Universidade de Aveiro Departamento de Biologia
2007

VÂNIA SOFIA DE SOUSA BESSA

DINÂMICA DE BACTERIOPLÂNCTON DURANTE A OCORRÊNCIA DE BLOOM DE FITOPLÂNCTON

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia, realizada sob a orientação científica do Doutor Mário Jorge Pereira, Professor Auxiliar, e da Doutora Maria Ângela Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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agradecimentos

Esta é uma altura privilegiada para demonstrar o meu reconhecimento a todos aqueles que contribuíram para a realização deste trabalho.

Ao Doutor Mário Jorge Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, orientador desta tese, pelos conhecimentos científicos transmitidos, pelo sentido critico, rigor cientifico, pelas apreciações pertinentes, pelas sugestões valiosas, incentivo e amizade demonstrados ao longo de todo o trabalho.

À Doutora Maria Ângela Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, co-orientadora desta tese, pelo acompanhamento critico e activo do desenvolvimento dos trabalhos, pelos conselhos pertinentes e valiosos, rigor cientifico, incentivo e amizade demonstrados ao longo de todo o trabalho.

À Licenciada Luísa Santos, pelo apoio laboratorial demonstrado diariamente e ensinamentos de técnicas úteis no desenvolvimento do trabalho experimental.

Às Licenciadas Ana Rita Lopes e Catarina Pereira, por estarem sempre presentes quando necessitei de apoio e pelo bom ambiente que sempre envolveu o nosso grupo de trabalho.

Aos meus pais, por sempre me incentivarem e motivarem a evoluir, por me incutirem desde sempre o espírito do trabalho por objectivos, por compreenderem o meu reduzido tempo para a família e pelos mimos sempre disponíveis.

Aos meus amigos de quatro patas, por me proporcionarem bons momentos de brincadeira e carinhos.

Ao Ricardo Resendes, pelo carinho e paciência que demonstrou a longo destes dois anos, por estar sempre presente quando precisava de desabafar e pelos bons momentos de distracção proporcionados.

A eles, e aos muitos amigos que acompanharam o desenvolvimento deste trabalho, só não posso prometer que a vossa paciência não voltará a ser testada…

palavras-chave *Eutrofização, Bacterioplâncton, Cyanoprokariota, virus, dinâmica de populações, lagos pouco prufundos*

resumo

O presente trabalho pretendeu avaliar a estrutura das comunidades virais, bacterianas e fitoplanctónicas durante um período de crescente temperatura (Primavera), num lago pouco profundo (Lagoa da Vela – Portugal).
Numa primeira fase, foi avaliada a dinâmica plantónica bem como a importância da população viral sobre a comunidade fitoplanctónica. Foi possível verificar que a comunidade fitoplanctónica foi dominada por taxa da Divisão Cyanobacteria e Chlorophyta assim como a existência de uma correlação positiva entre a abundância viral e a do bacterioplancton.
Numa segunda fase, foi avaliada a composição, diversidade e abundância da comunidade de bacterioplâncton. Esta comunidade procariota mostrou-se maioritariamente constituída pelo Domínio *Bacteria*, estando as *Archaea* representadas em todas as amostras. Dentro do primeiro domínio, os filos *Cytophaga* e *Proteobacteria* estiveram bem representados. As primeiras apresentaram elevadas abundâncias durante a fluorescência de fitoplâncton, o mesmo acontecendo a sub-grupos do *Proteobacteria*. A análise de regressão revelou que a variabilidade dos distintos grupos procariotas foi regulada por factores químicos, interacções biológicas e por regulação dupla de factores bióticos e abióticos.

keywords *Eutrophication, Bacterioplankton, Virus, CyanoproKariota, population dynamics, shallow lake*

abstract

The present study aimed to assess the structure of viral, bacterial and phytoplankton communities during a period of increasing temperature (spring), in a shallow lake (Lagoa da Vela – Portugal).

On a first phase, the plankton dynamics were assessed, as well as the impact of the viral population in the phytoplankton community. It was observed that the phytoplankton community was dominated by species of Cyanobacteria as well as a positive correlation between viral and bacterioplankton abundance was also found.

Secondly, the bacterioplancton community composition, diversity and abundance were evaluated. This community was dominated by the *Bacteria* domain, being *Archaea* present in all samples. Within the Bacteria domain, the phylum *Cytophaga* and *Proteobacteria* were well represented. The first ones had high abundance during the phytoplankton bloom, as would the Proteobacteria sub-groups. The regression analysis showed that the variability of different prokaryote groups was governed by chemical, biological interactions and dual regulation of biotic and abiotic factors.

ÍNDICE

Capítulo I	Introdução geral	06
	1. Lagos profundos *Vs* lagos pouco profundos	07
	2. Interacções tróficas como mecanismos reguladores	07
	3. Fitoplancton	09
	4. Bacterioplancton	10
	4.1. Papel ecológico do bacterioplancton	10
	4.2. Diversidade e abundância do bacterioplancton	11
	4.3. Factores que afectam o crescimento do bacterioplancton	11
	4.3.1. Disponibilidade de nutrientes organicos e inorganicos	12
	4.3.2. Propriedades da água	13
	4.3.3. Mortalidade através de infecção e predação	14
	5. Análise por "Fluorescence In Situ Hybridization"	15
	6. Objectivos e estrutura da tese	16
	7. Referências	17
Capítulo II	Water column environment of a eutrophic shallow temperate lake in late spring: pelagic communities of phytoplankton, cyanobacteria and associated heterotrophic bacteria and viruses	29
Capítulo III	Bacteripoplankton dynamics during the development of a cyanobacteria bloom in a temperate shallow lake	56
Capítulo IV	Discussão geral	87
	Referências	92

Capítulo I

Introdução geral

1. Lagos profundos *Vs* lagos pouco profundos

Os lagos oligotróficos, normalmente denominados lagos profundos, são caracterizados por uma zona hipolímnica, pouco iluminada ou mesmo inteiramente escura, pobre em fitoplâncton, cuja temperatura varia pouco durante o ano, sendo a produtividade muito reduzida. Em contraste, os lagos eutróficos (ou pouco profundos) são caracterizados por temperaturas mais elevadas e elevada produtividade.

No sul da Europa, os lagos pouco profundos (influenciados pelo clima Mediterrâneo) estão sujeitos a efeitos *top-down* (Blanco *et al.*, 2003; Gyllstrom *et al.*, 2005), pelo que se encontram mais vulneráveis a processos de eutrofização. Embora estes habitats suportem uma considerável biodiversidade, o seu valor está normalmente comprometido devido ao enriquecimento em nutrientes (estimulado pelas actividades humanas). Um bom exemplo desta problemática é a Lagoa da Vela, local escolhido como objecto do presente estudo. Este pequeno lago natural (resultante da acumulação de água numa depressão superficial) e permanente, outrora transparente e com grande variedade de peixes, encontra-se actualmente altamente turbido, sob forte carga de nutrientes (Abrantes *et al.*, 2006) e presença recorrente de cianobactérias, muitas das quais tóxicas (de Figueiredo *et al.*, 2006). Este sistema é utilizado para fins recreacionais, bem como para actividades agrícolas. Como o solo circundante é arenoso, o lixiviamento de nutrientes e tóxicos é potenciado.

2. Interacções tróficas como mecanismos reguladores

A regulação dos sistemas aquáticos estabelece uma interacção entre as condicionantes externas (factores abióticos) e os processos internos (interacções bióticas). Os mecanismos de regulação *top-down* (do topo da cadeia trófica para a sua base) inferem que os predadores podem ter efeitos determinantes nos níveis tróficos inferiores (Brooks & Dodson, 1965; Hall *et al.*, 1976), em oposição aos mecanismos de regulação *bottom-up* que resultam da competição pela disponibilidade de nutrientes ou alimento. Da relação entre estes dois mecanismos resulta o equilíbrio ecológico da cadeia trófica de um lago (Brönmark & Hansson , 1998).

Paine (1980) denominou de cadeia trófica ao conjunto de efeitos recíprocos do predador sobre a presa alterando a sua abundância, biomassa ou produtividade de uma

população, comunidade ou nível trófico, afectando mais do que um elo da teia trófica. Os microorganismos são membros activos nesta complexa rede. Como defende Sherr & Sherr (1988), o *loop* microbiano (Figura 1) deve ser considerado como um componente integral de uma grande rede alimentar, que inclua todos os organismos unicelulares pro e eucariotas, tanto auto como heterotróficos, estando a cargo da cadeia alimentar microbiana suportar a cadeia alimentar dos metazoários. Por outro lado, a presença de populações virais numa comunidade aquática pode afectar a sua composição, comportamento e estrutura (Chattopadhyay *et al.,* 2003). Vários estudos (Bergh *et al.,* 1989; Suttle *et al.*, 1990; Tarutani *et al.,* 2000; Wommack & Colwell, 2000) têm demonstrado a presença de vírus patogénicos nas comunidades de fitoplâncton tendo sido descritas para muitas algas eucariotas (van Etten *et al*., 1991; Reisser, 1993), cianobactérias (Suttle & Chan, 1993) e comunidades naturais de fitoplâncton (Peduzzi & Weinbauer, 1993). No entanto, apesar do crescente número de trabalhos, o papel das infecções virais na população de fitoplâncton continua longe de estar completamente entendido.

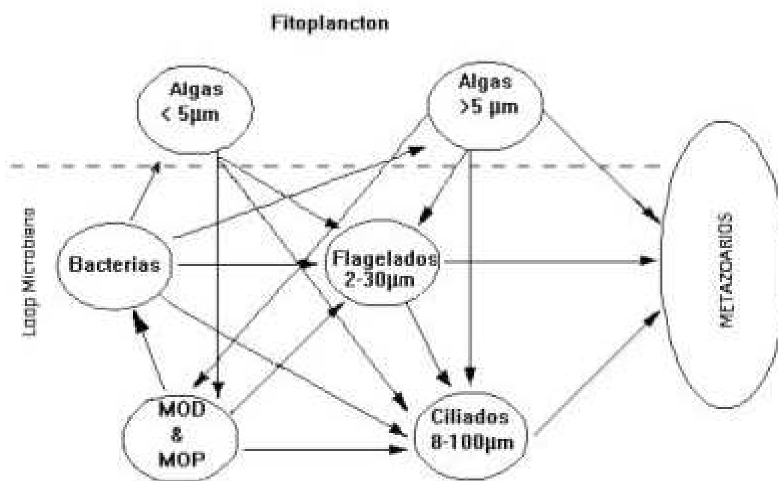

Figura 1: Esquema simplificado do *loop* microbiano. MOD - Matéria Organica Dissolvida; MOP - Matéria Orgânica Particulada. (Adaptado de Madigan *et al*., 2000)

3. Fitoplâncton

O plâncton é a base de toda a cadeia alimentar aquática, ocupando o fitoplâncton o primeiro nível trófico (produtores) (Chattopadhyay *et al.,* 2003). Tal como as plantas terrestres, o fitoplâncton necessita de luz solar, água e nutrientes para a fotossíntese. Através da conversão da energia solar em energia química, via fotossíntese, produzem a energia necessária para toda a cadeia alimentar aquática (Moss, 1998). Uma característica comum das populações de fitoplâncton, dadas as condições ecológicas apropriadas (elevados níveis de nutrientes, calor e sol), é a sua capacidade de rápido crescimento conduzindo a elevadas densidades celulares, ou *blooms*, de uma ou mais espécies (Solé *et al.*, 2006). Normalmente, esta situação é benéfica para a aquacultura e actividade piscatória. No entanto, a proliferação de algumas espécies pode ser responsável pela ocorrência de efeitos nocivos (quer por toxicidade directa ou por outros mecanismos), tanto na cadeia alimentar, na saúde pública como em actividades socio-económicas (Solé *et al.*, 2006). Este tipo de florescimento é denominado por HABs (*Harmful Algal Blooms*) (Smayda, 1997). O desenvolvimento de elevadas concentrações celulares está dependente de variáveis físicas (ex. elevados tempos de residência), químicas (ex. disponibilidade de nutrientes) e biológicas (ex. baixa predação) (Solé *et al.*, 2006). HABs podem ser causados por uma grande variedade de grupos de algas, mas são normalmente os dinoflagelados os maiores causadores. A propriedade chave que favorece a acumulação de elevadas biomassas destes organismos é a sua mobilidade, que lhes permite migrar e interagir com padrões da circulação da água (Smayda, 1997). Uma característica particular de algumas espécies presentes de HAB é a sua capacidade de produzir toxinas que afectam outros organismos (Smayda, 1997).

4. Bacterioplâncton

Papel ecológico do bacterioplâncton

As bactérias são os componentes biológicos mais importantes e abundantes envolvidos na renovação (transformação e remineralização) da matéria organica em sistemas aquáticos (Pomeroy, 1991). As bactérias heterotróficas estão particularmente especializadas na transformação de matéria orgânica. Elas hidrolisam matéria organica dissolvida e particulada, podem utilizar substratos de difícil de degradação e utilizar diferentes compostos alóctones como fonte de carbono organico. Elas convertem carbono organico dissolvido, que iria ser, inevitavelmente, perdido para outros membros da cadeia alimentar, em carbono particulado que assim se torna potencialmente disponível para níveis tróficos superiores (*loop* microbiano) (Sherr & Sherr, 1988). Esta capacidade de recuperar o carbono organico dissolvido é significativa, uma vez que representa uma ligação entre as fontes em diversos níveis e os consumidores superiores (Azam & Hodson, 1977; Azam, 1998). Por outro lado, estudos em vários ambientes aquáticos indicam que o *loop* microbiano (fitoplâncton → DOC → bactérias → protozoários → metazoários) (Figura 1) pode processar aproximadamente tanta energia como a cadeia alimentar clássica (fitoplâncton → animais herbívoros → animais carnívoros) (Riemann & Sondergaard, 1986).

Através da remineralização da matéria organica, as bactérias regeneram nutrientes *in situ*, que são depois utilizados pelos produtores primários. As bactérias heterotróficas convertem carbono organico em inorganico a elevadas taxas, tornando a respiração bacteriana a maior fracção da respiração total medida na maioria dos sistemas aquáticos (Williams, 1981). Cole e colaboradores (1988) estimaram, numa vasta extensão de condições tróficas, que a respiração bacteriana é, no mínimo, tão grande como a do zooplâncton, atingindo valores até 40% da respiração planctónica total.

Compreender o carácter dual da transformação e remineralização das bactérias planctónicas em sistemas aquáticos é um paradigma central da ecologia microbiana contemporanea (del Giorgio & Cole, 1998).

Diversidade e abundância do bacterioplankton

Desde 1970 tem-se tornado cada vez mais evidente que o bacterioplâncton contribui significativamente para a biomassa planctónica (del Giorgio & Cole, 1998). As propriedades físicas e químicas da coluna de água, assim como a abundância e produtividade da comunidade bacteriana variam nos sistemas aquáticos de acordo com a amplitude de temperaturas (di Servi *et al*., 1995) e profundidade da coluna de água (Pace & Cole, 1994; Talbot *et al*., 1997). As bactérias são os componentes planctónicos menos variáveis em termos de densidade total e biomassa, contribuindo cerca de 20% para a biomassa planctónica total (Williams, 1984).

A estrutura das comunidades de fito e zooplanctónica têm sido vastamente analisadas. No entanto, o mesmo não se verifica com as estruturas das comunidades de bacterioplâncton e a sua dinâmica espaço-temporal (Hobbie, 1988). Esta falha no conhecimento ecológico existe porque as bactérias não podem ser morfologicamente identificadas e porque uma taxonomia geralmente aceite não existia ate muito recentemente (Höfle *et al*., 1999). O aparecimento, na última década, dos métodos moleculares para a taxonomia de bactérias, tornou possível uma taxonomia filogenética valida para bactérias que permite a análise das estruturas taxonómicas das comunidades microbianas naturais (Olsen *et al*., 1986; Pace *et al*., 1986).

Factores que afectam o crescimento do bacterioplâncton

Uma vez que, nos sistemas aquáticos, uma elevada fracção de carbono flúi através das bactérias, o conhecimento dos factores que controlam a produção de bacterioplâncton torna-se relevante no conhecimento do funcionamento dos ciclos biogeoquímicos e, particularmente, na previsão da sua evolução após perturbação.

Os factores ambientais interagem na regulação da actividade de bacterioplâncton de tal forma que, frequentemente, se torna complicado fazer a descriminação entre efeitos individuais e avaliar a sua contribuição relativa (Pomeroy & Wiebe, 2001).

Os principais factores que regulam o crescimento bacteriano incluem diferentes variáveis, nomeadamente: nutricionais (disponibilidade de nutrientes organicos e

inorganicos), físicos (temperatura), químicos (salinidade) e biológicos (predação e lise viral).

4.3.1 Disponibilidade de nutrientes orgânicos e inorgânicos

O fornecimento de substratos há muito é reconhecido como um factor dominante na regulação da abundância e produção do bacterioplâncton em sistemas aquáticos (Shiah *et al.*, 1999). No entanto, vários estudos têm sugerido que as forças de controlo *bottom-up* podem ser dependentes do sistema e também podem variar dentro do sistema (Wiebe & Pomeroy, 1992; Shiah & Ducklow, 1995; Shiah *et al.*, 1999).

A forte correlação positiva, observada em estudos laboratoriais e de campo, entre a biomassa de fitoplâncton e bacterioplâncton e a produção bacteriana sugerem que o fitoplâncton pode ser uma força autóctone importante de substratos de crescimento para bactérias (Williams, 1990; White *et al.*, 1991; Panzenbock *et al.*, 2000). O fitoplâncton pode, directamente, fornecer matéria organica às bactérias, através da exsudação de células saudáveis e lise de células em senescencia ou mortas (Vadstein *et al.*, 1993; Panzenbock *et al.*, 2000). Fornecimentos indirectos ocorrem através de lise viral ou predação por zooplâncton herbívoro (Peduzzi & Herndl, 1992; Strom *et al.*, 1997; Bratbak *et al.*, 1998; Hasegawa *et al.*, 2000).

As bactérias respondem a alterações na disponibilidade de matéria organica por alternância entre níveis de actividade. Como tendência geral, aumentos nas taxas de produção de biomassa bacteriana, actividade ectoenzimática e taxas de *uptake*, têm sido amplamente observadas durante *blooms* de fitoplâncton (Chróst *et al.*, 1989; Chróst, 1991; Middelboe *et al.*, 1995), na proximidade de fontes de POM (Tholosan *et al.*, 1999; Grossart & Ploug, 2001) ou ao longo de gradientes crescentes de eutrofização (Hoppe *et al.*, 1998).

Adições experimentais de matéria organica a comunidades de bacterioplâncton, demonstram que as respostas não estão somente relacionadas com a concentração do carbono organico disponível, mas também com a qualidade dos substratos. Descobriu-se que os detritos terrestres são relativamente refractários, enquanto que materiais derivados de algas estimulam significativamente o crescimento (Wehr *et al.*, 1999). Os substratos

dissolvidos são mais estimulantes do que a matéria particulada de idêntica fonte (Ferrier-Pagès *et al*., 2000).

A concentração de substratos inorganicos é também um factor de controlo da produção bacteriana (Torréton *et al*., 2000; Ferrier-Pagès & Furla, 2001). No entanto, ainda não está completamente esclarecido até que extensão os substratos organicos e inorganicos podem limitar a produção de bacterioplâncton (Toolan *et al*., 1991). Enquanto que se tem pensado que a disponibilidade do carbono organico é um factor chave na limitação do crescimento bacteriano, os elevados requisitos de nutrientes inorganicos (Vadstein *et al*., 1988) e a elevada percentagem de *uptake* detectável para as bactérias (Currie & Kalff, 1984) sugerem que o fornecimento de nutrientes inorganicos também podem limitar a abundância e produção bacteriana. De facto, vários estudos têm demonstrado que o crescimento bacteriano aumenta com o aumento da disponibilidade de nutriente inorganicos (Wikner *et al*., 1999; Torréton *et al*., 2000). Por outro lado, tem sido demonstrado que as bactérias heterotróficas competem, com sucesso, por nutrientes inorganicos com o fitoplâncton (Thingstad *et al*., 1993). Blackburn *et al*., (1998) demonstrou que as bactérias têm, aproximadamente, potenciais de *uptake* de nutrientes 100 vezes mais rápidos que o fitoplâncton. A baixas concentrações de nutrientes inorganicos, a vantagem competitiva do bacterioplâncton sobre o fitoplâncton, é uma ideia consensual (Torréton *et al*., 2000).

4.3.2 Propriedades da água

O crescimento de bactérias em sistemas aquáticos é afectado pela temperatura *in situ*, como foi concluído pelas correlações positivas geralmente encontradas entre produção bacteriana e temperatura (Shiah & Ducklow, 1997). Tem sido demonstrado que as diferentes temperaturas óptimas de crescimento bacteriano reflectem a amplitude de temperaturas no local (Simon & Wunsch, 1998). Alguns estudos têm demonstrado que a variação nas taxas de actividade bacteriana em ecossistemas eutróficos podem ser primeiramente regulados pela temperatura, passando a disponibilidade de substratos para um papel secundário, durante as estações frias (Shiah & Ducklow, 1995). Um efeito indirecto importante da temperatura é que afecta a afinidade dos sistemas enzimáticos. A baixas temperaturas, a afinidade dos sistemas enzimáticos diminui significativamente,

possibilitando a acumulação de substratos lábeis no ambiente uma vez que se tornam menos biodisponiveis (Pomeroy & Wiebe, 2001).

4.3.3 Mortalidade através de infecção e predação

A descoberta de que a abundância de vírus livres pode exceder em 1 a 2 ordens de magnitude o número de bactérias platónicas (Maranger & Bird, 1995; Weinbauer & Peduzzi, 1995a), motivou a pesquisa do impacto das infecções virais nos microorganismos aquáticos. Vários estudos, numa ampla variedade de ecossistemas aquáticos, têm indicado que os bacteriófagos podem ser importantes no controlo do crescimento bacteriano (Maranger & Bird, 1995; Weinbauer & Peduzzi, 1995b). Tem sido demonstrado que até 30% das bactérias planctónicas estão infectadas por vírus líticos (Fuhrman & Suttle, 1993; Suttle, 1994). Estimativas da mortalidade bacteriana devido a lise viral, indicam que os fagos podem ser responsáveis por 1 a 100% da mortalidade bacteriana observada (Weinbauer & Peduzzi, 1995b; Suttle, 1994), dependendo das condições ambientais e da estrutura da comunidade hospedeira (Wommack & Colwell, 2000).

Os vírus também podem afectar a estrutura das comunidades de bactérias uma vez que são específicos para um hospedeiro (Wommack *et al.*, 1999). Por outro lado, podem influenciar a diversidade bacteriana ao nível genético, dado que podem mediar trocas genéticas através da transducção (Miller & Sayler, 1992; Paul, 1999).

A predação tem sido reportada como um dos maiores factores de mortalidade das comunidades bacterianas (Weisse & Muller, 1998). Os nanoflagelados heterotróficos e os ciliados são os predadores mais importantes tanto em sistemas marinhos (Wikner *et al.*, 1990) como nos de água doce (Sanders *et al.*, 1989). Vários estudos têm demonstrado que a predação por protistas influencia a distribuição celular (Hahn & Hofle, 1998) e a estrutura taxonómica (Hahn & Hofle, 1998; Suzuki, 1999) das comunidades de bactérias.

5. Análise por Fluorescence In Situ Hybridization

Até recentemente, os microbiólogos que estudavam bactérias aquáticas enfrentavam um dilema: podiam ou medir a abundância, a biomassa, a taxa de crescimento, a actividade, da "média" bacteriana em condições *in situ* (Fuhrman *et al.,* 1989; Pernthaler *et al*., 1998), ignorando a diversidade filogenética e fisiológica das comunidades microbianas, ou podiam isolar e caracterizar fisiologicamente estirpes bacterianas individuais (Shut *et al.,* 1993; Pernthaler *et al*., 1998), mas depois não eram capazes de dizer se estes microorganismos também eram comuns no ambiente. Por conseguinte, poucos conhecimentos foram gerados sobre as variações espaciais e temporais de abundância de grupos filogenéticos definidos e espécies bacterianas individuais em habitats naturais.

Técnicas de biologia molecular utilizadas para identificar microorganismos em amostras ambientais têm, recentemente, fornecido novas ferramentas para estudar a biodiversidade do bacterioplâncton (Acinas *et al*., 1997; DeLong e*t al.,* 1993; Fuhrman *et al.,* 1988; Giovannoni *et al*., 1995; Hiorns *et al.,* 1997; Pernthaler *et al*., 1998) e as abundâncias de bactérias e Archaea *in situ* que não podiam ser, anteriormente, adequadamente distinguidas (Alfreider *et al.,* 1996; Amann *et al*., 1990; Amann *et al*., 1995; Murray *et al*., 1998; Pernthaler *et al*., 1998). Agora, os microbiólogos encontram-se em posição de esclarecer a biogeografia (Pernthaler *et al*., 1998), a dinâmica populacional, e as sucessões (Pinhassi & Hagström, 1997; Pernthaler *et al*., 1998) não de apenas alguns microorganisms morfologicamente distintos, mas de um grande número de espécies. Fluorescence in situ hybridization (FISH), com sondas oligonucleotídicas dirigidas selectivamente ao rRNA, visualiza células bacterianas com afiliações definidas filogeneticamente (Amann *et al.,* 1995; Amann *et al*., 1997; Pernthaler *et al*., 1998).

Ao contrário de outras abordagens de identificação, o FISH conserva grande parte da forma dos microorganismos alvo, i.e., a sua morfologia, o tamanho celular (Ramsing *et al.*, 1996; Pernthaler *et al*., 1997; Pernthaler *et al*., 1998) e o conteúdo celular em rRNA (Boyle *et al*., 1995; Poulsen *et al.,* 1993; Pernthaler *et al*., 1998). Portanto, apesar das limitações do método (como discutido por Amann *et al*., 1995), o seu potencial para a identificação e análise microscópica de microorganismos planctónicos estão agora reconhecidos.

6. Objectivos e estrutura da tese

Devido às características inerentes aos lagos pouco profundos, este trabalho pretendeu estudar as interacções tróficas na Lagoa da Vela. Desta forma, os objectivos primordiais do presente estudo foram:

- Avaliar a dinâmica plantónica num lago Mediterrânico, em território Português;
- Avaliar a importância dos vírus na dinâmica bacteriana;
- Interpretar a variabilidade na composição da comunidade bacteriana, durante um *bloom* de fitoplâncton, num lago pouco profundo (Lagoa da Vela);
- Analisar os parâmetros químicos e biológicos que regulam a comunidade procariota, em diferentes fases do *bloom* de fitoplâncton, com especial ênfase nas interacções entre cianobactérias e outros grupos procariotas.

A presente dissertação assenta numa estrutura dividida em capítulos, que representam unidades com objectivos próprios, integrados no âmbito global do trabalho. Os capítulos 2 e 3 correspondem a um artigos a submeter para publicação. Por este motivo estão escritos na língua inglesa. No 1º Capítulo, é apresentada uma Introdução geral, na qual se abordam os principais aspectos analisados experimentalmente, à luz dos conhecimentos científicos actuais, onde são definidos os objectivos e o âmbito do presente trabalho. O Capítulo 2 (Water column environment of a eutrophic shallow temperate lake in late spring: pelagic communities of phytoplankton, cyanobacteria and associated heterotrophic bacteria and viruses) avalia a dinâmica plantónica do local em estudo, bem como a importância dos vírus na dinâmica bacteriana. A variabilidade na composição da comunidade bacteriana, durante um *bloom* de fitoplâncton, assim como os parâmetros químicos e organicos que regulam a comunidade procariota, em diferentes fases do *bloom* de fitoplâncton, com especial ênfase nas interacções entre cianobactérias e outros grupos procariotas são apresentados no Capítulo 3. A última secção da tese tenta discutir, de forma integrada e global, os resultados obtidos.

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Capítulo II

Water column environment of a eutrophic shallow temperate lake in late spring: pelagic communities of phytoplankton, cyanobacteria and associated heterotrophic bacteria and viruses

Water column environment of a eutrophic shallow temperate lake in spring: pelagic communities of phytoplankton, cyanobacteria and associated heterotrophic bacteria and viruses

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"This paper has not been submitted elsewhere in identical or similar form, nor will it be during the first three months after submission to Hydrobiologia."

Abstract: The high viral counts reported in aquatic systems suggest that viral infection may be an important factor controlling bacterial populations in natural waters. In order to test the importance of viruses on bacterial dynamics, we have taken advantage of the spring phytoplankton bloom, which occurs regularly in the shallow freshwater Vela Lake, causing large natural fluctuations in the density of pelagic viruses and bacteria. Viral particles were counted by epifluorescence microscopy after staining with SYBR gold. Total bacterial number was determined by epifluorescence microscopy after staining with acridine orange. Phytoplankton composition, viral and bacterial densities were determined every second day between April and June of 2006 in surface water samples (32 samples). The concentration of viruses through the spring bloom decrease from 2×10^{12} viruses L^{-1} after the Chlorophyta bloom to 5×10^{10} viruses L^{-1} during the peak of Cyanobacteria, with a mean of 2×10^{11} viruses L^{-1}. Bacterial numbers followed the same pattern of variation, decreasing during the bloom from 3.3×10^{11} cells L^{-1} to 1.1×10^{11} cells L^{-1} with a mean of 1.6×10^{11} cells L^{-1}. Although viruses counts were, on average, higher than bacterial counts, yielding mean virus-to-bacterium ratio of 1.4 (range 0.3 to 5.7), the virus-to-bacterium ratio was frequently low, showing values below 1 in 57 % of the cases. The same pattern of variation of viruses and bacteria during the phytoplankton bloom as well as the significant correlation between viruses and bacteria (p 0.05) suggest that a significant fraction of viruses are bacteriophages. However, the frequent low viruses-to-bacteria ratios seem to indicate that during a bloom a large fraction of planktonic viruses results from phytoplankton cells infection.

Keywords: shallow lake, eutrophication, phytoplankton dynamics, environmental parameters, virioplankton, bacterioplankton

Introduction

Many ecosystems that are impacted by nutrients are shallow, and in contrast to deep plankton-dominated ecosystems they are capable of supporting a variety of autotrophs. These autotrophs compete for nutrients, light, and space, and have other complex ecological interactions that may influence how the ecosystem as a whole responds to nutrient stress (Havens et al. 2001). Environmental conditions such as increased temperatures and pH, low turbulence and enhanced nutrient loading, stimulate the development of planktonic cyanobacteria in lakes, leading to the formation of blooms (de Figueiredo et al. 2004[a]) by the bloom-forming genera including *Anabaena*, *Aphanizomenon* and *Microcystis* (Simis et al. 2005; de Figueiredo et al. 2006). Cyanobacterial-microbial associations occur during both active growth and senescent phases of bloom cyanobacteria. These interactions may lead to parallel optimization of growth and bloom potentials among microbial epiphytes and cyanobacterial hosts acting as consortia, and may also reflect mutually beneficial physiological and ecological adaptations and exchange processes allowing cyanobacteria to periodically dominate planktonic communities (de Figueiredo et al. 2006). Microalgae are known to produce a variety of composts that are referred in the collective as secondary metabolites. This substances are diverse in there chemical structure and physiological function (Skulberg 2000), namely antibiotics (Piccardi et al. 2000) and antibacterial (Jaki et al. 1999; Østensvik et al. 1998), antiviral (Hayashi & Hayashi 1996; Mundt et al. 2001; Nowotny et al. 1997), antialgal (Kodani et al. 2002; Volk 2005), enzyme inhibitors (Doan et al. 2000; Kós et al. 1995; Matsuda et al. 1996; Mundt et al. 2001; Murakami et al. 1995; Sano et al. 2005) and stimulators of enzymes involved in the degradation of reactive aerobic species activity (peroxidase; glutathione S- transferase; catalase) (Mitrovic et al. 2004;

Pflugmacher et al. 1999). These substances produced by cyanobacteria, influence their aquatic environment reducing the number of virus, bacteria and other microorganims (Mundt et al. 2001).

There is a lack of recently published information concerning planktonic dynamics in Mediterranean lakes, especially in Portuguese territory where there is a strong climatic influence from the Atlantic Ocean. Lake Vela is a polymictic shallow lake exhibiting some characteristics typical of an advanced trophic state, namely, the permanently turbid water, the reduction in biodiversity, and the recurrent occurrence of Cyanobacteria blooms (Abrantes et al. 2006; de Figueiredo et al. 2006). For Vela Lake the works of Antunes et al. (2003), Abrantes et al. (2006) and de Figueiredo et al. (2006), establish that the phytoplankton assemblages are highly correlated with temperature and nutrient concentrations (particularly phosphorus); Chlorophytes dominate in early spring and cyanobacteria from early spring until the beginning of autumn (low nutrient levels, along with high temperatures).

New techniques of enumeration have shown that viruses are abundant in marine and freshwaters. As summarized by Wommack & Colwell (2000), the abundance of viruses ranged from $<10^4$ to $>10^8$ ml^{-1} in the studied aquatic systems. Recent estimates reveal ca 10^{10} viruses per litre which are far higher than previous estimates (Bergh et al. 1989; Maranger & Bird 1995; Fuhrman 1999; Wommack & Colwell 2000; Zhang 2002; Liu et al. 2006). They can regulate biomass production, species diversity, population dynamics and community structure in the aquatic microbial communities, influence carbon and nutrient recycling in food chain and water environment, and even have implications on the global climate (Bergh et al. 1989; Fuhrman 1999; Bratbak & Heldal 2000; Bettarel et al. 2004). The high viral counts reported in aquatic systems (Liu et al. 2006) suggest that viral infection may be an important factor controlling bacterial populations in natural

waters, and can match grazing by protists as a source of mortality for bacteria (Weinbauer et al. 2003).

Since the ecological significance of virioplankton in aquatic ecosystems must be realized by their interaction with the hosts (e.g. bacteria, cyanobacteria and algae), enumeration of viruses and their hosts in water samples is an important first step elucidating the virus-host interaction. In order to test the importance of viruses on bacterial dynamics, we have taken advantage of the spring phytoplankton bloom, which occurs regularly in the shallow freshwater Vela Lake.

Materials and Methods

Studied area and sampling

Vela Lake (44º58'N, 5º18'W) is a shallow eutrophied freshwater body (mean depth = 0.9 m, maximum depth = 2.4 m, with 70 ha of floodable surface area and a total volume of 70 $\times 10^4$ m^3) (Abrantes et al. 2006; de Figueiredo et al. 2006) located in Quiaios (Figueira da Foz, Central Portugal). The water volume is predominantly influenced by the variation of groundwater levels and rainfall, which makes the lake very susceptible to drought during summer months. This lake is classified as eutrophic since 1960 (Nauwerck 1960), showing high N and P levels typical of eutrophic systems (Antunes et al. 2003). The increase of phytoplankton biomass, with the presence of algal blooms, the depletion of submerged macrophytes, the increase in turbidity, and a subsequent reduction in biodiversity are some of the characteristics associated with the eutrophication process in Lake Vela (de Figueiredo et al. 2006). The organic matter and nutrient inputs come mainly from human activities (such as agriculture and modification of land) in surrounding areas. Antunes et al. (2003) has reported an increase of the nutrient levels during the last decade.

The study started at April 18th and ended at June 19th of 2006 (during the spring period). Samples were collected every two days (32 samples). Dissolved oxygen (O_2 mg.L^{-1} and O_2%), conductivity, water, air temperature and pH were determined *in situ* (WTW multiliner P4).

The samples were collected just bellow the water surface; a 1,5 L plastic bottle and a 1L glass bottle with Lugol (1% v/v) were used: the content of the first was filtered and used for the determination of chemical parameters (phosphates, nitrates and ammonia), organic mater (total and volatile solids) and chlorophyll *a* in the laboratory; and the second, was used for the phytoplankton analyses. A sterile analyses flask was also filled with water for bacterial analyses. Glutaraldehyfe-fixed samples for virus and bacteria enumeration were also collected. Sampling occurred always in the same location and during the morning period (10±2h).

The samples were maintained in the obscurity and processed after an hour, in the laboratory. The processing consisted in the sample filtration using Whatman GF/C filters, the filters used for the evaluation of organic material were previously dried at 103ºC until they obtain a constant weight (APHA 1992). After the processing, the filtered water was used for determination of chemical parameters and the filters were used for determination of chlorophyll *a* and organic matter (which were conserved a -20ºC).

Environmental parameters and chlorophyll a

Phosphate, nitrate and ammonia concentrations were determined spectrophotometrically (Genesys 6 Thermo spectronic): phosphate was assayed using the stannous chloride method, described in APHA (1992); nitrate was determined applying the sodium salicilate

method described in Rodier (1984) and ammonia was quantified as described by Hall & Lucas (1981).

Quantification of organic matter (total and volatile solids) was made as described in APHA (1992). Chlorophyll *a* was determined after maceration and extraction with acetone 90%, overnight at 4ºC, centrifugation at 3000rpm during 10min, as described by Lorenzen (1967).

Phytoplankton diversity and abundance

For the phytoplankton quantification, samples were fixed with (1% v/v) Lugol and the enumeration was done in agreement with Lund et al. (1958). During enumeration, were considered as unit: colony for colonial organisms; filament for filamentous organisms and cell for unicellular organisms.

The more important groups in this study were: Chlorophyta; Cryptophyta; Cyanoprokariota and Euglenophyta, because of their predominance (diatoms were rare, not only in terms of species number, but also in terms of abundance and were not identified and quantified). The identification of phytoplankton species, belonging to these groups, was made by observation through a light microscope using different references for: Chlorophyta (Chlorococcales) (Komárek & Fott 1983); Cryptophyta (Fott 1968); Cyanoprokaryota (Geitler 1932; Komárek & Anagnostidis 1989; Komárek & Anagnostidis 1999) and Euglenophyta (Huber-Pertolozi 1961).

Evaluation and characterization of bacterial dynamics

Total bacterial number was determined by epifluorescence microscopy after staining with acridine orange (Hobbie et al. 1977).

Heterotrophic aerobic bacteria quantification was done by pour plate using Tryptic Soy Agar (TSA) as culture medium, with 96h incubation at 25 ºC (P selecta, Hotcold - M);

isolation was done by streak-plate technique. The isolated strains have grown in 10mL TSB (Tryptic Soy Broth), overnight at 37ºC and with a rotation of 100rpm (Sanyo orbital incubation – orbisafe Tsnetwise). After this period, 6mL of the inoculum was transferred for a new recipient, at which was added 3mL of glycerol 45% (v/v). After this, 5 aliquots (1mL) of the mixture were transferred to a new sterile eppendorf and conserved at -80ºC (Sanyo ultra low temperature freezer, MDF 382AT) (for a primary characterization of the isolated bacteria gram stain, catalase and oxidase tests have been performed: they were Gram negative, catalase and oxydase positive and had mobility by polar flagella) (Rodier 1984).

Evaluation and characterization of viral dynamics

Viral particles were counted by epifluorescence microscopy after staining with SYBR gold.

Statistical analysis

A canonical correspondence analysis or CCA (Ter Braak 1995) was used to ascertain the relationships between phytoplankton, bacteria, virus and environmental variables along the study period. CCA extracts synthetic gradients from the biotic and environmental matrices and the explanatory variables are quantitatively represented by arrows in graphical biplots. The arrow direction indicates positive or negative correlations and their length is relative to the importance of the explanatory variable in the ordination. A Monte Carlo permutation test was used to assess the significance of the relationships between the biotic data and the explanatory variables.

Results

Environmental parameters

During the sampling period water temperature showed a minimum of 17.2 ºC and a maximum value of 27.5 ºC, with an average value of 22.5±2.4 ºC. Water conductivity ranged between 566 and 641 µS cm^{-1}, and an average value of 606±21 µS cm^{-1}. pH values ranged between 7.75 and 9.82 during the sampling period. Dissolved O_2 showed a minimum of 4.75 mg l^{-1} and 58.4% and a maximum of 13.7 mg l^{-1} and 173.3% (Table 1). The higher values obtained for pH and dissolved O_2 were measured in the same day (5th of June of 2006).

Chlorophyll *a* concentration ranged between 0.005 and 0.098 µg l^{-1} with an average value of 0.035±0.028 µg l^{-1}. Total solids ranged between 12.700 and 52.900 mg l^{-1} and volatile solids showed a minimum value of 12.700 and a maximum value of 47.900 mg l^{-1}; the average values were 28.624±12.453 mg l^{-1} and 27.384±11.220 mg. l^{-1}, respectively (Table 1).

NH_4^+ concentration ranged between 0.002 and 0.131 mgN l^{-1}. NO_3^- ranged between 0.004 and 0.017 mgN l^{-1}, with an average value of 0.008±0.003 mgN l^{-1}. Phosphate concentration (detection level: 0.003 mg l^{-1}) reached a maximum of 0.009 mg l^{-1}, during a very rainy period (≤ 0,003 mg l^{-1}) (Table 1).

Phytoplankton

The more representative *taxa* were Chlorophyta and Cyanoprokariota, while the Cryptophyta and Euglenophyta were less representative, i.e., had a lower frequency and number of effectives (Fig. 1).

It was observed that Euglenophyta had the lowest expression, during the studied period. However, between May 26th and June 19th of 2006, its expression was higher, showing the higher percentage in May 28th (1643 cells ml^{-1}), due to the species *Euglena viridis* (1559 cells ml^{-1}) and in 5 June (1348 cells ml^{-1}), due to the genus *Trachelomonas* (*T. nigra*; *T. stoweri*; *T. volvocina*) (337 cells ml^{-1}). Although Cryptophyta "division" had a lower expression than the Chlorophyta and Cyanobacteria, it was always represented during the sampling period; the exception occurred between May 26th and June 7th. The major abundance due to *Cryptomonas* sp. was observed during these days: April 20th (1067 cells ml^{-1}) and June 11th and 17th (1685 and 1541 cells ml^{-1}, respectively).

It is also visible that Chlorophyta division was more abundant during the initial period. Then there was a shift in the dominant division, leading to Cyanoprokariota dominance. This variation occurred as the water temperature, conductivity, pH and dissolved oxygen were getting higher.

During the sampling period, it was observed a great diversity in species and their abundance within Chlorophyta. The species that during this period contributed for this group abundance, belong to the following genera: *Phacotus* (*Phacotus lenticularis*) *Coelastrum*, (*Coelastrum pseudomicroporum* and *Coelastrum reticulatum*) *Pediastrum* (*Pediastrum boryanum*, *Pediastrum duplex*, *Pediastrum simplex* and *Pediastrum tetras*) *Scenedesmus* (*Scenedesmus acuminatus*, *Scenedesmus acutus* and *Scenedesmus oaauensis*) and *Tetraedron* (*Tetraedron caudatum* and *Tetraedron minimum*). The higher values observed between the April 18th and 24th of 2006 occurred due to a bloom of *Scenedesmus* spp., being the maximum observed in April 20th (27079 CU ml^{-1}). A new increase was observed in May 22nd (10322 CU ml^{-1}), with *Pediastrum* spp., *Tetraedron* spp. and a still higher number of *Scenedesmus* spp.. In June 3rd was observed a new peak (17522 CU ml^{-1}) due to a *Tetraedron* spp. bloom (8199 cells ml^{-1}). Species from the *Coelastrum* genera

have been always present during the studied period; its higher expression occurred between April 22nd and 28th of 2006 with a maximum in April 26th (506 CU ml^{-1}) and between the June 3rd and 13th of 2006, where the highest value was obtained in June 9th (337 CU ml^{-1}) (Fig. 1c). In figure 1 is possible to observe that, although Cyanobacteria division had always an elevated cellular concentration, it only became dominant after April 20th of 2006. This shift followed a decrease in total cellular concentration of Chlorophyta and an increase in cellular concentration of cyanobacteria species *Aphanizomenon flos-aquae*. During the sampling period, there were two cyanobacterial species that have been present since the beginning of the study (*Microcystis* cf. *pulveria* and *Chroococcus limneticcus*), being the responsible for the elevated cellular level observed. After the 19th sample (May 24th of 2006), the observed pattern for this group follows the bloom-forming filamentous cyanobacteria (*Aphanizomenon flos-aquae*), detectable between May 22nd and June 19th of 2006, with an exception in the following days June 11th and 13th. In this way the peaks observed in May 6th, 12th and 18th (23635 CU ml^{-1}, 25236 CU ml^{-1} and 24899 CU ml^{-1}, respectively) were in majority caused by the species *Microcystis* sp. and the peaks observed in June 1st and 9th (25489 CU ml^{-1} and 26753 CU ml^{-1}, respectively) were mainly caused by the filamentous species. The highest cellular concentration obtained for these *taxa* occurred in June 9th, being coincident with the day in which the highest cellular concentration for the filamentous species was observed (8342 CU ml^{-1}) (Fig. 1). The appearance of the bloom forming filamentous cyanobacteria *Aphanizomenon flos-aquae* occurred simultaneously with an increase in phosphate concentration in the water (May 30th to June 19th), after a very rainy period; and with a low level of nitrogen source compounds (NO_3^- and NH_4^+).

Bacteria, Virus and virus-to-bacterium ratio

The concentration of viruses through the spring bloom decrease from 2 x 10^{12} viruses L^{-1} after the Chlorophyta bloom to 5 x 10^{10} viruses L^{-1} during the peak of Cyanobacteria, with a mean of 2 x 10^{11} viruses L^{-1}. Bacterial numbers followed the same pattern of variation, decreasing during the bloom from 3.3 x 10^{11} cells L^{-1} to 1.1 x 10^{11} cells L^{-1} with a mean of 1.6 x 10^{11} cells L^{-1} (Fig. 2). Although viruses counts were, on average, higher than bacterial counts, yielding mean virus-to-bacterium ratio of 1.4 (range 0.3 to 5.7), the virus-to-bacterium ratio was frequently low, showing values below 1 in 57 % of the cases.

The canonical correspondence analysis

Results from CCA ordination of most abundant phytoplankton species, bacteria, virus and environmental variables (Fig. 3a) lead to the conclusion that water temperature (WT) and volatile solids (VS) were strongly correlated with the first CCA axis while dissolved oxygen (O_2), pH, ammonia (NH_4^+), phosphates (PO_4^{3-}) and nitrates (NO_3^-) were the most correlated with the second axis. These two axes alone explained 48,5% of the total phytoplankton, bacterial and viral variance (Table 2a). On the negative side, the first axis is defined by the green algae *Tetraedron minimum* (TETm), *Scenedesmus oahuensis* (SCEo), *Scenesdesmus acuminatus* (SCEa), *Pediastrum boryanum* (PEDb), *Scenedesmus* sp. (SCE), *Scenesdesmus spinosus* (SCEs), but also by the cyanobacteria *Microcystis* sp. (MYCS) and total viral concentration (Vir). The positive side of the first axis is defined by the euglenophyte *Euglena* sp. (EUG.), *Lepocinclis salina* (LEP.), *Trachelomonas* sp. (TRA) and *Trachelomonas nigra* (TRAn), the chlorophyte *Botriococcus brauni* (BATR), *Pediastrum tetras* (PEDt), and the cyanobacteria *Aphanizomenon flos-aquae* (APHA) and *Merismopedia* sp. (MERI), but also by the total bacteria concentration (Bac). Along the

second axis, the positive area is defined by the cyanobacteria *Microcystis* sp. (MYCS) and by the cryptophyte *Cryptomonas* sp. (CRYP) and the negative side is defined by chlorophyte: *Coelastrum pseudomicroporum* (COEL), *Scenedesmus disciformes* (SCEd), *Pediastrum duplex* (PEDd) and *Tetraedron caudatum* (TETc).

In the second CCA virus densities were used as explanatory variables (Table 2c and Fig. 3b). The first two axes explained 50.6% (a higher percentage then the obtained for the first diagram) of the total phytoplankton and bacterial variance. This analysis was preceded by a CCA (Table 2b) using the same data (without virus) (the first two axes explained 45.2% of the total variance). The ordination diagram obtained (Fig. 3b) shows that water temperature and volatile solids are still strongly correlated with the first CCA axis, but immediately followed by viral abundance. The second axis shows correlations with pH, dissolved oxygen and conductivity.

Discussion

De Figueiredo et al. (2006) described the dominance of cyanobacteria from late spring until early autumn (less nutrient availability and higher temperatures) in this shallow lake, eutrophic system (a massive cyanobacterial bloom of *Aphanizomenon flos-aquae* occurred early in May 2001 and was preceded by the lowest nitrogen levels measured in the water during all the study period). The development of the dense *A. flos-aquae* bloom was preceded by the lowest concentrations of nitrogen, indicating that this cyanobacterial strain is not very dependent on nitrogen availability, probably due to its N-fixing capability. However, the availability of phosphate appeared to be required for the bloom development. This phosphorus dependence has been reported for this species (Teubner et al. 1999; Dokulil & Teubner 2000; de Figueiredo et al., 2004b; 2006).

Loss of biodiversity and increase of algal blooms are the most evident negative ecological impacts of human activities on the microbial level in aquatic systems. The dominance of Cyanobacteria, and the presence of the genus *Microcyctis* and *Aphanizomenon* follows a pattern described for shallow Mediterranean lakes (Naselli-Flores & Barone 2003; de Figueiredo et al. 2006; Moustaka-Gouni et al. 2007). This pattern may indicate relations between climate factors and phytoplankton dynamics (Naselli-Flores & Barone 2003; de Figueiredo et al. 2006; Moustaka-Gouni et al. 2007). If the smoother seasonal changes in irradiance in lower latitudes compared to those in higher latitudes, in combination with other properties of warmer climates, are assumed to result in smoother changes in physical conditions of the lakes, these may allow persistence of Cyanobacteria (Naselli-Flores et al. 2003; Moustaka-Gouni et al. 2007). These observations may have implications for future investigation of climate impacts on phytoplankton dynamics due to global warming (Moustaka-Gouni et al. 2007).

The abundance of heterotrophic bacteria in the water column depends on the availability of organic matter and is indirectly linked to the chlorophyll concentration. Ammonia- and nitrite-oxidising bacteria together are responsible for the process of nitrification (which is the oxidation of ammonium to nitrate). The highest appearing of heterotrophic aerobic bacteria was observed during the bloom, which match the high values of organic matter. The maximum values of chlorophyll *a*, total and volatile solids were observed during the bloom period, decreasing when the senescence period began (Fig. 4).

In most instances, viral abundance was significantly correlated with bacterioplankton abundance (Wommack & Colwell 2000; Fischer & Velimirov 2002; Bettarel et al. 2003; Vrede et al. 2003), but in a few studies, it showed significant correlation with chlorophyll a concentration (Boehme et al 1993; Maranger & Bird 1995; Weinbauer et al. 1995). The same pattern of variation of viruses and bacteria during the phytoplankton bloom as well as

the significant correlation between viruses and bacteria ($p<0.05$) suggest that a significant fraction of viruses are bacteriophages. However, the frequent low viruses-to-bacteria ratios seem to indicate that during a bloom a large fraction of planktonic viruses results from phytoplankton cells infection; phytoplankton might represent an important host reservoir for the virioplankton which is consistent with the results reported by Maranger & Bird (1995). Laboratory experiments with whole water-columns from shallow, eutrophic lakes repeatedly showed collapse of the predominant filamentous cyanobacteria; the collapse could be due to viral activity (Gons et al. 2006). VBR is a common index used to examine viral-bacterial dynamics in aquatic systems (Maranger & Bird 1995). Generally, high VBR values may have resulted from either a high infection rate or a long persistence of viruses in the plankton (Laybourn-Parry et al. 2001). In addition, high concentration phytoplankton and cyanobacteria may also result in high VBR value (Maranger & Bird 1995). The mean VBR of 1.4 (range 0.3 to 5.7) is somehow below the general range (i.e., 3 to 10) reported for pelagic environments (Wommack & Colwell 2000). The possible explanation for our results is that viral dynamics might result from bacteriophages, cyanophages, algal viruses and other viruses together.

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Table 1. Maximum (max), minimum (min), average and standard errors (σ) values for each measured parameter. Sampling was performed every second day between April and June of 2006 (32 samples of Vela Lake).

Air temp. ºC		**Water temp. ºC**		**Cond. µS cm^{-1}**		**pH**		**Dissolved O_2 mg l^{-1}**		**Dissolved O_2 %sat**	
min	**max**	**min**	**max**	**min**	**máx**	**min**	**max**	**min**	**max**	**min**	**max**
14,7	28,2	17,2	27,5	566	641	7,75	9,82	4,75	13,7	58,4	173,3
average ± σ		**average ± σ**		**average ± σ**		**average ± σ**		**average ± σ**		**average ± σ**	
20,7±3,4		22,5±2,4		606±21		8,63±0,55		9,53±1,80		110,9±22,9	
Chl *a* µg l^{-1}		**TS mg l^{-1}**		**VS mg l^{-1}**		**C P µg l^{-1}**		**C N(NH_4^+) mg l^{-1}**		**C N(NO_3^-) mg l^{-1}**	
min	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**
0,005	0,098	12,700	52,900	12,700	47,900	0,000	0,009	0,002	0,131	0,004	0,017
average ± σ		**average ± σ**		**average ± σ**						**average ± σ**	
0,035±0,028		28,624±12,453		27,384±11,220						0,008±0,003	

Table 2. a) Summary of canonical correspondence analysis between most abundant phytoplankton species, bacteria, virus and physico-chemical parameters; b) Summary of CCA analysis between most abundant phytoplankton species, bacteria and -chemical parameters (without virus); c) Summary of CCA analysis between most abundant phytoplankton species, bacteria and physico-chemical parameters (plus virus as explanatory variable) . The study period was from 18th April to 19th June of 2006.

	a)		b)		c)	
	Axes 1	Axes 2	Axes 1	Axes 2	Axes 1	Axes 2
Eigenvalues	0,139	0,067	0,137	0,055	0,151	0,064
Species-environment correlations	0,897	0,824	0,893	0,780	0,926	0,799
Cumulative percentage variance						
of species data	32,8	48,6	32,3	45,2	35,5	50,7
of species-environment relation	51,3	76,0	56,4	79	52,8	75,3
Sum of all unconstrained eigenvalues	0,425		0,425		0,425	
Sum of all canonical eigenvalues	0,272		0,243		0,286	
Variance explained by the CCA	**64,0%**		**57,2%**		**67,3%**	
Variance explained by the first two axes	**48,5%**		**45,2%**		**50,6%**	

Figures

Figure 1. Abundances distribution of the main phytoplankton groups and *A. flos-aquae* dynamics every second day between April and June of 2006 in surface water samples (32 samples) of Vela Lake.

Figure 2. Variation of bacterial and viral abundance every second day between April and June of 2006 in surface water samples (32 samples) of Vela Lake.

Figure 3. Results from canonical correspondence analysis for Vela Lake between 18th April and 19th June of 2006: a) biplot for most abundant phytoplankton species, bacteria, virus and environmental variables; b) biplot for most abundant phytoplankton species, bacteria and environmental variables plus virus (as explanatory variable).

Figure 4. Time courses of phytoplankton biomass as chlorophyll a (Chl-*a*), organic matter every second day between April and June of 2006 in surface water samples (32 samples) of Vela Lake.

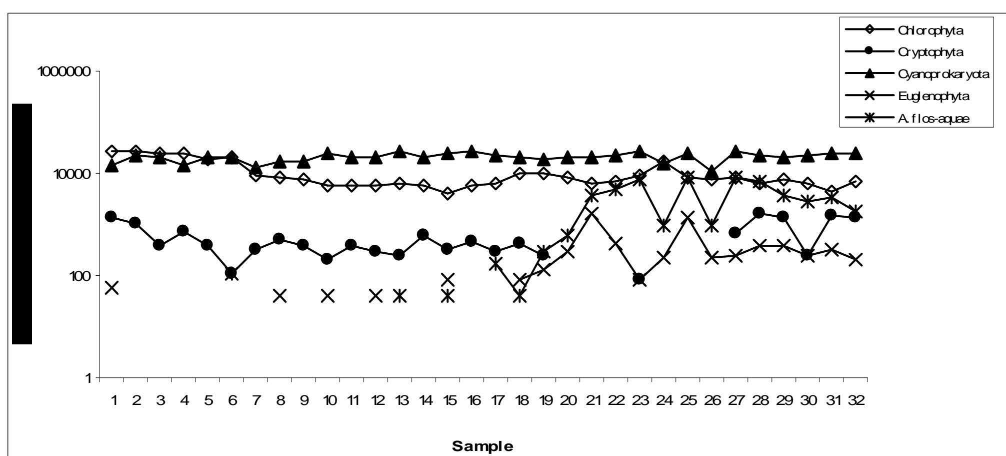

Fig. 1

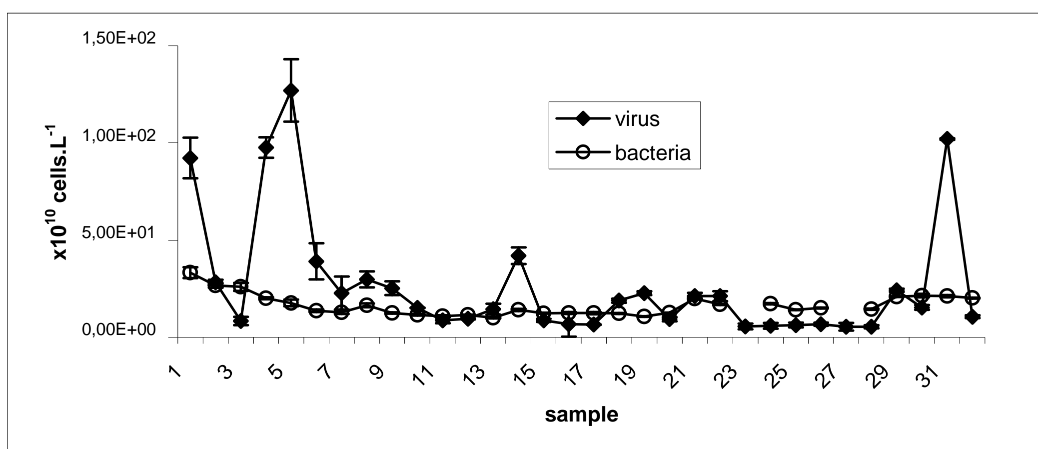

Fig. 2

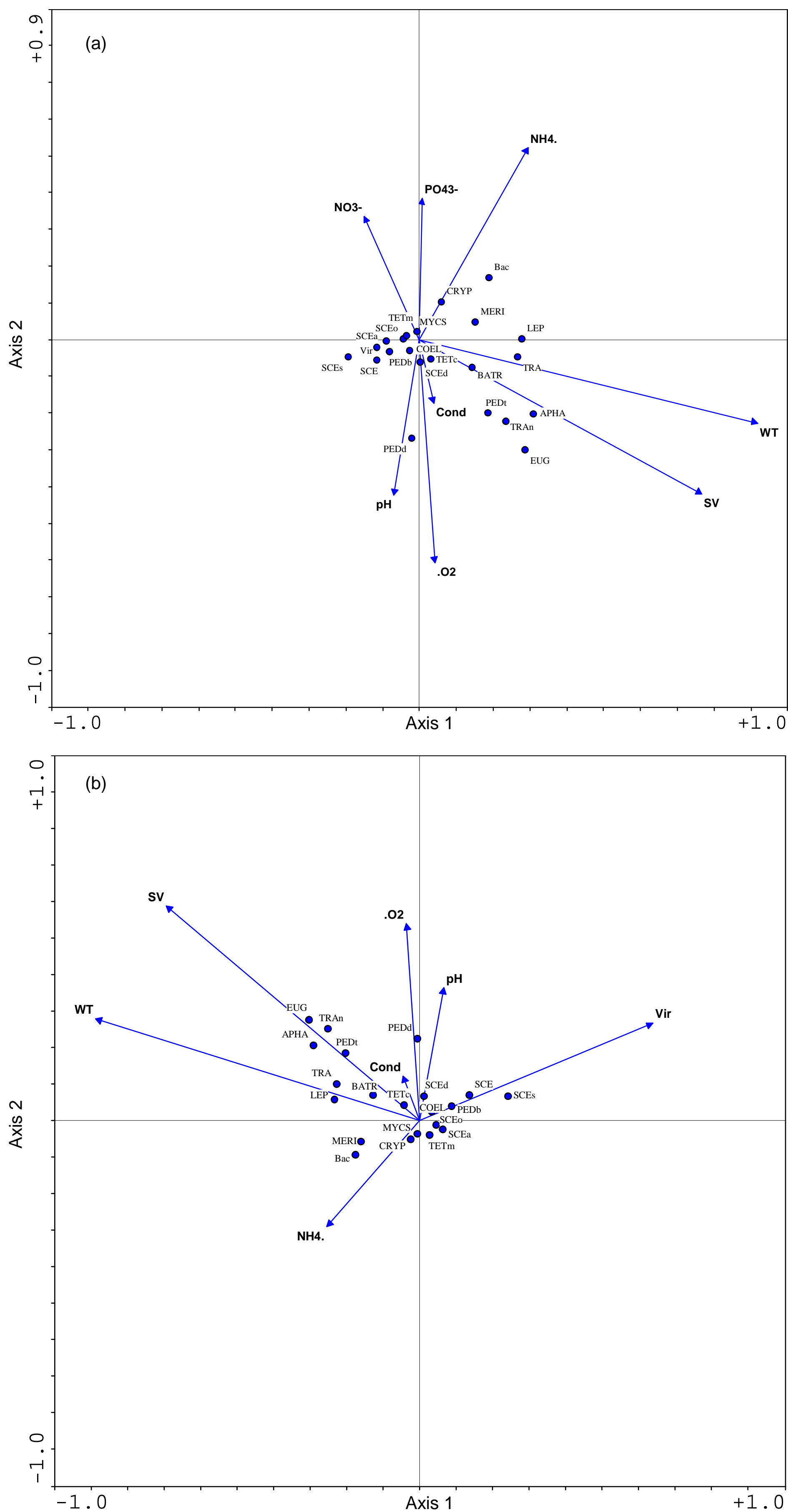

Fig. 3

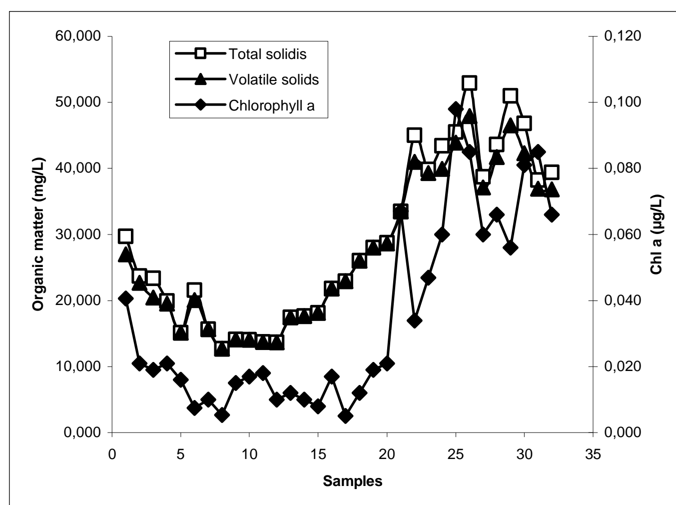

Fig. 4

Capítulo III

Bacterioplankton dynamics during the development of a phytoplankton bloom in a temperate shallow lake

Bacterioplankton dynamics during the development of a phytoplankton bloom in a temperate shallow lake

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Journal *(Paper submitted for publication in the Microbial Research)*

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Bacterioplankton dynamics during the development of a phytoplankton bloom in a temperate shallow lake

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Abstract

Phytoplankton blooms are common in eutrophic freshwaters, being Cyanobacteria frequently reported as the major intervenient in bloom formation. Some information about the dynamics of bacterioplankton communities during phytoplankton blooms is currently available but little is known about phytoplankton influence on prokaryote diversity and the relation between cyanobacterial development and the dynamics of other prokaryote groups.

The aim of this work was the characterization of freshwater planktonic prokaryote communities during a phytoplankton bloom and the identification of major environmental factors regulating prokaryote dynamics during these events.

The prokaryote community was described in terms the total culturable fraction (colony counts) and the relative abundance of selected phylogenetic groups assessed by Fluorescence *In Situ* Hybridization. Probes for *Archaea, Eubacteria, α, β* and *γ–Proteobacteria, Cyanobacteria*, *Actinobacteria*, and *Cytophaga-Flavobacteria* were used. Physical and chemical water properties were also described.

The culturable fraction (323-15000 $CFU.mL^{-1}$) was mainly composed of gram-negative, catalase positive strains.

The culture independent approach revealed that the prokaryote community was diverse, although mainly composed by *Eubacteria*, and that its structure was highly variable during the study period. The abundance of *Cyanobacteria* did not correlate significantly with the abundance of any other of the analyzed prokaryote groups but it correlated positively with PO_4^{3-}. Conductivity correlated positively with the abundance of *Archaea*, *β* and *γ–Proteobacteria*. The results indicate an association of these groups with organic matter, derived from primary production.

INTRODUCTION

Freshwater habitats play an important role in global biogeochemical cycles regulating the flux of dissolved and particulate matter from the terrestrial environment to the sea. Bacteria are involved in the most significant biogeochemical processes. However, little is known about the identity and function of the groups that inhabit freshwater environments as well as about their relation with phytoplankton and chemical and biological parameters. Recent studies of comparative analysis of 16S rRNA clone libraries have revealed that lakes and rivers contain many bacterial groups that appear to be phylogenetically distinct from those found in other environments (e.g. in soils and oceans; Eiler and Bertilsson, 2004).

Eutrophication is frequently a result of nutrient unbalance associated to the release of sewage effluent and run-off from lawn fertilizers into natural waters, although it may also occur naturally in situations where nutrients accumulate (e.g. depositional environments) (Spatharis *et al.*, 2007). Human activities can accelerate the rate at which nutrients enter ecosystems (for example: runoff from agriculture and development, pollution from septic systems and sewers, and other human-related activities), increasing the flux of both inorganic nutrients and organic substances into the ecosystem.

In freshwater systems, phosphorus and nitrogen often act as limiting nutrients – the levels of these two nutrients determine the biological productivity of the lake. A sharp increase in one or both of these elements can result in an algal bloom (de Figueiredo *et al.*, 2006). There are several direct and indirect costs associated with algal blooms. As the algae population proliferates, waters become increasingly more turbid, especially in shallow lakes (Schäfer *et al.*, 2001; Haukka, *et al.*, 2006).

Phytoplankton and cyanobacterial blooms are common in eutrophic freshwaters often producing elevated levels of noxious compounds that compromise water quality. Transfer of photosynthetically fixed organic carbon to heterotrophic bacteria can occur by bacterial degradation and utilization of dead cyanobacteria. Enzymatic attack and predation on living phytoplankton by heterotrophic bacteria can liberate dissolved organic matter for bacterial bloom conditions. Cyanobacterial dominated phytoplankton is the main source of readily available DOM. However, in the post-bloom, when some bacterial groups are actively using dead cell material, the total bacterial numbers were found to decrease (Kisand and Tammert, 2000) indicating a shift in the structure of the heterotrophic fraction of bacterioplankton.

Since the introduction of culture-independent methods for the identification and classification of microorganisms in different environments, our knowledge of the diversity of Bacteria and Archaea has significantly increased. This is also true for bacterioplankton communities of lakes (Hiorns *et al*., 1997; Methé and Zehr, 1999; Zwart *et al*., 1998; Lindström, 2000). However, only a few attempts have been made to relate community composition to biological, chemical and physical parameters in the lakes (e.g., Höfle *et al*., 1999; Lindström, 1998; Methé and Zehr, 1999; Lindström, 2000). Consequently, little is known about the factors that may have a significant impact on the phylogenetic structure of lake bacterioplankton.

The present work aimed to interpret the variability of the composition of the bacterial community in a shallow lake (Vela Lake), a eutrophic freshwater system, during a phytoplankton bloom. In different bloom phases, the major chemical and organic parameters regulating prokaryote dynamics were analyzed with a particular focus on search for interactions between cyanobacteria and other prokaryote groups.

MATERIAL AND METHODS

Study site

Vela Lake (44º58'N, 5º18'W) is one of the few natural shallow lakes (average depth *circa* 1m) existing in the west coast of Portugal, lying in a transition zone between Mediterranean (southwards) and Atlantic (northwards) bioclimates (Abrantes *et al.*, 2006). Vela Lake is a small (60-70ha), shallow (0,9m average depth; 2,4 m maximum depth) water body, being the largest of a cluster of small shallow lakes located within a coastal dune system (de Figueiredo *et al.*, 2006).

Agriculture, livestock activities and, to a lesser extend, domestic runoffs from adjacent human settlements supply regular nutrient inputs to the lake, originating high primary productivity and turbity (Abrantes *et al.*, 2006).

Phytoplankton and cyanobacterial blooms are frequent and occasional episodes of oxygen shortage have occurred in recent years (Antunes *et al.*, 2003).

Sampling Strategy

Samples were collected just below surface every second day, from April 18th to June 19th, 2006 (spring time), during the morning period. Samples were enumerated according to the sequential days of sampling. In each sampling day, water was collected for chemical (phosphates, nitrates, ammonia) parameters, particulate matter (volatile and total solids), chlorophyll *a* (as an estimate of algal biomass) and prokaryote dynamics analyses. All the samples were kept in the dark, being processed in the laboratorium within one hour.

During the sampling period, several parameters were evaluated *in situ*: pH, O_2 concentration ($mg.L^{-1}$) and percentage of saturation (%), conductivity and water temperature (WTW multiliner P4).

Physical and chemical parameters

For the determination of total and volatile solids, aliquots of variable volume (depending on the trophic status) were filtered through GF/C precombusted filters. Total solids were calculated by gravimetry and volatile solids were calculated by weight loss after incineration (APHA, 1992).

Inorganic nutrients were determinated colorimetrically (6 Genesys Thermo spectronic) following the stannous chloride method for phosphate (APHA, 1992), the sodium salicylate method for nitrate (described by Rodier, 1984) and the indophenol blue method was used for ammonia (described by Hall & Lucas, 1981).

Chlorophyll *a* concentration was determined spectophotometrically (Lorenzen, 1967), after filtration of a proper volume (depending on the trophic status), extraction with acetone 90%, overnight at 4ºC and centrifugation at 3000 rpm during 10min.

Phytoplankton abundance and diversity

For the phytoplankton quantification, samples were fixed in Lugol solution (1% v/v) being the enumeration performed according to the described by Lund *et al*., (1958), after adequate concentration.

During the quantification, it was considered as unit: the colony in the case of colonial organisms; the filament for filamentous organisms and the cell for unicellular organisms.

Chlorophyta, Cryptophyta, Cyanobacteria and *Euglenophyta* were the relevant groups and green and blue-green algae the dominants. For the identification of different species and genera different flora were used: *Chlorophyta* (Chlorococcales) (Komárek and Fott, 1983); *Cryptophyta* (Fott, 1968); *Cyanobacteria* (Geitler, 1932; Komárek and Anagnostidis, 1989; Komárek and Anagnostidis, 1999) and *Euglenophyta* (Huber- Pestalozzi, 1961).

Culturable Bacteria

Culturable bacteria were enumerated by the pour-plate method of the convenient dilution, using TSA (Tryptic Soy Agar) as culture medium and Ringer solution as dilution liquid, and incubation at 25ºC for 96h. Some colony types were selected according to colony morphology

and cultural characteristics for further characterization. The selected colonies were isolated in TSA plates, using the streaking method (Madigan *et al.,* 2000).

The isolates were stored in glycerol until analysis (Madigan *et al.*, 2000).

Each isolate was characterized as to catalase and oxidase activities as well as Gram type. The presence of motility was observed on the microscope in wet mounts (Madigan *et al.*, 2000).

Fluorescence *In Situ* Hybridization (FISH)

The phylogenetic structure of prokaryotic planktonic communities was determined by Fluorescence *In situ* Hybridization (FISH) with Cy3-labeled oligonucleotide probes (MWG Biotech). Sample fixation was performed according to the method described by Glöckner *et al*. (1996). Briefly, samples were filtered through 0.2 µm polycarbonate filters (GE Osmonics) and fixed during 30 m with 4 % paraformaldehyde (Sigma, Aldrich).

For the hybridization with the oligonucleotide probes, some sample were selected according with to profiles of variation of chemical and biological descriptors, in order to represent different phases of the bloom development.

Domain and group-specific probes used were Eub338-II-III for Bacteria, Alf968 for *α-proteobacteria*, Bet42a for *β-proteobacteria*, Gam42a for *γ-proteobacteria*, CF319a for *Cytophaga-Flavobacterium*, HGC236 for *Actinobacteri*a, Cya361 for *Cyanobacteria* and Arch915 for *Archaea*. Unlabeled competitor probes were also used for distinguished *Betaproteobateria* and *Gammaproteobacteria*. A negative control probe (Bet42aNM and Gam42NM) was used to examine non-specific binding. The probe sequences and references are given in Table 1. For each probe, triplicate filter pieces were placed on a Parafilm-covered glass slide and overlaid with 30µl of hybridization solution with 3ng.μL^{-1} of probe (final concentration). The hybridization solution contained 0.9M NaCl, 20mM Tris-HCl (pH 7.4), 0.01% SDS, and the optimum concentration of formamide for each probe. Filters were incubated in sealed chambers at 46°C for 90m. After hybridization, filters were washed for 15 min at 48°C in wash solution (20mM Tris HCl pH 7.4, 5mM ethylenediaminetetraacetic acid, 0.01% SDS, and the appropriate concentration of NaCl) (Pernthaler *et al*., 2002).

After rinsed and dried, the filter pieces were stained with 2 µg.mL^{-1} DAPI (final concentration), mounted with Vectashield and Citifluor (1:4) and examined under the epifluorescence microscope (Leica, DMLS) equipped with a 50-W high-pressure mercury

bulb and appropriate filter sets for DAPI and CY3 fluorescence. Each microscopic field was first viewed with the CY3 filter set before switching to the DAPI filter set, to avoid fading of CY3 fluorescence during the DAPI examination. More than 1500 of DAPI-stained cells were counted per sample.

Table 1: Oligonucleotide probes used in the study.

Specificity	Probe	Reference
Archea	Arch 915	Stahl and Amann, 1991
Bacteria	Eub 338	Amann *et al*., 1990
Negative control	Non 338	Wallner *et al*., 1993
Planctomycetales	Eub 338 II	Daims *et al*., 1999
Verrucomicrobiales	Eub III	Daims *et al*., 1999
α-Proteobacteria	Alf 918	Manz *et al*., 1992
β-Proteobacteria	Bet 42a and Bet 42a NM	Manz *et al*., 1992
γ-Proteobacteria	Gam 42a and Gam 42aNM	Manz *et al*., 1992
Cytophaga-Flavobacterium	CF 319a	Manz *et al*., 1992
Actinobacteria 16S	HGC 236	Erhart *et al*., 1997
Cyanobacteria	Cya 361	Schönhuber *et al*., 1999

Statistical analysis

In order to assess environmental dynamics during a cyanobacterial bloom, principal component analysis was performed using FISH absolute abundance data, physical and chemical parameters (Aguilera *et al*., 2007). With the aim of identifying the major environmental factors regulating prokaryote dynamics, a stepwise multiple regression analysis was performed using conductivity, PO_4^{3-}, NO_3^-, total solids, volatile solids, chlorophyll *a*, total DAPI, *Archaea*, *α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria* and *Actinobacteria* and absolute counts as independent variables and biological parameters (Total DAPI counts, *Archaea*, Bacteria, *α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria* and *Cyanobacteria* counts) as dependent variables (Pallant, 2004). Collinearity was verified by the Spearman's test. All the statistical analysis were performed using SPSS 14.0 package.

RESULTS

Physical and chemical parameters

During the sampling period, the water temperature presented an average value of 22.5±2.4ºC, varying between 17.2 and 27.5 ºC. The values of conductivity varied between 566 and 641μS.cm^{-1}, being the average value of 606±21 μS.cm^{-1} (Figure 1). The recorded pH values varied from 7.75 to 9.82, presenting an average value of 8.63±0.55 (Figure 2). The percentage saturation of dissolved O_2 showed wide amplitude (minimum of 58.4% and a maximum of 173.3%) with a value of 110.9±22.9% (Figure 2). The highest pH value was observed in the same day as the maximum of dissolved oxygen, at the end of the sampling period (sample 25, June 5th 2006).

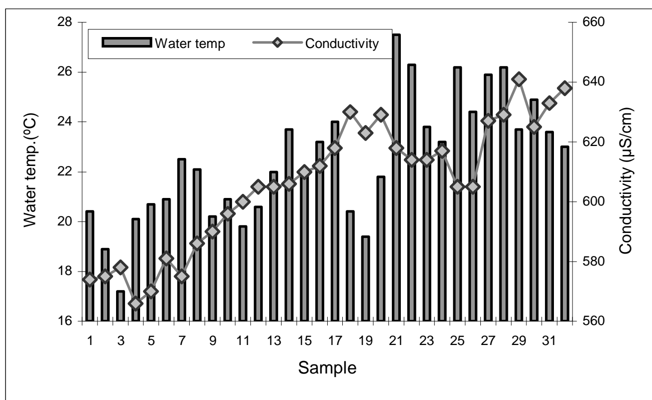

Figure 1 – Variation of water temperature and conductivity in Lagoa da Vela, during the sampling period.

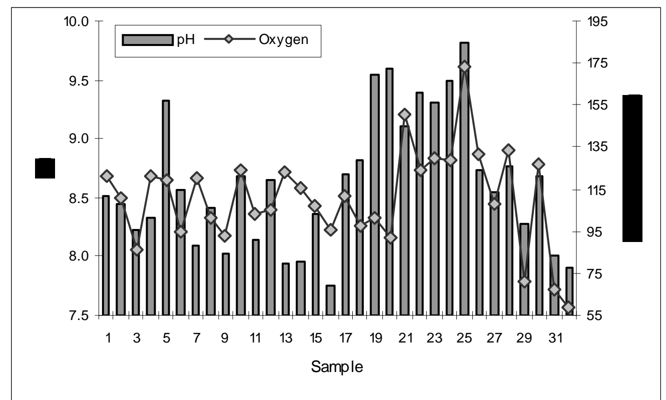

Figure 2 – Variation of pH and oxygen concentration (saturation %), in Lagoa da Vela, during the sampling period.

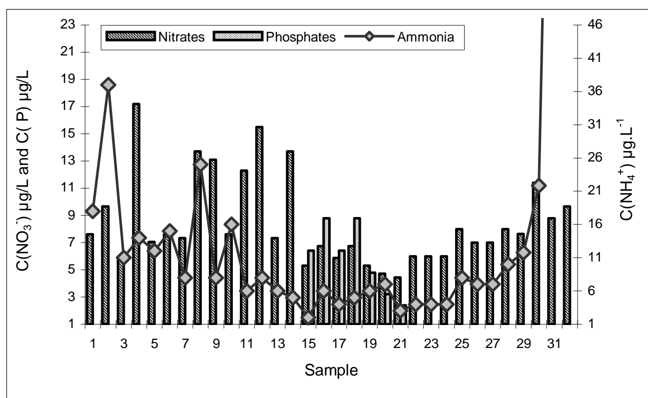

Figure 3 – Variation of total and volatile solids and chlorophyll *a*, in Lagoa da Vela, during the sampling period.

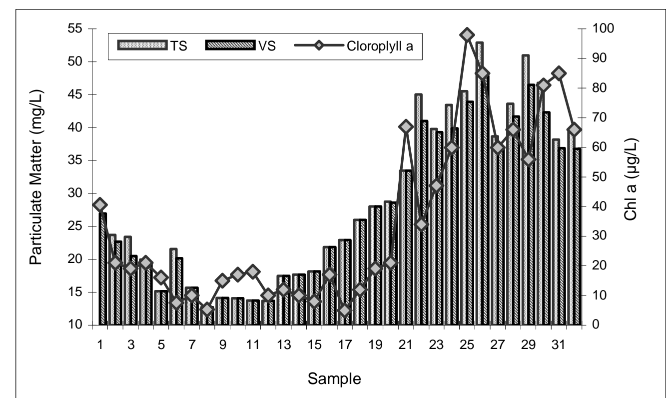

Figure 4 – Variation of nitrate, phosphate and ammonia, in Lagoa da Vela, during the sampling period.

The chlorophyll *a* concentration varied between 5 and 98 µg.L^{-1}, with an average value of 35±28 µg.L^{-1}. The total solids varied from 12.7 to 52.9 mg.L^{-1}, with an average value of 28.6±12.5 mg.L^{-1}. The volatile solids, as well as the total solids, showed a wide range of values during the sampling period, varying between 12.7 and 47.9 mg.L^{-1}. The average value was 27.4±11.2 mg.L^{-1} (Figure 3).

The concentrations of ammonia and nitrates (NH_4^+ and NO_3^-, respectively) oscillate throughout the sampling period. Ammonia concentrations varied between 2 and 131 µgN.L^{-1}, with an average value of 16 µgN.L^{-1}. Nitrate concentration wasn't as variable as NH_4^+, ranging between 4 and 17 µgN.L^{-1}, with an average value of 8±3 µgN.L^{-1}. The concentration of phosphate (PO_4^{3-}) was, during almost all the sampling period, below the limit of quantification (3 µg.L^{-1}). A maximum of 9 µg.L^{-1} was observed during a rainy period, being the average value of this period of 5.8 µg.L^{-1} (Figure 4).

Biological Parameters

The abundance of culturable bacteria varied widely during the study period. The highest values were observed in June 15th and 17th (sample 30 and 31, respectively) (maximum of 15000 CFU.mL^{-1}) and the lowest values occurred in April 28 (sample 6) and May 8th and 16th (sample 11 and 15, respectively). The minimum was observed in May 8 (323 CFU.mL^{-1}) (Figure 5). The highest CFU abundance occurred during a peak of ammonia concentration (June 17).

Throughout the sampling period 66 isolates were obtained being 97% Gram negative, 91% mobile, 94% positive catalase and 70% oxidase negative.

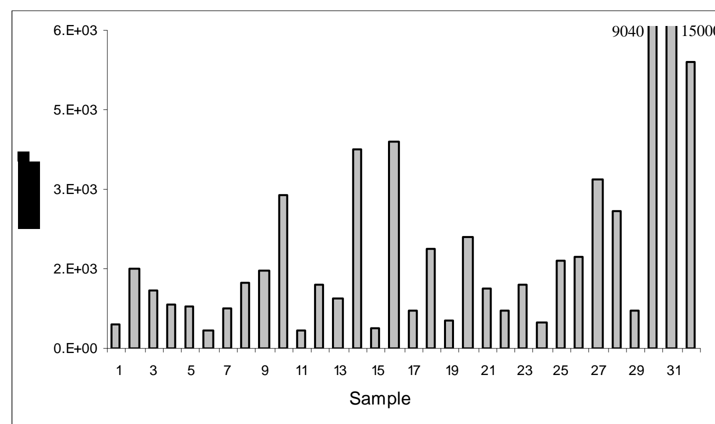

Figure 5 – Abundance of culturable bacteria (colony counts), during the sampling period.

Phytoplankton abundance and diversity

The variation of different phytoplankton groups during the sampling period is showed in Figure 6. The most representative divisions were Cyanobacteria and Chlorophyta, whereas the Cryptophyta and Euglenophyta had a lower representation and frequency.

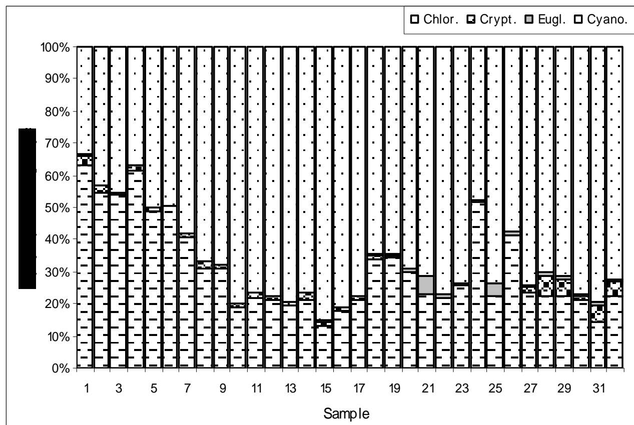

Figure 6 – Seasonal dynamics of Chlorophyta, Cryptophyta, Cyanobacteria and Euglenophyta, during the sampling period.

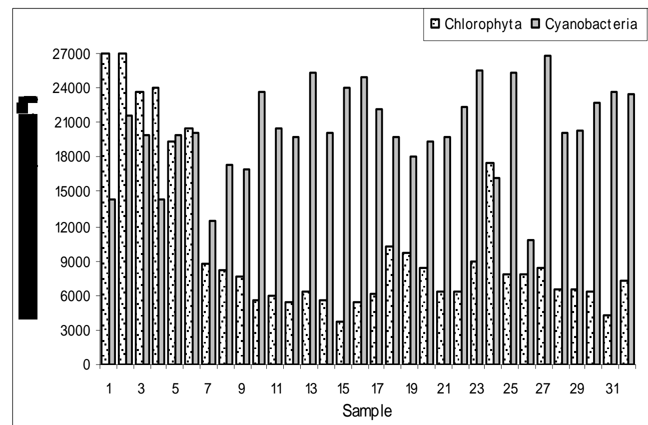

Figure 7 – Variation of the absolute abundance of Chlorophyta and Cyanobacteria, during the sampling period.

Early in the sampling period, Chlorophyta division was predominant, being this predominance progressively transferred to Cyanobacteria (Figure 6 and 7), while the values of water temperature, conductivity, pH and dissolved oxygen (Figure 1 and 2) were increasing. Within Chlorophyta division, there was a variation in species composition, being the genus *Phacotus* (*Phacotus lenticularis*), *Coelastrum* (*Coelastrum pseudomicroporum*, *Coelastrum reticulatum*), *Pediastrum* (*Pediastrum boryanum*, *Pediastrum duplex*, *Pediastrum simplex*, *Pediastrum tetras*), *Scenedesmus* (*Scenedesmus acuminatus*, *Scenedesmus acutus*, *Scenedesmus oahuensis*) and *Tetraedron* (*Tetraedron caudatum*, *Tetraedron minimum*) the major representatives. The maximum value of Chlorophyta abundance was 27079 cells.mL^{-1} (sample 2, April 20th) and the minimum was 3707 cells.mL^{-1} (sample 15, May 16th) (Figure 7).

The Cyanobacteria division began to predominate only when Chlorophyta started to decline, i. e., after April 20th (sample 2). *Microcystis* cf. *pulveria* and *Chroococcus limneticcus* were present throughout the sampling period. In June 9th (sample 27), a maximum value of Cyanobacteria abundance was observed (8342 cels.mL^{-1}) (Figure 7), coinciding with an *Aphanizomenon flos-aquae* maximum.

Abundance and composition of prokaryote groups

Total cell counts, accessed by epifluorescence counts after DAPI staining, varied from 3.03×10^{10} cells.L^{-1} (sample 3, April 22nd) to 9.07×10^{10} cells.L^{-1} (sample 29, June 13th) with an average value of 6.14×10^{10} cell.L^{-1} (Figure 8).

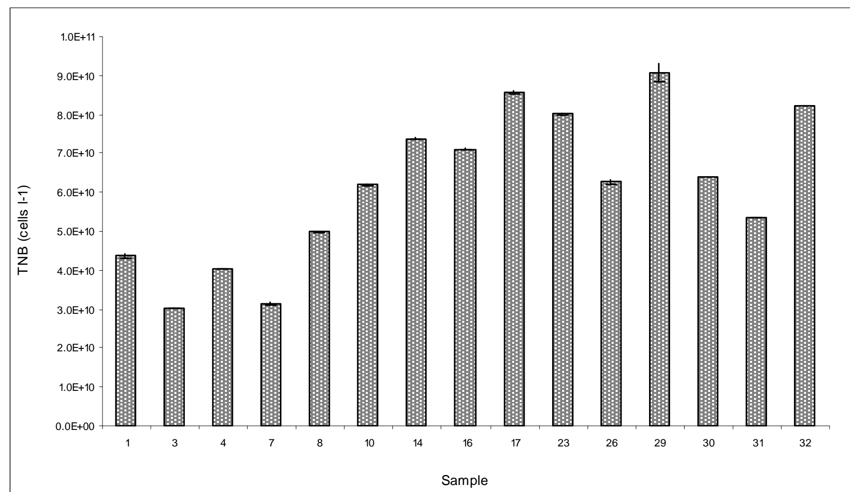

Figure 8 – Variation of the total cell counts accessed by DAPI staining, during the sampling period.

The community was dominated by *Bacteria* which, relatively to DAPI counts, varied between 20.7% (May 6th, sample 10) and 75% (June 17th, sample 31), with an average value of 50.1% (Table 2 and Figure 9). The lowest value found with Eub338 probe was observed in a period of very low total and volatile solids concentration (Figure 3). The June 17th (sample 31) showed the highest bacteria abundance (Eub 338 Probe) (Figure 8 and 9), corresponding to conditions of high ammonia (Figure 4), low oxygen (% saturation) (Figure 2), high conductivity (Figure 1), high chlorophyll *a* values (Figure 3), total and volatile solids concentration in the beginning of a declining phase of the bloom (Figure 3).

Archaea were detected in all samples, ranging from 2.7% (May 6th, sample 10) to 12.2% (June 7th), with an average value of 8.0% (Table 2 and Figure 9). Higher *Archaea* abundance occurred during the decline of the Cyanobacterial bloom (Figure 12a).

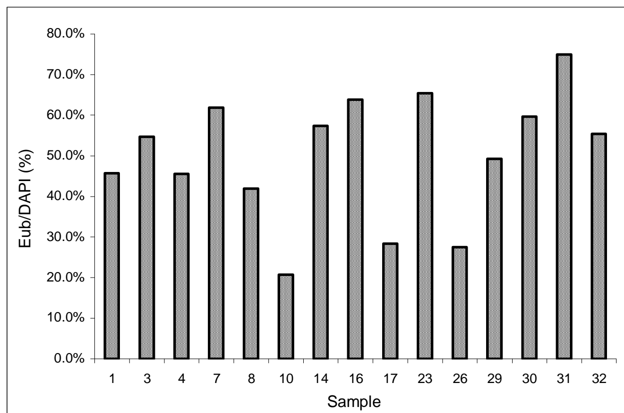

Figure 9 – Percentage of *Bacteria* detected by Probe Eub 338 relatively to DAPI, during the sampling period.

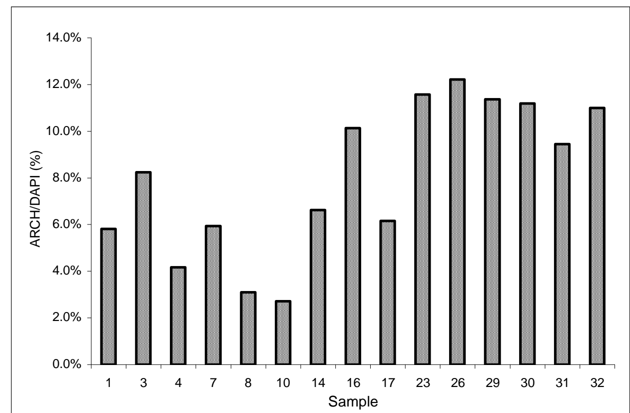

Figure 10 – Percentage of *Archaea* detected by Probe Arch 915 relatively to DAPI, during the sampling period.

Figures 9 and 10 show that the relative abundance of Bacteria and Archaea varied considerably during the sampling period.

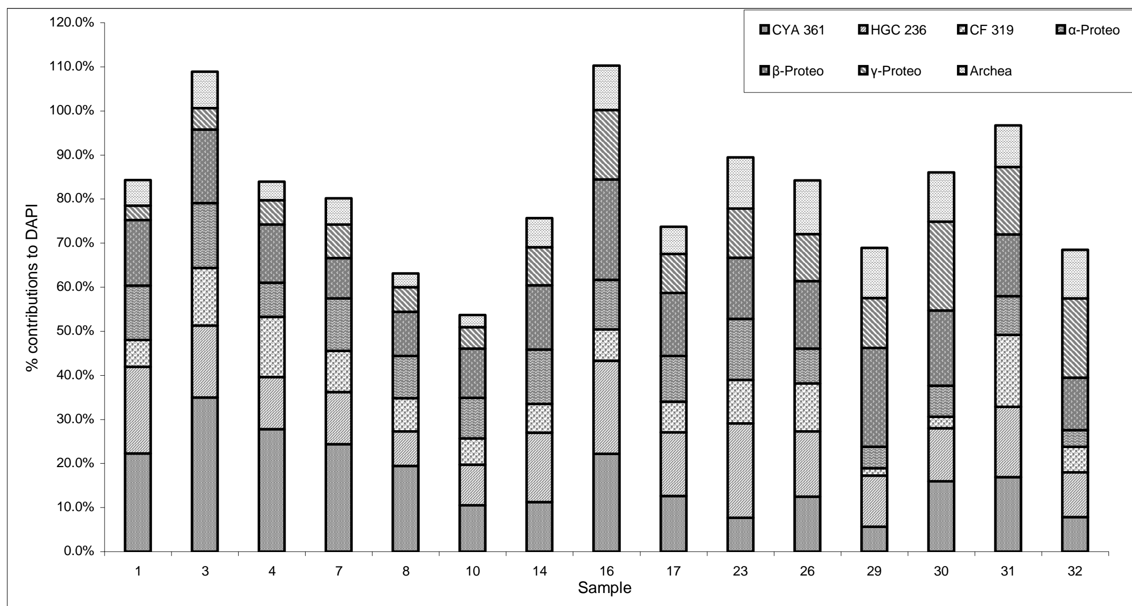

Figure 11 – Relative DAPI counts of Archaea and Bacteria groups: *Cyanobacteria* (Probe CYA361), *Actinobacteria* (Probe HGC236), *Cytophaga-Flavobacterium* (Probe CF319), *Alfaproteobacteria* (Probe ALFA918), *Betaproteobacteria* (Probe Bet42a) and *Gammaproteobacteria* (ProbeGAM42a).

The results of the relative abundance (%DAPI counts) of the specific phylogenetic groups are presented in the table 2.

Table 2: DAPI and probe specific counts relatively to DAPI, during the sampling period.

Day	Sample number	Total cell counts (10^{10}cell.L^{-1})	Fraction (%) of total cells (mean±SD) detected with probe[a]							
			Arc915	**Eub338, II & III**	**Alf918**	**Bet42**	**Gam42**	**CF319**	**HGC236**	**Cya361**
18-April	1	4.4 ± 7.0E+08	5.8±0.7	45.7±9.8	12.3±1.1	14.9±1.1	3.3±0.1	6.1±0.3	19.7±6.2	22.3±2.2
22- April	3	3.0 ± 0.6E+08	8.2±0.6	54.7±14.7	14.7±1.6	16.7±1.7	4.9±0.4	13.0±0.3	16.3±0.4	34.9±1.4
24- April	4	4.0 ± 0.4E+08	4.2±0.7	45.5±2.8	7.8±0.1	13.2±1.1	5.6±0.8	13.6±3.1	11.8±2.1	27.8±0.2
30-Abr	7	3.1 ± 3.7E+08	5.9±0.3	61.9±14.2	11.9±1.0	9.1±0.9	7.6±0.6	9.4±0.4	11.8±0.6	24.4±7.4
02-May	8	5.0 ± 2.3E+08	3.1±0.3	42.0±14.6	9.6±0.8	10.0±0.3	5.6±0.3	7.6±0.4	7.8±0.2	19.4±4.2
06-May	10	6.2 ± 1.5E+08	2.7±0.1	20.7±4.2	9.2±0.3	11.2±0.5	4.9±0.2	5.9±0.2	9.2±0.5	10.5±1.8
14-May	14	7.4 ± 2.8E+08	6.6±0.3	57.3±1.7	12.3±0.7	14.6±0.8	8.6±0.3	6.6±0.2	15.7±0.2	11.2±1.4
18-May	16	7.1 ± 1.2E+08	10.1±0.4	63.8±0.7	11.3±0.5	22.7±0.6	15.7±0.4	7.1±0.4	21.1±0.5	22.2±0.3
20-May	17	8.6 ± 4.6E+08	6.2±0.1	28.4±9.0	10.4±0.2	14.3±1.0	8.9±0.6	6.9±0.2	14.5±0.4	12.6±0.5
01-June	23	8.0 ± 0.3E+08	11.6±0.3	65.4±0.8	13.8±0.4	13.9±0.6	11.2±0.4	9.9±0.1	21.4±0.8	7.7±0.3
07-June	26	6.3 ± 5.2E+08	12.2±0.5	27.5±0.5	7.9±0.2	15.3±0.3	10.6±1.2	10.9±1.2	14.8±0.9	12.5±0.6
13-June	29	9.1 ± 23.4E+08	11.4±0.5	49.3±2.0	4.9±0.2	22.4±0.8	11.4±0.1	1.6±0.1	11.6±0.3	5.7±0.4
15-June	30	6.4 ± 0.3E+08	11.2±0.5	59.6±1.3	7.1±0.2	17.1±0.7	20.1±1.4	2.6±0.1	12.0±0.2	15.9±0.8
17-June	31	5.3 ± 0.5E+08	9.5±0.5	75.0±5.2	8.8±0.4	14.0±1.1	15.3±3.1	16.3±0.1	16.0±0.3	16.9±0.1
19-June	32	8.2 ± 0.3E+08	11.0±0.2	55.4±3.5	3.8±0.2	11.8±0.7	18.1±0.8	5.8±0.3	10.2±0.8	7.8±5.7
Mean ± σ		6.1 ± 1.9	8.0 ± 3.2	50.14 ± 16.3	9.7 ± 3.1	14.8 ± 3.9	10.1 ± 5.1	8.2 ± 4.0	14.3± 4.3	16.8 ± 8.4

a Percent of DAPI counts. Numbers have been corrected by subtracting NON338 counts.

The detailed analysis of the Bacteria domain (Figures 11 and 12) showed that *Cyanobacteria* detected with the Cya361 probe varied, relatively to DAPI counts, between 6.7 (June 13th) and 34.9 (April 22nd), being the average value of 16.8% (Table 2 and Figure 11). In terms of absolute counts, it was observed a maximum abundance value of 1.58x10^{10} cells.L^{-1} (sample 16, May 18th) and a minimum of 5.14x10^{9} cells.L^{-1} (sample 29, June 13th). An initial peak was observed between April 18th and May 18th, decreasing until June 13th, where a new peak occurs, diminishing again (Figure 12a).

Actinobacteria showed an increasing trend until June 1st, decreasing until the end of the study period. The minimum absolute value was observed in April 30th (3.7x10^{9} cells.L^{-1}) and the maximum in June 1st (1.71x10^{10} cells.L^{-1}) (Figure 12b). This sub-group accounted for a minimum of 7.8% (May 2nd) and the maximum of 21.4% (June 1st) of DAPI counts, being the average value of 14.3% (Table 2 and Figure 11).

The *Cytophaga-Flavobacterium* (Probe CF319) showed an oscillatory trend, with three increasing periods: from April 18th to April 24th, from April 26th to June 1st and from June 13th to June 17th, being the minimum absolute value observed in June 13th (1.49x10^{9} cells.L^{-1}) and the maximum in June 17th (8,73x10^{9} cells.L^{-1}) (Figure 12c). The relative abundance varied from a minimum of 2.6 (June 15th) to a maximum of 16.3 (June 17th), being 8.2% the average value (Table 2 and Figure 11).

In terms of relative abundance, *α-Proteobacteria* contribution varied between 3.8% (June 19th) and 14.7% (April 22nd) with an average value of 9.7% (Table 2 and Figure 11). It was verified an increasing trend until June 1st (1.10x10^{10} cells.L^{-1}) then decreasing to a value that stayed almost constant until the end of the study period (3.11x10^{9} cells.L^{-1}) (Figure 12d).

β-Proteobacteria showed two periods of increasing abundance (from April 18th to May 18th and from May 20th to June 13th), decreasing soon after (Figure 12e). The maximum value was observed after the second increase, in June 13th (2.03x10^{10} cells.L^{-1}) and the minimum in April 30th (2.86x10^{9} cells.L^{-1}) (Figure 12e). This sub-group minimum contribution to the total Bacteria counts was 9.1% and 22.7% the maximum. The average value was 14.8% (Table 2 and Figure 11).

γ-Proteobacteria presented an increasing trend until May 18th, maintaining constant levels up to the end of the study period (Figure 12f). On the first sample (April 18th) the lowest abundance value was registered, 1.43x10^{9} cells.L^{-1}. The highest was observed in the last sample (June 19th), 1.49x10^{10} cells.L^{-1} (Figure 12f). This sub-group had a minimum

contribution of 3.3% (April 18th) and a maximum contribution of 20.1% (June 1st) (average value of 10.1) to the whole bacteria population (Table 2 and Figure 12f).

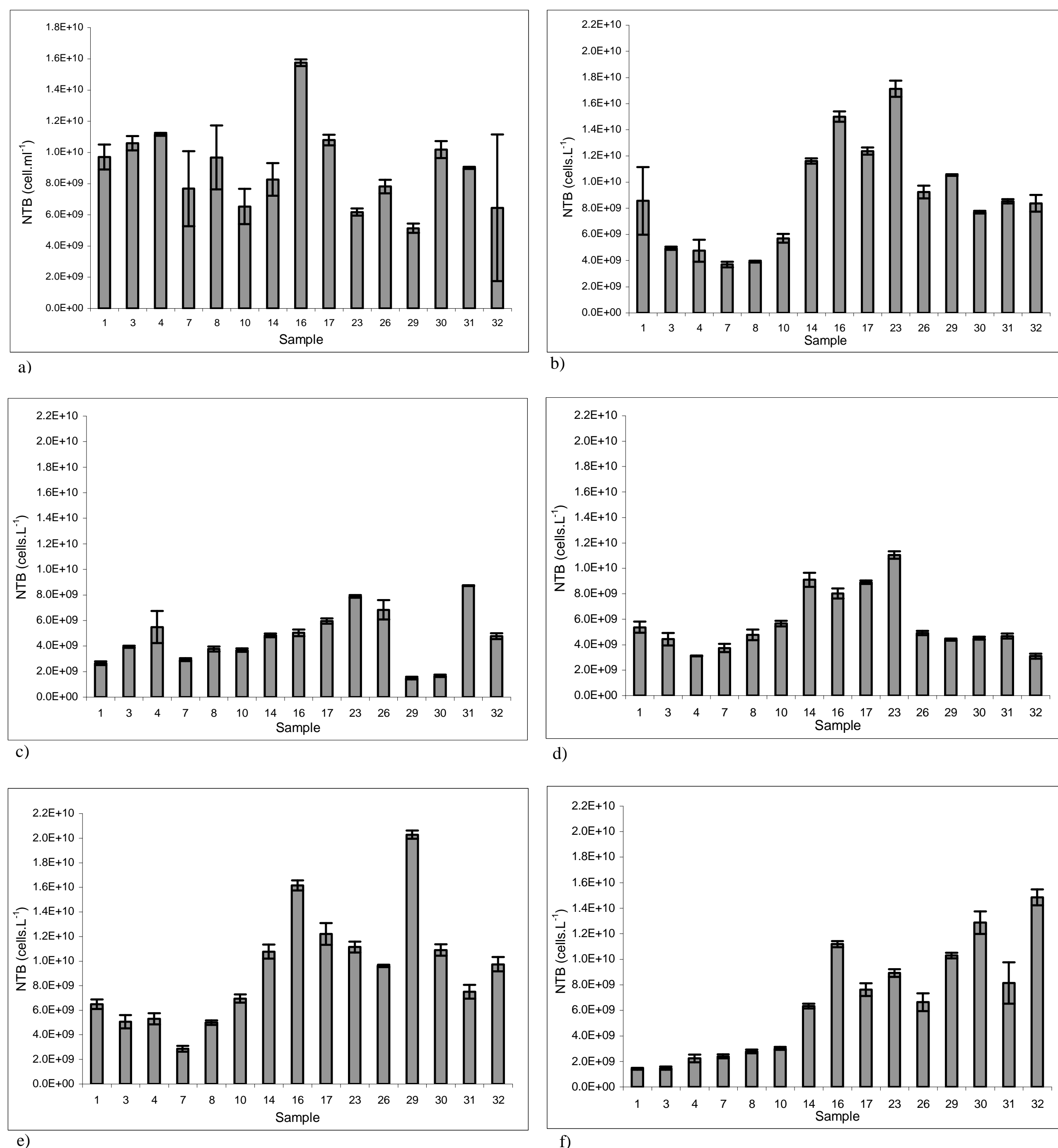

Figure 12 - Bacteria total numbers for each probe [a) Cya 361; b)HGC 236; c) CF 319; d) Alf918; e) Bet42a; f) Gam42], detected by FISH, during the sampling period.

Principal component analysis

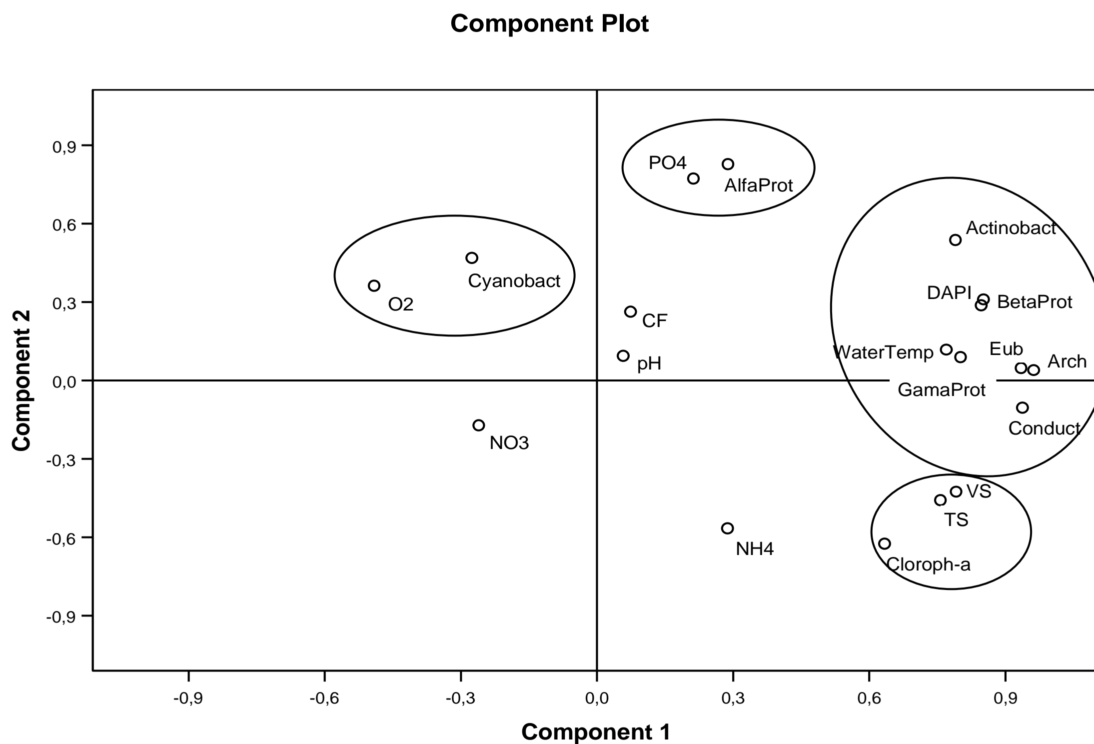

Figure 13 – Principle Component Analysis between procaryte total numbers for each probe assessed by epifluorescence microscopy and FISH and physical-chemical parameters, during the sampling period.

The ordination analysis by PCA (Figure 13) showed that dissolved oxygen and the abundance of prokaryote groups, with the exception of *Cyanobacteria*, were mostly associated to the first axis. This axis is associated to the bloom temporal sequence. On the negative side are represented the more oxic conditions, characterized by high *Cyanobacteria* abundance, dissolved oxygen and nitrate concentration (the bloom fase). *Cyanobacteria* had a peak of abundance in May 18th (sample16, Figure 12a), corresponding to low levels of dissolved oxygen (Figure 2) and nitrates (Figure 4). The positive side describes the less oxic conditions, characterized by higher bacterial abundance (*Actinobacteria*, *β* and *γ-Proteobacteria*, *Archaea*), water temperature, organic matter, conductivity, ammonia and phosphates (Figure 4) (indicating the bloom decline fase). *Actinobacteria*, α, *β* and *γ-Proteobacteria* (Figure 12b), d) e) and f)) showed peaks of abundance in samples from 14 to 29. Comparing with the PCA analysis it can be assumed that in this period the decline of the bloom phase started. *Cyanobacteria* had a peak on sample 16 (Figure 12a). *Cytophaga-Flavobacterium* showed a very oscillatory behaviour (Figure 12c). In the PCA analysis this sub-group was placed near the origin centre of both axis, indicating that it’s regulation was

rather insensitive to the environmental conditions associated to axis 1 and 2 (Figure 13). The relative abundance of *Cytophaga-Flavobacterium* group increased with an increasing concentration of total and volatile solids (Figure 3).

The second axis was mainly related to the concentration of inorganic nutrients, NO_3^- and PO_4^{3-}. From the interpretation of the second axis, situations of high nitrate concentration, associated with high content in total and volatile solids and high concentrations of chlorophyll *a* the contrast with high phosphate conditions and high *α-Proteobacteria* abundance. The two first axes explained 60.7% of the total variability.

Table 3: Principal component matrix of the analysis of abundance total numbers of each phylogenetic groups, as well as physical and chemical data, during the sampling period.

Variables	**Component**	
	1	**2**
Log DAPI	0.847	0.287
Log *Eubacteria*	0.801	0.089
Log *Cyanobacteria* (Probe Cya361)	-0.276	0.468
Log *Actinobacteria*	0.790	0.537
Log Cytophaga-Flavobacterium	0.074	0.262
Log *α-Proteobacteria*	0.288	0.827
Log *β-Proteobacteria*	0.852	0.310
Log *γ-Proteobacteria*	0.934	0.047
Log Archaea	0.962	0.039
Water Temperature	0.769	0.117
Conductivity	0.937	-0.104
pH	0.057	0.094
Dissolved oxygen	-0.491	0.362
Chlorophyll *a*	0.633	-0.624
Total solids	0.757	-0.458
Volatile solids	0.791	-0.426
PO_4^{3-}	0.212	0.772
NH_4^+	0.287	-0.566
NO_3^-	-0.261	-0.172

Stepwise multiple regressions

The attempt to identify mechanistic relations between different prokaryote groups and environmental variables by stepwise multiple regressions is summarized in Table 4. The variability associated to *Cytophaga-Flavobacterium* could not be explained by none of the used variables. The percentage of variation that could be explained with this set of independent variables was high (60 – 90%) for subgroups of Bacteria domain and 50 – 65% for total cell counts, total Bacteria and *Actinobacteria*.

Table 4: Regression equations for the explanation of the variation of bacterioplankton abundance using Stepwise multiple regressions analysis.
Dependent variables = total DAPI (TDAPI), Eub (Eubacteria), Cya (Cyanobacteria), HGC (Actinobacteria), α-Proteo (Alfaproteobacteria), β-Proteo (Betaproteobacteria), γ-Proteo (Gamaproteobacteria), Arch (Archea), CF (Cytophaga-Flavobacterium); independent variables = Conduct (conductivity), PO_4^{3-} (phosphates), NO_3^- (nitrates), TS (total solids), VS (volatile solids), Chlor a (chlorophyll *a*) TDAPI, HGC, Arch, γ-Proteo, β-Proteo, α-Proteo.

Dependent variable	**Independent variables**	**Regression equation**	**Adj. R^2**
TDAPI	Conduct (β=0.817; p=0.000)	TDAPI= $-3x10^{11} + 6x10^8$Conduct	0.64
Arch	γ-Proteo (β=0.546; p=0.000)	Arch= $-2x10^9$ + 0.387γ-Proteo + $3x10^8$VS – $7x10^{10}$Chlor a	0.95
	VS (β=1.082; p=0.000)		
	Chlor a (β=-0.676; p=0.000)		
Bacteria	Conduct (β=0.749; p=0.001)	Eub= $-2x10^{11} + 4x10^8$Conduct	0.53
α-Proteo	HGC (β=1.272; p=0.000)	α-Proteo= $2x10^9$ + 0.746HGC – 0.488Arch	0.85
	Arch (β=-0.635; p=0.001)		
β-Proteo	Arch (β=0.756; p=0.000)	β-Proteo= $3x10^9$ + 1.120Arch + $6x10^{11}$ PO_4^{3-}	0.72
	PO_4^{3-} (β=0.333; p=0.038)		
Log γ-Proteo	Conduct (β=0.922; p=0.000)	Logγ-Proteo=1.855 + 0.013 Conduct	0.84
LogHGC	Conduct (β=0.571; p=0.010)	LogHGC= 7.026 + 0.005 Conduct + 32.066 PO_4^{3-}	0.52
	PO_4^{3-} (β=0.429; p=0.041)		
Cya	PO_4^{3-} (β=0.783; p=0.001)	Cya= $3x10^{10} + 8x10^{11}$ PO_4^{3-} - $4X10^7$Conduct	0.62
	Conduct(β=-0.407; p=0.031)		

Proteobacteria group was well represented in this study. *Gamma-Proteobacteria* was related to conductivity, which explained 84% of the variability of the relative abundance of this group. More than 70% of the variability of *β-Proteobacteria* was explained by the relation with the abundance of *Archaea* and the availability of PO_4^{3-} (Table 4). *AlphaProteobacteria* was mainly regulated by biotic factors through interactions with other phylogenetic groups, which explained 85% of the variability. In this study, the organisms whose DNA contains high (>55%) levels of guanine plus cytosine residues (HGC) were also counted, accounting for 14% of the total counts (Table 2). Conductivity and PO_4^{3-} concentration explained 52% of the observed variability, indicating that it responds mainly to shifts in the chemical environment.

DISCUSSION

Cyanobacteria were generally well represented. *Aphanizomenon flos-aquae* and others nitrogen fixing cyanobacteria are highly dependent of phosphorus availability, high water temperature and conductivity (de Figueiredo *et al*., 2004). In this study, the cyanobacterial populations that could be detected with the Cya361 probe were mostly regulated by the chemical environment since the concentration of PO_4^{3-} and conductivity explained 62% of the variability (Table 4). Cyanobacterial blooms are associated not only to the increased availability of organic matter but also to the production of toxic substances and therefore the interaction with other prokaryote groups may be extremely complex (Pflugmacher, 2002). The major development of the bloom of primary producers occurred during the second half of the sampling period with the occurrence of peaks of chlorophyll *a*, total and volatile solids (Figure 3) and an increase in water temperature and conductivity (Figure 1). Nitrate concentration (Figure 4) was reduced to low levels during the first days of the development of the bloom and oversaturation of dissolved oxygen was also observed (Figure 2). In the phytoplankton quantification obtained by direct microscope counts it was not verified a marked variation in the cells number during the sampling period (Figures 6 and 7). This is probably due to two major technical aspects: the sample sedimentation, so the fluctuant phytoplankton organisms are not counted; the organism size must be higher then 5µm, thus all the smaller organisms were not visualized and therefore not accounted. This approach only demonstrated the phytoplankton successions during the study period.

During this study were found a marked increase in the number of culturable bacteria (Figure 5) associated with the peak of chlorophyll *a*, which indicated the establishment of bloom conditions during the second half of the sampling period. This development was associated to an increase availability of organic matter that can also be inferred from the high concentration of volatile solids (Figure 3).

Culture independent approaches such as FISH allow a deeper insight into dynamics of the whole prokaryote assemblage during the phytoplankton bloom. Total prokaryote abundance counts (DAPI) increased just before the bloom, probably responding to an increase in conductivity and in the availability of inorganic nutrients. The values of abundance remained high during the bloom when algal biomass were probably used as a major source of substrates for heterotrophic bacteria. The prokaryote community was dominated by *Bacteria*.

Archaea were present in all samples in low numbers such as generally found in surface waters of freshwaters systems (Briee *et al.,* 2007). The negative relation between *Archaea* and chlorophyll *a* in the corresponding regression model, may indicate that the former decrease in relative importance under bloom conditions. Since Bacteria account for majority of DAPI counts it is expected that more then 50% of the variability in both counts can be explained by the same parameter (conductivity).

Heterotrophic bacteria constitute an important part of the pelagic food web by mineralizing dissolved organic carbon and converting dissolved matter into particulate biomass available to higher trophic levels through the microbial loop (Azam *et al*., 1983) with bacterial activity levels and population dynamics embedded in a web of changing ecological and biological relationships. Factors such as the concentration of inorganic nutrients (Toolan *et al*., 1991; Thingstad and Lingnell, 1993; Torréton *et al*. 2000, Ferrier-Pagès and Furla, 2001) and the phytoplankton biomass (Williams, 1990; White *et al.*, 1991; Panzenbock *et al*., 2000) are involved in the regulation of bacterial growth and abundance, influencing the success of different groups and ultimately the structure of the prokaryote communities. Several field and laboratory studies have shown that during the distinct phases of phytoplankton blooms considerable shifts in bacterioplankton community dynamics can occur (Fandino *et al.*, 2001, Pinhassi *et al*., 2004, Riemann *et al*., 2000). *Cytophaga* are known to be important degraders of biomacromolecules. Due to their high degradative capacity, they are potentially the major contributors to the organic matter degradation, being possible that different types were physiologically better able to utilize organic matter available at different bloom stages (Fandino *et al.*, 2001). During a phytoplankton bloom, where high intense polymer and particle enrichment are expected, high abundance of this group is accepted. This fact is in accordance with the results obtained in this study, although the authors did not related with the biotic and abiotic factors studied. *Alpha, β* and *γ -Proteobacteria* include nitrifying bacteria, which are able to grow chemolithotrophically at the expense of reduced inorganic compounds. The nitrification process of ammonia, in nature, results from the sequential action of two separate groups of organisms, the ammonia-oxidizing bacteria (Nitrosomonas and Nitrosococcus) and the nitrite-oxidizing bacteria (*Nitrobacter*). Nitrifying bacteria (such as *α-Proteobacteria*) are widespread in habitats where considerable amounts of ammonia are present, such as sites where expensive protein decomposition occurs (ammonification) (Madigan *et al.*, 2000). Pinhassi *et al.* (2004) observed that *α-Proteobacteria* respond positively to nutrient increase. This fact is in accordance with the

results obtained in the present study, since this group gains importance when the concentrations of total and volatile solids, chlorophyll *a* (Figure 3) and phytoplankton (Figure 6 and 7) are rising.

The regression analysis revealed that the variability in the abundance of distinct prokaryote phylogenetic groups could, in some cases, be mostly regulated by chemical factors, such as for the globality of the bacterial domain (*Eubacteria*) the *γ* subdivision of *Proteobacteria*, the *Actinobacteria* group and the *Cyanobacteria* or, in other cases, by biological interactions with other groups such as for *α-Proteobacteria* or even by both type of factors, such as for Archaea and *β-Proteobacteria*, indicating a dual regulation by biotic and abiotic factors. Remarkingly, the abundance of cyanobacteria was not a relevant factor in the regression models used for the explanation of the variability of other prokaryote groups. This can be interpreted as an indication that, in this study, during the phytoplankton bloom, prokaryote communities interact with primary producers mostly by using DOM-rich exudates, inferred from significant relations found with chlorophyll *a*, or by competing with bloom primary producers for inorganic nutrients and phosphate.

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Capítulo IV

Discussão geral

A eutrofização de sistemas aquáticos é frequentemente causada por excesso em nutrientes associados a descargas de efluentes e à lixiviação de fertilizantes agrícolas. No entanto, também pode ocorrer naturalmente, quando existe acumulação de nutrientes (Spatharis *et al*., 2007). As actividades humanas podem acelerar este processo, dado que aumentam o fluxo de nutrientes inorganicos e organicos para o ecossistema.

Os lagos pouco profundos encontram-se especialmente em risco de se tornarem eutróficos, uma vez que estão sujeitos a efeitos *top-down* (Blanco *et al*., 2003; Gyllstrom *et al*., 2005).

No trabalho realizado foi possível observar as relações tróficas existentes entre fitoplancton, bacterioplancton e vírus e avaliar quais os factores reguladores do fitoplancton, no sistema eutrofizado da Lagoa da Vela. Assim, podem ser enumerados três factores: nutricionais, alelopáticos e virais.

- **Factores nutricionais**

Vários estudos laboratoriais e de campo (Williams, 1990; White *et al*., 1991; Panzenbock *et al*., 2000) correlacionam a biomassa de fitoplâncton, bacterioplâncton e produção bacteriana. Panzenbock e colaboradores (2000) concluíram que as bactérias podem, receber directamente matéria orgânica do fitoplâncton, quer através da exsudação por parte de células saudáveis, quer através da lise celular. Não obstante, estes mesmos factores influenciam o sucesso de determinados grupos e, consequentemente, a estrutura da comunidade procariota.

Utilizando abordagens independentes de cultivo bacteriano, como a técnica de FISH, é possível avaliar a dinâmica da comunidade procariota de uma forma mais abrangente. Assim, foi possível verificar um aumento da abundância de procariotas após o *bloom*, período em que também foi verificado um aumento da condutividade e dos nutrientes disponíveis. Dado que estes valores se mantiveram em níveis elevados durante o *bloom*, é possível inferir que a biomassa de fitoplâncton serviu de fonte primordial de substratos para as bactérias heterotróficas. Esta tendência está de acordo com o observado em outros estudos (Sundh, 1992) que verificaram que o carbono orgânico dissolvido (DOC) libertado pelo fitoplâncton é uma importante fonte de carbono para as bactérias heterotróficas.

Tem sido demonstrado que as bactérias heterotróficas competem, com sucesso, por nutrientes inorgânicos com o fitoplâncton (Thingstad *et al*., 1993). As bactérias têm, aproximadamente, potenciais de absorção de nutrientes inorgânicos 100 vezes mais elevados que o fitoplâncton (Blackburn *et al*., 1998) com baixas concentrações de nutrientes inorgânicos, a vantagem competitiva do bacterioplâncton sobre o fitoplâncton, é uma ideia consensual (Torréton *et al*., 2000). A competição entre bactérias e fitoplâncton por nutrientes inorgânicos dissolvidos levanta algumas questões, apontadas por Kirchman (1994). Limitações nutritivas severas podem resultar na produção (ou eventual libertação) de matéria organica com elevados rácios de C:N e C:P. A consequente utilização destes materiais pelas bactérias plantónicas irá gerar uma necessidade intracelular de azoto (ou fósforo), estimulando assim a absorção de nutrientes orgânicos dissolvidos pelas bactérias (Caron, 1994). Assim, dependendo da velocidade a que a absorção ocorre, a vantagem será ou das bactérias ou do fitoplâncton.

Associações entre fitoplâncton e bactérias ocorrem quer durante a fase crescimento, quer durante a fase de senescência de um *bloom*. Estas interacções podem conduzir a uma optimização paralela dos potenciais de crescimento entre bactérias e fitoplâncton, podendo também reflectir adaptações ecológicas e fisiológicas mutuamente benéficas (Gons *et al.* 2002; de Figueiredo *et al.,* 2006). O fitoplâncton, e em particular as cianobactérias, pode também tirar vantagem da associação, uma vez que é sensível à deficiente disponibilidade de CO_2 (Pearl, 1996). As bactérias associadas à ficoesfera, podem suprir esta deficiência através do processo de mineralização, que recicla o CO_2. As bactérias da ficoesfera podem ainda aumentar os potenciais de fixação de N_2 de cianobactérias fixadoras de azoto (Pearl, 1996), tornando-as assim mais eficazes sobre outras que também tenham a mesma capacidade, mas não tão apurada.

Durante diferentes fases do *bloom*, ocorrem alterações na dinâmica da comunidade de bacterioplâncton (Fandino *et al*., 2001; Pinhassi *et al*., 2004; Riemann *et al*., 2000). O género *Cytophaga*, conhecido pela sua elevada capacidade de degradação de matéria orgânica em diferentes fases de *bloom* (Fandino *et al.,* 2001), manteve-se bem representado durante todo o período de amostragem.

O Filo *Proteobacteria* inclui as bactérias nitrificantes (*α, β e γ*). Estas estão amplamente distribuídas em habitats onde existam elevadas quantidades de amónia, ou seja, onde ocorra elevada decomposição proteica (Madigan *et al*., 2000). Durante o período

de amostragem, *α-Proteobacteria* apresentou abundância crescente coincidente com aumentos de sólidos voláteis e totais e abundância de fitoplâncton. Isto está de acordo com observações de outros autores (Pinhassi *et al.,* 2004) que demonstram relações positivas deste subgrupo com aumentos de nutrientes inorgânicos.

A análise de regressão permite inferir que, durante o *bloom*, as comunidades procariotas interagem com os produtores primários usando os exsudados de matéria orgânica fornecidos pela comunidade fitoplanctónica.

- **Factores virais**

Os vírus são parasitas obrigatórios que podem determinar a diversidade das espécies bacterianas (Weinbauer & Rassoulzadegan, 2004). Embora sejam agentes infecciosos de pequenas dimensões, a sua elevada abundância atribui-lhes um papel importante no controlo estrutura e função das comunidades bacterianas dos ecossistemas de água doce (Manage *et al*., 1999; Weinbauer, 2004). A sua especificidade em relação aos hospedeiros implica que possam alterar significativamente a estrutura das comunidades de bactérias e microalgas.

Devido a grandes avanços técnicos durante os anos 90, verificou-se que, nos sistemas aquáticos, estes organismos constituem uma componente dinâmica e significativa das comunidades microbianas (Suttle *et al.,* 1990). O número de vírus num sistema aquático normalmente excede de 10 a 100 vezes mais o numero de bactérias (Hennes *et al.*, 1995). Estima-se que, diariamente, aproximadamente 20% de bactérias heterotróficas e 51% de cianobactérias são destruídas por lise viral (Fuhrman & Suttle, 1993; Suttle, 1993). Desta forma, os vírus desempenham papéis importantes no ciclo de nutrientes e na estruturação da comunidade microbiana (Fuhrman, 1999; Fuhrman, 2000).

Durante o *bloom* de fitoplâncton, verificou-se uma correlação positiva entre a abundância de vírus e bactérias, tal como o descrito para outros sistemas aquáticos (Wommack & Colwell, 2000; Fischer & Velimirov, 2002; Bettarel *et al.,* 2003; Vrede *et al*., 2003). Por outro lado, existem vários estudos (Boehme *et al.,* 1993; Maranger & Bird, 1995; Weinbauer *et al*., 2004) que apontam uma correlação entre os vírus e a concentração de clorofila *a*. Baixos rácios de vírus/bactérias, frequentemente observados neste estudo, parecem indicar que, durante um *bloom*, uma grande fracção de vírus infecta células de

fitoplâncton. Desta forma, e de acordo com o descrito por Maranger & Bird (1995), o fitoplâncton pode representar um reservatório hospedeiro importante para o virioplâncton. No entanto, os valores médios de viruses-to-bacteria ratios (VBR) obtidos encontram-se muito abaixo do esperado para este tipo de sistema (Wommack & Colwell, 2000). Assim, é possível aferir que a dinâmica viral pode ter resultado da contribuição conjunta de bacteriófagos, cianófagos e vírus parasitas de algas.

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