial DGGE bands shown in Fig. 3.

DGGE band	NCBI Accession Nº	Phylogenetic affiliation	Closest relatives (accession Nº)	Origin	Percentage similarity (%)
22_57LV_AG05	submitted	*Verrrucomicrobia*	Uncultured Verrucomicrobiales bacterium clone TH3-89 (AM690977)	Lake Taihu, China	99
24_ex61LV_ST05	submitted	*Cyanobacteria*	*Synechococcus* sp. KORDI-78 (FJ497748)	Seawater of the East China	100
28_LVAG_cl7	submitted	*Betaproteobacteria*	*Polynucleobacter cosmopolitanus* MWH-T1W13 (AJ550662)	Freshwater pond, China	100
29B_ex1LV.JN05	submitted	*Cyanobacteria*	*Aphanizomenon aphanizomenoides* ANA235C (FJ234896)	Menindee Lake, Australia	100
			Aphanizomenon aphanizomenoides UADFA3 (FJ895118)	Vela Lake, Portugal	100
31_ex12LV_JN05	submitted	*Betaproteobacteria*	Uncultured Burkholderiaceae bacterium clone LW18m-2-18 (EU642357)	Lake Michigan, USA	99
			Polynucleobacter sp. TEGAF013 (AB470448)	Lake Teganuma, Japan	99
32_ex2LV_JN05	submitted	*Cyanobacteria*	*Microcystis viridis* NIES102 (U40332)	Lake Kasumigaura, Japan	99
33_LVJN_cl38	submitted	*Cyanobacteria*	*Aphanizomenon gracile* UADFA12 (FJ895127)	Vela Lake, Portugal	100
48_ex10LV_JN05	submitted	*Actinobacteria*	Uncultured Actinomycetales bacterium clone Gap-1-32 (EU117986)	Milwaukee harbor, USA	100
			Uncultured freshwater bacterium isolate DGGE band 38LV (DQ900045)	Vela Lake, Portugal	100
43_ex30LV_JL05	submitted	*Actinobacteria*	Uncultured Microbacteriaceae bacterium clone CB_054 (DQ881441)	Salt marsh, Georgia, USA	100
46_ex33LV_JL05	submitted	*Cyanobacteria*	*Phormidium* sp. 0417 (DQ408370)	Thermal spring, Arshan, Russia	99

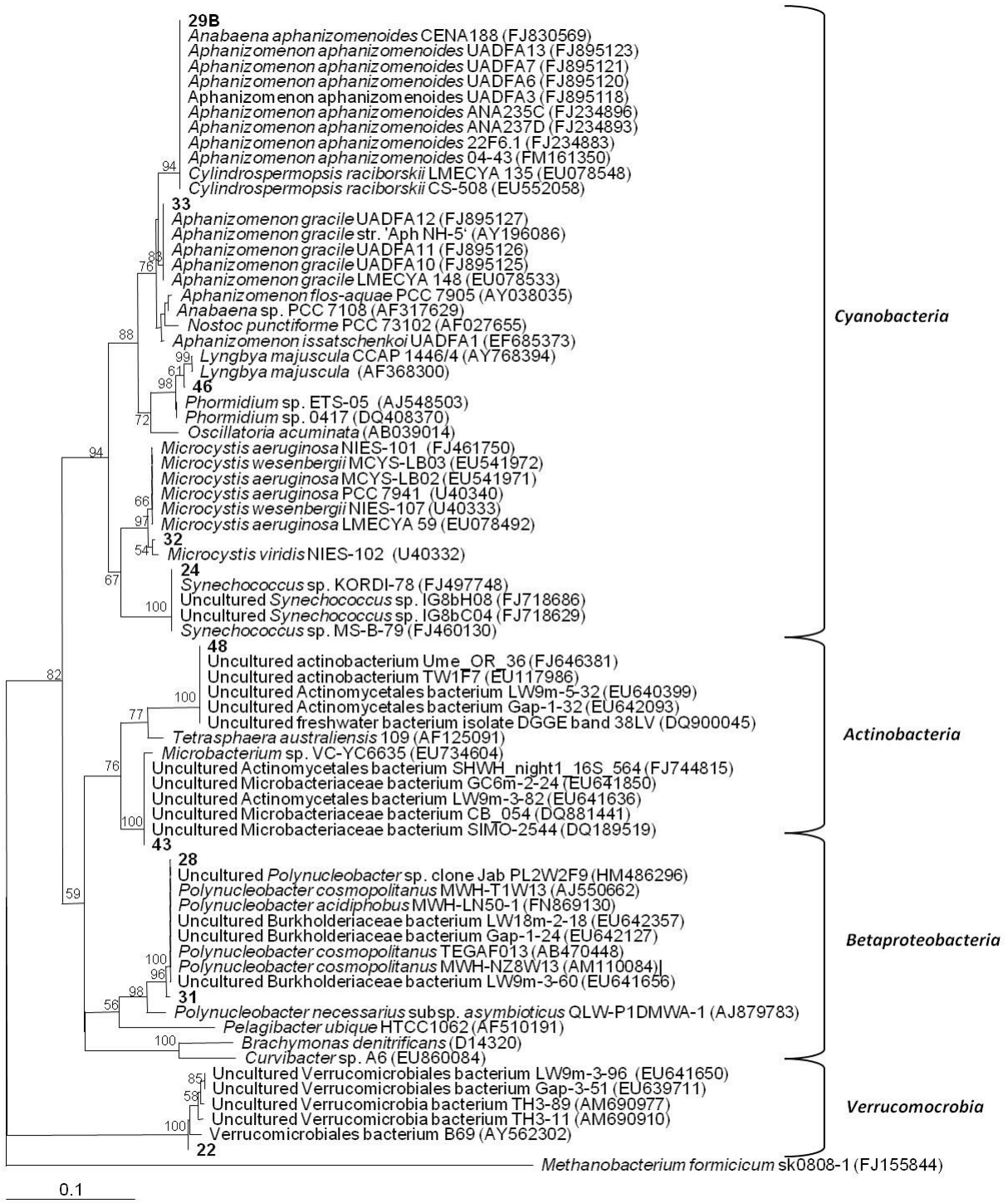


Fig. 5. Evolutionary tree showing the phylogenetic affiliations of the partial bacterial *16S rRNA* gene sequences obtained from DNA fragments excised from the DGGE gel (Fig. 3). The archaeal sequence from *Methanobacterium formicicum* strain sk0808-1 was used as outgroup. Scale bar indicates 0.1 substitutions per site. Bootstrap values (1000 replicates) that were > 50 are placed at the nodes of the branches.

sample AG5 (and band 12). This axis was mostly related to *16S rRNA* bands 3 (0.81), 16 (0.69), 30 (0.50) and 36B (-0.48) as well as to the environmental factors TSS (-0.53) and chl *a* (-0.37).

Discussion

Environmental parameters

In Portugal, 2005 was considered having one of the most intense droughts over the last half century, according to the Water Resources Information National System (http://snirh.pt/) with eutrophication enhancement effects on water bodies recorded already in winter 2005 (de Figueiredo et al., 2007). The undetectable concentrations of nitrite and phosphate are frequent during summer at Vela Lake (de Figueiredo et al., 2006) although nitrate and ammonium levels could be recorded. However, in summer 1993 there were in all samples detectable concentrations of all tested nutrient forms (Barros, 1994). In general, in summer months 2005, Vela Lake showed dissolved oxygen and TSS levels within the range of previous summer records (Barros, 1994; de Figueiredo et al., 2006) but pH, chl *a* and conductivity showed increased values and, in July and August, the lake water achieved temperatures way above the ones recorded in

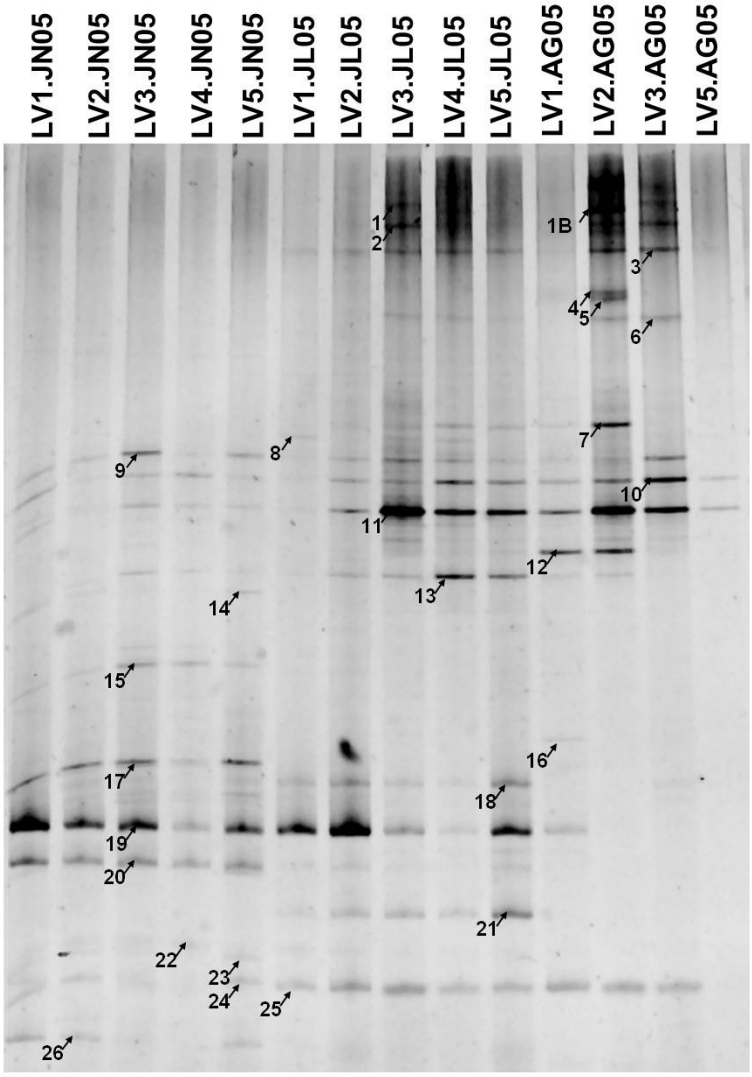
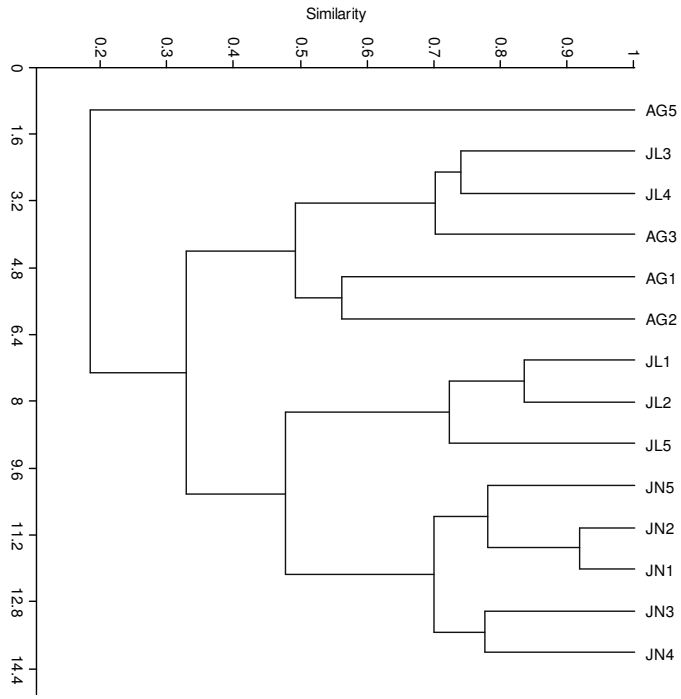


Fig. 6. Negative image of an ethidium bromide-stained DGGE pattern of the bacterioplankton assemblage at the five sampling sites in Vela Lake in June, July and August 2005. The codes at the top of the image refer to the sampling site and date. The circles refer to the excised and sequenced bands presented in Table 2.

(a)



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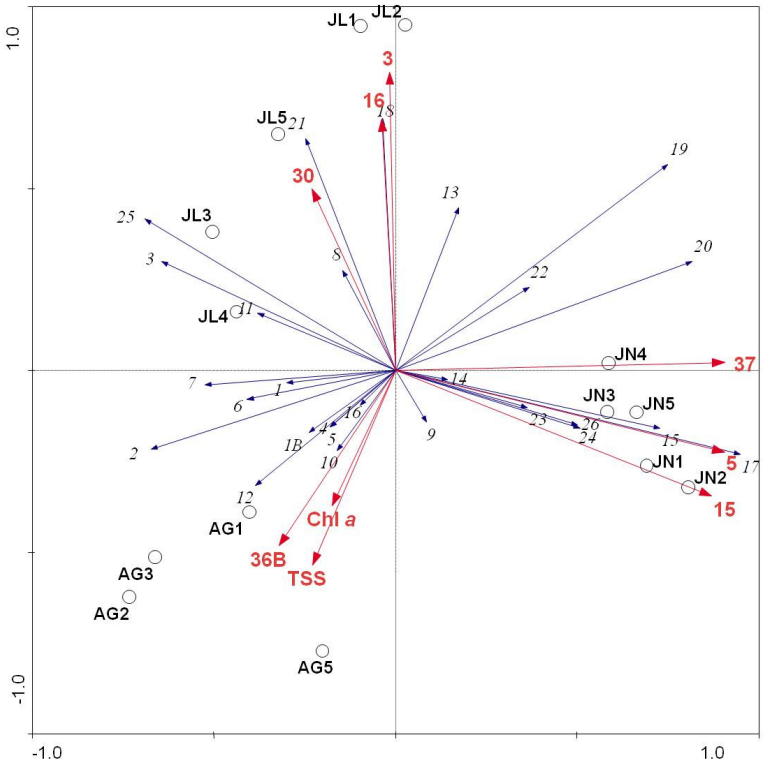

Fig. 7. (a) dendrogram of sampled water bodies according to *mcyA*-DGGE band patterns and (b) RDA ordination triplot of samples, *mcyA*-DGGE bands and environmental data along with 16S rDNA-DGGE bands (in red) from summer samples 2005 (see sample codes in table 1).

1993 and 2001 (Barros, 1994; de Figueiredo et al., 2006). This points out to a deterioration of the trophic status of the lake, as recorded over the past twenty years. In 1989, Vela lake was considered oligo- to β-mesosaprobic (Calado, 1990) but in 1993 its eutrophic condition was already reported based on nutrient levels, phytoplankton densities and cyanobacterial massive development (Barros et al., 1993). Results showed that spatial samples were identical amongst them concerning environmental data and DGGE patterns in spring 2005 (data not shown). However, amongst summer samples locations, there was an important environmental variation, sometimes even more important than sampling dates, which highlights the enhanced effect of drought during summer. Particularly in July and August, those differences were accentuated with a distinct discrimination between the clustered western sampling sites 1 and 2, and the eastern sites mainly due to the highest Chl *a* and ammonium concentrations. Sites 1 and 2 showed to have similar characteristics by appearing repeatedly clustered together. The East sampling sites 3, 4 and 5 located near the agriculture fields showed a higher differentiation among them and a higher sensitivity to drought conditions. Site 5 from August sampling was associated with the highest TSS and conductivity whereas site 3 was related with the highest pH, oxygen values and temperature. In general, there was an increase in temperature, pH, conductivity and oxygen levels between June and August. Interestingly, the maximum TSS value did not correspond to the maximum chl *a* concentration suggesting they were not mainly due to phytoplankters. In fact, the water level was decreased severely and sample locations from the East side of the lake began to dry (in September these dried completely). Thus, it is possible that sediment suspension got to the water column very easily (by wind, for instance) and increased the TSS levels. Based on chl *a* levels, all samples could be considered hypereutrophic.

Environmental parameters and dominant bacterial phylotypes

Cyanobacteria may produce toxins that affect many aquatic organisms (de Figueiredo et al., 2004b), including other bacteria (Casamatta & Wickstrom, 2000; Mankiewicz et al., 2002) Dixon et al., 2004). However, some bacteria may enhance cyanotoxin production while others degrade cyanotoxins (Christoffersen et al., 2002; Bourne et al., 2006; Berg et al., 2008). There is still a need for more environmental studies on the overall bacterial community during cyanobacterial bloom development. Studies using culturing techniques have retrieved bacteria from phyla such as *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* associated with cyanobacterial blooms and enhancing cyanobacterial growth (Berg et al., 2008; Pope & Patel, 2008). Thus, the dynamics of the bacterial assemblage in a natural aquatic system must be affected by cyanobacterial blooms with the increase / decrease of some key bacterial *taxa*. More, environmental studies show that, as the *Microcystis* spp. abundance increases into a bloom, there is also a significant decrease in bacterioplankton diversity (Xing et al., 2007). In the present study, the phylogenetic affiliation of the dominant DGGE bands corresponded to the groups *Cyanobacteria*, *Actinobacteria* and

Betaproteobacteria which are commonly found in shallow eutrophic and hypertrophic lakes (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; Wu et al., 2007b).

Cyanobacterial phylotypes corresponded to *Aphanizomenon aphanizomenoides*, *Aphanizomenon gracile*, *Microcystis viridis*, *Phormidium* sp. and *Synechococcus* sp.. The sequence of *Aph. gracile* showed total match with sequences retrieved from Vela Lake in previous years, namely during bloom episodes (de Figueiredo et al., 2006); this highlights this species persistence and bloom-forming potential at this lake. The *Aph. aphanizomenoides* phylotype showed total match with sequences from Australian and Brazilian to German (Stüken et al., 2009) and Portuguese water bodies, including strains isolated from Vela Lake during summer 2004 (de Figueiredo et al., 2010a), indicating its persistent occurrence at this water body. This species has been considered invasive in Europe (Stefaniak & Kokociński, 2005; Stüken et al., 2006). The dominance of *Aph. aphanizomenoides* showed to be significantly negatively correlated with conductivity and ammonium. It was dominant during all summer with co-dominance of Burkholderiaceae (*Betaproteobacteria*) and Actinomycetales (*Actinobacteria*) phylotypes, and also the cyanobacterium *Microcystis viridis* (in June). Actinomycetales and Burkholderiales bacteria have proven to enhance the growth of cyanobacteria belonging to cyanobacteria from the genera *Microcystis* and *Anabaena* (Berg et al., 2008). The dominance of cyanobacteria during late spring and summer months is recurrent at Vela Lake, but there is usually a succession in the bloom-forming species (Barros et al., 1993; de Figueiredo et al., 2006). The development of *Aphanizomenon* spp. blooms is associated with very low concentrations or absence of nitrogen sources (nitrate, nitrite and ammonium) due to N-fixing capability, but under phosphate availability (Teubner *et al.*, 1999; Kahru *et al.*, 2000; de Figueiredo *et al.*, 2004). *M. aeruginosa* dominates cyanobacterial blooms during late summer at Vela Lake (de Figueiredo et al., 2006).

The *Actinobacteria* phylotypes recorded were affiliated with Actinomycetales bacteria, one of them to Microbacteriaceae, which has shown to enhance the growth of *Microcystis* and *Anabaena* strains (Berg et al., 2008). In fact, these actinobacterial phylotypes were well represented during the dominance of *Aph. aphanizomenoides* along with *Microcystis viridis*. Interestingly, one of the Actinomycetales phylotypes had already been recorded as dominant at Vela Lake during winter (de Figueiredo et al., 2007), indicating its persistence throughout the year. *Actinobacteria* are frequent in lentic water bodies such as lakes and reservoirs (De Wever et al., 2005; Van Der Gucht et al., 2005; de Figueiredo et al., 2007).

The *Betaproteobacteria* phylotype recorded in this study was similar to Burkholderiaceae sequences and co-dominated during *Aph. aphanizomenoides* dominance but without *Microcystis viridis* dominance. Interestingly, laboratory results have shown that members of Burkholderiales inhibit the growth of *Microcystis* strains (Berg et al., 2008) and lyse *Microcystis* cells (Yamamoto

et al., 1993) although enhance the growth of filamentous cyanobacteria (Berg et al., 2008). This may justify the absence of *Microcystis* phylotype under this betaproteobacterium dominance.

A Verrucomicrobiales phylotype was also recorded and had a high similarity with bacteria from the eutrophic Taihu Lake, in China (Wu et al., 2007a). In fact, *Actinobacteria*, *Verrucomicrobia* and *Betaproteobacteria* have shown to be present during blooms of *Aphanizomenon* spp. (Pope & Patel, 2008) which is in accordance to our results.

Our results showed that the bacterial communities clearly differed among the East and Western sampling sites, particularly under drought intensification. Thus, the sampling location was an important factor for variation among samples and could even surpass the influence of sampling date in July and August. Eastern sampling sites (with lower depth) were particularly vulnerable to drought which must explain the segregation of sampling sites 1 and 2 *vs* sites 3, 4 and 5. Actually, these differences reached their extreme in September, when sampling sites 3, 4 and 5 all dried (data not shown). Oxygen levels proved to be the most important factor for explaining the spatial bacterioplankton diversity observed, followed by water temperature and conductivity. Other studies have also shown the importance of dissolved oxygen, temperature and ammonium concentrations for spatial bacterioplankton variation in freshwater lakes (Wei et al., 2008). Zeng et al. (2009) also found ammonium levels as an important BCC modulator (Zeng et al., 2009) as well as pH, as previously reported (Lindström et al., 2005; Yannarell & Triplett, 2005; de Figueiredo et al., 2007). Although not considered in the present study, factors such as the wind may be of great importance for the spatial differences observed, as previously observed for other shallow water bodies (Izydorczyk & Tarczynska, 2005), given that the surface blooms are easily transported on the water due to aerotopes and buoyancy regulation of cyanobacteria such as *Microcystis* spp..

Spatial and temporal diversities could be also recorded for *mcyA*-DGGE profiles. In spite of the segregation of sample AG05 in cluster analysis based on the Bray-Curtis similarity, RDA analysis showed its proximity from the other August samples and showed to be most related to TSS and Chl *a* levels, but also to the dominance of band 36B from 16S rDNA-DGGE profiles. In fact, a clear temporal succession for *mcyA*- phylotypes could be evidenced. June *mcyA* profiles showed to be related with higher densities of 16S rDNA bands 5, 15 and 37 whereas July profiles were mostly related with the dominance of 16S rDNA bands 3, 16 and 30. Further investigation, namely using more statistical exploitation of DGGE profiles and sequencing the key *mcyA* and 16S rDNA bands in these relationships, should be conducted and could give interesting information for the ecology of potentially toxic cyanobacterial blooms.

Conclusion

This study showed there were pronounced spatial differences in the BCC at Vela Lake during the dry summer of 2005. This spatial BCC variation was mainly related to oxygen levels, water temperature and conductivity. The bacterial phylotypes retrieved from sequencing dominant

bands in the DGGE profiles showed the co-dominance of members of the *Cyanobacteria*, *Actinobacteria*, *Verrucomicrobia* and *Betaproteobacteria* groups. Blooms of *Aphanizomenon aphanizomenoides*, *Aphanizomenon gracile* and *Microcystis viridis* could be detected using this methodology. From the results from the present study, one could conclude that during summer drought, the samples location was an important factor for variation among samples, sometimes even more important than sampling date. After the results obtained during the study period concerning the presence and dominance of potentially toxic cyanobacteria (as shown by the amplification of the *mcyA* region of the microcystin synthetase operon), one should consider as very important a future investigation on the spatial monitoring and quantification of toxins, namely microcystins, at Vela Lake. This would help to evaluate the risk level for lake users in each point of the lake during bloom occurrence in a dry summer. In general, by comparing to data from previous studies, there was an evident deterioration of the trophic status of the lake during 2005 drought. A proper and effective water management is also necessary to prevent these potentially toxic cyanobacterial blooms from developing.

References

Abrantes N., Pereira R., de Figueiredo D. R., Marques C. R., Pereira M. J. & Gonçalves F. (2009). A whole sample toxicity assessment to evaluate the sub-lethal toxicity of water and sediment elutriates from a lake exposed to diffuse pollution. *Environmental Toxicology* 24: 259-270.

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Barros P., Implicações ecotoxicológicas de cianobactérias em cladóceros. Faculty of Sciences and Technology, Vol. MSc. University of Coimbra, Coimbra, 1994.

Barros P., Silveira S., Ribeiro R., Gonçalves F., Vasconcelos V. & Soares A. M. V. M. (1993). Estrutura populacional fitoplanctónica nas lagoas das Braças, Vela e Mira (região centro-litoral). Resultados preliminares. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 1-8.

Berg K. A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P. & Rapala J. (2008). High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME Journal* 3: 314-325.

Bourne D. G., Blakeley R. L., Riddles P. & Jones G. J. (2006). Biodegradation of the cyanobacterial toxin microcystin LR in natural water and biologically active slow sand filters. *Water Research* 40: 1294-1302.

Calado A. J., Fundamentação da aplicação do sistema de saprobia às algas de água doce do Centro de Portugal. Department of Biology. University of Aveiro, Aveiro, 1990.

Casamatta D. A. & Wickstrom C. E. (2000). Sensitivity of two disjunct bacterioplankton communities to exudates from the cyanobacterium *Microcystis aeruginosa* Kützing. *Microbial Ecology* 40: 64-73.

Christoffersen K., Lyck S. & Winding A. (2002). Microbial activity and bacterial community structure during degradation of microcystins. *Aquatic Microbial Ecology* 27: 125-136.

Clarke K. R. & Gorley R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Codd G. A., Morrison L. F. & Metcalf J. S. (2005). Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology* 203: 264-272.

de Figueiredo D. R., Alves A., Pereira M. J. & Correia A. (2010). Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). *Journal of Plankton Research* 32: 239-252.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Izydorczyk K. & Tarczynska M. (2005). The influence of wind on cyanobacterial bloom development in shallow, lowland reservoir in central Poland. *Ecohydrology and Hydrobiology* 5: 195-203.

Jacoby J. M., Collier D. C., Welch E. B., Hardy F. J. & Crayton M. (2000). Environmental factors associated with a toxic bloom of Microcystis aeruginosa. *Canadian Journal of Fisheries and Aquatic Sciences* 57: 231-240.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Mankiewicz J., Walter Z., Tarczynska M., Palyvoda O., Wojtysiak-Staniaszczyk M. & Zalewski M. (2002). Genotoxicity of cyanobacterial extracts containing microcystins from Polish water reservoirs as determined by SOS chromotest and comet assay. *Environmental Toxicology* 17: 341-350.

Mazur-Marzec H. (2006). Characterization of phycotoxins produced by cyanobacteria. *Oceanological and Hydrobiological Studies* 35: 85-109.

Mischke U. (2003). Cyanobacteria associations in shallow polytrophic lakes: influence of environmental factors. *Acta Oecologica* 24.

Muylaert K., Van der Gucht K., Vloemans N., De Meester L., Gillis M. & Vyverman W. (2002). Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. *Applied and Environmental Microbiology* 68: 4740-4750.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Nixdorf B., Mischke U. & Rücker J. (2003). Phytoplankton assemblages and steady state in deep and shallow eutrophic lakes - An approach to differentiate the habitat properties of Oscillatoriales. *Hydrobiologia* 502: 111-121.

Oliver R. L. & Ganf G. G., 2000. Freshwater blooms. In: B. A. Whitton & M. Potts, (eds.), The Ecology of Cyanobacteria. Kluwer Academic Publishers: 149-194.

Paerl H. W. & Huisman J. (2008). Climate: Blooms like it hot. *Science* 320: 57-58.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Pope P. B. & Patel B. K. C. (2008). Metagenomic analysis of a freshwater toxic cyanobacteria bloom. *FEMS Microbiology Ecology* 64: 9-27.

Pouria S., De Andrade A., Barbosa J., Cavalcanti R. L., Barreto V. T. S., Ward C. J., Preiser W., Poon G. K., Neild G. H. & Codd G. A. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 352: 21-26.

Rodier J., 1996. L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Saitou N. & Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Saker M. L., Nogueira I. C. G., Vasconcelos V. M., Neilan B. A., Eaglesham G. K. & Pereira P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55: 243-250.

Stefaniak K. & Kokociński M. (2005). Occurrence of invasive cyanobacteria species in polimictic lakes of the Wielkopolska Region (Western Poland). *Oceanological and Hydrobiological Studies* 34: 137-148.

Stüken A., Campbell R. J., Quesada A., Sukenik A., Dadheech P. K. & Wiedner C. (2009). Genetic and morphologic characterization of four putative cylindrospermopsin producing species of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Journal of Plankton Research* 31: 465-480.

Stüken A., Rücker J., Endrulat T., Preussel K., Hemm M., Nixdorf B., Karsten U. & Wiedner C. (2006). Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. *Phycologia* 45: 696-703.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

Thompson J. D., Higgins D. G. & Gibson T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

Ueno Y., Nagata S., Tsutsumi T., Hasegawa A., Watanabe M. F., Park H.-D., Chen G.-C., Chen G. & Yu S.-Z. (1996). Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* 17: 1317-1321.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Vasconcelos V. M., Campos T., Amorim A. & Soares A. M. V. M. (1993). Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 193-201.

Wei C., Bao S., Zhu X. & Huang X. (2008). Spatio-temporal variations of the bacterioplankton community composition in Chaohu Lake, China. *Progress in Natural Science* 18: 1115-1122.

Wiedner C., Rücker J., Brüggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.

Wilson A. E., Sarnelle O., Neilan B. A., Salmon T. P., Gehringer M. M. & Hay M. E. (2005). Genetic Variation of the Bloom-Forming Cyanobacterium *Microcystis aeruginosa* within and among Lakes: Implications for Harmful Algal Blooms. *Applied and Environmental Microbiology* 71: 6126-6133.

Wu Q. L., Zwart G., Wu J., Kamst-Van Agterveld M. P., Liu S. & Hahn M. W. (2007a). Submersed macrophytes play a key role in structuring bacterioplankton community composition in the large, shallow, subtropical Taihu Lake, China. *Environmental Microbiology* 9: 2765-2774.

Wu X., Xi W., Ye W. & Yang H. (2007b). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Xing P., Kong F., Cao H., Zhang M. & Tan X. (2007). Variations of bacterioplankton community composition during *Microcystis* spp. blooms in a shallow eutrophic lake. *Journal of Freshwater Ecology* 22: 61-67.

Yamamoto Y., Nizuma S., Kuroda N. & Sakamoto M. (1993). Occurrence of hetrotrophic bacteria causing lysis of cyanobacteria in a eutrophic lake. *Japanese Journal of Phycology* 41: 215-220.

Yannarell A. C. & Triplett E. W. (2005). Geographic and Environmental Sources of Variation in Lake Bacterial Community Composition. *Appl. Environ. Microbiol.* 71: 227-239.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology*: 1-10.

Chapter IV

Vela Lake – a model case study for the characterization of bloom-forming cyanobacterial strains

Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal)

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Abstract

The diversity within the genus *Aphanizomenon* (Nostocales, Cyanobacteria) is still controversial and more studies are needed to clarify its heterogeneity. *Aphanizomenon* strains isolated from the eutrophic shallow Vela Lake (Centralwestern Portugal) during bloom formation in summer months from 2001 to 2007 were characterized combining phenotypic and molecular approaches. The molecular affiliation of the strains was achieved through sequencing of the 16S rDNA, as well as *nifH* and *hetR* genes fragments, and inter-specific genetic variability was checked through repetitive sequences fingerprinting. The morphological characters fell into the descriptions of *Aph. issatschenkoi* (*Cuspidothrix issatschenkoi*), *Aphanizomenon gracile* and *Aph. aphanizomenoides*. Molecular phylogenetic results corroborated the morphological identification and fingerprinting showed a persistent occurrence of *Aph. gracile* and *Aph. aphanizomenoides* strains over the years but also indicated that *Aph. aphanizomenoides* strains are not clonal within a same bloom. Partial *nifH* and *hetR* genes sequences of the cyanobacterial strains showed a high phylogenetic differentiation capacity, even at the strain level.

Keywords

Vela Lake, *Aphanizomenon* spp., repetitive PCR fingerprinting, *16S rRNA*, *nifH*, *hetR*.

Introduction

The development of cyanobacterial blooms in freshwater shallow lakes occurs worldwide (Dokulil & Teubner, 2000) and frequently leads to toxic outcomes (Codd, 2000; de Figueiredo et al., 2004b). At Vela Lake (Figueira da Foz, Western Central Portugal), blooms of toxic cyanobacteria are recurrent and usually dominated by *Microcystis* spp. although species identified as *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* and *Cylindrospermopsis raciborskii* may also achieve high densities (Vasconcelos et al., 1993a; Saker et al., 2003; de Figueiredo et al., 2006). For several genera such as *Aphanizomenon* a correct identification through phenotypical characters is often compromised due to the ambiguity of morphological features (Hindák, 2000; Li et al., 2000; Komárek & Komárková, 2006), especially when handling strains maintained under laboratory conditions for a long time (Palinska *et al.*, 1996; Nübel *et al.*, 1997; Zapomělová, 2006). Cases of misidentifications of *Aphanizomenon* spp. based on morphological features have been reported (Li *et al.*, 2000; Li *et al.*, 2003; Wood *et al.*, 2007). Thus, it is essential to combine molecular and morphological data to allow an accurate cyanobacterial taxonomic analysis as discussed over the past years (Komárek, 2006). Methods based on PCR amplification and the use of cyanobacteria-specific primers targeting highly conserved domains inside the *16S rRNA* gene (Nübel *et al.*, 1997) have contributed to a more objective identification and discrimination of cultured cyanobacterial strains. The use of specific primers is important since it can be very difficult to obtain axenic cultures from cyanobacteria, particularly filamentous strains (Abed & Köster, 2005; Bruno et al., 2006). Complementary to this, partial sequences of protein-coding genes such as *hetR*, coding for a serine type protease essential in heterocyst differentiation (Janson *et al.*, 1998; Schiefer *et al.*, 2002; Zhang *et al.*, 2006), and *nifH*, coding for the dinitrogenase reductase (Zehr & McReynolds, 1989), also give relevant information for phylogenetic differentiation (Dyble *et al.*, 2002; Haande *et al.*, 2008). Fingerprinting based on repetitive sequences such as short tandemly repeated repetitive (STRR) (Mazel *et al.*, 1990), and highly iterated palindrome (HIP1) (Smith *et al.*, 1998) sequences, which are present in many cyanobacteria, has been also a very useful technique to assess genetic variability among cyanobacterial strains even using non-axenic cultures (Rasmussen & Svenning, 1998; Lehtimäki et al., 2000; Bruno et al., 2006).

During the occurrence of cyanobacterial blooms between 2001 and 2007 at Vela Lake, eleven isolates of *Aphanizomenon* spp. were obtained. The present study deals with their morphological identification, their characterization through molecular approaches by targeting regions of the genes *16S rRNA*, *hetR* and *nifH*, and their fingerprinting using repetitive sequences in order to check for intra-specific genetic variability.

Materials and methods

Sampling, isolation and culturing of cyanobacteria

Water samples for isolation of *Aphanizomenon* strains were collected at the eutrophic Vela Lake located in Quiaios, Figueira da Foz (40° 16' N, 8° 47' W, Western Central Portugal) during the occurrence of cyanobacterial blooms in spring/summer of 2001, 2004, 2005, 2006 and 2007 (see Table I) using sterilized glass bottles. This freshwater body has an area of approximately 0.7 km^2 and it is mainly used as an irrigation source for agriculture but also as a recreational area. The *Aphanizomenon gracile* strain UADFA11 also included in this study was isolated from Monte Novo reservoir at the Guadiana River Basin (38º 30' N, 7º 42' W, Southern Portugal) in the dry summer of 2005. It was used as an *Aph. gracile* external geographical reference. Other heterocystous cyanobacterial isolates (*Anabaena cylindrica* UTAD_A212 and *Nostoc* sp. UTAD_N213) and Chrooccocales cyanobacteria (*Synechococcus* sp. UADFSYN1 and *Microcystis* sp. UADFM10) were also used as external references for the fingerprinting analyses (Table I). Nonaxenic unicyanobacterial cultures were established through micromanipulation by repeated isolation steps in sterilized liquid MBL – Woods Hole culture medium until only one filament was detectable. The cultures were maintained in 250 mL erlenmeyers containing 100 mL of MBL medium in an incubation chamber with 20±2ºC and a $16h^L$:$8h^D$ photoperiod of 130 $\mu E.m^{-2}.s^{-1}$ provided by cool white fluorescent tubes. The taxonomic identification was made using descriptions from Hindák (2000), Rajaniemi et al. (2005), Komárek (2006), Komárek and Komárková (2006) and Willame et al. (2006).

DNA extraction and PCR amplifications

The DNA from cyanobacterial cultures was extracted after centrifugation of 2 mL of the culture and resuspension in 200 µL of TE buffer [10 mM Tris HCl, 1 mM EDTA, pH 8.0]. Lysis was performed by adding 1 $mg.mL^{-1}$ lysozyme and incubating at 37 ºC for 1h. DNA extraction and purification was performed using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and DNA was resuspended in TE buffer and stored at -20 ºC. PCR amplification of *16S rRNA* gene fragments was performed combining the bacterial universal primer 27F (Lane, 1991) with the cyanobacteria-specific reverse primer CYA781R (Nübel *et al.*, 1997) and the cyanobacteria-specific forward primers CYA106F (Nübel *et al.*, 1997) and CYA738F (Valério *et al.*, 2005) along with the bacterial universal primer 1492R (Lane, 1991). The *hetR* gene region was targeted using the primers hetr1 / hetr2 (Janson et al., 1998) and *nifH* gene fragments were amplified using the degenerate primers designed by Zehr and McReynolds (1989). Fingerprinting was performed using the primer STRR1A (Rasmussen & Svenning, 1998) and Hip1 altered primers HipCA / HipTG with two bases added to the 3' end (Smith *et al.*, 1998) derived from the Hip1

Table I. Phenotypic identification, origin and sampling date of the studied strains. *Aphanizomenon* strains have also the accession number, closest relative (after a BLAST search) and corresponding percentage similarity for the obtained 16S rDNA partial sequences (1420 bp).

			16S rRNA partial sequences (1420bp)		
Strain	Phenotypic identification	Origin and sample date	NCBI access. Nº	Closest relatives (accession Nº)	Percentage similarity (%)
UADFA1	*Aphanizomenon issatschenkoi* (Usačev) Proškina-Lavrenko / *Cuspidothrix issatschenkoi* (Usačev)	Vela Lake, August 2004	EF685373	*Aphanizomenon issatschenkoi* 0tu37s7 (AJ630446)	99
UADFA2	*Aphanizomenon gracile* (Lemmerm.) Lemmerm.	Vela Lake, May 2001	FJ895124	*Aphanizomenon* cf. *gracile* 271 (AJ293125)	99
UADFA3	*Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek	Vela Lake, August 2004	FJ895118	*Aphanizomenon aphanizomenoides* 04-43 (FM161350)	99
UADFA5	*Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek	Vela Lake, August 2004	FJ895119	*Aphanizomenon aphanizomenoides* 04-43 (FM161350)	99
UADFA6	*Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek	Vela Lake, August 2004	FJ895120	*Aphanizomenon aphanizomenoides* 04-43 (FM161350)	99
UADFA7	*Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek	Vela Lake, August 2004	FJ895121	*Aphanizomenon aphanizomenoides* 04-43 (FM161350)	99
UADFA8	*Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek	Vela Lake, August 2004	FJ895122	*Aphanizomenon aphanizomenoides* 04-43 (FM161350)	99
UADFA10	*Aphanizomenon gracile* (Lemmerm.) Lemmerm.	Vela Lake, August 2005	FJ895125	*Aphanizomenon* cf. *gracile* 271 (AJ293125)	99
UADFA11	*Aphanizomenon gracile* (Lemmerm.) Lemmerm.	Monte Novo reservoir, July 2005	FJ895126	*Aphanizomenon* cf. *gracile* 271 (AJ293125)	99
UADFA12	*Aphanizomenon gracile* (Lemmerm.) Lemmerm.	Vela Lake, July 2006	FJ895127	*Aphanizomenon* cf. *gracile* 271 (AJ293125)	99
UADFA13	*Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek	Vela Lake, July 2006	FJ895123	*Aphanizomenon aphanizomenoides* 04-43 (FM161350)	99
UADFA16	*Aphanizomenon gracile* (Lemmerm.) Lemmerm.	Vela Lake, June 2007	FJ895128	*Aphanizomenon gracile* 219 (AJ293124) *Aphanizomenon* cf. *flos-aquae* PMC9501 (AJ293128)	99 99
UTADA212	*Anabaena cylindrica* Lemmerm.	Mondego River, rice paddy, spring 2006	(Galhano et al., 2009)		
UTADN213	*Nostoc* sp.	Mondego River, rice paddy, spring 2006	(Galhano et al., 2010)		
UADFSYN1	*Synechoccocus* sp.	Vela Lake, April 2004	This study		
UADFM10	*Microcystis* sp.	Vela Lake, August 2006	This study		

consensus (Robinson *et al.*, 1995) to overcome potential confusing patterns containing many amplification fragments due to the high frequency of Hip1 sequence in the cyanobacteria genome. All primers were commercially synthesized (STABVida, Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 15 pmol of each primer, 1 U of *Taq* DNA polymerase and 50-200 ng of template DNA. The PCR programs for all primer sets included an initial denaturation at 94°C for 5 min and a final extension step for 10 min at 72ºC. For 27F / CYA781R and CYA106F / 1492R there were also included 30 cycles consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; for the primer pair CYA738F / 1492R there were included 35 cycles of 1 min at 94°C, 30 s at 51°C, and 1 min at 72°C; for *hetR* amplification there were performed 35 cycles consisting of 1 min at 94°C, 2 min at 52°C and 1 min at 72°C; for *nifH* amplification the PCR program included 30 cycles of 72 s at 93°C, 1 min at 50°C, and 90 s at 72°C. Cyanobacteria fingerprinting with STRR1A followed the original procedure with an annealing temperature of 56ºC (Rasmussen & Svenning, 1998) and the program for Hip1 primers included 95°C for 5 min and 30 cycles of 10 s at 95°C, 20 s at 40°C and 1 min at 72°C. Negative and positive control reactions were carried out simultaneously. PCR products were checked by electrophoresis on 1.5 % agarose gels at 80 V for 1 h 30 min, following ethidium bromide staining and visualization on a UV transilluminator. For fingerprinting profiles, PCRs were performed at least three times and electrophoresis was carried out on 1.5 % agarose gels for 3 h at 80 V and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder).

Sequencing, alignment of sequences and phylogenetic analyses

All amplicons were purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) and sequenced by STABVida (Portugal). The nucleotide sequences determined in the present study were deposited in the GenBank database under the accession numbers EF685373 and FJ895118 to FJ895128 for the *16S* rDNA, EF531706 and FJ895140 to FJ895150 for the *nifH* gene, and EF531705 and FJ895129 to FJ895139 for the *hetR* gene. A BLAST search was performed against all sequences deposited in the GenBank database. ClustalX version 1.83 (Thompson *et al.*, 1997) was used to align sequences and reconstruct trees using the neighbour-joining method with bootstrap values from 1000 NJ bootstrap replicates. Trees were visualized using the program TreeView (Page, 1996). The *hetR* and *nifH* sequences were translated using the Translate program from Expasy (http://www.expasy.org) and ClustalX was also used to align the amino acid sequences.

Results

Morphological identification of cyanobacterial strains

Following morphological criteria, the studied strains fell into the descriptions made by Komárek and Komárková (2006) for two morphotypes of the classical *Aphanizomenon* genus: *Aph. gracile* (which includes the morphospecies *Aph. aphanizomenoides*) and *Aph. issatschenkoi*. The strain UADFA1 (Fig. 1a

and b) showed to belong to the species *Aphanizomenon issatschenkoi* (Usačev) Proškina-Lavrenko 1968 (Komárek & Komárková, 2006; Willame et al., 2006) recently proposed as forming a new genus called

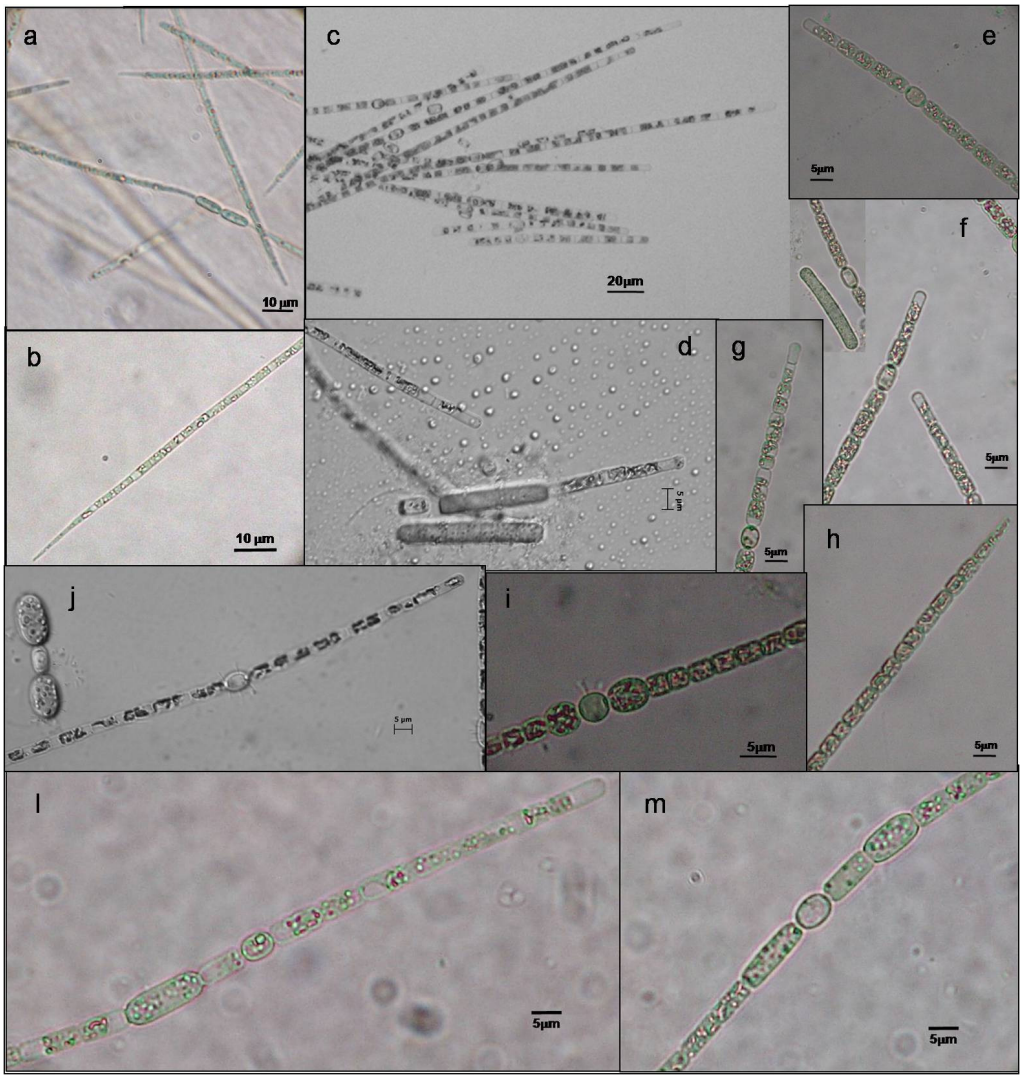

Fig. 1. Photomicrographs of trichomes of the *Aphanizomenon* morphospecies: a and b) *Aph. issatschenkoi* UADFA1; c and d) *Aph. gracile* UADFA12; e) *Aph. gracile* UADFA2; f) *Aph. gracile* UADFA10; g) *Aph. gracile* UADFA11; h and i) *Aph. aphanizomenoides* UADFA3; j) *Aph. aphanizomenoides* UADFA13; l and m) *Aph. gracile* UADFA16.

Cuspidothrix (Rajaniemi *et al.*, 2005b) with the type species *C. issatschenkoi* (*Aph. issatschenkoi* Otu37s7). This species is characterized by possessing planktonic solitary free-floating trichomes with cylindrical vegetative cells and distinguishably lengthened, pointed and hyaline apical cells; cylindrical akinetes (solitary or in pairs) are distant from heterocysts (Komárek & Komárková, 2006). Strains UADFA2 (Fig. 1e), UADFA10 (Fig. 1f), UADFA11 (Fig. 1g), UADFA12 (Fig. 1c and d) and UADFA16 (Fig. 1l and m) were found to belong to the species *Aphanizomenon gracile* (Lemmerm.) Lemmerm. 1907 [also known as *Aphanizomenon flos-aquae* f. *gracile* (Lemmerm.) Elenkin 1938 and anciently known as *Anabaena flos-aquae* var. *gracile* Lemmerm. 1898] (Hindák, 2000). This species has planktonic free-floating straight solitary trichomes with barrel-shaped vegetative cells (with aerotopes always present) and narrowed towards the ends with apical cells rounded or with an encircling or "drop-like" tip; cylindrical and usually

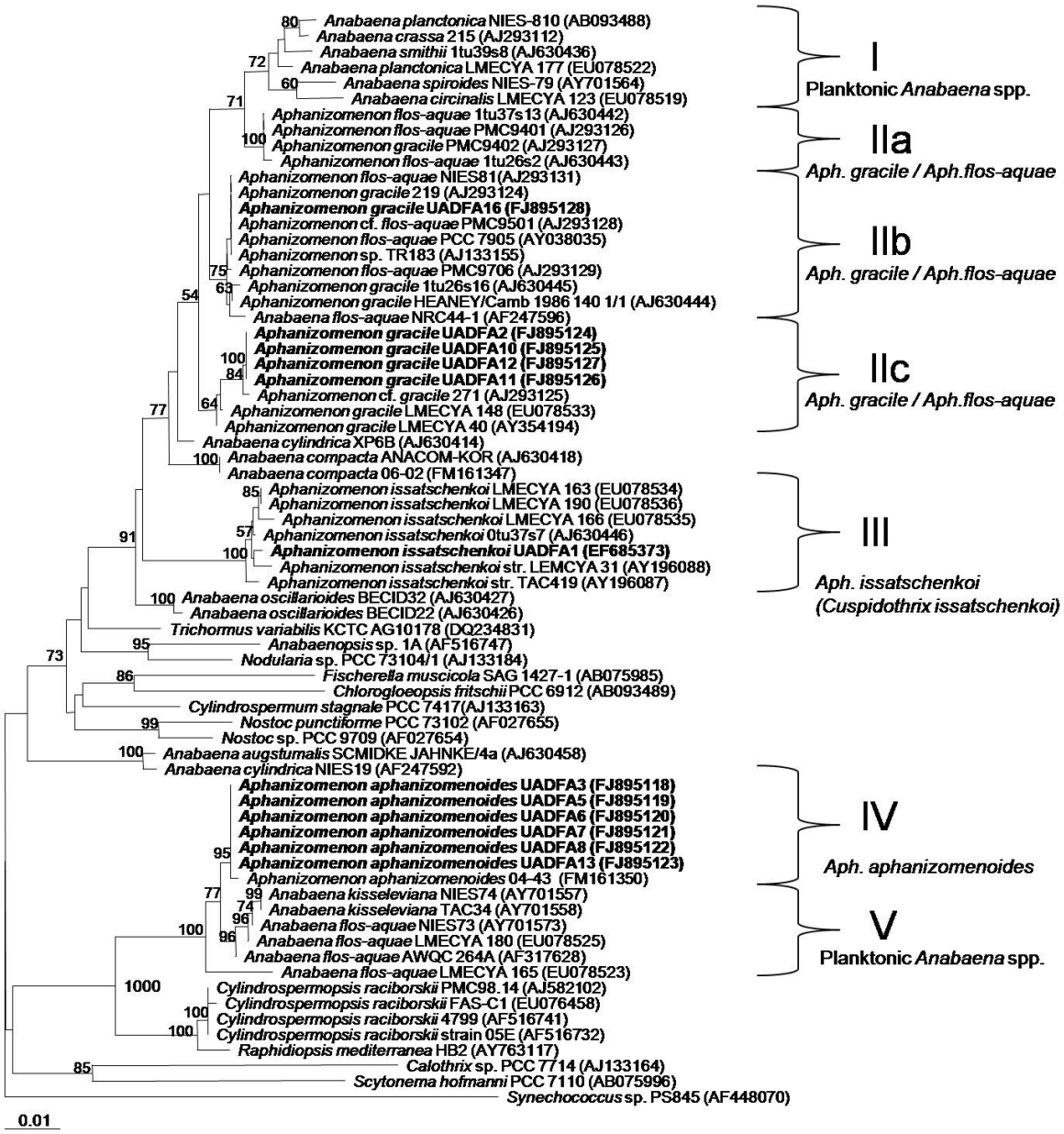

Fig. 2. Neighbour-joining tree constructed using *16S rRNA* gene partial sequences (approximately 650 bp) from cultured cyanobacterial isolates (sequences with accession numbers). Sequence from a *Synechococcus* sp. was used as the outgroup. Percentages of 1000 bootstraps replicates are indicated near the nodes (only those values > 50 % are shown).

solitary akinetes are distant from heterocysts (Komárek & Komárková, 2006). However, *Aph. gracile* may be confused with *Aph. flos-aquae* although the later has thicker trichomes and apical cells more vacuolated and elongated towards the ends besides forming fascicle-like colonies (Komárek & Kováčik, 1989). The strains UADFA3 (Fig. 1h and i), UADFA5, UADFA6, UADFA7, UADFA8 and UADFA13 (Fig. 1j) showed to belong to the species *Aphanizomenon aphanizomenoides* (Forti) Horecká et Komárek 1979 (also

described as *Anabaena aphanizomenoides* Forti 1912) (Hindák, 2000; Stefaniak & Kokociński, 2005). According to Komárek and Komárková (2006) this planktonic species (also included in the *Aph. gracile* morphotype group) has *Anabaena*-like features such as rounded akinetes (solitary or in two on each side of the heterocyst) (see Figs. 1j and i); trichomes are straight, planktonic and free-floating with barrel-shaped vegetative cells (with aerotopes always present); terminal cells are slightly narrowed towards the end (as shown in Fig. 1h).

16S rRNA gene sequences for the studied strains

The *16S rRNA* gene sequences (Table I, based on 1420 bp) and phylogenetic analysis (Fig. 2, based on a 650 fragment to include a wider diversity of sequences) showed UADFA1 strain belonged to the *Aph. issatschenkoi* / *Cuspidothrix issatschenkoi* clade (cluster III in Fig. 2) which is distinctly separated from the clades containing other *Aphanizomenon* or *Anabaena* species. The highest similarity (99%) was observed with several *Aph. issatschenkoi* strains such as the type strain 0tu37s7 isolated from a eutrophic lake in Northern Europe (Finland) (Rajaniemi *et al.*, 2005a) and the strain LMECYA 31 isolated from the Portuguese Montargil reservoir (Li *et al.*, 2003). UADFA2, UADFA10, UADFA11 and UADFA12 sequences showed 99% similarity with the sequence of *Aphanizomenon* cf. *gracile* PH271, isolated from a Danish lake (Lyra *et al.*, 2001; Gugger *et al.*, 2002). However, strain UADFA16 achieved the highest sequence similarity (99%) with strains such as *Aph.* cf. *flos-aquae* PMC9501, *Aph. flos-aquae* PCC 7905 (type strain) and *Aph. gracile* PH219 isolated from lakes in France, The Netherlands and Denmark, respectively (Gugger *et al.*, 2002). This genetic variability was sufficient to locate this strain in a clade separated from the other *Aph. gracile* UADFA strains suggesting this species is polyphyletic (clusters IIa, b and c in Fig. 2). All the studied *Aph. aphanizomenoides*-like strains clustered with planktonic *Anabaena* species within an independent clade (clusters IV and V in Fig. 2), clearly separated from the clades of *Aph. issatschenkoi* and *Aph. gracile* strains. The studied strains showed 99% sequence similarity with *Aphanizomenon aphanizomenoides* 04-43, isolated from an eutrophic freshwater fishpond in Svet (South Bohemia, Czech Republic). Yet, UADFA3 and UADFA13 sequences were 99% similar to *Anabaena flos-aquae* NIES73 and *Anabaena kisseleviana* NIES74 (both isolated from Kasumigaura Lake in Japan (Lyra *et al.*, 2001)) as well as *Anabaena flos-aquae* AWQC 264A; UADFA5 showed 99, 98 and 98% similarities with the sequences of these strains, respectively while sequences from the remaining strains (UADFA6, UADFA7 and UADFA8) showed 98, 99 and 99% similarities with the strains mentioned above, correspondingly. This suggests a genetic variability among the isolated strains as confirmed by fingerprinting and 16S rDNA sequence alignments (see sections ahead).

Nucleotide sequences of hetR gene fragments

Table II. Accession number, closest relative (after a BLAST search) and corresponding percentage similarity for the partial sequences of *hetR* and *nifH* genes from the isolated *Aphanizomenon* strains.

Strain	*hetR* partial sequences			*nifH* partial sequences		
	NCBI Accession Nº	Closest relatives (accession Nº)	Percentage similarity (%)	NCBI Accession Nº	Closest relatives (accession Nº)	Percentage similarity (%)
Aphanizomenon issatschenkoi						
UADFA1	EF531705	*Anabaena* sp. M14-4 (AF314179)	90	EF531706	Uncultured cyanobacterium clone FALLSnif01B02 (DQ398373)	97
Aphanizomenon gracile						
UADFA2	FJ895136	*Aphanizomenon* sp. TR183 (AF364338) *Anabaena flos-aquae* SAG30.87 (AY222367)	95 95	FJ895146	Uncultured cyanobacterium clone FALLSnif01B02 (DQ398373)	99
UADFA10	FJ895135	*Aphanizomenon* sp. TR183 (AF364338) *Anabaena flos-aquae* SAG30.87 (AY222367)	95 95	FJ895147	Uncultured cyanobacterium clone FALLSnif01B02 (DQ398373)	99
UADFA11	FJ895138	*Aphanizomenon* sp. TR183 (AF364338) *Anabaena flos-aquae* SAG30.87 (AY222367)	95 95	FJ895149	Uncultured cyanobacterium clone FALLSnif01B02 (DQ398373)	99
UADFA12	FJ895137	*Aphanizomenon* sp. TR183 (AF364338) *Anabaena flos-aquae* SAG30.87 (AY222367)	95 95	FJ895148	Uncultured cyanobacterium clone FALLSnif01B02 (DQ398373)	99
UADFA16	FJ895139	*Anabaena flos-aquae* SAG30.87 (AY222367) *Aphanizomenon* sp. TR183 (AF364338)	**98** **97**	FJ895150	Uncultured cyanobacterium clone Gt1463 (DQ250430)	98
Aph. aphanizomenoides						
UADFA3	FJ895129	*Anabaena* sp. M14-4 (AF314179) *Calothrix* PCC7507 (AF135805)	83 82	FJ895140	*Anabaena aphanizomenoides* NRE2 (AY461416)	98
UADFA5	FJ895130	*Cylindrospermum licheniforme* UTEX 2014 (AB075810) *Anabaena* sp. M14-4 (AF314179)	**84** **83**	FJ895141	*Anabaena aphanizomenoides* NRE2 (AY461416)	**97**
UADFA6	FJ895131	*Anabaena* sp. M14-4 (AF314179) *Calothrix* PCC7507 (AF135805)	83 82	FJ895142	*Anabaena aphanizomenoides* NRE2 (AY461416)	98
UADFA7	FJ895132	*Anabaena* sp. M14-4 (AF314179) *Calothrix* PCC7507 (AF135805)	83 82	FJ895143	*Anabaena aphanizomenoides* NRE2 (AY461416)	98
UADFA8	FJ895133	*Anabaena* sp. M14-4 (AF314179) *Calothrix* PCC7507 (AF135805)	83 82	FJ895144	*Anabaena aphanizomenoides* NRE2 (AY461416)	98
UADFA13	FJ895134	*Anabaena* sp. M14-4 (AF314179) *Calothrix* PCC7507 (AF135805)	83 82	FJ895145	*Anabaena aphanizomenoides* NRE2 (AY461416)	98

The *hetR* fragment from UADFA1 yielded only a maximum of 90% similarity with sequences from *Anabaena* strains M14-2 and M14-4 (see Table II and Fig. 3) which highlights the lack of published *hetR* sequences. The sequences of the *hetR* gene from UADFA2, UADFA10,

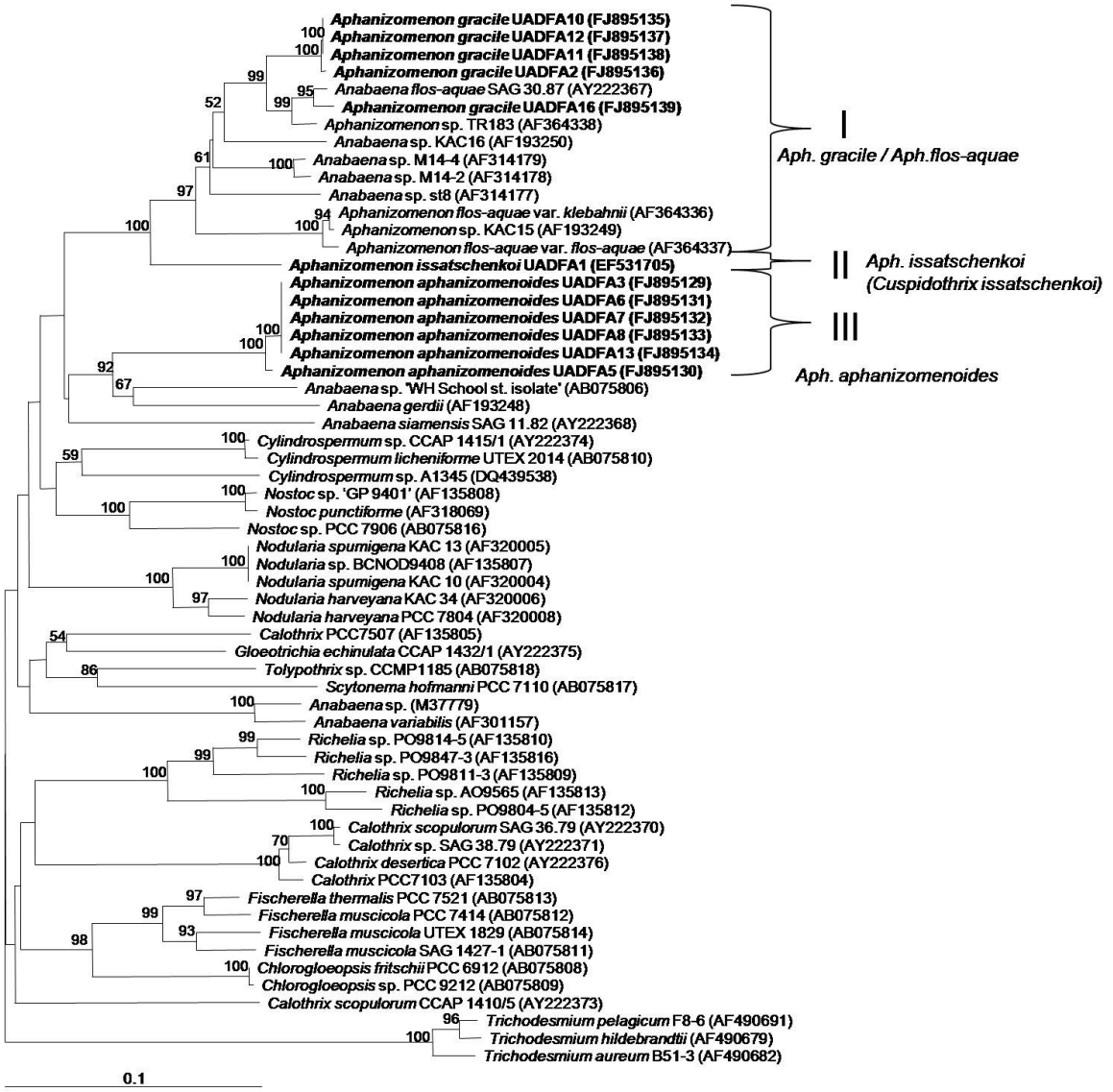

Fig. 3. Neighbour-joining tree constructed using *hetR* gene partial sequences (450 bp) from cultured cyanobacterial isolates (sequences with accession numbers). Sequences from *Trichodesmium* spp. were used as the outgroup. Percentages of 1000 bootstraps replicates are indicated near the nodes (only those values > 50 % are shown). The scale bar represents 0.1 substitutions per site.

UADFA11 and UADFA12 showed 95% similarity with sequences from *Aphanizomenon* sp. TR18 (isolated from the Baltic Sea) and *Anabaena flos-aquae* SAG 30.87 (isolated from Burton Lake, Canada) whereas UADFA16 showed 97 and 98% similarities with these same strains, respectively. Strains UADFA3, UADFA6, UADFA7, UADFA8 and UADFA13 all showed 86, 85 and 84% similarities with *hetR* sequences from *Anabaena gerdii*, *Anabaena* sp. 'WH School st. isolate' and *Cylindrospermum licheniforme* UTEX 2014, respectively. However, UADFA5 showed a point mutation in the *hetR* gene which altered the percentage similarities for 86, 85 and 83%,

respectively. The results obtained above for the *hetR* sequences suggest their potential for intra-specific phylogenetic discrimination.

Nucleotide sequences of nifH gene fragments

The partial *nifH* gene sequence from UADFA1 attained the highest similarity (97%) with an uncultured cyanobacterium FALLSnif01B02 detected at a meso to eutrophic lake in North Carolina (USA) and 95% similarity with *Aphanizomenon* sp. KAC15 isolated from the Baltic Sea (Table II and Fig. 4). All *Aph. gracile*-like strains showed *nifH* fragments 99% similar to cyanobacterium

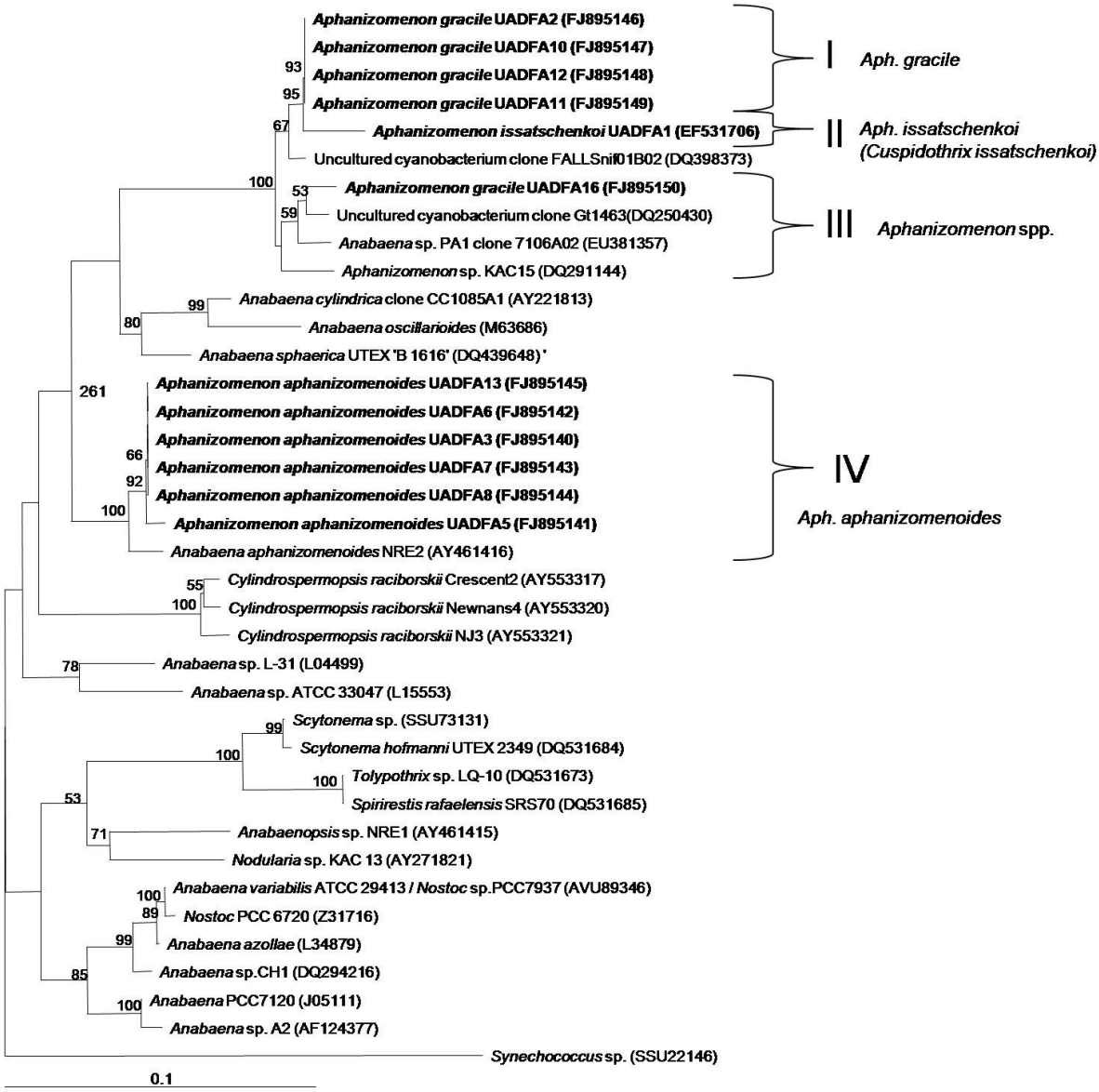

Fig. 4. Neighbour-joining tree constructed using *nifH* gene partial sequences (approximately 300 bp) from cultured cyanobacterial isolates (sequences with accession numbers). The sequence from a *Synechococcus* sp. was used as the outgroup. Percentages of 1000 bootstraps replicates are indicated near the nodes (only those values > 50 % are shown). The scale bar represents 0.1 substitutions per site.

FALLSnif01B02 (already mentioned above) except for UADFA16 which showed the highest similarity (98%) with an uncultured cyanobacterium clone Gt1463 isolated from the Baltic Sea, in

Sweden. In fact, from the *nifH* phylogenetic tree UADFA16 does not cluster together within the other *Aph. gracile* strains (Fig. 4) supporting the genetic variability inside the species. The *nifH* sequences from *Aph. aphanizomenoides* strains clustered, once more, distinctly separated from the other *Aphanizomenon* spp.. UADFA3, UADFA6, UADFA7, UADFA8 and UADFA13 sequences showed 98% similarity with *Anabaena aphanizomenoides* strain NRE2 isolated from Neuse River Estuary (North Carolina, USA) whereas UADFA5 showed only 97% similarity with this same strain, confirming once more the intra-specific genetic variation among *Aph. aphanizomenoides* strains.

Repetitive sequences fingerprinting of Aph. gracile and Aph. aphanizomenoides strains

In order to further explore the genetic variability among the strains from each studied species, repetitive sequences genetic fingerprinting was performed. Hip1 fingerprinting (Fig. 5) showed identical patterns for isolates UADFA2 and UADFA10 but differences in relation to UADFA11 and UADFA12 profiles; more, a clear distinct pattern was observed for UADFA16. The results suggest that *Aph. gracile* has a persistent occurrence at Vela Lake throughout different years (2001, 2005, 2006 and 2007) but not in a clonal mode over that period. More, Hip1 fingerprinting patterns of UADFA11 (from Monte Novo reservoir) showed to be different from those retrieved from Vela Lake strains, indicating geographical differences of *Aph. gracile* strains over the Portuguese territory. Hip1 fingerprinting for *Aph. aphanizomenoides* strains showed identical patterns for UADFA6, UADFA7 and UADFA8, which showed to be different from the other strains. UADFA5 displayed a pattern identical to UADFA13 while UADFA3 and UADFA13 showed a minor difference in their profiles. Nevertheless, results from STRR1A fingerprinting (Fig. 6) and 16S rDNA sequences alignment (see supplementary data online Fig. 7b) suggest these strains were identical. More information could be retrieved from STRR1A fingerprinting profiles: UADFA6

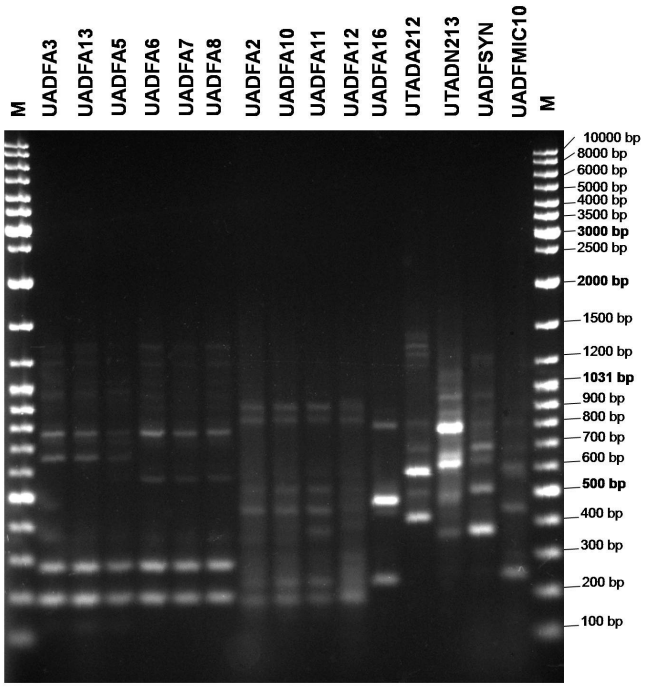

Fig. 5. Hip-CA/TG fingerprinting patterns of genomic DNA of *Aphanizomenon aphanizomenoides* and *Aph. gracile* strains. Strains UADFMIC10, UADFSYN, UTADA212 and UTADN213 were used as external controls. Lanes M correspond to DNA the molecular weight standard GeneRuler™ 1 kb DNA ladder.

and UADFA7 also showed identical fingerprinting patterns but strains UADFA5 and UADFA8 showed unique and distinct patterns. These results suggest clonal diversity within a same bloom of *Aph. aphanizomenoides* at Vela Lake (differences in strains collected in 2004). Although STRR1A fingerprinting yielded a better discrimination than the HipCA/HipTG fingerprinting for *Aph. aphanizomenoides* strains, STRR1A primer was not suitable for *Aph. gracile* strains and suggest that STRR sequences may be absent or have a different nucleotide composition in these strains.

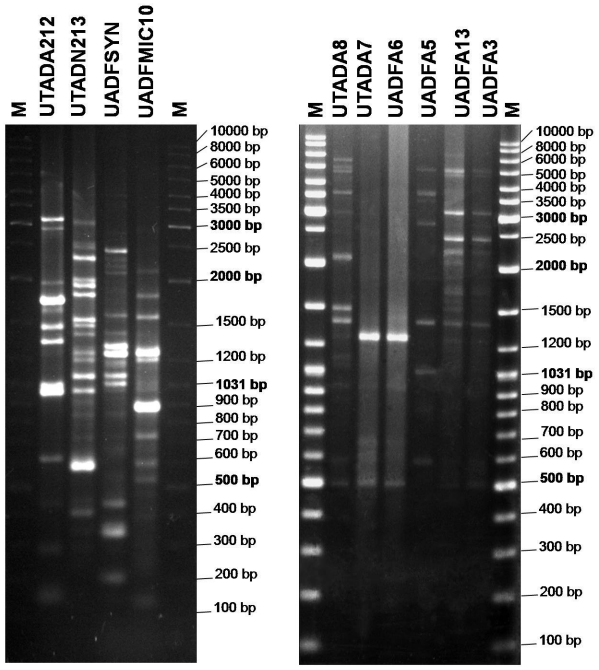

Fig. 6. STRR1A fingerprinting patterns of genomic DNA of *Aphanizomenon aphanizomenoides* strains. Strains UADFMIC10, UADFSYN, UTADA212 and UTADN213 were used as external controls. Lanes M correspond to DNA the molecular weight standard GeneRuler™ 1 kb DNA ladder.

Discussion

Morphological identification of *Aphanizomenon* spp. may be frequently unfeasible (Komárek & Komárková, 2006). More, laboratory culturing may also change the original cyanobacterial morphological features (Pearson & Kingsbury, 1966; Palinska et al., 1996; Nübel et al., 1997; Zapomělová, 2006) making even harder a correct identification. Thus, it is essential a detailed genetic characterization along with the morphological description for a more accurate identification, as proposed by Komárek (2006). In this study, information retrieved from *16S rRNA* gene corroborated the morphological identification of the studied strains.

In general, the trend for phylogenetic clustering observed in the *16S rRNA* gene sequences tree (Fig. 2) was also evidenced in the trees using partial sequences from the *nifH* and *hetR* genes (Figs. 3 and 4) confirming their potential for phylogenetic discrimination (Dyble *et al.*, 2002; Haande *et al.*, 2008). However, there is still a lack of sequences (particularly for *hetR* and *nifH* genes) on GenBank for the species characterized in the present study; this leads to low similarity percentages with database sequences and hinders to go further in phylogenetic analysis. The *16S rRNA* gene phylogenetic analysis suggests the *Aphanizomenon* genus is very heterogeneous and polyphyletic. The *Aph. issatschenkoi* / *Cuspidothrix issatschenkoi* strains are distinctly separated from the other *Aphanizomenon* or *Anabaena* species, supporting the separation of *Aph. issatschenkoi* into the new *Cuspidothrix* genus as previously reported (Rajaniemi et al., 2005b; Komárek & Komárková, 2006; Willame et al., 2006). *Aph. issatschenkoi* (*C. issatschenkoi*) is

characteristic from stagnant freshwaters in temperate regions (Rajaniemi *et al.*, 2005b) and it is known to occur in Spanish (Moreno *et al.*, 2005) and lentic Portuguese water bodies (Li *et al.*, 2003; Valério *et al.*, 2005) such as Vela Lake.

The genetic variability found between *Aph. gracile* strains (namely the strains from this study) suggests that this species is polyphyletic and 16S rDNA has intra-specific discrimination potential for *Aph. gracile* strains (see supplementary data online Fig. 7a). Results from partial sequencing and deduced amino acid sequences of *hetR* and *nifH* genes (see also supplementary data online Fig. 8a) and from Hip1 fingerprinting corroborate that strain UADFA16 was indeed very different from the other studied *Aph. gracile* strains (which showed similar fingerprinting patterns). However, 16S rDNA alignments confirmed that the strain from Monte Novo reservoir (UADFA11) was different from Vela Lake strains (UADFA2, UADFA10, UADFA12 and UADFA16). Both these results suggest that *Aph. gracile* has a non-clonal persistent occurrence at Vela Lake throughout different years and at different geographic locations in Portugal. Although morphological criteria for distinguishing *Aph. gracile* from *Aph. flos-aquae* are well established, the phylogenetic results obtained support the idea that there is not a clear separation between strains identified as *Aph. flos-aquae* or *Aph. gracile* as reported by other authors (Stüken *et al.*, 2009). Further phylogenetic studies should explore these two *taxa*; however, there may be misidentifications based on non up-to-date references (e.g. *Anabaena flos-aquae* NRC44-1 in cluster I of Fig. 2). *Aph. gracile* is known to be planktonic in water bodies with high water retention times (Komárek & Komárková, 2006) including Portuguese lakes and reservoirs (Pereira *et al.*, 2004) similar to Vela Lake. However, there may have been misidentifications of *Aph. gracile* as *Aph. flos-aquae* such as in the case of the bloom-forming strain UADFA2 (this study) isolated in 2001 at Vela Lake (de Figueiredo *et al.*, 2006).

Interestingly, 16S rDNA phylogenetic analysis showed that all *Aph. aphanizomenoides*-like strains clustered with planktonic *Anabaena* species within a clade clearly separated from the clades including *Aph. issatschenkoi* and *Aph. gracile* strains, suggesting a higher similarity with sequences of *Anabaena* spp. such as *An. kisseleviana* and *An. flos-aquae* rather than with typical *Aphanizomenon* spp.. This seems to be in accordance with its initial description as an *Anabaena* sp. which still generates a controversial discussion. Both fingerprinting results showed to be complementary, with different discriminating potential for intra-specific genetic variability of strains as confirmed by 16S rDNA sequences alignment (see supplementary data online Fig. 7b). Thissuggests there should be always used more than one fingerprinting analysis. In general, results from fingerprinting and *16S rRNA*, *hetR* and *nifH* partial sequencing and alignment of deduced translation results (supplementary data online Fig. 8b) indicate there is not a clonal persistence of *Aph. aphanizomenoides* strains at Vela Lake over the years neither within a same bloom; this has been reported before for other cyanobacteria, namely *Microcystis* spp. (Yoshida *et al.*, 2005;

Kardinaal *et al.*, 2007), which may lead to repercussions on the overall development and toxicity of the bloom. However, it should be kept in mind that spontaneous mutants are known to occur among cultured cyanobacteria under certain media conditions (Jeeji-Bai, 1976; Singh, 1976), namely in *Anabaena aphanizomenoides* strains (Das & Singh, 1977), which may also be behind the intra-specificity detected among the studied strains. Nevertheless, for an efficient management of bloom development at a water body the identification of the bloom-forming cyanobacteria must be accurate and thus molecular approaches are essential. *Aph. aphanizomenoides* is a planktonic species which has been considered an invader of the Northern Europe along with other cyanobacteria such as *Cylindrospermopsis raciborskii* (Stefaniak & Kokociński, 2005; Stüken et al., 2006) which highlights the importance to study its occurrence patterns. It develops in eutrophic lakes and reservoirs under high water temperatures, typically in tropical and subtropical regions; however, in recent years the occurrence of this species has been increasingly reported for several water bodies from temperate regions (Horecká & Komárek, 1979; Komárek & Komárková, 2006; Stüken et al., 2006) including several Iberian water bodies such as the Portuguese Montijo reservoir (Moreno *et al.*, 2005) and Vela Lake as reported in the present study. Besides water temperature, environmental conditions such as high light intensity and nutrient deficiency favour the massive development of *Aph. aphanizomenoides* (Sabour *et al.*, 2005).

Conclusions

According to both phenotypic and molecular results, the studied strains could be identified as belonging to the species *Aphanizomenon issatschenkoi* (*Cuspidothrix issatschenkoi*), *Aph. gracile* and *Aph. aphanizomenoides*.

The phylogenetic trees retrieved from partial sequences of *16S rRNA, nifH* and *hetR* genes showed a similar clustering trend confirming their potential for phylogenetic discrimination as previously proposed (Dyble *et al.*, 2002; Haande *et al.*, 2008). More, the phylogenetic results suggest that *Aph. gracile* is clearly polyphyletic but there is not an obvious separation from *Aph. flos-aquae* strains suggesting these should be considered as only one species as recently reported by Stüken et al. (2009). The molecular results from the present study also imply that *Aph. aphanizomenoides* is closer to *Anabaena* spp. rather than to *Aphanizomenon* spp. which may raise the debate about its allocation from the *Anabaena* genus. Furthermore, results showed an inter-annual persistence of *Aph. gracile* and *Aph. aphanizomenoides* strains as well as intra-specific genetic variability of *Aph. aphanizomenoides* within a bloom which may impact the blooms' development.

The information retrieved from this study is intended to contribute for the development of a database on the occurrence of bloom-forming cyanobacteria at Vela Lake and similar water bodies in the Portuguese territory which may be an important help for the development of future water management strategies.

References

Abed R. M. M. & Köster J. (2005). The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. *International Biodeterioration and Biodegradation* 55: 29-37.

Bruno L., Billi D., Albertano P. & Urzi C. (2006). Genetic characterization of epilithic cyanobacteria and their associated bacteria. *Geomicrobiology Journal* 23: 293-299.

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Das B. & Singh P. K. (1977). Isolation, growth and nitrogen fixation of a gas vacuole-less mutant of the blue green alga *Anabaena aphanizomenoides*. *Cytobios* 17: 151-158.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Dyble J., Paerl H. W. & Neilan B. A. (2002). Genetic characterization of *Cylindrospermopsis raciborskii* (cyanobacteria) isolates from diverse geographic origins based on nifH and cpcBA-IGS nucleotide sequence analysis. *Applied and Environmental Microbiology* 68: 2567-2571.

Galhano V., Peixoto F., Gomes-Laranjo J. & Fernández-Valiente E. (2009a). Comparative toxicity of bentazon and molinate on growth, photosynthetic pigments, photosynthesis, and respiration of the Portuguese ricefield cyanobacterium *Nostoc muscorum*. *Environmental Toxicology* 9999: NA.

Galhano V., Peixoto F., Gomes-Laranjo J. & Fernández-Valiente E. (2009b). Differential effects of bentazon and molinate on *Anabaena cylindrica* , an autochthonous cyanobacterium of Portuguese rice field agro-ecosystems. *Water, Air, & Soil Pollution* 197: 211-222.

Gugger M., Lyra C., Henriksen P., Coute A., Humbert J. F. & Sivonen K. (2002). Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *International Journal of Systematic and Evolutionary Microbiology* 52: 1867-1880.

Haande S., Rohrlack T., Ballot A., RÃ¸berg K., Skulberg R., Beck M. & Wiedner C. (2008). Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) isolates from Africa and Europe. *Harmful Algae* 7: 692-701.

Hindák F. (2000). Morphological variation of four planktic nostocalean cyanophytes - Members of the genus *Aphanizomenon* or *Anabaena*? *Hydrobiologia* 438: 107-116.

Horecká M. & Komárek J. (1979). Taxonomic position of three planktonic blue- green algae from the genera *Aphanizomenon* and *Cylindrospermopsis*. *Preslia* 51: 289-312.

Janson S., Matveyev A. & Bergman B. (1998). The presence and expression of *hetR* in the non-heterocystous cyanobacterium *Symploca* PCC 8002. *FEMS Microbiology Letters* 168: 173-179.

Jeeji-Bai N. (1976). Morphological variation of certain blue-green algae in culture: *Scytonema stuposum* (Kütz.) Born. *Schweizerische Zeitschrift für Hydrologie* 38: 55-62.

Kardinaal W. E. A., Tonk L., Janse I., Hol S., Slot P., Huisman J. & Visser P. M. (2007). Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. *Applied and Environmental Microbiology* 73: 2939-2946.

Komárek J. (2006). Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. *Algae* 21: 349-375.

Komárek J. & Komárková J. (2006). Diversity of *Aphanizomenon*-like cyanobacteria. *Czech Phycology* 6: 1-32.

Komárek J. & Kováčik L. (1989). Trichome structure of *fourAphanizomenon* taxa (Cyanophyceae) from Czechoslovakia, with notes on the taxonomy and delimitation of the genus. *Plant Systematics and Evolution* 164: 47-64.

Lane D. J., 1991. 16S/23S rRNA sequencing. In: E. Stackebrandt & M. Goodfellow, (eds.), Nucleic acid techniques in bacterial systematics. Wiley & Sons, Chichester, United Kingdom: 115-175.

Lehtimäki J., Lyra C., Suomalainen S., Sundman P., Rouhiainen L., Paulin L., Salkinoja-Salonen M. & Sivonen K. (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *International Journal of Systematic and Evolutionary Microbiology* 50: 1043-1053.

Li R., Carmichael W. W. & Pereira P. (2003). Morphological and 16s rRNA gene evidence for reclassification of the paralytic shellfish toxin producing *Aphanizomenon flos-aquae* LMECYA 31 as *Aphanizomenon issatschenkoi* (Cyanophyceae). *Journal of Phycology* 39: 814-818.

Li R., Watanabe M. & Watanabe M. M. (2000). Taxonomic studies of planktic species of *Anabaena* based on morphological characteristics in cultured strains. *Hydrobiologia* 438: 117-138.

Lyra C., Suomalainen S., Gugger M., Vezie C., Sundman P., Paulin L. & Sivonen K. (2001). Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. *International Journal of Systematic and Evolutionary Microbiology* 51: 513-526.

Mazel D., Houmard J., Castets A. M. & Tandeau de Marsac N. (1990). Highly repetitive DNA sequences in cyanobacterial genomes. *Journal of Bacteriology* 172: 2755-2761.

Moreno I., Repetto G., Carballal E., Gago A. & Cameán A. M. (2005). Cyanobacteria and microcystins occurrence in the Guadiana River (SW Spain). *International Journal of Environmental Analytical Chemistry* 85: 461-474.

Nübel U., Garcia-Pichel F. & Muyzer G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology* 63: 3327-3332.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Palinska K. A., Liesack W., Rhiel E. & Krumbein W. E. (1996). Phenotype variability of identical genotypes: The need for a combined approach in cyanobacterial taxonomy demonstrated on *Merismopedia*-like isolates. *Archives of Microbiology* 166: 224-233.

Pearson J. E. & Kingsbury J. M. (1966). Culturally induced variation in four morphological diverse bluegreen algae. *American Journal of Botany* 53: 192-200.

Pereira P., Li R. H., Carmichael W. W., Dias E. & Franca S. (2004). Taxonomy and production of paralytic shellfish toxins by the freshwater cyanobacterium *Aphanizomenon gracile* LMECYA40. *European Journal of Phycology* 39: 361-368.

Rajaniemi P., Hrouzek P., Kastovska K., Willame R., Rantala A., Hoffmann L., Komarek J. & Sivonen K. (2005a). Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *International Journal of Systematic and Evolutionary Microbiology* 55: 11-26.

Rajaniemi P., Komárek J., Willame R., Hrouzek P., Kaštovská K., Hoffmann L. & Sivonen K. (2005b). Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. *Algological Studies, Archiv für Hydrobiologie* 117: 371-91.

Rasmussen U. & Svenning M. M. (1998). Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. *Applied and Environmental Microbiology* 64: 265-272.

Robinson N. J., Robinson P. J., Gupta A., Bleasby A. J., Whitton B. A. & Morby A. P. (1995). Singular over-representation of an octameric palindrome, HIP1, in DNA from many cyanobacteria. *Nucleic Acids Research* 23: 729-735.

Sabour B., Loudiki M., Oudra B., Vasconcelos V., Oubraim S. & Fawzi B. (2005). Dynamics and toxicity of *Anabaena aphanizomenoides* (Cyanobacteria) waterblooms in the shallow brackish Oued Mellah lake (Morocco). *Aquatic Ecosystem Health and Management* 8: 95-104.

Saker M. L., Nogueira I. C. G., Vasconcelos V. M., Neilan B. A., Eaglesham G. K. & Pereira P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55: 243-250.

Schiefer W., Schütz K., Hachtel W. & Happe T. (2002). Molecular cloning and characterization of hetR genes from filamentous cyanobacteria. *Biochimica et Biophysica Acta - Gene Structure and Expression* 1577: 139-143.

Singh P. K. (1976). Growth and nitrogen fixation of mutants defective in heterocyst differentiation in the blue green alga *Cylindrospermum* sp. *Zeitschrift fur Allgemeine Mikrobiologie* 16: 453-463.

Smith J. K., Parry J. D., Day J. G. & Smith R. J. (1998). A PCR technique based on the Hip1 interspersed repetitive sequence distinguishes cyanobacterial species and strains. *Microbiology* 144: 2791-2801.

Stefaniak K. & Kokociński M. (2005). Occurrence of invasive cyanobacteria species in polimictic lakes of the Wielkopolska Region (Western Poland). *Oceanological and Hydrobiological Studies* 34: 137-148.

Stüken A., Campbell R. J., Quesada A., Sukenik A., Dadheech P. K. & Wiedner C. (2009). Genetic and morphologic characterization of four putative cylindrospermopsin producing species of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Journal of Plankton Research* 31: 465-480.

Stüken A., Rücker J., Endrulat T., Preussel K., Hemm M., Nixdorf B., Karsten U. & Wiedner C. (2006). Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. *Phycologia* 45: 696-703.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. & Higgins D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Valério E., Pereira P., Saker M. L., Franca S. & Tenreiro R. (2005). Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. *Harmful Algae* 4: 1044-1052.

Vasconcelos V. M., Campos T., Amorim A. & Soares A. M. V. M. (1993). Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 193-201.

Willame R., Boutte C., Grubisic S., Wilmotte A., Komárek J. & Hoffmann L. (2006). Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. *Journal of Phycology* 42: 1312-1332.

Wood S. A., Rasmussen J. P., Holland P. T., Campbell R. & Crowe A. L. M. (2007). First report of the cyanotoxin anatoxin-a from *Aphanizomenon issatschenkoi* (cyanobacteria). *Journal of Phycology* 43: 356-365.

Yoshida M., Yoshida T., Takashima Y., Kondo R. & Hiroishi S. (2005). Genetic diversity of the toxic cyanobacterium *Microcystis* in Lake Mikata. *Environmental Toxicology* 20: 229-234.

Zapomělová E. (2006). Current taxonomic issues with planktonic representatives of the genus *Anabaena* (Cyanobacteria) with special reference to their morphological features; literary review. *Czech Phycology* 6: 33-47.

Zehr J. P. & McReynolds L. A. (1989). Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Applied and Environmental Microbiology* 55: 2522-2526.

Zhang C. C., Laurent S., Sakr S., Peng L. & Bédu S. (2006). Heterocyst differentiation and pattern formation in cyanobacteria: A chorus of signals. *Molecular Microbiology* 59: 367-375.

Effects of different nutrient levels on growth of bloom-forming *Aphanizomenon* strains isolated from a eutrophic temperate shallow lake – importance of intra-specific variation

In: de Figueiredo D. R., Gonçalves A. M. M., Castro B. B., Gonçalves F., Pereira M. J. & Correia, A. (submitted). Effects of different nutrient levels on growth of bloom-forming *Aphanizomenon* strains isolated from a eutrophic temperate shallow lake – importance of intra-specific variation.

Abstract

In Vela Lake (Figueira da Foz, Western Central Portugal), cyanobacterial blooms of *Aphanizomenon* spp. frequently occur during spring and summer months with deleterious effects over the aquatic communities. The present work aimed to investigate the effects of different phosphorus and nitrogen levels on the growth of *Aphanizomenon* bloom-forming strains isolated from Vela Lake. Algal inhibition tests were also performed in order to assess the potential allelopathic effects of the strains' filtrates over the growth of four green algae (*Pseudokirchneriella subcapitata, Chlorella vulgaris, Pandorina morum* and *Coelastrum astroideum*). Phosphorus concentration had a significant effect over the growth of all the tested strains. In general, orthophosphate concentrations equal to or below 0.3 mg PO_4^{3-} L^{-1} led to a decrease of more than 50 % in the cyanobacterial growth of most strains. The growth of *Aph. gracile* strains was unaffected by the presence or absence of aqueous nitrogen ($N\text{-}NO_3^-$), confirming their ability to fix atmospheric nitrogen; however, some strains (*Aph. issatschenkoi* UADFA1 and *Aph. aphanizomenoides* UADFA6, UADFA7 and UADFA13) were moderately to extremely sensitive to the absence of nitrate in the test medium. Culturing under saturation conditions and mutations were found as explanations for the morphological and physiological changes observed. Results strengthen the idea that the massive development of these cyanobacteria may be controlled through the management of nutrient-rich inputs, particularly phosphorus. However, intra-specific variation may play an important role for bloom formation. Allelopathic assays revealed a significant inhibition of microalgal growth only for filtrates of *Aph. gracile* strains UADFA2 and UADFA10. On the other hand, a stimulatory effect on microalgal growth was recorded for filtrates of *Aph. aphanizomenoides* strains.

Keywords

Aphanizomenon issatschenkoi (*Cuspidothix issatschenkoi*), *Aph. gracile, Aph. aphanizomenoides*, nutrient assays, allelopathic assays.

Introduction

Climatic changes are enhancing the occurrence of cyanobacterial blooms worldwide with effects on the aquatic organisms but also with risks for water consumers (Pouria et al., 1998; Codd, 2000; de Figueiredo et al., 2004b). Thus, it is important to characterize the conditions that may lead to the massive development of bloom-forming cyanobacteria as well as their potential effects over the aquatic communities. Cyanobacteria are known to develop into blooms under low N/P ratios (Dokulil & Teubner, 2000; Jacoby et al., 2000; de Figueiredo et al., 2006). It has been recently proven that the growth of diazotrophic filamentous cyanobacteria, in particular, will be enhanced by the expected global warming, with nutrient inputs playing a major role (Markensten et al., 2010). Species such as *Cylindrospermopsis raciborskii* (Woloszyńska) Seenayya & Subba Raju, *Aphanizomenon issatschenkoi* (Usačev) Proškina-Lavrenko / *Cuspidothrix issatschenkoi* (Usačev) and *Aphanizomenon aphanizomenoides* (Forti) Horecká and Komárek have already been considered invasive in Europe (Stüken et al., 2006; Wiedner et al., 2007; Kaštovský et al., 2010). Allelopathy seems also to be important to explain this expansion of invasive nitrogen-fixing filamentous species (Figueredo et al., 2007). However, the intra-specific diversity among strains needs more investigation, although there is evidence that it may play an important role for the success of a bloom, namely concerning toxicity (Neilan et al., 2003; Chonudomkul et al., 2004). The phytoplankton community, in particular, is strongly affected by cyanobacterial blooms, not only due to the advantageous competition of cyanobacteria over microalgae (by nitrogen fixation, phosphorus storage and/or buoyancy regulation for achieving better light and nutrients conditions) but also to the effect of cyanotoxins' release as well as a reduced grazing by zooplankton (Dokulil & Teubner, 2000; Kearns & Hunter, 2001; de Figueiredo et al., 2004a).

In the freshwater shallow Vela Lake (Figueira da Foz, Western Central Portugal), the development of cyanobacterial blooms during warmer months is frequent, with associated neuro and hepatotoxic risks (Vasconcelos et al., 1993a; Saker et al., 2003). The cyanobacteria responsible for blooms at this shallow freshwater lake have been identified as belonging to *Microcystis* spp., as well as some filamentous forms such as *Aphanizomenon flos-aquae* Ralfs ex Born. et Flah and *Anabaena flos-aquae* (Lyngb.) de Breb. (Vasconcelos et al., 1993a; Vasconcelos et al., 1996; Vasconcelos, 2001; de Figueiredo et al., 2006).

In the present work, an experimental laboratory approach was conducted with the purpose of assessing intra-specific variation in the growth requirements and allelopathic power of bloom-forming strains of the diazotrophic species *Aphanizomenon* (*Cuspidothrix*) *issatschenkoi*, *Aph. aphanizomenoides* and *Aph. gracile* (Lemmerm.) Lemmerm. isolated from Vela Lake. The strains were studied regarding: 1) the effect of nitrogen depletion and different phosphorus concentrations (in combination) on their growth; 2) the allelopathic potential (assessed by growth inhibition tests) over four green algae (*Pandorina morum* (Müller) Bory, *Pseudokirchneriella subcapitata*

(Korshikov) Hindak, *Chlorella vulgaris* Beijerinck and *Coelastrum astroideum* De Notaris). Intra-specific differential responses were investigated and correlated to morphological variability of strains.

Materials and methods

Cyanobacteria isolation and culturing

The shallow eutrophic Vela Lake (40º16'12''N, 8º47'30''W) is located in Figueira da Foz (Western Central Portugal) and occupies a floodable area of 70 ha (Castro & Gonçalves, 2007; Abrantes et al., 2009a). It is used as an irrigation source for agriculture but also as a swimming, sailing and recreation area. High nutrient inputs from agriculture occur, especially during raining seasons, leading to high levels of nitrate (up to 6.6 mg L^{-1}) and SRP (up to 1.7 mg L^{-1}); however, during summer months, the nitrate and phosphate levels severely decrease to almost undetectable values (de Figueiredo et al., 2006). During the summers of 2001, 2004, 2005 and 2006, several bloom-forming cyanobacteria were isolated from this lake (de Figueiredo et al., 2010a). Water samples were collected in sterilized glass bottles and nonaxenic unicyanobacterial cultures were

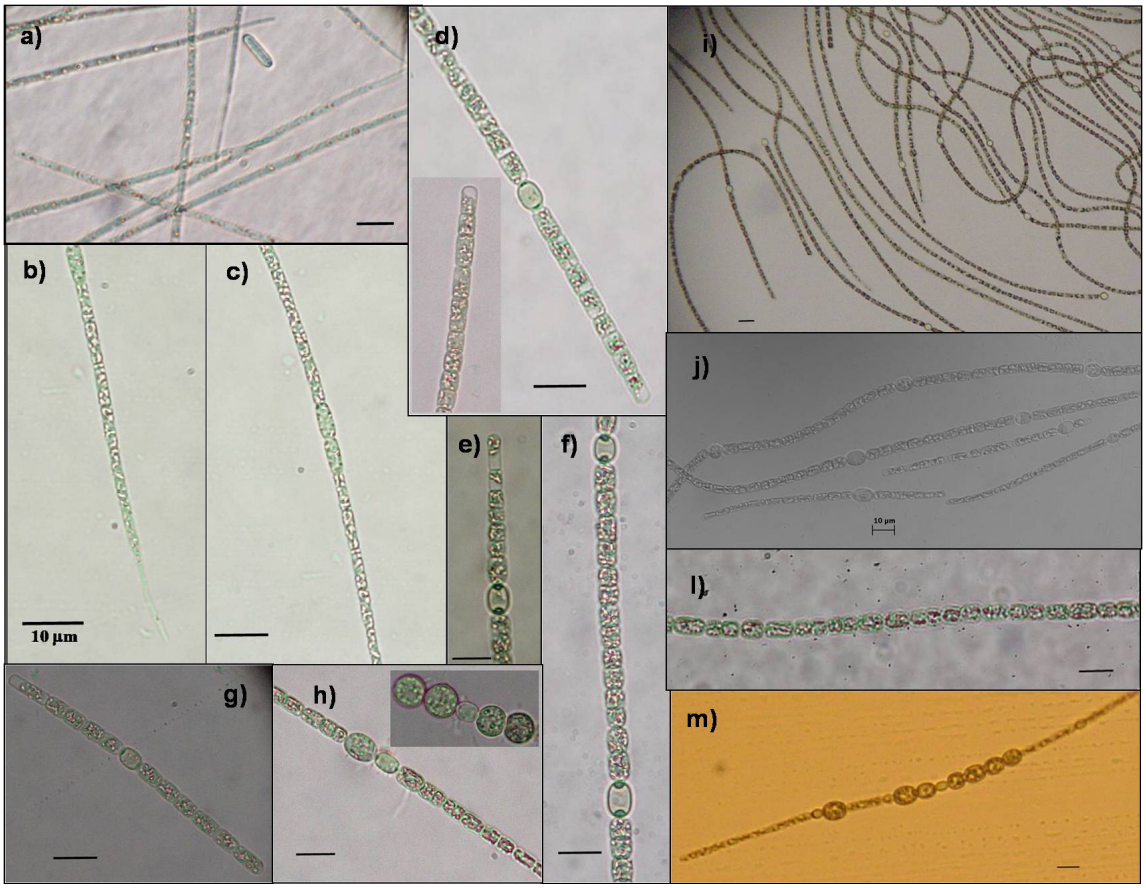

Fig. 1. Photomicrographs of trichomes belonging to: a, b and c) *Aphanizomenon issatschenkoi* (*Cuspidothrix issatschenkoi*) UADFA1 (Ai1); d) *Aph. gracile* UADFA2 (Ag2); e and f) *Aph. gracile* UADFA10 (Ag10); g) *Aph. gracile* UADFA12 (Ag12); h and m) *Aph. aphanizomenoides* UADFA3 (Aa3); i) *Aph. aphanizomenoides* UADFA6 (Aa6); j) *Aph. aphanizomenoides* UADFA13 (Aa13); l) *Aph. aphanizomenoides* UADFA7 (Aa7). The scale bar proportionally corresponds to 10 µm.

established through micromanipulation by repeated isolation steps in sterilized liquid MBL – Woods Hole culture medium. The strains were maintained as semi-continuous batch cultures in 250 mL erlenmeyers containing 100 mL of Woods Hole (MBL) culture medium (Nichols, 1973) in an incubation chamber with controlled temperature (20±2ºC), photoperiod regime ($16h^L$:$8h^D$), and light conditions (130 $\mu E.m^{-2}.s^{-1}$, provided by cool white fluorescent tubes).

Information on sampling date and taxonomic identification based on morphological (Fig. 1) and genetic information was previously published (de Figueiredo et al., 2010a). The strains were named as follows: *Aphanizomenon issatchenkoi* UADFA1 (Ai1); *Aphanizomenon gracile* UADFA2, UADFA10 and UADFA12 (Ag2, Ag10 and Ag12, respectively); and *Aphanizomenon aphanizomenoides* UADFA3, UADFA6, UADFA7 and UADFA13 (Aa3, Aa6, Aa7 and Aa13, in that order). It was noticed that the average number of heterocysts considerably varied among the *Aph. aphanizomenoides* strains in culture (Fig. 1). The frequency of heterocysts was determined under a microscope by counting their number per unit length of filament (a 100 cells segment) in a minimum of 30 filaments.

Nutrient assays

The cyanobacteria were grown in triplicate in several concentrations of the molecule PO_4 (from 0.0 to 8.7 mg PO_4^{3-} L^{-1} – Table 1) in 100 mL Erlenmeyer flasks with 40 mL of sterilized growth medium (modified MBL medium – see below), under nitrate saturated (85N) or nitrate depleted (0N) conditions, corresponding to 85 and 0 mg NO_3^- L^{-1}. All nutrient concentrations are expressed relatively to the respective molecule (PO_4^{3-} and NO_3^-). A preliminary test was performed to see if there were differences between the growth in "full" MBL medium (with 8.7 mg PO_4^{3-} L^{-1} and 85.0 mg NO_3^- L^{-1}) and modified MBL medium (containing 4.4 mg PO_4^{3-} L^{-1} and 8.5 mg NO_3^- L^{-1}) which

Table 1 Concentrations of nitrate and phosphate used in the nutrient assays, with respective codes.

Nitrate concentration (mg NO_3^- L^{-1})	**Phosphate concentration (mg PO_4^{3-} L^{-1})**						
	0.0	0.3	0.5	1.1	2.2	4.4	8.7
85.0	**0P**	**0.3P**	**0.5P**	**1P**	**2P**	**4P**	**9P (MBL)**
0.0	**0P0N**	**0.3P0N**	**0.5P0N**	**1P0N**	**2P0N**	**4P0N**	**9P0N**

is closer to realistic nutrient saturation situations in the lake during winter months, when precipitation is intense (de Figueiredo et al., 2006). All tests were performed during 7 days in an incubation chamber under controlled laboratory conditions (continuous agitation in an orbital shaker at 100 rpm, 20±2ºC and $16h^L$:$8h^D$ photoperiod with a light intensity of 130 $\mu mol.m^{-2}.s^{-1}$). Pre-cultures

prepared from stock cultures were grown under these conditions and retrieved at the beginning of the exponential phase in order to be used an inoculum for the tests. Initial cell density in each test corresponded to an optical density value of 0.0010 (which showed to be equivalent to an average of about 10^5 cells mL^{-1} for all strains).Optical density (measured spectrophotometrically at 440 nm) was the method chosen for biomass determination (de Figueiredo et al., 2004c). In order to demonstrate the robustness of using this rapid methodology, for some samples biomass was also assessed by cell counting (under a Sedgewick-Rafter counting chamber and extrapolating the total cell number by counting filaments and using a median cell number *per* filament) and chlorophyll *a* (filtering through a Whatman GF/C filter, extracting pigments with 90 % acetone and measuring chlorophyll *a* spectrophotometrically, according to the trichromatic method).

Allellopathic assays

Cyanobacteria were grown in nutrient saturated medium ("full" MBL) under the same temperature and light conditions of the former tests and harvested in a late exponential growth phase when it is admissible that levels of cyanotoxins may be higher, due to the beginning of cell lysis, as for other filamentous cyanobacteria (Rapala & Sivonen, 1998). The potential inhibitory effects of the cyanobacterial cell-free filtrates on the growth of the green algae were assessed. Commercial strains of *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Chlorella vulgaris* Beijerinck (unicellular species also present in Vela Lake) as well as strains of the colonial species *Pandorina morum* (Müller) Bory and *Coelastrum astroideum* De Notaris (isolated from Vela Lake) were used. The exudates or filtrates (growth medium and dissolved cyanobacterial products) were obtained after filtration of cultures through a Whatman GF/C filter. The algae were exposed to the exudates, during a 96 hours period, using a methodology previously described (de Figueiredo et al., 2004c; Gonçalves et al., 2005). Briefly, the treatments were performed with 40 % of the filtrate added to 60 % of MBL medium while control experiments were simultaneously performed with 40 % of distilled water added to 60 % MBL (in order to exclude any possible effect from nutrient deficiency due to the dilution caused by the added volume of filtrate); the tests were conducted in triplicate in 100 mL Erlenmeyer flasks with 40 mL of final test solution. All culture handling was aseptic and the vessels were randomly located in the growth chamber (as in the nutrient assays) under the same conditions previously described. Growth was quantified by measuring the algal biomass with chlorophyll *a* for *P. morum* and optical density at 440 nm for the other three algae as described by Gonçalves et al. (2005).

Statistical analysis

In order to compare the different methods of cyanobacterial biomass determination (optical density, cell counting and chlorophyll *a*), the Pearson correlation coefficient was applied. A two-way analysis of variance (ANOVA) was used to assess significant differences among the nutritional treatments (N and P), and to assess the significance of their potential interaction. Whenever an interaction was found, a test for simple main effects (similar to one-way ANOVA) of

P concentration within each N scenario (depletion or saturation) was carried out using the residual error term of the two-way ANOVA as the denominator for the F test (Quinn & Keough, 2002). Occasionally, deviations from homogeneity of variances or normality were detected, but we assumed the ANOVAs to be robust to these deviations. Experimental treatments for each cyanobacterial strain were analysed independently.

For each microalgal growth inhibition test (allelopathic assays), an independent t-test was performed to screen for significant differences between the cyanobacterial filtrates and the respective control. A fixed significance level (□) of 0.05 was used in all statistical analyses.

Results

Nutrient assays

In the nutrient assays, it was possible to record an intra-specific variation in the responses to the tested phosphate and nitrate concentrations. No significant interactions between N and P were recorded for three strains (Ag2, Aa3 and Aa6), suggesting a proportional growth under the same P concentrations, independently of N saturation or absence (Table 2 and Fig. 2). The significant effect of N reveals that, for the same level of P, the biomass of Ag2, Aa3, and Aa6 was significantly higher under N saturation conditions, which is most evident for Aa6 (Fig. 2; note that the absence or presence of N explained most of the variation in the biomass data of Aa6 – see mean squares (MS) partition in ANOVA table). Significant interactions between both P and N were observed in the biomass of the remaining strains (Table 2), which means that growth curves as a function of P concentration were not consistent between N-saturated and N-depleted conditions (Fig. 2). For Ag10, Ag12, and Aa13, P concentration contributed most to the variability of the data (see MS partition) and was found to be significant under both N scenarios (simple main effects, $P < 0.001$). In these cases, the significant interactions found are explained by a significantly higher (Ag10 and Ag12) or lower (Aa13) biomass in the absence of N, only at high P levels (Fig. 2). These three strains were able to grow in N-free medium, but their intrinsic variability (in terms of N preferences) becomes evident when P is no longer limiting (> 1.1 mg PO_4^{3-} L^{-1}).

A distinct scenario was recorded for strains Ai1 and Aa7 (Table 2), where the N x P interaction recorded suggests an apparent inability to fix nitrogen and grow under N depleted conditions (Fig. 2). A large contribution of N to the overall model variance was highlighted by the high F value (see also MS partition in Table 2). The analysis of simple main effects showed that the growth of these strains is only dependent of P concentration when N is available ($P < 0.001$), whereas in N-free medium there is no effect of P concentration ($P = 1.0$), because N becomes the limiting factor. The absence of growth in N-free medium, irrespective of P concentration, is an unexpected result for filamentous, heterocyst-bearer cyanobacteria (*i.e.*, putative diazotrophic species).

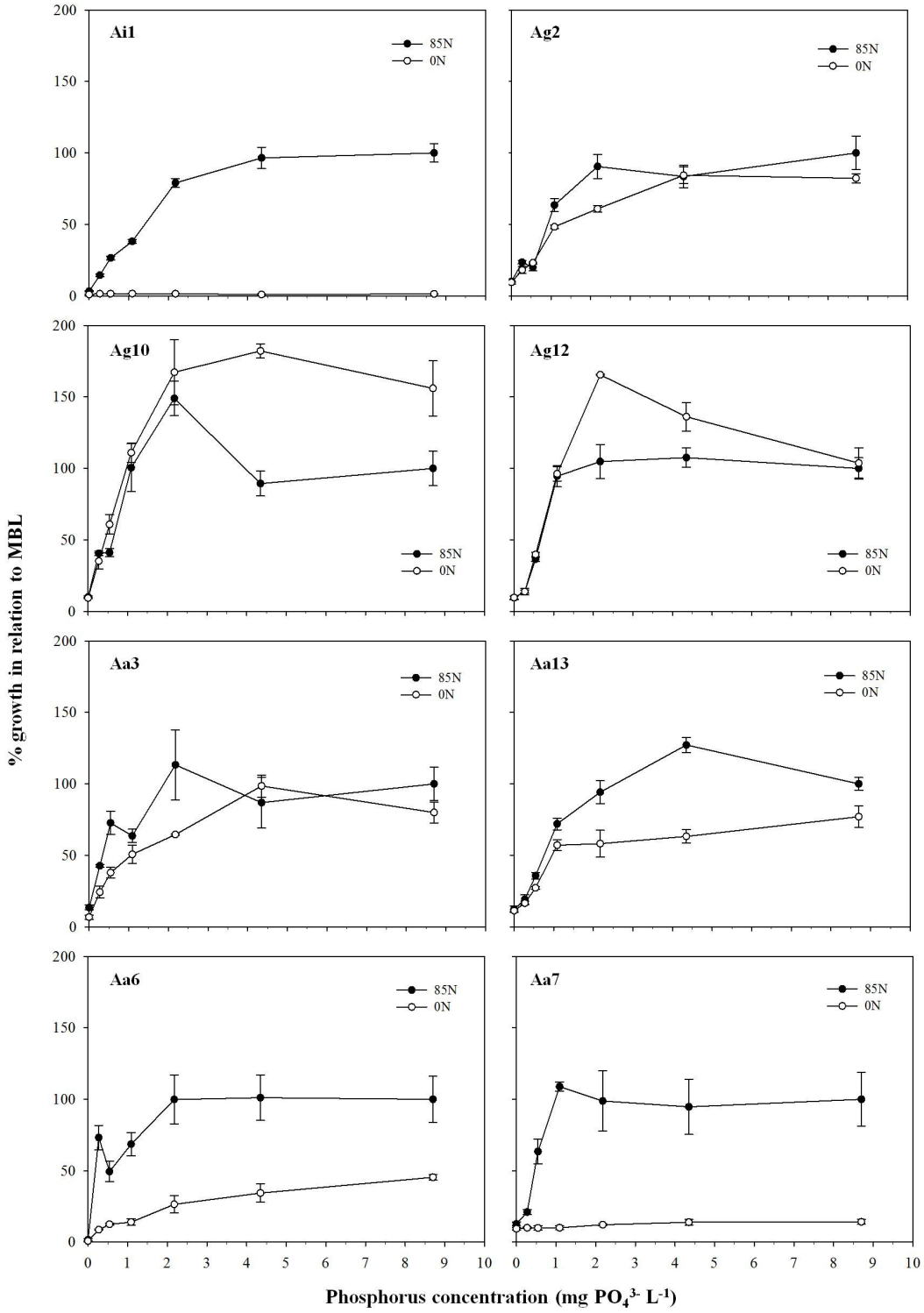

Fig. 2. Effects of different concentrations of phosphorus (8.7, 4.4, 2.2, 1.1, 0.5, 0.3 and 0.0 mg PO_4^{3-} L^{-1}), under nitrogen-saturated (85N - 85 mg L^{-1}) and nitrogen-depleted (0N - 0 mg L^{-1}) conditions, on the growth of *Aphanizomenon* strains. Data are the mean relative biomass (measured in terms of optical density) of three replicates and error bars represent standard error. In order to facilitate the visualization and comparison of all treatments and strains, data are presented as % biomass relatively to nutrient saturation conditions ("full" MBL). The corresponding maximum biomass recorded in the test series for each strain corresponded to 0.71, 0.40, 0.50, 0.36,

Morphologically, although Aa13 and Aa3 cultures showed short trichomes (< 380 μm long) with frequent and regularly spaced heterocysts (about 7 for a 100 cells trichome segment), Aa6 and

Table 2 Two-way ANOVA summary table for the nutrient growth assays performed with cyanobacterial strains of *Aphanizomenon issatschenkoi* (Ai1), *Aph. gracile* (Ag2, Ag10, Ag12) and *Aph. aphanizomenoides* (Aa3, Aa13, Aa6, Aa7). P includes 0P, 0.3P, 0.5P, 1P, 2P, 4P and 9P treatments whereas N refers to 85N and 0N treatments (Table 1).

Strain	Source of variation	df	MS	*F*	Significance
UADFA1 (Ai1)	P	6	0.24	67.6	***
	N	1	2.6	731	***
	Interaction (P x N)	6	0.24	67.8	***
	Residual	28	0.0035		
UADFA2 (Ag2)	P	6	0.68	59.3	***
	N	1	0.088	7.73	**
	Interaction (P x N)	6	0.022	1.89	NS
	Residual	28	0.012		
UADFA10 (Ag10)	P	6	1.9	35.1	***
	N	1	0.78	14.4	***
	Interaction (P x N)	6	0.18	3.39	*
	Residual	28	0.05		
UADFA12 (Ag12)	P	6	1.6	92.0	***
	N	1	0.21	11.6	**
	Interaction (P x N)	6	0.079	4.45	**
	Residual	28	0.018		
UADFA3 (Aa3)	P	6	0.60	14.0	***
	N	1	0.36	8.43	**
	Interaction (P x N)	6	0.057	1.32	NS
	Residual	28	0.043		
UADFA13 (Aa13)	P	6	0.71	66.1	***
	N	1	0.48	44.9	***
	Interaction (P x N)	6	0.075	7.01	***
	Residual	28	0.011		
UADFA6 (Aa6)	P	6	0.37	7.88	***
	N	1	2.5	54.2	***
	Interaction (P x N)	6	0.090	1.94	NS
	Residual	28	0.047		
UADFA7 (Aa7)	P	6	0.25	5.83	***
	N	1	3.7	86.4	***
	Interaction (P x N)	6	0.22	5.12	**
	Residual	28	0.043		

*** $P \leq 0{,}001$; ** $P \leq 0{,}01$; * $P \leq 0{,}05$; NS – non significant

Aa7 both presented longer trichomes (up to 3500 µm long for Aa6 and 800 µm long for Aa7) and a much lower average frequency of heterocysts formation, averaging 2 and 0.2 for Aa6 and Aa7, respectively, in a 100-cell trichome segment (Fig. 1).In general, orthophosphate concentrations equal to or below 0.5 mg PO_4^{3-} L^{-1} led to a decrease of more than 50 % in the cyanobacterial growth (for Ag2, Ag12 and Aa13; and Aa3_0N and Ag10_85N) when compared to the growth under P saturated conditions (MBL) (Fig. 2). However, Ai1 proved to be more sensitive to low P levels and concentrations of 1.2 mg PO_4^{3-} L^{-1} proved to inhibit its growth in more than 50 %. This same level of growth inhibition was only achieved at 0.3 mg PO_4^{3-} L^{-1} for Aa3_85N, Aa7_85N and Ag10_0N. Interestingly, Aa7_0N and Aa6 proved to be more tolerant to low P concentrations, with growth levels above 50 % even at 0.3 mg PO_4^{3-} L^{-1}. This may suggest a "compensation behaviour" by these strains.

For all strains, the combination of 4.36 mg PO_4^{3-} L^{-1} and 8.5 mg NO_3^- L^{-1} (modified MBL) did not have a significant effect over the growth of the tested cyanobacteria in comparison with growth in "full" MBL (data not shown). Clearly, these can be also considered saturation conditions that may lead to the cyanobacterial dominance during most of the year if temperature increases during winter (as already observed during 2007/2008 at Vela Lake – unpublished data).

For the three species, the optical density (at 440 nm) chosen to measure the cyanobacterial biomass showed significant positive correlations ($P < 0.05$) with cell counts ($r = 0.81$, $n = 9$ for *Aph. issatschenkoi*; $r = 0.97$, $n = 20$ for *A. gracile*; and $r = 0.94$, $n = 21$ for *Aph. aphanizomenoides*) and chlorophyll *a* concentration ($r = 0.97$, $n = 9$ for *Aph. issatschenkoi*; $r = 0.76$, $n = 20$ for *A. gracile*; and $r = 0.66$, $n = 15$ for *Aph. aphanizomenoides*).

Allelopathic assays

Significant inhibition of the microalgal growth could be observed for exudates from strains of *Aph. issatschenkoi* (Ai1), *Aph. aphanizomenoides* (Aa7) and *Aph. gracile* (Ag2, Ag10 and Ag12) (Table 3). No allelopathic effects were observed for *Aph. aphanizomenoides* strains Aa3, Aa6, and Aa13. The growth of *P. subcapitata* was significantly inhibited by Ag10 and Aa7 exudates, whereas filtrates of Ag2 and Ag10 inhibited the growth of *C. vulgaris*. An inhibitory effect on growth of *P. morum* was recorded for filtrates of Ai1 and Ag10, while Ag2 and Ag10 negatively affected the growth of *C. astroideum*. The most relevant allelopathic effects were observed for Ag10, whose exudates caused significant growth impairment in all four microalgae (achieving 85 and 92% inhibition in *C. vulgaris* and *P. morum*). On the other hand, a significant stimulatory effect was observed for the filtrate of Ag12 on the growth of *C. astroideum.*

Discussion

The studied strains belonged to diazotrophic species that should be capable of fixing atmospheric nitrogen (N_2) when the ammonia or nitrate levels are low. Blooms of *Aph. gracile* and

Table 3 Effect of the cyanobacterial strains' filtrates over the microalgae *Pseudokirchneriella subcapitata*, *Chlorella vulgaris*, *Pandorina morum* and *Coelastrum astroideum*. Significant effects (inhibition or stimulation), relatively to the control, are presented in boldface.

Cyanobacterial strain	Microalgal species tested	t_s	df	Effect on microalgal growth ($P \leq 0.05$)
UADFA1 (Ai1)	*P. subcapitata*	-0.364	4	No effect
	C. vulgaris	1.34	4	No effect
	P. morum	3.13	4	**Inhibitory effect (38%)**
	C. astroideum	1.48	4	No effect
UADFA2 (Ag2)	*P. subcapitata*	2.05	4	No effect
	C. vulgaris	2.83	4	**Inhibitory effect (47%)**
	P. morum	0.930	4	No effect
	C. astroideum	3.42	4	**Inhibitory effect (66%)**
UADFA10 (Ag10)	*P. subcapitata*	4.57	4	**Inhibitory effect (27%)**
	C. vulgaris	14.3	4	**Inhibitory effect (85%)**
	P. morum	7.58	4	**Inhibitory effect (92%)**
	C. astroideum	3.33	4	**Inhibitory effect (63%)**
UADFA12 (Ag12)	*P. subcapitata*	-2.10	4	No effect
	C. vulgaris	-1.32	4	No effect
	P. morum	-0.995	4	No effect
	C. astroideum	-2.17	4	**Stimulatory effect (51%)**
UADFA3 (Aa3)	*P. subcapitata*	-0.77	4	No effect
	C. vulgaris	-2.30	4	No effect
	P. morum	-0.256	4	No effect
	C. astroideum	1.16	4	No effect
UADFA13 (Aa13)	*P. subcapitata*	1.33	4	No effect
	C. vulgaris	-1.55	4	No effect
	P. morum	1.07	4	No effect
	C. astroideum	-2.60	4	No effect
UADFA6 (Aa6)	*P. subcapitata*	0.276	4	No effect
	C. vulgaris	0.378	4	No effect
	P. morum	0.085	4	No effect
	C. astroideum	-0.633	4	No effect
UADFA7 (Aa7)	*P. subcapitata*	3.33	4	**Inhibitory effect (37%)**
	C. vulgaris	-2.00	4	No effect
	P. morum	0.894	4	No effect
	C. astroideum	-0.773	4	No effect

Aph. aphanizomenoides are known to occur under nitrogen depletion (Sabour et al., 2005; de Figueiredo et al., 2006). The growth of strains of *Aph. aphanizomenoides* (Aa3) and *Aph. gracile* (Ag2, Ag10 and Ag12) showed not to be affected by nitrate unavailability, as it should be expected, but a significant inhibitory effect was recorded over the growth of strains of *Aph. issatschenkoi* (Ai1) and *Aph. aphanizomenoides* (Aa6, Aa7 and Aa13). In fact, heterocysts from *Aph. issatschenkoi* UADFA1 were never seen in culture (Fig. 1) and the frequency of heterocysts in filaments also showed to decrease from *Aph. aphanizomenoides* strain Aa13 to strains Aa6 and Aa7, in this order. Aa13 (similarly to Aa3) had short trichomes with frequent and regularly spaced heterocysts and akinetes (in pairs joined to both sides of the heterocysts) while Aa6 and Aa7 both presented longer trichomes but with very low average frequency of heterocysts. For Aa7, heterocysts were almost absent, thus disabling this strain for fixing nitrogen and grow under nitrogen-depleted conditions. Akinetes were also very rare for both strains Aa6 and Aa7. Morphological changes are known to occur after cultivation in laboratory (Palinska et al., 1996; Zapomělová, 2006), namely concerning heterocyst differentiation (Pearson & Kingsbury, 1966; Jeeji-Bai, 1976; Singh, 1976). Furthermore, spontaneous and induced mutants with partial and complete loss of heterocyst differentiation have been recorded in nostocacean cyanobacteria such as *Cylindrospermum* sp. (Singh, 1976). These mutants also presented morphological changes such as the formation of longer filaments and the lower or null frequency of heterocysts which prevented growth under nitrogen-free conditions (Singh, 1976). Interestingly, since 1912, the *Aph. aphanizomenoides* (formerly named *Anabaena aphanizomenoides*) species has been described with specimens from different origins having some variations concerning morphometry (Horecká & Komárek, 1979). One cannot exclude that these variations may represent different strains from the same species and that changes may have occurred during the culturing period. Horecká and Komárek (1979) reviewed that the *A. aphanizomenoides* firstly described (by Forti, in 1912) showed long trichomes with 2000 μm (such as strain Aa6) while the strains described by Hegewald at al. (in 1975) and by Horecká (in 1979) showed short trichomes with 400 μm and less than 300 μm long, respectively (such as Aa3 or Aa13). Thus, genetic information is essential not only to assist in the identification of a strain (through phylogenetic affiliation) but also to discriminate between strains within the species. For example, in the present study, we *a priori* established three morphotypes which proved to belong to *Aph. aphanizomenoides* (according to sequencing results) – but only two different genotypes were recorded (as proven by STRR1A fingerprinting (de Figueiredo et al., 2010a)). Aa3 and Aa13 have shown to be identical strains but different from Aa6 and Aa7 (de Figueiredo et al., 2010a); the latter strains can be considered mutants as they differed from the type phenotypic characteristics of the species. The combination of both phenotypic and

genotypic approaches is therefore necessary to conveniently characterize and discriminate cyanobacterial strains.

In spite of the absence of heterocysts, the *hetR* gene (coding for a serine type protease essential for heterocyst differentiation) was present in all strains (de Figueiredo et al., 2010a). It is possible that these strains suffered some mutation in one of the *het* genes involved in heterocyst differentiation (Golden & Yoon, 2003) as intra-specific variation in *hetR* sequence, for instance, has been recorded within *Aph. aphanizomenoides* (de Figueiredo et al., 2010a). Further tests under anaerobic conditions could clarify if the problem is effectively related to the expression of the genes responsible for heterocyst differentiation. Without the morphologically and physiologically adapted heterocysts (avoiding the presence of oxygen inside the cells), the nitrogenase is not able to act in aerobic conditions due to the oxygen sensitivity. Although not explored, it should be kept in mind that the cultures were nonaxenic, since cyanobacteria are cultivated more easily when accompanied by heterotrophic bacteria and purification is a difficult and time-consuming procedure and not always successful (Nübel et al., 1997). Thus, the influence of associated bacteria should not be neglected in laboratory assays and may help explain the differences in morphology and nitrogen sensitivity. In fact, in spite of genetically identical (de Figueiredo et al., 2010a), cultures of Aa6 and Aa7 showed differences in their bacterial composition, as assessed by DGGE (data not shown). This fact should be further explored in future studies.

All studied strains showed to be dependent on phosphorus availability to grow, as observed for other N-fixing filamentous cyanobacteria (Lehtimaki et al., 1997; Rapala et al., 1997a; Saadoun et al., 2001; de Figueiredo et al., 2004c; Degerholm et al., 2006). In general, concentrations equal to or below 0.3 mg PO_4^{3-} L^{-1} led to a decrease of more than 50 % in the cyanobacterial growth of all tested strains by comparing to MBL phosphorus saturated conditions. However, the *Aph. issatschenkoi* strain (Ai1) proved to be more sensitive to low P concentrations whereas the mutant *Aph. aphanizomenoides* (Aa6 and Aa7) proved to be more tolerant to low P levels (suggesting a "survival compensation mechanism"). Nonetheless, this highlights the need for controlling phosphorus inputs, in particular, to restrain the massive growth of these bloom-forming cyanobacteria.

Effects of cyanobacterial exudates on growth of four green algae

Cell-free filtrates from cultures of nostocacean cyanobacterial strains have shown to cause effects over the plankton community (Suikkanen et al., 2005). For microalgae, in particular, effects such as stabilization or inhibition of growth have been reported (Keating, 1978; Kearns & Hunter, 2001; Suikkanen et al., 2004). In the present study, 4 out of 8 strains affected the growth of at least one green microalga and the other 4 did not affect growth of any of the microalgae. *Aph. gracile* strain Ag10 significantly inhibited growth of all the microalgae tested. Many cyanobacteria produce secondary metabolites that have an allelopathic function towards microalgae (Kearns & Hunter, 2001; Leflaive & Ten-Hage, 2007). The production of toxins has been reported for *Aph.*

issatschenkoi (Nogueira et al., 2004; Wood et al., 2007), *Aph. gracile* (Ballot et al., 2010) and *Aph. aphanizomenoides* (Sabour et al., 2005). However, the inhibition of microalgal growth has been also recorded using cell-filtrates from *Aphanizomenon* strains considered as non-toxic (Suikkanen et al., 2004); this effect may have been due to the production of other growth inhibitors such as cyanobacterial chelators (Murphy et al., 1976). In ecological studies, alellopathic effects of cyanobacteria towards microalgae such as chlorophytes and diatoms have been also suggested (Kozak, 2005; de Figueiredo et al., 2006). Nevertheless, the enhancement of growth for some microalgae has been also reported (Suikkanen et al., 2005), as recorded in the present study for *C. astroideum* (with *Aph. aphanizomenoides* UADFA12 filtrate).

Different sensitivities among the tested microalga were observed which highlights the need of using a battery of assays to determine eventual effects over the growth of target phytoplanktonic organisms. More, it should also be kept in mind that ultrastructural effects may occur without changing significantly growth (Valdor & Aboal, 2007).

Once more, the influence of associated bacteria should not be neglected in these assays. However, the potential effects of exudates of heterotrophic bacteria over the microalgae growth, by using non-axenic cyanobacterial cultures, has previously shown not to be relevant (Suikkanen et al., 2004).

Conclusion

The present work highlights the need for a polyphasic approach to characterize cyanobacterial strains, namely within the *Aphanizomenon* genus. Besides the fact that it is a very heterogeneous genus (Komárek & Komárková, 2006; de Figueiredo et al., 2010a), with cases of misidentifications (Li et al., 2003; Wood et al., 2007), morphological changes and mutation in culture may further hinder a correct morphological identification of strains. Thus, it is important to combine not only morphological and genetic data but also ecophysiological data, as morphotypes, genotypes and ecotypes may not be coincident. Thus, caution must be taken when assuming that one genotype will have a single answer under certain environmental conditions or stress. Also, it should be kept in mind that heterotrophic bacteria associated in cyanobacterial cultures may have a role in the morphological and ecological differences found between identical genotypes. The analysis of optimal ecological preferences such as nutrient requirements is important to establish management strategies to reduce their massive development in lakes; this is particularly relevant if we take into account climatic changes that are modifying typical seasonal patterns and allowing the persistence of blooms of filamentous cyanobacteria throughout the year. Our results indicated that phosphorus, in particular, is very important for the growth control of these filamentous strains. Allelopathic assays revealed a significant inhibition of microalgal growth for some cyanobacterial

filtrates suggesting that, depending on the bloom-forming cyanobacterial strain, there may be different effects over the phytoplankton community composition.

References

Abrantes, N., R. Pereira, D. R. de Figueiredo, C. R. Marques, M. J. Pereira & F. Gonçalves, 2009. A whole sample toxicity assessment to evaluate the sub-lethal toxicity of water and sediment elutriates from a lake exposed to diffuse pollution. Environmental Toxicology 24: 259-270.

Ballot, A., J. Fastner & C. Wiedner, 2010. Paralytic Shellfish Poisoning Toxin-Producing Cyanobacterium *Aphanizomenon gracile* in Northeast Germany. Applied and Environmental Microbiology 76: 1173-1180.

Castro, B. B. & F. Gonçalves, 2007. Seasonal dynamics of the crustacean zooplankton of a shallow eutrophic lake from the Mediterranean region, with particular emphasis on *Daphnia*. Fundamental and Applied Limnology - Archiv für Hydrobiologie 169: 189-202.

Chonudomkul, D., W. Yongmanitchai, G. Theeragool, M. Kawachi, F. Kasai, K. Kaya & M. M. Watanabe, 2004. Morphology, genetic diversity, temperature tolerance and toxicity of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) strains from Thailand and Japan. FEMS Microbiology Ecology 48: 345-355.

Codd, G. A., 2000. Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. Ecological Engineering 16: 51-60.

de Figueiredo, D. R., A. Alves, M. J. Pereira & A. Correia, 2010. Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). Journal of Plankton Research 32: 239-252.

de Figueiredo, D. R., S. C. Antunes, M. J. Pereira & F. Gonçalves, 2004a. Chronic effects of *Aphanizomenon flos-aquae* on the survival and reproduction of daphnids. Fresenius Environmental Bulletin 13: 665-670.

de Figueiredo, D. R., U. M. Azeiteiro, S. M. Esteves, F. J. M. Gonçalves & M. J. Pereira, 2004b. Microcystin-producing blooms - A serious global public health issue. Ecotoxicology and Environmental Safety 59: 151-163.

de Figueiredo, D. R., U. M. Azeiteiro, F. Gonçalves & M. J. Pereira, 2004c. *Aphanizomenon flos-aquae* grown under different nutrient concentrations and the effects of its exudates on growth of two green algae. Fresenius Environmental Bulletin 13: 657-664.

de Figueiredo, D. R., A. S. S. P. Reboleira, S. C. Antunes, N. Abrantes, U. Azeiteiro, F. Gonçalves & M. J. Pereira, 2006. The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. Hydrobiologia 568: 145-157.

Degerholm, J., K. Gundersen, B. Bergman & E. Söderbäck, 2006. Phosphorus-limited growth dynamics in two Baltic Sea cyanobacteria, *Nodularia* sp. and *Aphanizomenon* sp. FEMS Microbiology Ecology 58: 323-332.

Dokulil, M. T. & K. Teubner, 2000. Cyanobacterial dominance in lakes. Hydrobiologia 438: 1-12.

Figueredo, C. C., A. Giani & D. F. Bird, 2007. Does allelopathy contribute to *Cylindrospermopsis raciborskii* (cyanobacteria) bloom occurrence and geographic expansion? Journal of Phycology 43: 256-265.

Golden, J. W. & H. S. Yoon, 2003. Heterocyst development in *Anabaena*. Current Opinion in Microbiology 6: 557-563.

Gonçalves, A. M., D. R. de Figueiredo & M. J. Pereira, 2005. A low-cost methodology for algal growth inhibition tests using three freshwater green algae. Fresenius Environmental Bulletin 14: 1192-1195.

Horecká, M. & J. Komárek, 1979. Taxonomic position of three planktonic blue- green algae from the genera *Aphanizomenon* and *Cylindrospermopsis*. Preslia 51: 289-312.

Jacoby, J. M., D. C. Collier, E. B. Welch, F. J. Hardy & M. Crayton, 2000. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. Canadian Journal of Fisheries and Aquatic Sciences 57: 231-240.

Jeeji-Bai, N., 1976. Morphological variation of certain blue-green algae in culture: *Scytonema stuposum* (Kütz.) Born. Schweizerische Zeitschrift für Hydrologie 38: 55-62.

Kaštovský, J., T. Hauer, J. Mareš, M. Krautová, T. Bešta, J. Komárek, B. Desortová, J. Heteša, A. Hindáková, V. Houk, E. Janeček, R. Kopp, P. Marvan, P. Pumann, O. Skácelová & E. Zapomělová, 2010. A review of the alien and expansive species of freshwater cyanobacteria and algae in the Czech Republic. Biological Invasions DOI 10.1007/s10530-010-9754-3.

Kearns, K. & M. Hunter, 2001. Toxin-producing *Anabaena flos-aquae* induces settling of *Chlamydomonas reinhardtii*, a competing motile alga. Microbial Ecology 42: 80-86.

Keating, K. I., 1978. Blue-Green Algal Inhibition of Diatom Growth: Transition from Mesotrophic to Eutrophic Community Structure. Science 199: 971-973.

Komárek, J. & J. Komárková, 2006. Diversity of *Aphanizomenon*-like cyanobacteria. Czech Phycology 6: 1-32.

Kozak, A., 2005. Seasonal changes occurring over four years in a reservoir's phytoplankton composition. Polish Journal of Environmental Studies 14: 451-465.

Leflaive, J. & L. Ten-Hage, 2007. Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshwater Biology 52: 199-214.

Lehtimaki, J., P. Moisander, K. Sivonen & K. Kononen, 1997. Growth, Nitrogen Fixation, and Nodularin Production by Two Baltic Sea Cyanobacteria. Applied and Environmental Microbiology 63: 1647-1656.

Li, R., W. W. Carmichael & P. Pereira, 2003. Morphological and 16s rRNA gene evidence for reclassification of the paralytic shellfish toxin producing *Aphanizomenon flos-aquae* LMECYA 31 as *Aphanizomenon issatschenkoi* (Cyanophyceae). Journal of Phycology 39: 814-818.

Markensten, H., K. Moore & I. Persson, 2010. Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate. Ecological Applications 20: 752-767.

Murphy, T., D. Lean & C. Nalewajko, 1976. Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. Science 192: 900-902.

Neilan, B. A., M. L. Saker, J. Fastner, A. Törökné & B. P. Burns, 2003. Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. Molecular Ecology 12: 133-140.

Nichols, H. W., 1973. Growth media - freshwater. In: J. R. Stein, (ed.), Handbook of Phycological Methods. Cambridge University Press, Cambridge: 7-24.

Nogueira, I. C. G., P. Pereira, E. Dias, S. Pflugmacher, C. Wiegand, S. Franca & V. M. Vasconcelos, 2004. Accumulation of paralytic shellfish toxins (PST) from the cyanobacterium *Aphanizomenon issatschenkoi* by the cladoceran *Daphnia magna*. Toxicon 44: 773-780.

Nübel, U., F. Garcia-Pichel & G. Muyzer, 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Applied and Environmental Microbiology 63: 3327-3332.

Palinska, K. A., W. Liesack, E. Rhiel & W. E. Krumbein, 1996. Phenotype variability of identical genotypes: The need for a combined approach in cyanobacterial taxonomy demonstrated on *Merismopedia*-like isolates. Archives of Microbiology 166: 224-233.

Pearson, J. E. & J. M. Kingsbury, 1966. Culturally induced variation in four morphological diverse bluegreen algae. American Journal of Botany 53: 192-200.

Pouria, S., A. De Andrade, J. Barbosa, R. L. Cavalcanti, V. T. S. Barreto, C. J. Ward, W. Preiser, G. K. Poon, G. H. Neild & G. A. Codd, 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352: 21-26.

Quinn, G. & M. Keough, 2002. Experimental design and data analysis for biologists. Cambridge University Press, Cambridge.

Rapala, J. & K. Sivonen, 1998. Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. Microbial Ecology 36: 181-192.

Rapala, J., K. Sivonen, C. Lyra & S. I. Niemelä, 1997. Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. Applied and Environmental Microbiology 63: 2206-2212.

Saadoun, I. M. K., K. K. Schrader & W. T. Blevins, 2001. Environmental and nutritional factors affecting geosmin synthesis by Anabaena SP. Water Research 35: 1209-1218.

Sabour, B., M. Loudiki, B. Oudra, V. Vasconcelos, S. Oubraim & B. Fawzi, 2005. Dynamics and toxicity of *Anabaena aphanizomenoides* (Cyanobacteria) waterblooms in the shallow brackish Oued Mellah lake (Morocco). Aquatic Ecosystem Health and Management 8: 95-104.

Saker, M. L., I. C. G. Nogueira, V. M. Vasconcelos, B. A. Neilan, G. K. Eaglesham & P. Pereira, 2003. First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. Ecotoxicology and Environmental Safety 55: 243-250.

Singh, P. K., 1976. Growth and nitrogen fixation of mutants defective in heterocyst differentiation in the blue green alga *Cylindrospermum* sp. Zeitschrift fur Allgemeine Mikrobiologie 16: 453-463.

Stüken, A., J. Rücker, T. Endrulat, K. Preussel, M. Hemm, B. Nixdorf, U. Karsten & C. Wiedner, 2006. Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. Phycologia 45: 696-703.

Suikkanen, S., G. O. Fistarol & E. Granéli, 2004. Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. Journal of Experimental Marine Biology and Ecology 308: 85-101.

Suikkanen, S., G. O. Fistarol & E. Granéli, 2005. Effects of cyanobacterial allelochemicals on a natural plankton community. Marine Ecology Progress Series 287: 1-9.

Valdor, R. & M. Aboal, 2007. Effects of living cyanobacteria, cyanobacterial extracts and pure microcystins on growth and ultrastructure of microalgae and bacteria. Toxicon 49: 769-779.

Vasconcelos, V., 2001. Freshwater cyanobacteria and their toxins in Portugal. In: I. Chorus, (ed.), Cyanotoxins - Occurrence, Causes, Consequences. Springer, Berlin: 62-67.

Vasconcelos, V. M., T. Campos, A. Amorim & A. M. V. M. Soares, 1993. Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. Boletim UCA, Universidade do Algarve, UCTRA 1: 193-201.

Vasconcelos, V. M., K. Sivonen, W. R. Evans, W. W. Carmichael & M. Namikoshi, 1996. Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. Water Research 30: 2377-2384.

Wiedner, C., J. Rücker, R. Brüggemann & B. Nixdorf, 2007. Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia 152: 473-484.

Wood, S. A., J. P. Rasmussen, P. T. Holland, R. Campbell & A. L. M. Crowe, 2007. First report of the cyanotoxin anatoxin-a from *Aphanizomenon issatschenkoi* (cyanobacteria). Journal of Phycology 43: 356-365.

Zapomělová, E., 2006. Current taxonomic issues with planktonic representatives of the genus *Anabaena* (Cyanobacteria) with special reference to their morphological features; literary review. Czech Phycology 6: 33-47.

Molecular characterization and toxic potential of *Microcystis* strains isolated from Vela Lake (Western Central Portugal)

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Abstract

Cyanobacterial blooms occur all over the world and are frequently dominated by *Microcystis* spp. which can produce potent hepatotoxins named microcystins. Morphological identification of these cyanobacteria can be sometimes difficult. Toxic and non-toxic strains are found within the same species. For those reasons molecular approaches are essential to detect and identify potentially toxic strains. Bloom-forming *Microcystis* spp. were extemporarily isolated from the shallow Vela Lake (Figueira da Foz, Portugal) frequently suffering from cyanobacterial blooms. Their molecular affiliation was achieved through amplification and partial sequencing of the 16SrRNA gene. The potential for microcystin-production was analyzed using primers targeting *mcyA* and *mcyE* regions of the microcystin synthetase gene cluster. The isolated strains belonged to species such as *M. aeruginosa* and *M. viridis*. Forty percent of the *Microcystis* strains showed both *mcyA* and *mcyE* amplifications (indicating their microcystin-producing potential); *mcyA* sequences, in particular, allowed a differentiation of the strains, suggesting a phylogenetic discrimination potential.

Keywords

Vela Lake, *Microcystis* spp., microcystins, *mcyA*, *mcyE*.

Introduction

The development of cyanobacterial blooms in freshwaters is recorded worldwide and it is frequently related to toxicity caused by *Microcystis* spp. which may produce secondary metabolites such as the hepatotoxic microcystins, posing risks to Public Health (Pouria et al., 1998; de Figueiredo et al., 2004b). From an ecological point of view, microcystins have also proven to cause noxious effects over several organisms, from bacteria and phytoplankton to other vertebrates (de Figueiredo et al., 2004b; Wiegand & Pflugmacher, 2005; Valdor & Aboal, 2007). Over the last years, studies have reported intra-specific genetic variation within *Microcystis* spp. (Yoshida et al., 2008) and the problem associated with the fact that there are toxic and non-toxic strains, sometimes co-existing in a same water body and even within a same bloom, determining its toxicity (Kardinaal et al., 2007). Classical techniques based on observation of morphological characters may not be sufficient to correctly identify *Microcystis* spp., particularly if these are cultured for a long time (Komárek, 1991; Komárek & Anagnostidis, 1999; Bittencourt-Oliveira et al., 2001), and certainly are not suitable to assess the strains' toxicity. Thus, molecular approaches have been increasingly used due to its accuracy and fastness in determining the toxic potential of an isolated strain as well as providing information for its taxonomical identification (Bittencourt-Oliveira et al., 2001; Innok et al., 2005; Saker et al., 2005a; Wilson et al., 2005; Komárek, 2006).

The formation of blooms by toxic *M. aeruginosa* strains has been widely reported in Portuguese water bodies used for water supply and/or recreation (Vasconcelos et al., 1993a; Vasconcelos et al., 1993b; Vasconcelos et al., 1995; Vasconcelos et al., 1996; Vasconcelos et al., 2001; Saker et al., 2005a) with average microcystins levels above the maximum concentration of 1 $\mu g.L^{-1}$ (for MC-LR) established by the World Health Organization (WHO, 1998; Vasconcelos et al., 2001; de Figueiredo et al., 2004b). At the freshwater shallow Vela Lake (Figueira da Foz, Portugal), in particular, the development of summer cyanobacterial blooms dominated by *Microcystis* spp. has been reported since long time (Vasconcelos et al., 1993a; de Figueiredo et al., 2006).

In the present study, bloom-forming cyanobacteria (mostly *Microcystis* strains) were isolated from Vela Lake during summer blooms occurrence. The strains were molecularly characterized through partial 16S rRNA gene sequencing, and their microcystin-producing potential was assessed by targeting the *mcyA* and *mcyE* regions of the microcystin synthetase gene cluster (Kaebernick & Neilan, 2001).

Materials and methods

Sampling, isolation and culturing of cyanobacteria

Vela Lake is a shallow eutrophic water body (approximately with 70 ha) located at Figueira da Foz (Centralwestern Portugal) and it is mainly used as a recreational area and as an irrigation

source for agriculture (Abrantes et al., 2009b). Water samples from the lake were collected using sterilized glass bottles during cyanobacterial blooms in the summers of 2004, 2005 and 2006. Two strains (see Table 1) were obtained from shallow waters located in Porto Santo (Madeira, Portugal) and in Aveiro (Portugal) and were used, respectively, as negative and positive controls for the presence of the targeted *mcyA* and *mcyE* fragments. Nonaxenic unicyanobacterial cultures were established through micromanipulation by repeated isolation steps in sterilized liquid MBL – Woods Hole culture medium. The cultures were maintained in 250 mL erlenmeyers containing 100 mL of MBL medium in an incubation chamber with 20±2ºC and a $16h^{L}$:$8h^{D}$ photoperiod of 130 $\mu E.m^{-2}.s^{-1}$ provided by cool white fluorescent tubes. The taxonomic identification was made using recent descriptions (Komárek, 1991; Komárek & Anagnostidis, 1999).

DNA extraction and PCR amplifications

The DNA from each cyanobacterium culture was extracted after centrifugation of 2 mL of the culture, resuspension in 200 µL of TE and lysis by adding 1 $mg.mL^{-1}$ lysozyme and incubating at 37 ºC for 1h. DNA extraction and purification was performed using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and DNA was resuspended in TE buffer and stored at -20 ºC. PCR amplification of 16S rRNA gene fragments from all cultures was performed combining the bacterial universal primer 27F (Lane, 1991) with the cyanobacteria-specific primer CYA781R (Nübel et al., 1997). The amplification of *Microcystis* spp. partial sequences of *mcyA* and *mcyE* regions of microcystin synthetase was achieved using the primers QmetF/QmetR (Wilson et al., 2005) and mcyE-F2/mcyE-R4 (Rantala et al., 2004), respectively. All primers were commercially synthesized (STABVida, Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Hercules, California, USA) with 50 µL reaction mixtures each containing 3 mM $MgCl_2$, 200 µM of each nucleotide, 1x PCR buffer with $(NH_4)_2SO_4$, 5% dimethylsulfoxide (DMSO), 15 pmol of each primer, 1 U of *Taq* DNA polymerase and 50-200 ng template DNA. The PCR programs for the primer set targeting the 16S rRNA gene fragment included an initial denaturation at 94°C for 5 min, followed by 30 cycles consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a final extension step for 10 min at 72ºC. The *mcyA* amplification was run with an initial denaturation at 95°C for 2 min followed by 30 cycles of 94°C for 10 s, 45°C for 20 s, and 72°C for 30 s, according to Wilson et al. (2005), but a final elongation step of 72°C for 10 min was added. The *mcyE* amplification included an initial denaturation at 95ºC for 3 min and 30 cycles of 94ºC for 30 s, 56ºC for 30s and 72ºC for 1 min, with a final step of 10 min at 72ºC. Negative (without any template DNA) and positive control reactions were carried out simultaneously. PCR products were checked by electrophoresis on 1.5 % agarose gels at 80 V for 1h 30min. A molecular weight marker (GeneRuler™ 1 kb DNA ladder) was run in each gel. The staining of the gels was performed with ethidium bromide and visualization was made on a UV transilluminator.

Sequencing and phylogenetic analysis

The retrieved amplicons were purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) before they were commercially sequenced (STABVida, Portugal) using the reverse primers from the original PCR. A BLAST search was performed against all sequences deposited in the GenBank database and ClustalX version 1.83 (Thompson et al., 1997) was used to align sequences and reconstruct trees using the neighbour-joining method with bootstrap values from 1000 NJ bootstrap replicates. Trees were visualized using the program TreeView (Page, 1996). The cyanobacterial partial sequences determined in the present study were were deposited in the GenBank database under the accession nos. HM854730 to HM854749.

Results and discussion

According to the descriptions made by Komárek (Komárek, 1991) and Komárek and Anagnostidis (Komárek & Anagnostidis, 1999), the strains were identified (Table 1) as belonging to the *taxa Microcystis aeruginosa* (Kütz.) Kütz. 1846 (strains UADFM1, 2, 3 and 7), *Microcystis viridis* (A.Br.) Lemmerm. (strains UADFM5 and 6) and *Microcystis* sp. (strains UADFM4, 8 and 10). Phylogenetic affiliation (see Table 1) generally corroborated the achieved morphological identification. A combined morphological and molecular approach allows a more correct identification of cyanobacterial strains since some species and even genera can be very similar phenotypically (Komárek, 2006). The *16S rRNA* partial sequence (of about 715 bp) of UADFM1 showed the highest similarity (99%) with sequences retrieved from *M. aeruginosa* strains such as UTEX 'LB 2388' or NIES-298. The strains UADFM2, UADFM3 and UADFM7 showed to sequences 99% similar to sequences from other *M. aeruginosa* strains, namely strain 0BB35S02. UADFM4 partial 16S rDNA sequence had total match with sequences from *Microcystis* sp. strains (GL260735 and UWOCC Q) and 99% similarity with *M. aeruginosa* strains such as PCC 7806, UWOCC 023 or 0BF29S03. Strains UADFM5 and UADFM6 had partial 16S rDNA sequences 99% similar to sequences of strains from *M. viridis* (such as NIES-1058 e NIES-102) but also from *M. aeruginosa* (such as NIES-843 e 1BB38S07). In spite of considered as *Microcystis* sp. strains, UADFM8 and UADFM10 showed *16S rRNA* partial sequences with lower similarity values to sequence NCBI database. UADFM8 sequence had the highest similarity (97%) with a cyanobacterium sp. (IW11) whereas UADFM10 showed to be 92% similar to an uncultured cyanobacterium (ND2_CYA_1_8).

The *mcyA* sequences (about 180 bp) were compared with GenBank sequence database and showed the highest similarities (between 99 and 100%) with sequences from *Microcystis* strains (Table 2 and Figure 1). UADFM4 showed total match with *M. novacekii* strain (T20-3) and 99% similarity with *M. aeruginosa* strains (such as PCC 7806 or UWOCC 019). A 99% similarity was found between *mcyA* partial sequences of UADFM5 and UADFM6 and a *Microcystis aeruginosa*

Table 1. Phenotypic identification, origin, sampling date, potential for microcystin production (amplification of *mcyA* and *mcyE*) and accession number, closest relative (after a BLAST search) and corresponding percentage similarity for the 16S rDNA partial sequences obtained from the studied strains.

Strain	Phenotypic identification	Origin and sampling date	*mcyA**	*mcyE**	16S rDNA NCBI Accession Nº	Closest relatives (accession Nº)	Percentage similarity (%) (*c.* 720 bp)
UADFM1	*Microcystis aeruginosa* (Kütz.) Kütz.	Porto Santo (Madeira), summer 2002	-	-	HM854730	*Microcystis aeruginosa* UTEX 'LB 2388' (DQ648030)	99
UADFM2	*Microcystis aeruginosa* (Kütz.) Kütz.	Vela Lake, August 2004	-	-	HM854731	*Microcystis aeruginosa* 0BB35S02 (AJ635430)	99
UADFM3	*Microcystis aeruginosa* (Kütz.) Kütz.	Vela Lake, April 2004	-	-	HM854732	*Microcystis aeruginosa* 0BB35S02 (AJ635430)	99
UADFM4	*Microcystis* sp.	Vela Lake, August 2004	+	+	HM854733	*Microcystis* sp. GL260735 (AY439282)	100
UADFM5	*Microcystis viridis* (A.Br.) Lemmerm.	Vela Lake, April 2004	+	+	HM854734	*Microcystis viridis* NIES-1058 (DQ648029)	99
UADFM6	*Microcystis viridis* (A.Br.) Lemmerm.	Urban lake, Aveiro, July 2001	+	+	HM854735	*Microcystis viridis* NIES-1058 (DQ648029)	99
UADFM7	*Microcystis aeruginosa* (Kütz.) Kütz.	Vela Lake, August 2004	-	-	HM854736	*Microcystis aeruginosa* 0BB35S02 (AJ635430)	99
UADFM8	*Microcystis* sp.	Vela Lake, August 2005	+	+	HM854737	Cyanobacterium sp. IW11 (AJ565868)	97
UADFM10	*Microcystis* sp.	Vela Lake, summer 2006	-	-	HM854738	Uncultured cyanobacterium clone H-A02 (DQ181685)	92

* Presence (+) or absence (-) of targeted fragments of the microcystin synthetase gene cluster.

Table 2. Accession number, closest relative (after a BLAST search) and corresponding percentage similarity for the obtained *mcyA* and *mcyE* partial sequences.

Strain	*mcyA* NCBI Accession Nº	Closest relatives for *mcyA* fragments (accession Nº)	Percentage similarity (%) (182 bp)	*mcyE* NCBI Accession Nº	Closest relatives for *mcyE* fragments (accession Nº)	Percentage similarity (%) (760 bp)
UADFM4	HM854739	*Microcystis novacekii* T20-3 (AB110113)	100	HM854743	*Microcystis aeruginosa* NIES-843 (AP009552)	97
UADFM5	HM854740	*Microcystis* sp. MCS3 (AB110110)	99	HM854744	*Microcystis aeruginosa* PCC 7806 (AF183408)	98
UADFM6	HM854741	*Microcystis* sp. MCS3 (AB110110)	99	HM854745	*Microcystis aeruginosa* PCC 7806 (AF183408)	98
UADFM8	HM854742	*Microcystis aeruginosa* PCC 7806 (AM778952)	99	HM854746	*Microcystis aeruginosa* NIES-843 (AP009552)	97

strain (MCS3) (Tanabe et al., 2004); 98% similarity was retrieved with sequences from *M. botrys* (N-C 161/1) and only 97% similarity was found with strains of *M. viridis* (N-C 169/7, NIES 102, NIES 103 and CL4) and other *M. aeruginosa* strains (such as UWOCC 84/1). UADFM8 *mcyA*

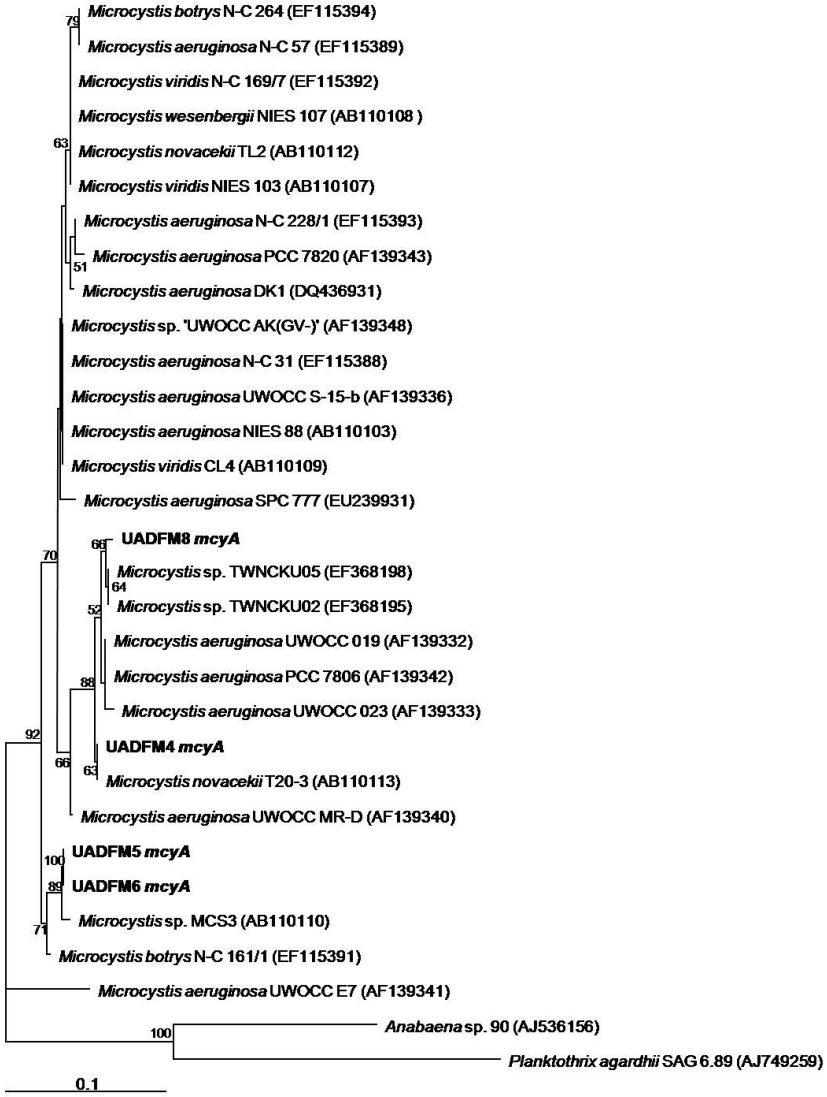

Fig. 1. Neighbour-joining tree constructed from the partial sequences (180 bp) of the *mcyA* region of the microcystin synthetase gene of the cultured cyanobacterial isolates. Sequences of *Anabaena* and *Planktothrix* spp. were used as outgroup. Percentages of 1000 bootstraps replicates are indicated near the nodes (only those values > 50 % are shown). The scale bar represents 0.1 substitutions per site. Sequences from GeneBank are marked with accession numbers.

sequence was 99% similar to sequences from *M. aeruginosa* strains (PCC 7806, UWOCC 019, UWOCC 017 and UWOCC 001). Results from *mcyE* sequencing (up to 760 bp) reinforced the phylogenetic segregation of the studied *M. viridis* strains from the remaining toxic strains but did not discriminate the *M. aeruginosa* strains from each other. UADFM4 and UADFM8 *mcyE* sequences showed 97% similarity with sequences from *Microcystis* sp. (IZANCYA5), *M. viridis* NIES 102 and *M. aeruginosa* strains (NIES 843 and NIES 89). Sequences from UADFM5 and UADFM6 achieved the highest similarity (98%) with *mcyE* sequence of *Microcystis aeruginosa* PCC 7806 and only 96% with a *M. viridis* strain (NIES 102).

Interestingly, in spite of short, the *mcyA* fragment allowed to differentiate the studied toxic strains not only at the species level but also between the *M. aeruginosa* strains. Nevertheless, future studies should include more toxic strains of the same species to explore its potential for intra-specific discrimination since the NCBI database is still short of *mcyA* (as well as of *mcyE*) sequences from identified cultures. From both the *mcyA* and *mcyE* trees (Figures 1 and 2) there is still a confounding mixture for species clustering. Can the phenotype lead to misidentification of

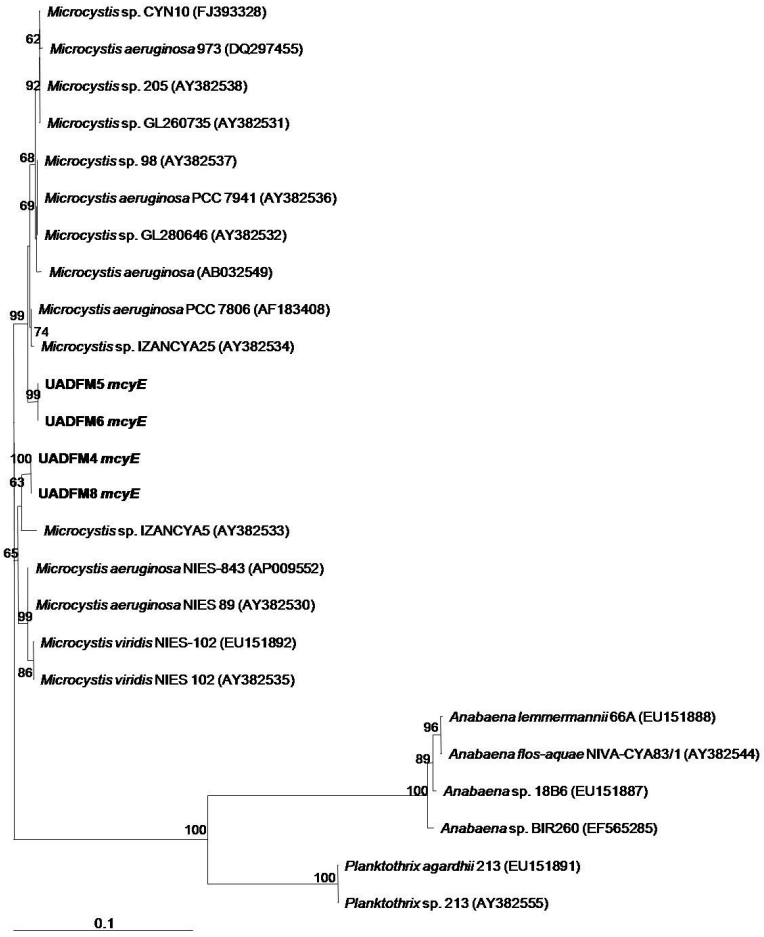

Fig. 2. Neighbour-joining tree constructed from the partial sequences (640 bp) of the *mcyE* region of the microcystin synthetase gene of the cultured cyanobacterial isolates. Sequences of *Anabaena* and *Planktothrix* spp. were used as outgroup. Percentages of 1000 bootstraps replicates are indicated near the nodes (only those values > 50 % are shown). The scale bar represents 0.1 substitutions per site. Sequences from GeneBank are marked with accession numbers.

the species or are there actually distinct strains possessing differences in these sequences within a same species? Definitely, more sequences are needed and more studies on this topic must be conducted. The fact that there is not a clear segregation of toxic *vs.* non-toxic strains using phylogenetic information has led to the theory that there was a common ancient origin for microcystins production (Rantala et al., 2004) but the unclear species clustering of toxic strains may also reinforce this assumption. However, there is also the possibility that partial fragments of

the microcystin synthetase gene cluster may have been more recently laterally transferred among strains inside the genus *Microcystis* (Tanabe et al., 2004).

The toxicity of the *Microcystis* spp. is very well documented, particularly due to the health risks for humans when microcystins levels rise in drinking water supplies (WHO, 1998; Fleming et al., 2002). Nevertheless, in lakes used for recreation health risks may still persist, particularly when massive cyanobacterial blooms develop and lake users get in direct contact with contaminated water. This way, in summers 2004 and 2005 the dense blooms recorded of the studied toxic strains UADFM4, UADFM5 and UADFM8 at Vela Lake were a concerning subject, particularly in 2005 when a strong drought considerably reduced the water level of the lake (de Figueiredo et al., unpublished). Furthermore, a relevant issue is that the water of Vela Lake is used for irrigation of crop fields and microcystin uptake and bioaccumulation by plants used for human consumption has already been reported (Codd et al., 1999). Thus, the importance of monitoring the development of microcystin-producing strains at water bodies used for human purposes is emergent for Public Health protection. The early detection of the presence of toxic strains is an important basis for management planning and control of bloom development.

Conclusions

The isolated strains were identified as belonging to *Microcystis* genus, namely to species such as *M. aeruginosa* and *M. viridis.* These two species showed strains with both *mcyA* and *mcyE* amplification suggesting their toxicity by having the potential to produce microcystins. For the species *M. aeruginosa* there were found toxic and non-toxic strains. The *mcyA* sequences, in particular, showed a high phylogenetic discrimination allowing inferring taxonomic differences among strains in spite of the lack of deposited sequences. The results from the present study will be used as a basis for future molecular studies allowing a quicker and more thorough monitoring of toxic *Microcystis* spp. development at Vela Lake.

References

Abrantes N., Pereira R., de Figueiredo D. R., Marques C. R., Pereira M. J. & Gonçalves F. (2009b). A whole sample toxicity assessment to evaluate the sub-lethal toxicity of water and sediment elutriates from a lake exposed to diffuse pollution. *Environmental Toxicology* 24: 259-270.

Bittencourt-Oliveira M. D. C., De Oliveira M. C. & Bolch C. J. S. (2001). Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (cpcBA). *Journal of Phycology* 37: 810-818.

Codd G. A., Metcalf J. S. & Beattie K. A. (1999). Retention of *Microcystis aeruginosa* and microcystin by salad lettuce (*Lactuca sativa*) after spray irrigation with water containing cyanobacteria. *Toxicon* 37: 1181-1185.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J. & Correia A. (unpublished). Spatial diversity of bacterioplankton at Vela Lake (Centralwestern Portugal) during a severe summer drought - cyanobacterial blooms and microcystin production potential.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

Fleming L. E., Rivero C., Burns J., Williams C., Bean J. A., Shea K. A. & Stinn J. (2002). Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. *Harmful Algae* 1: 157-168.

Innok S., Matsumura M., Boonkerd N. & Teaumroong N. (2005). Detection of *Microcystis* in lake sediment using molecular genetic techniques. *World Journal of Microbiology and Biotechnology* 21: 1559-1568.

Kaebernick M. & Neilan B. A. (2001). Ecological and molecular investigations of cyanotoxin production. *FEMS Microbiology Ecology* 35: 1-9.

Kardinaal W. E. A., Tonk L., Janse I., Hol S., Slot P., Huisman J. & Visser P. M. (2007). Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. *Applied and Environmental Microbiology* 73: 2939-2946.

Komárek J. (1991). A review of water-bloom forming *Microcystis* species, with regard to populations from Japan. *Archives of Hydrobiology* Suppl 92: 115-127.

Komárek J. (2006). Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. *Algae* 21: 349-375.

Komárek J. & Anagnostidis K., 1999. Cyanoprokariota - 1. Teil Choococcales. In: H. Ettl, G. Gärtner, H. Heynig & D. Mollenhauer, (eds.), Sübwasserflora von Mitteleuropa, Band 19/1. G. Fischer Verlag, Jena, Stuttgart, Lübeck, Ulm.

Lane D. J., 1991. 16S/23S rRNA sequencing. In: E. Stackebrandt & M. Goodfellow, (eds.), Nucleic acid techniques in bacterial systematics. Wiley & Sons, Chichester, United Kingdom: 115-175.

Nübel U., Garcia-Pichel F. & Muyzer G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology* 63: 3327-3332.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Pouria S., De Andrade A., Barbosa J., Cavalcanti R. L., Barreto V. T. S., Ward C. J., Preiser W., Poon G. K., Neild G. H. & Codd G. A. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 352: 21-26.

Rantala A., Fewer D. P., Hisbergues M., Rouhiainen L., Vaitomaa J., Börner T. & Sivonen K. (2004). Phylogenetic evidence for the early evolution of microcystin synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 101: 568-573.

Saker M. L., Fastner J., Dittmann E., Christiansen G. & Vasconcelos V. M. (2005). Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. *Journal of Applied Microbiology* 99: 749-757.

Tanabe Y., Kaya K. & Watanabe M. M. (2004). Evidence for recombination in the microcystin synthetase (mcy) genes of toxic cyanobacteria *Microcystis* spp. *Journal of Molecular Evolution* 58: 633-641.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. & Higgins D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Valdor R. & Aboal M. (2007). Effects of living cyanobacteria, cyanobacterial extracts and pure microcystins on growth and ultrastructure of microalgae and bacteria. *Toxicon* 49: 769-779.

Vasconcelos V., Oliveira S. & Teles F. O. (2001). Impact of a toxic and a non-toxic strain of *Microcystis aeruginosa* on the crayfish Procambarus clarkii. *Toxicon* 39: 1461-1470.

Vasconcelos V. M., Campos T., Amorim A. & Soares A. M. V. M. (1993a). Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 193-201.

Vasconcelos V. M., Evans W. R., Carmichael W. W. & Namikoshi M. (1993b). Isolation of microcystin-LR from a Microcystis (cyanobacteria) waterbloom collected in the drinking water reservoir for Porto, Portugal. *Journal of Environmental Science and Health - Part A Environmental Science and Engineering* 28: 2081-2094.

Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1995). Isolation and characterization of microcystins (heptapeptide hepatotoxins) from Portuguese strains of *Microcystis aeruginosa* Kütz. emend Elekin. *Arch Hydrobiol* 134: 295-305.

Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 30: 2377-2384.

WHO, 1998. Cyanobacterial toxins: microcystin-LR., Guidelines for drinking water quality, Addendum to Vol. 2, Health criteria and other supporting information. World Health Organization, Geneva, Switzerland: 95-110.

Wiegand C. & Pflugmacher S. (2005). Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicology and Applied Pharmacology* 203: 201-218.

Wilson A. E., Sarnelle O., Neilan B. A., Salmon T. P., Gehringer M. M. & Hay M. E. (2005). Genetic Variation of the Bloom-Forming Cyanobacterium *Microcystis aeruginosa* within and among Lakes: Implications for Harmful Algal Blooms. *Applied and Environmental Microbiology* 71: 6126-6133.

Yoshida M., Yoshida T., Satomi M., Takashima Y., Hosoda N. & Hiroishi S. (2008). Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. *Journal of Applied Microbiology* 105: 407-415.

General conclusions

General conclusions

Global climatic changes are increasing the occurrence and severity of precipitation and drought episodes, which endangers the maintenance of water quality in freshwater bodies used for drinking water supply or recreation (Charron et al., 2004; Bond et al., 2008a). The occurrence of summer droughts, in particular, can cause a rapid decrease in the water level of shallow water bodies; this leads to important changes in the water physical and chemical characteristics as well as in the composition of the aquatic communities, namely the bacterial community composition (BCC), with the development of groups such as *Cyanobacteria* and *Actinobacteria* (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; Wiedner et al., 2007). The occurrence of blooms of these groups may put at risk the water quality (Zaitlin & Watson, 2006; Smith et al., 2008). More, *Cyanobacteria* can also produce toxins that endanger the human health safety (Codd, 2000; de Figueiredo et al., 2004b; Mazur-Marzec, 2006). Therefore, the establishment of water management strategies is needed but requires previous knowledge on the ecological dynamics of target communities in the water bodies.

The information on the background BCC is almost inexistent for Portuguese freshwater bodies, hindering the evaluation of potential impact from atypical phenomena such as drought. Nevertheless, an enhancement of cyanobacterial blooms has been related to heatwaves and drought conditions in eutrophic water systems (Bouvy et al., 2000; Jöhnk et al., 2008; Moore et al., 2008). In the present work, several water bodies were studied concerning their BCC, with special regard to potential cyanobacterial blooms' occurrence and the environmental conditions that favour their development.

Results showed that, in general, there could be established typical winter *vs.* summer DGGE profiles in the studied water bodies but the occurrence of cyanobacterial blooms was related to lentic conditions. In fact, the BCC differed in the affiliation of dominant bacterial phylotypes between the chosen lotic and lentic systems. In lentic water bodies, dominant phylotypes affiliated with *Cyanobacteria* (unicelular, colonial and filamentous forms), phototrophic eukaryotes and *Actinobacteria* whereas BCC in the studied riverine systems showed to be mostly dominated by *Bacteroidetes* phylotypes but also *Betaproteobacteria*. These results are in accordance to data published for water bodies with similar abiotic and trophic characteristics (Eiler & Bertilsson, 2004; Van Der Gucht et al., 2005; de Figueiredo et al., 2007; Wiedner et al., 2007; Wu et al., 2007b).

In lentic systems, the most significant factors behind the seasonality in BCC showed to be water temperature, conductivity and chlorophyll *a*, in spite of the severe variation of precipitation levels throughout the study period; this suggests BCC may resist the severe shifts caused by

drought. In riverine systems, this seasonality was still mostly defined by water temperature but also factors such as ammonium levels.

Under a drought scenario, spatial variation of BCC in a range of Portuguese water bodies was assessed. At the pick of the drought, in summer 2005, there was observed a clear segregation of Alentejo (Southern Portugal) reservoirs, where the BCC was completely dominated by *Cyanobacteria* phylotypes followed by *Actinobacteria* and *Alphaproteobacteria*. Many phylotypes were common to Alentejo reservoirs. Nevertheless, DGGE patterns and sequencing results suggest the presence of ubiquitous phylotypes throughout the Portuguese territory, in water bodies geographically distant. In fact, it has been demonstrated that the variation in the composition of bacterial assemblages seems to be mainly explained by differences in environmental conditions rather than biogeography (Van Der Gucht et al., 2007), suggesting transversal global patterns for similar freshwater bodies under common environmental contexts.

In shallow lakes, such as Vela Lake, drought can lead to a rapid reduction in the water level which, consequently, leads to spatial differences in the BCC (and dominant bloom-forming cyanobacteria) but also in the toxin-producing potential, as recorded in the present study; this may have direct impact on the health of the lake users.

Results from the present work also showed that strains isolated from a bloom of *Aphanizomenon aphanizomenoides* were not clonal. The alternation of dominant cyanobacterial strains during bloom formation has been reported as having a major impact on the overall toxicity of a bloom (Kardinaal et al., 2007; Yoshida et al., 2008). Thus, background data about toxic strains from a water body may also facilitate the monitoring and control of toxic cyanobacteria, as the *Microcystis* strains isolated from Vela Lake.

As *Cylindrospermopsis raciborskii*, *Aph. aphanizomenoides* and *Aph. (Cuspidothrix) issatschenkoi* are considered invasive species in Europe, and the study of ecological preferences and potential impact on aquatic communities is very important. In the present study, phosphorus showed to be very important for all *Aph. issatschenkoi* and *Aph. aphanizomenoides* strains, as recorded for other diazotrophic filamentous cyanobacteria (Lehtimaki et al., 1997; Degerholm et al., 2006). However, different strains of *Aph. aphanizomenoides* belonging to different morphotypes and genotypes showed to have differences in their N-fixing potential which influenced their success under dissolved nitrogen unavailability.

Studies such as the present one are laborious and stretched out, but will surely be more frequent in the future. The need for interdisciplinary approaches is becoming essential in order to retrieve more information and conclusions from the obtained data as well as contextualize them. However, the better approach is to gather several fields of research expertise, with different researchers contributing to a same team, having the same goals. Thus, certainly this will be the approach for the development of further studies, whether on the scope of BCC diversity in

Portuguese water bodies, under impacts of climatic changes, or to go forward on the cyanobacterial blooms dynamics and toxicity in Portuguese water bodies. Nevertheless, the present work is an important contribution as a backgroug for those future approaches.

References

Bond N., Lake P. & Arthington A. (2008). The impacts of drought on freshwater ecosystems: an Australian perspective. *Hydrobiologia* 600: 3-16.

Bouvy M., Falcão D., Marinho M., Pagano M. & Moura A. (2000). Occurrence of Cylindrospermopsis (Cyanobacteria) in 39 Brazilian tropical reservoirs during the 1998 drought. *Aquatic Microbial Ecology* 23: 13-27.

Charron D. F., Thomas M. K., Waltner-Toews D., Aramini J. J., Edge T., Kent R. A., Maarouf A. R. & Wilson J. (2004). Vulnerability of waterborne diseases to climate change in Canada: A review. *Journal of Toxicology and Environmental Health - Part A* 67: 1667-1677.

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

Degerholm J., Gundersen K., Bergman B. & Söderbäck E. (2006). Phosphorus-limited growth dynamics in two Baltic Sea cyanobacteria, *Nodularia* sp. and *Aphanizomenon* sp. *FEMS Microbiology Ecology* 58: 323-332.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Kardinaal W. E. A., Tonk L., Janse I., Hol S., Slot P., Huisman J. & Visser P. M. (2007). Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. *Applied and Environmental Microbiology* 73: 2939-2946.

Jöhnk K. D., Huisman J., Sharples J., Sommeijer B., Visser P. M. & Stroom J. M. (2008). Summer heatwaves promote blooms of harmful cyanobacteria. *Global Change Biology* 14: 495-512.

Lehtimaki J., Moisander P., Sivonen K. & Kononen K. (1997). Growth, Nitrogen Fixation, and Nodularin Production by Two Baltic Sea Cyanobacteria. *Applied and Environmental Microbiology* 63: 1647-1656.

Mazur-Marzec H. (2006). Characterization of phycotoxins produced by cyanobacteria. *Oceanological and Hydrobiological Studies* 35: 85-109.

Moore S. K., Trainer V. L., Mantua N. J., Parker M. S., Laws E. A., Backer L. C. & Fleming L. E. (2008). Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environmental Health: A Global Access Science Source* 7.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

Van Der Gucht K., Cottenie K., Muylaert K., Vloemans N., Cousin S., Declerck S., Jeppesen E., Conde-Porcuna J. M., Schwenk K., Zwart G., Degans H., Vyverman W. & De Meester L. (2007). The

power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20404-20409.

Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.

Wiedner C., Rücker J., Brüggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.

Wu X., Xi W., Ye W. & Yang H. (2007). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Yoshida M., Yoshida T., Satomi M., Takashima Y., Hosoda N. & Hiroishi S. (2008). Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. *Journal of Applied Microbiology* 105: 407-415.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

All references cited in the thesis

Abed R. M. M. & Köster J. (2005). The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. *International Biodeterioration and Biodegradation* 55: 29-37.

Abrantes N., Nogueira A. J. A. & Gonçalves F. (2009a). Short-term dynamics of cladocerans in a eutrophic shallow lake during a shift in the cyanobacteria dominance. *Annales de Limnologie - International Journal of Limnology* 45: 237-245.

Abrantes N., Pereira R., de Figueiredo D. R., Marques C. R., Pereira M. J. & Gonçalves F. (2009b). A whole sample toxicity assessment to evaluate the sub-lethal toxicity of water and sediment elutriates from a lake exposed to diffuse pollution. *Environmental Toxicology* 24: 259-270.

Allgaier M., Brückner S., Jaspers E. & Grossart H. P. (2007). Intra- and inter-lake variability of free-living and particle-associated Actinobacteria communities. *Environmental Microbiology* 9: 2728-2741.

Allgaier M. & Grossart H. P. (2006a). Diversity and seasonal dynamics of *Actinobacteria* populations in four lakes in Northeastern Germany. *Applied and Environmental Microbiology* 72: 3489-3497.

Allgaier M. & Grossart H. P. (2006b). Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

Almeida A. S., Gonçalves A. M. M., Pereira J. L. & Gonçalves F. (2006). The impact of *Eichhornia crassipes* on green algae and cladocerans. *Fresenius Environmental Bulletin* 15: 1531-1538.

Almeida S. F. P. (2001). Use of diatoms for freshwater quality evaluation in Portugal. *Limnetica* 20: 205-213.

Altmann D., Stief P., Amann R., Beer D. d. & Schramm A. (2003). *In situ* distribution and activity of nitrifying bacteria in freshwater sediment. *Environmental Microbiology* 5: 798-803.

Amer R., Díez B. & El-Shehawy R. (2009). Diversity of hepatotoxic cyanobacteria in the Nile Delta, Egypt. *Journal of Environmental Monitoring* 11: 126-133.

Anderson-Glenna M. J., Bakkestuen V. & Clipson N. J. W. (2008). Spatial and temporal variability in epilithic biofilm bacterial communities along an upland river gradient. *FEMS Microbiology Ecology* 64: 407-418.

APHA (1992). Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

APHA (1995). Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Appelo C. A. J. & Postma D. (2005). Geochemistry, groundwater and pollution. A.A.Balkema Publishers, Amsterdam, the Netherlands.

Awadallah R. M., Soltan M. E., Shabeb M. S. A. & Moalla S. M. N. (1998). Bacterial removal of nitrate, nitrite and sulphate in wastewater. *Water Research* 32: 3080-3084.

Bacelar-Nicolau P., Nicolau L. B., Marques J. C., Morgado F., Pastorinho R. & Azeiteiro U. M. (2003). Bacterioplankton dynamics in the Mondego estuary (Portugal). *Acta Oecologica* 24: S67-S75.

Ballot A., Fastner J. & Wiedner C. (2010). Paralytic Shellfish Poisoning Toxin-Producing Cyanobacterium *Aphanizomenon gracile* in Northeast Germany. *Applied and Environmental Microbiology* 76: 1173-1180.

Barros P., Implicações ecotoxicológicas de cianobactérias em cladóceros. Faculty of Sciences and Technology, Vol. MSc. University of Coimbra, Coimbra, 1994.

Barros P., Silveira S., Ribeiro R., Gonçalves F., Vasconcelos V. & Soares A. M. V. M. (1993). Estrutura populacional fitoplanctónica nas lagoas das Braças, Vela e Mira (região centro-litoral). Resultados preliminares. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 1-8.

Bent S. J. & Forney L. J. (2008). The tragedy of the uncommon: Understanding limitations in the analysis of microbial diversity. *ISME Journal* 2: 689-695.

Berg K. A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P. & Rapala J. (2008). High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME Journal* 3: 314-325.

Berggren M., Laudon H. & Jansson M. (2009). Hydrological control of organic carbon support for bacterial growth in boreal headwater streams. *Microbial Ecology* 57: 170-178.

Bittencourt-Oliveira M. D. C., De Oliveira M. C. & Bolch C. J. S. (2001). Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (cpcBA). *Journal of Phycology* 37: 810-818.

Böckelmanna U., Manz W., Neu T. R. & Szewzyk U. (2000). Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe River of Germany by cultivation and molecular methods. *FEMS Microbiology Ecology* 33: 157-170.

Bond N., Lake P. & Arthington A. (2008a). The impacts of drought on freshwater ecosystems: an Australian perspective. *Hydrobiologia* 600: 3-16.

Bond N. R., Lake P. S. & Arthington A. H. (2008b). The impacts of drought on freshwater ecosystems: An Australian perspective. *Hydrobiologia*: 1-14.

Boucher D., Jardillier L. & Debroas D. (2006). Succession of bacterial community composition over two consecutive years in two aquatic systems: A natural lake and a lake-reservoir. *FEMS Microbiology Ecology* 55: 79-97.

Bourne D. G., Blakeley R. L., Riddles P. & Jones G. J. (2006). Biodegradation of the cyanobacterial toxin microcystin LR in natural water and biologically active slow sand filters. *Water Research* 40: 1294-1302.

Bouvy M., Falcão D., Marinho M., Pagano M. & Moura A. (2000). Occurrence of Cylindrospermopsis (Cyanobacteria) in 39 Brazilian tropical reservoirs during the 1998 drought. *Aquatic Microbial Ecology* 23: 13-27.

Brümmer I. H. M., Fehr W. & Wagner-Döbler I. (2000). Biofilm community structure in polluted rivers: Abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* 66: 3078-3082.

Brümmer I. H. M., Felske A. & Wagner-Döbler I. (2003). Diversity and Seasonal Variability of {beta}-Proteobacteria in Biofilms of Polluted Rivers: Analysis by Temperature Gradient Gel Electrophoresis and Cloning. *Appl. Environ. Microbiol.* 69: 4463-4473.

Bruno L., Billi D., Albertano P. & Urzi C. (2006). Genetic characterization of epilithic cyanobacteria and their associated bacteria. *Geomicrobiology Journal* 23: 293-299.

Burkert U., Warnecke F., Babenzien D., Zwirnmann E. & Pernthaler J. (2003). Members of a readily enriched β-proteobacterial clade are common in surface waters of a humic lake. *Applied and Environmental Microbiology* 69: 6550-6559.

Burrows W. D. & Renner S. E. (1999). Biological warfare agents as threats to potable water. *Environ Health Perspectives* 107: 975-984.

Cabecinha E., Cortes R., Cabral J. A., Ferreira T., Lourenço M. & Pardal M. A. (2009a). Multi-scale approach using phytoplankton as a first step towards the definition of the ecological status of reservoirs. *Ecological Indicators* 9: 240-255.

Cabecinha E., Cortes R., Pardal M. A. & Cabral J. A. (2009b). A Stochastic Dynamic Methodology (StDM) for reservoir's water quality management: Validation of a multi-scale approach in a south European basin (Douro, Portugal). *Ecological Indicators* 9: 329-345.

Cadel-Six S., Peyraud-Thomas C., Brient L., De Marsac N. T., Rippka R. & Me?jean A. (2007). Different genotypes of anatoxin-producing cyanobacteria coexist in the Tarn River, France. *Applied and Environmental Microbiology* 73: 7605-7614.

Caetano S., Miguel R., Mendes P., Galvão H. & Barbosa A. (2001). Cyanobacteria blooms and cyanotoxin occurrence in the Guadiana (SE Portugal) - preliminary results. *Ecotoxicology and Environmental Restoration* 4: 53-59.

Calado A. J., Fundamentação da aplicação do sistema de saprobia às algas de água doce do Centro de Portugal. Department of Biology. University of Aveiro, Aveiro, 1990.

Calado A. J., Almeida S. F. P. & Craveiro S. C. (2005). Phycological surveys and monitoring in Portugal - a summary. *ALGAS - Boletín de la Sociedad Española de Ficologia* Septiembre 2005: 36-41.

Calado A. J. & Craveiro S. C. (1995). Notes on the ecology of Synurophycean algae found in Portugal. *Nord J Bot* 15: 641-654.

Calado A. J., Freitas A. M. & Veloso V. M. (1991). Algas da Pateira de Fermentelos numa situação de Inverno. *Revista de Biologia da Universidade de Aveiro* 4: 55-71.

Casamatta D. A. & Wickstrom C. E. (2000). Sensitivity of two disjunct bacterioplankton communities to exudates from the cyanobacterium *Microcystis aeruginosa* Kützing. *Microbial Ecology* 40: 64-73.

Castro B. B. & Gonçalves F. (2007). Seasonal dynamics of the crustacean zooplankton of a shallow eutrophic lake from the Mediterranean region, with particular emphasis on *Daphnia*. *Fundamental and Applied Limnology - Archiv für Hydrobiologie* 169: 189-202.

Cerqueira M. A., Silva J. F., Magalhães F. P., Soares F. M. & Pato J. J. (2008). Assessment of water pollution in the Antuã River basin (Northwestern Portugal). *Environmental Monitoring and Assessment* 142: 325-335.

Cerqueira M. A., Vieira F. N., Ferreira R. V. & Silva J. F. (2005). The water quality of the Cértima River Basin (Central Portugal). *Environmental Monitoring and Assessment* 111: 297-306.

Chang H. Q., Yang X. E., Fang Y. Y., Pu P. M., Li Z. K. & Rengel Z. (2006). In-situ nitrogen removal from the eutrophic water by microbial-plant integrated system. *Journal of Zhejiang University. Science. B.* 7: 521-531.

Charron D. F., Thomas M. K., Waltner-Toews D., Aramini J. J., Edge T., Kent R. A., Maarouf A. R. & Wilson J. (2004). Vulnerability of waterborne diseases to climate change in Canada: A review. *Journal of Toxicology and Environmental Health - Part A* 67: 1667-1677.

Chonudomkul D., Yongmanitchai W., Theeragool G., Kawachi M., Kasai F., Kaya K. & Watanabe M. M. (2004). Morphology, genetic diversity, temperature tolerance and toxicity of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) strains from Thailand and Japan. *FEMS Microbiology Ecology* 48: 345-355.

Chorus I. & Fastner J. (2001). Recreational Exposure to Cyanotoxins. In: I. Chorus, (ed.), Cyanotoxins - occurrence, causes, consequences. Springer, Berlin, Germany: 190-199.

Christiansen G., Molitor C., Philmus B. & Kurmayer R. (2008). Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. *Molecular Biology and Evolution* 25: 1695-1704.

Christoffersen K., Lyck S. & Winding A. (2002). Microbial activity and bacterial community structure during degradation of microcystins. *Aquatic Microbial Ecology* 27: 125-136.

Clarke K. R. & Gorley R. N. (2006). PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Codd G. A. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16: 51-60.

Codd G. A., Azevedo S. M. F. O., Bagchi S. N., Burch M. D., Carmichael W. W., Harding W. R., Kaya K. & Utkilen H. C., CYANONET - A Global Network for Cyanobacterial Bloom and Toxin Risk Management - Initial Situation Assessment and Recommendations. IHP-VI Technical Documents in Hydrology, Vol. 76. UNESCO, Paris, 2005a.

Codd G. A., Edwards C., Beattie K. A., Lawton L. A., Campbell D. L. & Bell S. G. (1995). Toxins from cyanobacteria (blue-green algae). In: W. Wiessner, E. Schnepf & R. C. Starr, (eds.), Algae, Environment and Human Affairs. Biopress Ltd., Bristol, England: 1-17.

Codd G. A., Metcalf J. S. & Beattie K. A. (1999). Retention of *Microcystis aeruginosa* and microcystin by salad lettuce (*Lactuca sativa*) after spray irrigation with water containing cyanobacteria. *Toxicon* 37: 1181-1185.

Codd G. A., Morrison L. F. & Metcalf J. S. (2005b). Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology* 203: 264-272.

Cortecci G., Dinelli E., Bencini A., Adorni-Braccesi A. & La Ruffa G. (2002). Natural and anthropogenic SO4 sources in the Arno river catchment, northern Tuscany, Italy: a chemical and isotopic reconnaissance. *Applied Geochemistry* 17: 79-92.

Cottrell M. T., Waidner L. A., Yu L. & Kirchman D. L. (2005). Bacterial diversity of metagenomic and PCR libraries from the Delaware River. *Environmental Microbiology* 7: 1883-1895.

Craun G. F., Nwachuku N., Calderon R. L. & Craun M. F. (2002). Outbreaks in drinking-water systems, 1991-1998. *Journal of Environmental Health* 65: 16-23.

Crump B. C. & Hobbie J. E. (2005). Synchrony and seasonality in bacterioplankton communities of two temperate rivers. *Limnology and Oceanography* 50: 1718-1729.

Das B. & Singh P. K. (1977). Isolation, growth and nitrogen fixation of a gas vacuole-less mutant of the blue green alga *Anabaena aphanizomenoides*. *Cytobios* 17: 151-158.

de Figueiredo D. R., Alves A., Pereira M. J. & Correia A. (2010a). Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). *Journal of Plankton Research* 32: 239-252.

de Figueiredo D. R., Antunes S. C., Pereira M. J. & Gonçalves F. (2004a). Chronic effects of *Aphanizomenon flos-aquae* on the survival and reproduction of daphnids. *Fresenius Environmental Bulletin* 13: 665-670.

de Figueiredo D. R., Azeiteiro U. M., Esteves S. M., Gonçalves F. J. M. & Pereira M. J. (2004b). Microcystin-producing blooms - A serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.

de Figueiredo D. R., Azeiteiro U. M., Gonçalves F. & Pereira M. J. (2004c). *Aphanizomenon flos-aquae* grown under different nutrient concentrations and the effects of its exudates on growth of two green algae. *Fresenius Environmental Bulletin* 13: 657-664.

de Figueiredo D. R., Pereira M. J. & Correia A. (2010b). Seasonal modulation of bacterioplankton community at a temperate eutrophic shallow lake. *World Journal of Microbiology and Biotechnology* 26: 1067-1077.

de Figueiredo D. R., Pereira M. J. & Correia A. (unpublished). Spatial diversity of bacterioplankton at Vela Lake (Centralwestern Portugal) during a severe summer drought - cyanobacterial blooms and microcystin production potential.

de Figueiredo D. R., Pereira M. J., Moura A., Silva L., Bárrios S., Fonseca F., Henriques I. & Correia A. (2007). Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. *FEMS Microbiology Ecology* 59: 638-650.

de Figueiredo D. R., Reboleira A. S. S. P., Antunes S. C., Abrantes N., Azeiteiro U., Gonçalves F. & Pereira M. J. (2006). The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. *Hydrobiologia* 568: 145-157.

De Wever A., Muylaert K., Van Der Gucht K., Pirlot S., Cocquyt C., Descy J. P., Plisnier P. D. & Vyverman W. (2005). Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and Environmental Microbiology* 71: 5029-5037.

Degerholm J., Gundersen K., Bergman B. & Söderbäck E. (2006). Phosphorus-limited growth dynamics in two Baltic Sea cyanobacteria, *Nodularia* sp. and *Aphanizomenon* sp. *FEMS Microbiology Ecology* 58: 323-332.

Dokulil M. T. & Teubner K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia* 438: 1-12.

Domingues R. B., Barbosa A. & Galvão H. (2005). Nutrients, light and phytoplankton succession in a temperate estuary (the Guadiana, south-western Iberia). *Estuarine, Coastal and Shelf Science* 64: 249-260.

Dorigo U., Volatier L. & Humbert J. F. (2005). Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Research* 39: 2207-2218.

Dow C. S. & Swoboda U. K. (2000). Cyanotoxins. In: B. A. Whitton & M. Potts, (eds.), The Ecology of Cyanobacteria. Kluwer Academic Publishers, The Netherlands: 613-632.

Ducharne A., Baubion C., Beaudoin N., Benoit M., Billen G., Brisson N., Garnier J., Kieken H., Lebonvallet S., Ledoux E., Mary B., Mignolet C., Poux X., Sauboua E., Schott C., Théry S. & Viennot P. (2007). Long term prospective of the Seine River system: Confronting climatic and direct anthropogenic changes. *Science of the Total Environment* 375: 292-311.

Dyble J., Paerl H. W. & Neilan B. A. (2002). Genetic characterization of *Cylindrospermopsis raciborskii* (cyanobacteria) isolates from diverse geographic origins based on nifH and cpcBA-IGS nucleotide sequence analysis. *Applied and Environmental Microbiology* 68: 2567-2571.

Eiler A. & Bertilsson S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environmental Microbiology* 6: 1228-1243.

Falconer I. R. & Humpage A. R. (2006). Cyanobacterial (blue-green algal) toxins in water supplies: Cylindrospermopsins. *Environmental Toxicology* 21: 299-304.

Ferreira da Silva E., Serrano Pinto L., Patinha C. & Cardoso Fonseca E. (2004). Site-Specific Characterization of Castromil Brownfield Area Related to Gold Mining Activities. *Environmental Geochemistry and Health* 26: 13-25.

Ferreira F. M. B., Soler J. M. F., Fidalgo M. L. & Ferna?ndez-Vila P. (2001). PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal). *Toxicon* 39: 757-761.

Ferreira R. S. V., Avaliação hidroquímica e da qualidade da água na bacia do rio Cértima (Portugal). Department of Environment and Planning, Vol. MSc. University of Aveiro, Aveiro, 2007.

Figueredo C. C., Giani A. & Bird D. F. (2007). Does allelopathy contribute to *Cylindrospermopsis raciborskii* (cyanobacteria) bloom occurrence and geographic expansion? *Journal of Phycology* 43: 256-265.

Fitzgerald D. J. (2001). Cyanotoxins and Human Health - Overview. In: I. Chorus, (ed.), Cyanotoxins - occurrence, causes, consequences. Springer, Berlin, Germany: 179-190.

Fleming L. E., Rivero C., Burns J., Williams C., Bean J. A., Shea K. A. & Stinn J. (2002). Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. *Harmful Algae* 1: 157-168.

Flores E. & Herrero A. (2010). Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Micro* 8: 39-50.

Fromin N., Hamelin J., Tarnawski S., Roesti D., Jourdain-Miserez K., Forestier N., Teyssier-Cuvelle S., Gillet F., Aragno M. & Rossi P. (2002). Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environmental Microbiology* 4: 634-643.

Galhano V., Peixoto F., Gomes-Laranjo J. & Fernández-Valiente E. (2009). Differential effects of bentazon and molinate on *Anabaena cylindrica* , an autochthonous cyanobacterium of Portuguese rice field agro-ecosystems. *Water, Air, & Soil Pollution* 197: 211-222.

Galhano V., Peixoto F., Gomes-Laranjo J. & Fernández-Valiente E. (2010). Comparative toxicity of bentazon and molinate on growth, photosynthetic pigments, photosynthesis, and respiration of the Portuguese ricefield cyanobacterium *Nostoc muscorum*. *Environmental Toxicology* 25: 147-156.

Galvão H. M., Reis M. P., Valério E., Domingues R. B., Costa C., Lourenço D., Condinho S., Miguel R., Barbosa A., Gago C., Faria N., Paulino S. & Pereira P. (2008). Cyanobacterial blooms in natural waters in southern Portugal: A water management perspective. *Aquatic Microbial Ecology* 53: 129-140.

Gao G., Qin B., Sommaruga R. & Psenner R. (2007). The bacterioplankton of Lake Taihu, China: Abundance, biomass, and production. *Hydrobiologia* 581: 177-188.

Gehringer M. M., Govender S., Shah M. & Downing T. G. (2003). An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. *Environmental Toxicology* 18: 142-148.

Gil M. C., Estudo ecológico das Diatomáceas dos rios Águeda, Agadão e Alfusqueiro. Department of Biology, Vol. PhD. University of Aveiro, Aveiro, 1988, pp. 389.

Golden J. W. & Yoon H. S. (2003). Heterocyst development in *Anabaena*. *Current Opinion in Microbiology* 6: 557-563.

Gonçalves A. M., de Figueiredo D. R. & Pereira M. J. (2005). A low-cost methodology for algal growth inhibition tests using three freshwater green algae. *Fresenius Environmental Bulletin* 14: 1192-1195.

Greer A., Ng V. & Fisman D. (2008). Climate change and infectious diseases in North America: The road ahead. *Canadian Medical Association Journal* 178: 715-722.

Gugger M., Lyra C., Henriksen P., Coute A., Humbert J. F. & Sivonen K. (2002). Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *International Journal of Systematic and Evolutionary Microbiology* 52: 1867-1880.

Haande S., Rohrlack T., Ballot A., RÃ¸berg K., Skulberg R., Beck M. & Wiedner C. (2008). Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) isolates from Africa and Europe. *Harmful Algae* 7: 692-701.

Hall E. K. & Cotner J. B. (2007). Interactive effect of temperature and resources on carbon cycling by freshwater bacterioplankton communities. *Aquatic Microbial Ecology* 49: 35-45.

Harper D. (1992). Eutrophication of freshwaters: principles, problems and restoration. Chapman et Hall, London.

Haukka K., Kolmonen E., Hyder R., Hietala J., Vakkilainen K., Kairesalo T., Haario H. & Sivonen K. (2006). Effect of Nutrient Loading on Bacterioplankton Community Composition in Lake Mesocosms. *Microbial Ecology* 51: 137-146.

Hernández J. M., López-Rodas V. & Costas E. (2009). Microcystins from tap water could be a risk factor for liver and colorectal cancer: A risk intensified by global change. *Medical Hypotheses* 72: 539-540.

Hindák F. (2000). Morphological variation of four planktic nostocalean cyanophytes - Members of the genus *Aphanizomenon* or *Anabaena*? *Hydrobiologia* 438: 107-116.

Hollibaugh J. T., Wong P. S. & Murrell M. C. (2000). Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California. *Aquatic Microbial Ecology* 21: 103-114.

Horecká M. & Komárek J. (1979). Taxonomic position of three planktonic blue- green algae from the genera *Aphanizomenon* and *Cylindrospermopsis*. *Preslia* 51: 289-312.

Hullar M. A. J., Kaplan L. A. & Stahl D. A. (2006). Recurring seasonal dynamics of microbial communities in stream habitats. *Applied and Environmental Microbiology* 72: 713-722.

Innok S., Matsumura M., Boonkerd N. & Teaumroong N. (2005). Detection of *Microcystis* in lake sediment using molecular genetic techniques. *World Journal of Microbiology and Biotechnology* 21: 1559-1568.

Izaguirre G. & Taylor W. D. (1995). Geosmin and 2-methylisoborneol production in a major aqueduct system. *Water Science and Technology* 31: 41-48.

Izydorczyk K. & Tarczynska M. (2005). The influence of wind on cyanobacterial bloom development in shallow, lowland reservoir in central Poland. *Ecohydrology and Hydrobiology* 5: 195-203.

Jacoby J. M., Collier D. C., Welch E. B., Hardy F. J. & Crayton M. (2000). Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Canadian Journal of Fisheries and Aquatic Sciences* 57: 231-240.

Janson S., Matveyev A. & Bergman B. (1998). The presence and expression of *hetR* in the non-heterocystous cyanobacterium *Symploca* PCC 8002. *FEMS Microbiology Letters* 168: 173-179.

Jardillier L., Boucher D., Personnic S., Jacquet S., Thénot A., Sargos D., Amblard C. & Debroas D. (2005). Relative importance of nutrients and mortality factors on prokaryotic community composition in two lakes of different trophic status: Microcosm experiments. *FEMS Microbiology Ecology* 53: 429-443.

Jeeji-Bai N. (1976). Morphological variation of certain blue-green algae in culture: *Scytonema stuposum* (Kütz.) Born. *Schweizerische Zeitschrift für Hydrologie* 38: 55-62.

Jin X., Lu S., Hu X., Jiang X. & Wu F. (2008). Control concept and countermeasures for shallow lakes' eutrophication in China. *Frontiers of Environmental Science and Engineering in China* 2: 257-266.

Jöhnk K. D., Huisman J., Sharples J., Sommeijer B., Visser P. M. & Stroom J. M. (2008). Summer heatwaves promote blooms of harmful cyanobacteria. *Global Change Biology* 14: 495-512.

Kaebernick M. & Neilan B. A. (2001). Ecological and molecular investigations of cyanotoxin production. *FEMS Microbiology Ecology* 35: 1-9.

Kardinaal W. E. A., Tonk L., Janse I., Hol S., Slot P., Huisman J. & Visser P. M. (2007). Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. *Applied and Environmental Microbiology* 73: 2939-2946.

Kaštovský J., Hauer T., Mareš J., Krautová M., Bešta T., Komárek J., Desortová B., Heteša J., Hindáková A., Houk V., Janeček E., Kopp R., Marvan P., Pumann P., Skácelová O. & Zapomělová E. (2010). A review of the alien and expansive species of freshwater cyanobacteria and algae in the Czech Republic. *Biological Invasions* DOI 10.1007/s10530-010-9754-3.

Ke Z., Xie P. & Guo L. (2008). Controlling factors of spring-summer phytoplankton succession in Lake Taihu (Meiliang Bay, China). *Hydrobiologia* 607: 41-49.

Kearns K. & Hunter M. (2001). Toxin-producing *Anabaena flos-aquae* induces settling of *Chlamydomonas reinhardtii*, a competing motile alga. *Microbial Ecology* 42: 80-86.

Keating K. I. (1978). Blue-Green Algal Inhibition of Diatom Growth: Transition from Mesotrophic to Eutrophic Community Structure. *Science* 199: 971-973.

Kisand V., Cuadros R. & Wikner J. (2002). Phylogeny of Culturable Estuarine Bacteria Catabolizing Riverine Organic Matter in the Northern Baltic Sea. *Appl. Environ. Microbiol.* 68: 379-388.

Komárek J. (1991). A review of water-bloom forming *Microcystis* species, with regard to populations from Japan. *Archives of Hydrobiology* Suppl 92: 115-127.

Komárek J. (2006). Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. *Algae* 21: 349-375.

Komárek J. (2010a). Modern taxonomic revision of planktic nostocacean cyanobacteria: a short review of genera. *Hydrobiologia* 639: 231-243.

Komárek J. (2010b). Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). *Hydrobiologia* 639: 245-259.

Komárek J. & Anagnostidis K. (1999). Cyanoprokariota - 1. Teil Chroococcales. In: H. Ettl, G. Gärtner, H. Heynig & D. Mollenhauer, (eds.), Süβwasserflora von Mitteleuropa, Band 19/1. G. Fischer Verlag, Jena, Stuttgart, Lübeck, Ulm.

Komárek J. & Kaštovský J. (2003). Coincidences of structural and molecular characters in evolutionary lines of cyanobacteria. *Algological Studies (Cyanobacterial Research 4)* 109: 305-325.

Komárek J. & Komárková J. (2006). Diversity of *Aphanizomenon*-like cyanobacteria. *Czech Phycology* 6: 1-32.

Komárek J. & Kováčik L. (1989). Trichome structure of *fourAphanizomenon* taxa (Cyanophyceae) from Czechoslovakia, with notes on the taxonomy and delimitation of the genus. *Plant Systematics and Evolution* 164: 47-64.

Kong Y., Xia Y., Nielsen J. L. & Nielsen P. H. (2007). Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. *Microbiology* 153: 4061-4073.

Kozak A. (2005). Seasonal changes occurring over four years in a reservoir's phytoplankton composition. *Polish Journal of Environmental Studies* 14: 451-465.

Ladas N. P. & Papageorgiou G. C. (2000). The salinity tolerance of freshwater cyanobacterium *Synechococcus* sp. PCC 7942 is determined by its ability for osmotic adjustment and presence of osmolyte sucrose. *Photosynthetica* 38: 343-348.

Lane D. J. (1991). 16S/23S rRNA sequencing. In: E. Stackebrandt & M. Goodfellow, (eds.), Nucleic acid techniques in bacterial systematics. Wiley & Sons, Chichester, United Kingdom: 115-175.

Lee H.-W., Lee S.-Y., Lee J.-W., Park J.-B., Choi E.-S. & Park Y. K. (2002). Molecular characterization of microbial community in nitrate-removing activated sludge. *FEMS Microbiology Ecology* 41: 85-94.

Leflaive J. & Ten-Hage L. (2007). Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. *Freshwater Biology* 52: 199-214.

Lehtimäki J., Lyra C., Suomalainen S., Sundman P., Rouhiainen L., Paulin L., Salkinoja-Salonen M. & Sivonen K. (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *International Journal of Systematic and Evolutionary Microbiology* 50: 1043-1053.

Lehtimaki J., Moisander P., Sivonen K. & Kononen K. (1997). Growth, Nitrogen Fixation, and Nodularin Production by Two Baltic Sea Cyanobacteria. *Applied and Environmental Microbiology* 63: 1647-1656.

Li L., Li Y., Biswas D. K., Nian Y. & Jiang G. (2008). Potential of constructed wetlands in treating the eutrophic water: Evidence from Taihu Lake of China. *Bioresource Technology* 99: 1656-1663.

Li R., Carmichael W. W. & Pereira P. (2003). Morphological and 16s rRNA gene evidence for reclassification of the paralytic shellfish toxin producing *Aphanizomenon flos-aquae* LMECYA 31 as *Aphanizomenon issatschenkoi* (Cyanophyceae). *Journal of Phycology* 39: 814-818.

Li R., Watanabe M. & Watanabe M. M. (2000). Taxonomic studies of planktic species of *Anabaena* based on morphological characteristics in cultured strains. *Hydrobiologia* 438: 117-138.

Lindström E. S. (2000). Bacterioplankton community composition in five lakes differing in trophic status and humic content. *Microbial Ecology* 40: 104-113.

Lindström E. S., Kamst-Van Agterveld M. P. & Zwart G. (2005). Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Applied and Environmental Microbiology* 71: 8201-8206.

Liu W. & Qiu R. (2007). Water eutrophication in China and the combating strategies. *Journal of Chemical Technology and Biotechnology* 82: 781-786.

Lodders N., Stackebrandt E. & Nübel U. (2005). Frequent genetic recombination in natural populations of the marine cyanobacterium *Microcoleus chthonoplastes*. *Environmental Microbiology* 7: 434-442.

López-Archilla A. I., Gérard E., Moreira D. & López-García P. (2004). Macrofilamentous microbial communities in the metal-rich and acidic River Tinto, Spain. *FEMS Microbiology Letters* 235: 221-228.

Lyautey E., Lacoste B., Ten-Hage L., Rols J. L. & Garabetian F. (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: Methodological settings and fingerprints interpretation. *Water Research* 39: 380-388.

Lyra C., Suomalainen S., Gugger M., Vezie C., Sundman P., Paulin L. & Sivonen K. (2001). Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. *International Journal of Systematic and Evolutionary Microbiology* 51: 513-526.

Magnan P., Rodríguez M. A., Legendre P. & Lacasse S. (1994). Dietary variation in a freshwater fish species: relative contributions of biotic interactions, abiotic factors, and spatial structure. *Canadian Journal of Fisheries and Aquatic Sciences* 51: 2856-2865.

Mankiewicz J., Walter Z., Tarczynska M., Palyvoda O., Wojtysiak-Staniaszczyk M. & Zalewski M. (2002). Genotoxicity of cyanobacterial extracts containing microcystins from Polish water reservoirs as determined by SOS chromotest and comet assay. *Environmental Toxicology* 17: 341-350.

Mao J., Chen Q. & Chen Y. (2008). Three-dimensional eutrophication model and application to Taihu Lake, China. *Journal of Environmental Sciences* 20: 278-284.

Markensten H., Moore K. & Persson I. (2010). Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate. *Ecological Applications* 20: 752-767.

Marzorati M., Wittebolle L., Boon N., Daffonchio D. & Verstraete W. (2008). How to get more out of molecular fingerprints: Practical tools for microbial ecology. *Environmental Microbiology* 10: 1571-1581.

Masciopinto C., La Mantia R., Carducci A., Casini B., Calvario A. & Jatta E. (2007). Unsafe tap water in households supplied from groundwater in the Salento Region of Southern Italy. *Journal of Water and Health* 5: 129-148.

Mašín M., Jezbera J., Nedoma J., Straškrabová V., Hejzlar J. & Šimek K. (2003). Changes in bacterial community composition and microbial activities along the longitudinal axis of two canyon-shaped reservoirs with different inflow loading. *Hydrobiologia* 504: 99-113.

Mazel D., Houmard J., Castets A. M. & Tandeau de Marsac N. (1990). Highly repetitive DNA sequences in cyanobacterial genomes. *Journal of Bacteriology* 172: 2755-2761.

Mazur-Marzec H. (2006). Characterization of phycotoxins produced by cyanobacteria. *Oceanological and Hydrobiological Studies* 35: 85-109.

Mischke U. (2003). Cyanobacteria associations in shallow polytrophic lakes: influence of environmental factors. *Acta Oecologica* 24.

Mitraki C., Crisman T. L. & Zalidis G. (2004). Lake Koronia, Greece: Shift from autotrophy to heterotrophy with cultural eutrophication and progressive water-level reduction. *Limnologica - Ecology and Management of Inland Waters* 34: 110-116.

Monteiro M. T., Oliveira R. & Vale C. (1995). Metal stress on the plankton communities of Sado River (Portugal). *Water Research* 29: 695-701.

Moore S. K., Trainer V. L., Mantua N. J., Parker M. S., Laws E. A., Backer L. C. & Fleming L. E. (2008). Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environmental Health: A Global Access Science Source* 7.

Moreno I., Repetto G., Carballal E., Gago A. & Cameán A. M. (2005). Cyanobacteria and microcystins occurrence in the Guadiana River (SW Spain). *International Journal of Environmental Analytical Chemistry* 85: 461-474.

Moreno I. M., Pereira P., Franca S. & Camea?n A. (2004). Toxic cyanobacteria strains isolated from blooms in the Guadiana River (southwestern Spain). *Biological Research* 37: 405-417.

Mueller-Spitz S. R., Goetz G. W. & McLellan S. L. (2009). Temporal and spatial variability in nearshore bacterioplankton communities of Lake Michigan. *FEMS Microbiology Ecology* 67: 511-522.

Murphy T., Lean D. & Nalewajko C. (1976). Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. *Science* 192: 900-902.

Muylaert K., Van der Gucht K., Vloemans N., De Meester L., Gillis M. & Vyverman W. (2002). Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. *Applied and Environmental Microbiology* 68: 4740-4750.

Muyzer G., De Waal E. C. & Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G. & Smalla K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 73: 127-141.

Navarro J., Moser D., Flores A., Ross C., Rosen M., Dong H., Zhang G. & Hedlund B. (2009). Bacterial Succession within an Ephemeral Hypereutrophic Mojave Desert Playa Lake. *Microbial Ecology* 57: 307-320.

Neilan B. A., Saker M. L., Fastner J., Törökné A. & Burns B. P. (2003). Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. *Molecular Ecology* 12: 133-140.

Newton R. J., Jones S. E., Helmus M. R. & McMahon K. D. (2007). Phylogenetic ecology of the freshwater Actinobacteria acI lineage. *Applied and Environmental Microbiology* 73: 7169-7176.

Nichols H. W. (1973). Growth media - freshwater. In: J. R. Stein, (ed.), Handbook of Phycological Methods. Cambridge University Press, Cambridge: 7-24.

Nijburg J. W. & Laanbroek H. J. (1997). The influence of *Glyceria maxima* and nitrate input on the composition and nitrate metabolism of the dissimilatory nitrate-reducing bacterial community. *FEMS Microbiology Ecology* 22: 57-63.

Nixdorf B., Mischke U. & Rücker J. (2003). Phytoplankton assemblages and steady state in deep and shallow eutrophic lakes - An approach to differentiate the habitat properties of Oscillatoriales. *Hydrobiologia* 502: 111-121.

Nogueira I. C. G., Pereira P., Dias E., Pflugmacher S., Wiegand C., Franca S. & Vasconcelos V. M. (2004). Accumulation of paralytic shellfish toxins (PST) from the cyanobacterium *Aphanizomenon issatschenkoi* by the cladoceran *Daphnia magna*. *Toxicon* 44: 773-780.

Nübel U., Garcia-Pichel F. & Muyzer G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology* 63: 3327-3332.

Nürnberg G. K. (1996). Trophic State of Clear and Colored, Soft- and Hardwater Lakes with Special Consideration of Nutrients, Anoxia, Phytoplankton and Fish. *Lake and Reservoir Management* 12: 432 - 447.

Nürnberg G. K. (1999). Determining trophic state in experimental lakes. *Limnology and Oceanography* 44: 1176-1179.

O'Sullivan L. A., Weightman A. J. & Fry J. C. (2002). New degenerate *Cytophaga-Flexibacter-Bacteroides*-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in river taff epilithon. *Applied and Environmental Microbiology* 68: 201-210.

Oliver R. L. & Ganf G. G. (2000). Freshwater blooms. In: B. A. Whitton & M. Potts, (eds.), The Ecology of Cyanobacteria. Kluwer Academic Publishers: 149-194.

Paerl H. W., Dyble J., Moisander P. H., Noble R. T., Piehler M. F., Pinckney J. L., Steppe T. F., Twomey L. & Valdes L. M. (2003). Microbial indicators of aquatic ecosystem change: Current applications to eutrophication studies. *FEMS Microbiology Ecology* 46: 233-246.

Paerl H. W. & Huisman J. (2008). Climate: Blooms like it hot. *Science* 320: 57-58.

Page R. D. M. (1996). Tree View: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357-358.

Palinska K. A., Liesack W., Rhiel E. & Krumbein W. E. (1996). Phenotype variability of identical genotypes: The need for a combined approach in cyanobacterial taxonomy demonstrated on *Merismopedia*-like isolates. *Archives of Microbiology* 166: 224-233.

Pearce D. A., van der Gast C. J., Woodward K. & Newsham K. K. (2005). Significant changes in the bacterioplankton community structure of a maritime Antartic freshwater lake following nutrient enrichment. *Microbiology* 151: 3237-3248.

Pearson J. E. & Kingsbury J. M. (1966). Culturally induced variation in four morphological diverse bluegreen algae. *American Journal of Botany* 53: 192-200.

Peng X., Fanxiang K., Huansheng C. & Min Z. (2007). Relationship between bacterioplankton and phytoplankton community dynamics during late spring and early summer in Lake Taihu, China. *Acta Ecologica Sinica* 27: 1696-1702.

Pereira M. J., Utilização de algas na avaliação de contaminação de águas doces por metais. Department of Biology, Vol. PhD. University of Aveiro, Aveiro, 1999, pp. 611.

Pereira P., Li R. H., Carmichael W. W., Dias E. & Franca S. (2004). Taxonomy and production of paralytic shellfish toxins by the freshwater cyanobacterium *Aphanizomenon gracile* LMECYA40. *European Journal of Phycology* 39: 361-368.

Phung N. T., Lee J., Kang K. H., Chang I. S., Gadd G. M. & Kim B. H. (2004). Analysis of microbial diversity in oligotrophic microbial fuel cells using 16S rDNA sequences. *FEMS Microbiology Letters* 233: 77-82.

Pinho P., Praça F. & Torres A., Estudo para o ordenamento da área envolvente da Pateira de Fermentelos. In: R. e. C. d. N. Serviço Nacional de Parques, (Ed.). Departamento de Ambiente e Ordenamento da Universidade de Aveiro, Aveiro, 1988.

Pope P. B. & Patel B. K. C. (2008). Metagenomic analysis of a freshwater toxic cyanobacteria bloom. *FEMS Microbiology Ecology* 64: 9-27.

Possemiers S., Verthé K., Uyttendaele S. & Verstraete W. (2004). PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. *FEMS Microbiology Ecology* 49: 495-507.

Pouria S., De Andrade A., Barbosa J., Cavalcanti R. L., Barreto V. T. S., Ward C. J., Preiser W., Poon G. K., Neild G. H. & Codd G. A. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 352: 21-26.

Preußel K., Stüken A., Wiedner C., Chorus I. & Fastner J. (2006). First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. *Toxicon* 47: 156-162.

Quinn G. & Keough M. (2002). Experimental design and data analysis for biologists. Cambridge University Press, Cambridge.

Rajaniemi P., Hrouzek P., Kastovska K., Willame R., Rantala A., Hoffmann L., Komarek J. & Sivonen K. (2005a). Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *International Journal of Systematic and Evolutionary Microbiology* 55: 11-26.

Rajaniemi P., Komárek J., Willame R., Hrouzek P., Kaštovská K., Hoffmann L. & Sivonen K. (2005b). Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. *Algological Studies, Archiv für Hydrobiologie* 117: 371-91.

Ramette A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiology Ecology* 62: 142-160.

Rantala A., Fewer D. P., Hisbergues M., Rouhiainen L., Vaitomaa J., Börner T. & Sivonen K. (2004). Phylogenetic evidence for the early evolution of microcystin synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 101: 568-573.

Rapala J., Niemela M., Berg K. A., Lepisto L. & Lahti K., Removal of cyanobacteria, cyanotoxins, heterotrophic bacteria and endotoxins at an operating surface water treatment plant. Water Science and Technology, Vol. 54, 2006, pp. 23-28.

Rapala J. & Sivonen K. (1998). Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. *Microbial Ecology* 36: 181-192.

Rapala J., Sivonen K., Lyra C. & Niemelä S. I. (1997a). Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Applied and Environmental Microbiology* 63: 2206-2212.

Rapala J., Sivonen K., Lyra C. & NiemelÃ¤ S. I. (1997b). Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Applied and Environmental Microbiology* 63: 2206-2212.

Rasmussen U. & Svenning M. M. (1998). Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. *Applied and Environmental Microbiology* 64: 265-272.

Riemann L. & Winding A. (2001). Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microbial Ecology* 42: 274-285.

Rino J. M. A. & Gil M. C. P. (1987). Diatomáceas do rio Cértima - Estudo preliminar. *Revista de Biologia da Universidade de Aveiro* 1: 53 - 103.

Robinson N. J., Robinson P. J., Gupta A., Bleasby A. J., Whitton B. A. & Morby A. P. (1995). Singular over-representation of an octameric palindrome, HIP1, in DNA from many cyanobacteria. *Nucleic Acids Research* 23: 729-735.

Rocha C., Galvão H. & Barbosa A. (2002). Role of transient silicon limitation in the development of cyanobacteria blooms in the Guadiana estuary, south-western Iberia. *Marine Ecology Progress Series* 228: 35-45.

Rodier J. (1996). L'analyse de l'eau: Eaux Naturelles, Eaux Résiduaires, Eau de mer. Dunod, Paris.

Rooney-Varga J. N., Giewat M. W., Savin M. C., Sood S., Legresley M. & Martin J. L. (2005). Links between phytoplankton and bacterial community dynamics in a coastal marine environment. *Microbial Ecology* 49: 163-175.

Saadoun I. M. K., Schrader K. K. & Blevins W. T. (2001). Environmental and nutritional factors affecting geosmin synthesis by Anabaena SP. *Water Research* 35: 1209-1218.

Sabater S. & Munoz I. (1990). Successional dynamics of the phytoplankton in the lower part of the River Ebro. *Journal of Plankton Research* 12: 573-592.

Sabour B., Loudiki M., Oudra B., Vasconcelos V., Oubraim S. & Fawzi B. (2005). Dynamics and toxicity of *Anabaena aphanizomenoides* (Cyanobacteria) waterblooms in the shallow brackish Oued Mellah lake (Morocco). *Aquatic Ecosystem Health and Management* 8: 95-104.

Saitou N. & Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Saker M. L., Fastner J., Dittmann E., Christiansen G. & Vasconcelos V. M. (2005a). Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. *Journal of Applied Microbiology* 99: 749-757.

Saker M. L., Jungblut A. D., Neilan B. A., Rawn D. F. K. & Vasconcelos V. M. (2005b). Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium *Aphanizomenon flos-aquae*. *Toxicon* 46: 555-562.

Saker M. L., Nogueira I. C. G., Vasconcelos V. M., Neilan B. A., Eaglesham G. K. & Pereira P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55: 243-250.

Saksena D. N., Garg R. K. & Rao R. J. (2008). Water quality and pollution status of Chambal river in National Chambal sanctuary, Madhya Pradesh. *Journal of Environmental Biology* 29: 701-710.

Schembri M. A., Neilan B. A. & Saint C. P. (2001). Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermosis raciborskii*. *Environmental Toxicology* 16: 413-421.

Schiefer W., Schütz K., Hachtel W. & Happe T. (2002). Molecular cloning and characterization of hetR genes from filamentous cyanobacteria. *Biochimica et Biophysica Acta - Gene Structure and Expression* 1577: 139-143.

Silva J. F. & Oliveira F. (2007). Eutrophication in the River Vouga basin - Impacts on the quality of water for public supply. *IAHS-AISH Publication*: 139-147.

Šimek K., Horňák K., Jezbera J., Nedoma J., Znachor P., Hejzlar J. & Sed'a J. (2008). Spatio-temporal patterns of bacterioplankton production and community composition related to phytoplankton composition and protistan bacterivory in a dam reservoir. *Aquatic Microbial Ecology* 51: 249-262.

Šimek K., Pernthaler J., Weinbauer M. G., Horňák K., Dolan J. R., Nedoma J., Mašín M. & Amann R. (2001). Changes in Bacterial Community Composition and Dynamics and Viral Mortality Rates Associated with Enhanced Flagellate Grazing in a Mesoeutrophic Reservoir. *Applied and Environmental Microbiology* 67: 2723-2733.

Singh P. K. (1976). Growth and nitrogen fixation of mutants defective in heterocyst differentiation in the blue green alga *Cylindrospermum* sp. *Zeitschrift fur Allgemeine Mikrobiologie* 16: 453-463.

Smith J. K., Parry J. D., Day J. G. & Smith R. J. (1998). A PCR technique based on the Hip1 interspersed repetitive sequence distinguishes cyanobacterial species and strains. *Microbiology* 144: 2791-2801.

Smith J. L., Boyer G. L. & Zimba P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* 208: 5-20.

Stefaniak K. & Kokociński M. (2005). Occurence of invasive *Cyanobacteria* species in polimictic lakes of the Wielkopolska region (western Poland). *Oceanological and Hydrobiological Studies* 34: 137-148.

Steffensen D. A. (2008). Economic cost of cyanobacterial blooms. *Advances in experimental medicine and biology* 619: 855-865.

Stein L. Y., Duc M. T. L., Grundl T. J. & Nealson K. H. (2001). Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environmental Microbiology* 3: 10-18.

Stewart I., Webb P., Schluter P. & Shaw G. (2006). Recreational and occupational field exposure to freshwater cyanobacteria – a review of anecdotal and case reports, epidemiological studies and the challenges for epidemiologic assessment. *Environmental Health: A Global Access Science Source* 5: 6.

Stüken A., Campbell R. J., Quesada A., Sukenik A., Dadheech P. K. & Wiedner C. (2009). Genetic and morphologic characterization of four putative cylindrospermopsin producing species of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Journal of Plankton Research* 31: 465-480.

Stüken A., Rücker J., Endrulat T., Preussel K., Hemm M., Nixdorf B., Karsten U. & Wiedner C. (2006). Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. *Phycologia* 45: 696-703.

Suikkanen S., Fistarol G. O. & Granéli E. (2004). Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. *Journal of Experimental Marine Biology and Ecology* 308: 85-101.

Suikkanen S., Fistarol G. O. & Granéli E. (2005). Effects of cyanobacterial allelochemicals on a natural plankton community. *Marine Ecology Progress Series* 287: 1-9.

Takeyama H., Nakayama H. & Matsunaga T. (2000). Salinity-regulated replication of the endogenous plasmid pSY10 from the marine cyanobacterium *Synechococcus* sp. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology* 84-86: 447-453.

Tanabe Y., Kaya K. & Watanabe M. M. (2004). Evidence for recombination in the microcystin synthetase (mcy) genes of toxic cyanobacteria *Microcystis* spp. *Journal of Molecular Evolution* 58: 633-641.

Teles L. O., Vasconcelos V., Pereira E. & Saker M. (2006). Time series forecasting of cyanobacteria blooms in the Crestuma Reservoir (Douro River, Portugal) using artificial neural networks. *Environmental Management* 38: 227-237.

Teles M., Pacheco M. & Santos M. A. (2007). Endocrine and metabolic responses of *Anguilla anguilla* L. caged in a freshwater-wetland (Pateira de Fermentelos-Portugal). *Science of the Total Environment* 372: 562-570.

ter Braak C. J. & Verdonschot P. F. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquatic Sciences* 57: 255-289.

ter Braak C. J. F. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology* 67: 1167-1179.

ter Braak C. J. F. (1995). Ordination. In: R. H. G. Jongman, C. J. F. ter Braak & O. F. R. Tongeren, (eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, UK: 91-173.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. & Higgins D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Thompson J. D., Higgins D. G. & Gibson T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

Tian C., Tan J., Wu X., Ye W., Liu X., Li D. & Yang H. (2009). Spatiotemporal transition of bacterioplankton diversity in a large shallow hypertrophic freshwater lake, as determined by denaturing gradient gel electrophoresis. *J. Plankton Res.* 31: 885-897.

Tolotti M., Thies H., Cantonati M., Hansen C. M. E. & Thaler B. (2003). Flagellate algae (Chrysophyceae, Dinophyceae, Cryptophyceae) in 48 high mountain lakes of the Northern and Southern slope of the Eastern Alps: Biodiversity, taxa distribution and their driving variables. *Hydrobiologia* 502: 331-348.

Tong S. T. Y., Liu A. J. & Goodrich J. A. (2007). Climate change impacts on nutrient and sediment loads in a midwestern agricultural watershed. *Journal of Environmental Informatics* 9: 18-28.

Tooming-Klunderud A., Mikalsen B., Kristensen T. & Jakobsen K. S. (2008). The mosaic structure of the *mcyABC* operon in *Microcystis*. *Microbiology* 154: 1886-1899.

Ueno Y., Nagata S., Tsutsumi T., Hasegawa A., Watanabe M. F., Park H.-D., Chen G.-C., Chen G. & Yu S.-Z. (1996). Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in

Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* 17: 1317-1321.
Urbach E., Vergin K., Young L. & Morse A. (2001). Unusual bacterioplankton community structure in ultra-oligotrophic Crater Lake. *Limnology and Oceanography* 46: 557-572.
Valdor R. & Aboal M. (2007). Effects of living cyanobacteria, cyanobacterial extracts and pure microcystins on growth and ultrastructure of microalgae and bacteria. *Toxicon* 49: 769-779.
Valério E., Faria N., Paulino S. & Pereira P. (2008). Seasonal variation of phytoplankton and cyanobacteria composition and associated microcystins in six Portuguese freshwater reservoirs. *Annales de Limnologie* 44: 189-196.
Valério E., Pereira P., Saker M. L., Franca S. & Tenreiro R. (2005). Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. *Harmful Algae* 4: 1044-1052.
van Apeldoorn M. E., van Egmond H. P., Speijers G. J. A. & Bakker G. J. I. (2007). Toxins of cyanobacteria. *Molecular Nutrition & Food Research* 51: 7-60.
Van Der Gucht K., Cottenie K., Muylaert K., Vloemans N., Cousin S., Declerck S., Jeppesen E., Conde-Porcuna J. M., Schwenk K., Zwart G., Degans H., Vyverman W. & De Meester L. (2007). The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20404-20409.
Van der Gucht K., Sabbe K., De Meester L., Vloemans N., Zwart G., Gillis M. & Vyverman W. (2001). Contrasting bacterioplankton community composition and seasonal dynamics in two neighbouring hypertrophic freshwater lakes. *Environmental Microbiology* 3: 680-690.
Van Der Gucht K., Vandekerckhove T., Vloemans N., Cousin S., Muylaert K., Sabbe K., Gillis M., Declerk S., De Meester L. & Vyverman W. (2005). Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *FEMS Microbiology Ecology* 53: 205-220.
Vasconcelos V. (2001). Freshwater cyanobacteria and their toxins in Portugal. In: I. Chorus, (ed.), Cyanotoxins - Occurrence, Causes, Consequences. Springer, Berlin: 62-67.
Vasconcelos V., Oliveira S. & Teles F. O. (2001). Impact of a toxic and a non-toxic strain of *Microcystis aeruginosa* on the crayfish Procambarus clarkii. *Toxicon* 39: 1461-1470.
Vasconcelos V. M. (1999). Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research* 32: 249-254.
Vasconcelos V. M., Campos T., Amorim A. & Soares A. M. V. M. (1993a). Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. *Boletim UCA, Universidade do Algarve, UCTRA* 1: 193-201.
Vasconcelos V. M., Evans W. R., Carmichael W. W. & Namikoshi M. (1993b). Isolation of microcystin-LR from a Microcystis (cyanobacteria) waterbloom collected in the drinking water reservoir for Porto, Portugal. *Journal of Environmental Science and Health - Part A Environmental Science and Engineering* 28: 2081-2094.
Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1995). Isolation and characterization of microcystins (heptapeptide hepatotoxins) from Portuguese strains of *Microcystis aeruginosa* Kütz. emend Elekin. *Arch Hydrobiol* 134: 295-305.
Vasconcelos V. M., Sivonen K., Evans W. R., Carmichael W. W. & Namikoshi M. (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 30: 2377-2384.
Wei C., Bao S., Zhu X. & Huang X. (2008). Spatio-temporal variations of the bacterioplankton community composition in Chaohu Lake, China. *Progress in Natural Science* 18: 1115-1122.
WHO (1998). Cyanobacterial toxins: microcystin-LR., Guidelines for drinking water quality, Addendum to Vol. 2, Health criteria and other supporting information. World Health Organization, Geneva, Switzerland: 95-110.
WHO (2003). Algae and cyanobacteria in fresh water. Guidelines for safe recreational water environments. Vol. 1: Coastal and fresh waters. World Health Organization, Geneva, Switzerland: 136-158.
Wiedner C., Rücker J., Brüggemann R. & Nixdorf B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473-484.
Wiegand C. & Pflugmacher S. (2005). Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicology and Applied Pharmacology* 203: 201-218.
Willame R., Boutte C., Grubisic S., Wilmotte A., Komárek J. & Hoffmann L. (2006). Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. *Journal of Phycology* 42: 1312-1332.

Wilson A. E., Sarnelle O., Neilan B. A., Salmon T. P., Gehringer M. M. & Hay M. E. (2005). Genetic Variation of the Bloom-Forming Cyanobacterium *Microcystis aeruginosa* within and among Lakes: Implications for Harmful Algal Blooms. *Applied and Environmental Microbiology* 71: 6126-6133.

Wood S. A., Rasmussen J. P., Holland P. T., Campbell R. & Crowe A. L. M. (2007). First report of the cyanotoxin anatoxin-a from *Aphanizomenon issatschenkoi* (cyanobacteria). *Journal of Phycology* 43: 356-365.

Wu Q. L., Zwart G., Schauer M., Kamst-van Agterveld M. P. & Hahn M. W. (2006). Bacterioplankton Community Composition along a Salinity Gradient of Sixteen High-Mountain Lakes Located on the Tibetan Plateau, China. *Appl. Environ. Microbiol.* 72: 5478-5485.

Wu Q. L., Zwart G., Wu J., Kamst-Van Agterveld M. P., Liu S. & Hahn M. W. (2007a). Submersed macrophytes play a key role in structuring bacterioplankton community composition in the large, shallow, subtropical Taihu Lake, China. *Environmental Microbiology* 9: 2765-2774.

Wu X., Xi W., Ye W. & Yang H. (2007b). Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. *FEMS Microbiology Ecology* 61: 85-96.

Xi W., Wu X., Ye W. & Yang H. (2007). Changes in bacterial community structure during preceding and degraded period of cyanobacterial bloom in a bay of the Taihu Lake. *Chinese Journal of Applied and Environmental Biology* 13: 97-103.

Xing P. & Kong F. (2007). Intra-habitat heterogeneity of environmental factors regulating bacterioplankton community composition in Lake Taihu, China. *Aquatic Microbial Ecology* 48: 113-122.

Xing P., Kong F., Cao H., Zhang M. & Tan X. (2007). Variations of bacterioplankton community composition during *Microcystis* spp. blooms in a shallow eutrophic lake. *Journal of Freshwater Ecology* 22: 61-67.

Yamamoto Y., Nizuma S., Kuroda N. & Sakamoto M. (1993). Occurrence of hetrotrophic bacteria causing lysis of cyanobacteria in a eutrophic lake. *Japanese Journal of Phycology* 41: 215-220.

Yannarell A. C. & Triplett E. W. (2005). Geographic and Environmental Sources of Variation in Lake Bacterial Community Composition. *Appl. Environ. Microbiol.* 71: 227-239.

Ye C., Xu Q., Kong H., Shen Z. & Yan C. (2007). Eutrophication conditions and ecological status in typical bays of Lake Taihu in China. *Environmental Monitoring and Assessment* 135: 217-225.

Yoshida M., Yoshida T., Satomi M., Takashima Y., Hosoda N. & Hiroishi S. (2008). Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. *Journal of Applied Microbiology* 105: 407-415.

Yoshida M., Yoshida T., Takashima Y., Kondo R. & Hiroishi S. (2005). Genetic diversity of the toxic cyanobacterium *Microcystis* in Lake Mikata. *Environmental Toxicology* 20: 229-234.

Yu X., Nipper J. & Yan N. (2007). Recommendations of eutrophication control of Taihu Lake from an international view. *Acta Geographica Sinica* 62: 899-906.

Zaitlin B. & Watson S. B. (2006). Actinomycetes in relation to taste and odour in drinking water: Myths, tenets and truths. *Water Research* 40: 1741-1753.

Zapomělová E. (2006). Current taxonomic issues with planktonic representatives of the genus *Anabaena* (Cyanobacteria) with special reference to their morphological features; literary review. *Czech Phycology* 6: 33-47.

Zapomelová E., Jezberová J., Hrouzek P., Hisem D., Reháková K. & Komárková J. (2009). Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (Cyanobacteria) and their reclassification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana*). *Journal of Phycology* 45: 1363-1373.

Zehr J. P. & McReynolds L. A. (1989). Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Applied and Environmental Microbiology* 55: 2522-2526.

Zeng J., Yang L., Du H., Xiao L., Jiang L., Wu J. & Wang X. (2009). Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World Journal of Microbiology and Biotechnology* 25: 763-772.

Zhang C. C., Laurent S., Sakr S., Peng L. & Bédu S. (2006). Heterocyst differentiation and pattern formation in cyanobacteria: A chorus of signals. *Molecular Microbiology* 59: 367-375.

Zhou L., Yu H. & Chen K. (2002). Relationship between microcystin in drinking water and colorectal cancer. *Biomedical and Environmental Sciences* 15: 166-171.

Zwart G., Crump B. C., Kamst-van Agterveld M. P., Hagen F. & Han S. K. (2002). Typical freshwater bacteria: An analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* 28: 141-155.