Universidade de Aveiro Departamento de Biologia
2003

Daniela Rebelo de Figueiredo

Produção de cianotoxinas em meio limitado em nutrientes e acção alelopática em organismos de dois níveis tróficos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia Molecular, realizada sob a orientação científica do Prof. Doutor Mário Jorge Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

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resumo

O aumento da eutrofização nos sistemas hídricos superficiais devido à intensificação das actividades antropogénicas e às alterações climáticas (aumento da temperatura e luminosidade) tem favorecido o aparecimento de *blooms* (ou florescências) superficiais de cianobactérias. Estes *blooms* são potencialmente perigosos devido à capacidade de várias estirpes cianobacterianas produzirem toxinas nocivas para diversos grupos de organismos, desde bactérias até humanos. A microcistina, em particular, é uma hepatotoxina que coloca sérios riscos para a Saúde Pública devido à sua capacidade de promover cancro por ingestão crónica de pequenas quantidades na água para consumo humano. As estirpes produtoras de microcistina pertencem essencialmente aos géneros *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc*, *Anabaenopsis* e *Aphanizomenon*, sendo a sua toxicidade determinada primariamente pela diversidade genotípica entre estirpes, embora possa haver também influência de factores ambientais. O estudo laboratorial da ecologia de estirpes potencialmente produtoras de microcistina pode permitir o aperfeiçoamento de estratégias de gestão dos sistemas aquáticos, de forma a controlar, ou mesmo evitar, a ocorrência deste tipo de fenómenos. Uma primeira parte do presente trabalho consistiu numa pesquisa bibliográfica relativamente exaustiva acerca da investigação realizada nos domínios da ocorrência, toxicidade e síntese de cianotoxinas, com especial relevância para a microcistina. Uma segunda fase incluiu o estudo da dinâmica fitoplanctónica num sistema aquático eutrofizado (integrando os resultados com dados físico-químicos), em que ocorreu um grande *bloom* dominado pela cianobactéria *Aphanizomenon flos-aquae*, potencialmente produtora de microcistina. Uma terceira parte pretendeu estudar, em laboratório, a estirpe de *A. flos-aquae* isolada do *bloom*, relativamente ao crescimento em diferentes concentrações de nitratos e fosfatos. A parte final do trabalho referiu-se ao estudo dos efeitos desta estirpe no crescimento de microalgas (*Chlorella vulgaris* e *Pseudokirchneriella subcapitata*) e na sobrevivência e reprodução de cladóceros (*Dapnhia magna* e *D. longispina*). O desenvolvimento do *bloom* de *A. flos-aquae* foi relacionado com os valores mais baixos de nitratos, nitritos e amónia. Dos estudos laboratoriais de nutrição pôde concluir-se que o crescimento desta estirpe cianobacteriana depende da disponibilidade de fosfatos, mas não da de nitratos, provavelmente devido à sua capacidade de fixação de azoto. O crescimento das espécies fitoplanctónicas testadas parece ser afectado pelos exudatos de *A. flos-aquae*. Quando sujeitos a uma alimentação exclusiva com esta estirpe, os cladóceros mostraram ser afectados na sua sobrevivência e reprodução, principalmente com *A. flos-aquae* cultivada em concentrações superiores de fosfatos. A partir dos resultados obtidos neste estudo, pode sugerir-se que o desenvolvimento de *blooms* desta estirpe cianobacteriana é independente da indisponibilidade em nitratos, mas favorecido por elevadas concentrações em fosfatos. Assim, o controlo das entradas de fosfatos (provenientes de efluentes agro-pecuários, por exemplo) para os sistemas aquáticos seria um importante factor para evitar o desenvolvimento de *blooms* potencialmente tóxicos desta estirpe e suas consequências nos diversos níveis tróficos, nomeadamente aos níveis do fitoplâncton e do zooplâncton.

abstract

The increasing eutrophication of superficial water bodies, due to the intensification of anthropogenic activities, along with climate changes towards higher temperature and light conditions, enhance the massive development of cyanobacteria in water bodies leading to formation of blooms frequently accumulating as surface scum. Some cyanobacterial blooms may become dangerous because there are many strains of cyanobacteria capable of producing toxins that affect many organisms from bacteria to humans. Microcystin, in particular, is a hepatotoxin that poses a serious Public Health risk due to its potential of promoting cancer through chronic ingestion of small quantities in drinking water. The microcystin producing strains mainly belong to the genera *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc*, *Anabaenopsis* and *Aphanizomenon*, and its toxigenicity seems to be primarily determined by genotype diversity among strains, although some environmental factors are known to influence microcystin biosynthesis. The ecological laboratory studies of potentially microcystin producing cyanobacterial strains may allow the improvement of water management strategies to control or even avoid the occurrence of this kind of phenomena. The first step of the present work consisted in an exhaustive compilation of the investigation recently made on the occurrence, toxicity and synthesis of cyanotoxins, with special regard to microcystin. A second part of the work included the study of the phytoplankton dynamics in an eutrophied water body (integrating biological and physico-chemical data) where a bloom of the potentially microcystin producer *Aphanizomenon flos-aquae* occurred. On a third phase, in laboratory, the *A. flos-aquae* strain was isolated from the bloom and grown in different nitrate and phosphate concentrations. The final part of the study aimed to assess the effects of this strain on the growth of other microalgae (*Chlorella vulgaris* and *Pseukirchneriella* subcapitata) and on survival and reproduction of cladocerans (*Dapnhia magna* and *D. longispina*).The *A. flos-aquae* bloom development was related to the lowest values of nitrogen sources: nitrate, nitrite and ammonium. From the laboratory nutritional experiments it could be concluded that this cyanobacterial strain growth depends on the availability of phosphate but not nitrate, probably due to its nitrogen fixing capability. The growth of the microalgae tested showed to be affected by the exudates of *A. flos-aquae*. When this strain was given as an exclusive food source, the tested cladocerans showed to be affected in their survival and reproduction, particularly with *A. flos-aquae* grown with higher phosphate concentrations. After the results obtained in this work, it can be suggested that the bloom development of this cyanobacterial strain is independent of nitrate unavailability but favoured by high phosphate concentrations. Thus, the control of the phosphate inflow (from the agriculture and animal farming effluents, for example) into the superficial water bodies would be an important factor to avoid the development of potentially toxic blooms of this strain and its consequences at different trophic levels, namely at phytoplankton and zooplankton levels.

Aos que adoçam o meu lar...

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Capítulo I

Introdução geral

Introdução geral

1. Eutrofização, blooms de cianobactérias e cianotoxinas

Actualmente, o crescimento da população mundial e a consequente intensificação das actividades industrial e agrícola (Cooperband and Good, 2002; de Jonge *et al.*, 2002; Withers and Lord, 2002), juntamente com uma gestão ineficiente dos sistemas hídricos (Codd, 2000), têm sido os principais responsáveis pelo aumento da eutrofização nos sistemas aquáticos superficiais (muitos destes utilizados para fins recreativos ou como reservatórios de água para abastecimento público). Mas, além da poluição orgânica (com o enriquecimento em nutrientes), outros factores como valores elevados de temperatura e pH, luminosidade intensa e pouca turbulência, que coincidem com os meses mais quentes do ano, estimulam o desenvolvimento de microalgas planctónicas, nomeadamente cianobactérias (Hadas *et al.*, 1999; Jacoby *et al.*, 2000; Oliver and Ganf, 2000). Adicionalmente, muitas cianobactérias possuem algumas características como a fixação de azoto (Flores and Herrero, 1994), a regulação da flutuação (Brookes and Ganf, 2001; Porat *et al.*, 2001) e uma reduzida taxa de predação pelo zooplâncton (Kurmayer and Jüttner, 1999; Henning *et al.*, 2001), que lhes permitem ter sucesso sobre os outros grupos de microalgas, em condições menos favoráveis. Assim, estas microalgas multiplicam-se até formar *blooms* (ou florescências) que se acumulam à superfície da água numa espessa camada de material celular cianobacteriano de cor azul esverdeada. Estes *blooms* planctónicos resultam geralmente na redução da diversidade específica a diversos níveis tróficos devido à depleção do oxigénio dissolvido na água, à deterioração do habitat pelo aumento dos sólidos em suspensão, à produção de substâncias que dão mau gosto e odor à água e/ou ainda à produção de compostos tóxicos por certas estirpes cianobacterianas. São conhecidas mais de 40 espécies de cianobactérias que possuem estirpes produtoras de toxinas (Dow and Swoboda, 2000) e uma elevada percentagem dos *blooms* de cianobactérias que ocorrem nos sistemas de água superficiais em todo o mundo é tóxica (Codd *et al.*, 1995; WHO, 1998b; Codd, 2000; Dow and Swoboda, 2000). As cianotoxinas já provaram ser nocivas para muitos organismos, incluindo humanos (Gorham and Carmichael, 1988; Codd *et al.*, 1995; Pouria *et al.*, 1998; Codd, 2000). São normalmente classificadas em dermatotoxinas, neurotoxinas e hepatotoxinas (Kaebernick and Neilan, 2001), segundo o efeito que provocam em animais. Do ponto de vista químico, as dermatotoxinas podem ser lipopolissacáridos ou alcalóides (lingbiatoxina-a e aplisiatoxinas); as neurotoxinas incluem alcalóides (anatoxina-a, homoanatoxina-a, saxitoxinas e neo-saxitoxinas) e o organofosfato anatoxina-a(s); e, por fim, as hepatotoxinas, mais frequentes e perigosas, podem ser alcalóides (cilindrospermopsina)

ou péptidos cíclicos (nodularina ou microcistina) (Codd, 2000; Dow and Swoboda, 2000; Kaebernick and Neilan, 2001; Nicholson and Burch, 2001). Existem ainda outras cianotoxinas já conhecidas (Dow and Swoboda, 2000) mas, provavelmente, muitas outras haverá por conhecer.

A ocorrência de *blooms* de cianobactérias nos reservatórios de água utilizados para abastecimento público, em particular, acarreta consequências económicas significativas devidas à deterioração da qualidade da água (mau sabor e odor (Park, 2001)), bloqueio dos filtros utilizados no tratamento da água e necessidade de processos de tratamento da água mais eficientes (Rositano *et al.*, 2001; Maatouk *et al.*, 2002) para remover as cianotoxinas que possam colocar em perigo a saúde dos consumidores. Em águas utilizadas para fins recreativos, a ocorrência de *blooms* de cianobactérias conduz geralmente a um decréscimo do turismo nesses locais devido à perda da qualidade da água e ao perigo de intoxicações provocadas pelas cianobactérias se houver contacto directo com a água contaminada, pelo banho ou pela prática de desportos náuticos (WHO, 1998a). Existe ainda o perigo da bioacumulação de toxinas pelos produtos agrícolas utilizados para consumo humano (Codd *et al.*, 1999), quando a irrigação é feita com água proveniente de um sistema aquático em que ocorra um *bloom* de cianobactérias. Assim, torna-se essencial a monitorização regular das águas para prever e prevenir este tipo de fenómenos.

2. Microcistina

2.1. Caracterização estrutural

A microcistina é uma substância muito estável em água destilada ou esterilizada, resistindo à irradiação solar (Dawson, 1998), a valores extremos de pH e a temperaturas muito elevadas (>300ºC) (WHO, 1998b). É um heptapéptido cíclico cuja estrutura geral é *cyclo(-D-Ala-L-X-erythro-β-methyl-D-Adda-D-isoGlu-N-methyldehydro-ala)*, sendo X e Z L-aminoácidos variáveis e Adda (*3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid*) o aminoácido considerado responsável pela hepatotoxicidade da molécula (Dawson, 1998). São conhecidas mais de 60 isoformas da microcistina (Codd, 2000; Dow and Swoboda, 2000), em parte devido aos L-aminoácidos variáveis, sendo a microcistina-LR (MC-LR) a variante de microcistina mais frequente e estudada, com os aminoácidos leucina (L) e arginina (R).

2.2. Síntese – via de produção, espécies produtoras, factores influentes

A microcistina é um metabolito secundário produzido via não ribossomal e a sua síntese parece ser um processo dependente de energia (ATP) (Bickel and Lyck, 2001). O complexo enzimático da sintetase da microcistina é codificado por um *cluster* de genes *mcy* composto por dois operões (*mcyA-C* e *mcyD-J*) (Kaebernick and Neilan, 2001). Este *cluster* está presente nas estirpes tóxicas pertencentes ao género *Microcystis*, mas também em estirpes produtoras de microcistina dos géneros *Anabaena*, *Nostoc* (Neilan *et al*., 1999) e *Planktothrix* (Christiansen *et al.*, 2003), permitindo o desenvolvimento de métodos rápidos e sensíveis, baseados na PCR (*Polymerase Chain Reaction*), para a sua detecção directamente a partir das amostras ambientais (Tillet *et al.*, 2001; Pan *et al.*, 2002).

Relativamente à função desta cianotoxina, ainda existe pouca informação publicada, mas alguns resultados indicam que possa actuar como uma defesa química contra a predação pelo zooplâncton (Laurén-Määttä *et al.*, 1997; Kurmayer and Jüttner, 1999; Henning *et al.*, 2001) ou ter efeito alelopático sobre outras microalgas competidoras (Kearns and Hunter, 2001), podendo ainda funcionar como regulador endógeno das fosfatases ou utilizada como reserva de azoto.

As estirpes produtoras de variantes de microcistina ocorrem nos sistemas hídricos à escala global e pertencem geralmente aos *taxa Microcystis* spp., *Anabaena* spp., *Planktothrix/Oscillatoria* (*P. agardhii* e *P. rubescens*), *Anabaenopsis*, *Nostoc* (*N. rivulare*) e *Aphanizomenon* (*A. flos-aquae*), embora possam ser também produzidas por estirpes do género terrestre *Hapalosiphon* (Codd *et al.*, 1995; Dow and Swoboda, 2000; Kaebernick and Neilan, 2001).

Apesar dos resultados contraditórios obtidos por diferentes estudos, a síntese de microcistina parece ser influenciada por diversos factores ambientais como macronutrientes (Lee *et al.*, 2000; Kotac *et al.*, 2000; Long *et al.*, 2001; Vézie *et al.*, 2002), micronutrientes (Utkilen and Gjølme, 1995), temperatura (Rapala and Sivonen, 1998) e luminosidade (Kaebernick *et al.*, 2000; Wiedner *et al.*, 2003), embora a diversidade genotípica entre estirpes seja o factor mais determinante na distinção da toxicidade entre *blooms* da mesma espécie (Hesse and Kohl, 2001; Rohrlack *et al.*, 2001; Kurmayer *et al.*, 2002; Mikalsen *et al.*, 2003). Relativamente aos macronutrientes, em *M. aeruginosa* (espécie não fixadora de azoto), o conteúdo em MC parece aumentar para razões de N:P mais elevadas (Utkilen and Gjølme, 1995; Lee *et al.*, 2000), embora Long *et al.* (2001) tenham obtido resultados que mostram que, em condições de limitação em azoto, as células de *M. aeruginosa* atingem um tamanho menor, mas aumentam

consequentemente os valores de conteúdo celular em MC. Ainda para *M. aeruginosa*, o conteúdo em MC aumenta com concentrações de fósforo mais elevadas (Kotak *et al.*, 2000; Jacoby *et al.*, 2000), apesar dos resultados de um estudo efectuado por Oh *et al*. (2000) mostrarem que os valores mais elevados para o conteúdo em MC ocorriam em condições de limitação de fósforo. Embora existam resultados controversos, a síntese de microcistina em estirpes de *M. aeruginosa* tem mostrado ser influenciada pela variação das concentrações de ambos estes nutrientes, mas com diferentes respostas para cada estirpe (Vézie *et al.*, 2002). Para as espécies fixadoras de azoto pertencentes ao género *Anabaena*, o azoto sob a forma de nitratos parece aumentar a produção de MC (Rapala *et al.*, 1997), embora em meio com indisponibilidade de azoto estas espécies sejam capazes de continuar a produzir microcistina. No entanto, para *Anabaena* spp., o conteúdo em MC parece estar mais dependente da concentração de fósforo, aumentando com concentrações crescentes deste nutriente (Rapala *et al.*, 1997).

2.3. Toxicidade em organismos e perigo para a Saúde Pública

As variantes de microcistina podem ser tóxicas para muitos organismos (Hiripiri *et al.*, 1998; Fischer and Dietrich, 2000; McElhiney *et al.*, 2001; Liu *et al.*, 2002; Romanowska-Duda and Tarczynska, 2002; Hamvas *et al.*, 2003) e ser bioacumuladas por outros (Amorim and Vasconcelos, 1999; Codd *et al.*, 1999; Magalhães *et al.*, 2001; Pflugmacher *et al.*, 2001; Vasconcelos *et al.*, 2001; Wiegand and Pflugmacher, 2001; Mohamed *et al.*, 2003), sugerindo a possibilidade de transferência pela cadeia trófica e o risco de exposição humana à toxina por consumo de alimentos contaminados.

Particularmente relevantes para o presente trabalho são os efeitos da microcistina sobre os organismos fitoplanctónicos e zooplanctónicos, pertencentes a dois níveis tróficos distintos. Certas microalgas parecem sofrer um efeito alelopático por algumas estirpes produtoras de microcistina (Kearns and Hunter, 2001), embora não haja muitas publicações recentes acerca deste assunto. A clorófita *Chlamydomonas reinhadtii* é paralisada na presença de MC-LR, que favorece a sua sedimentação, permitindo às estirpes cianobacterianas produtoras desta variante de microcistina criar no sistema aquático uma zona livre de algas competidoras (Kearns and Hunter, 2001). Relativamente ao zooplâncton, existem diversos grupos que são afectados pela ocorrência de *blooms* de estirpes cianobacterianas produtoras de microcistina, seja pelas condições nutritivas desfavoráveis devidas à falta de alimento alternativo (outras microalgas), aquando da sua dominância (Kurmayer and Jüttner, 1999), e ao seu baixo valor nutritivo (Brett and Müller-Navarra, 1997; Brett *et al.*, 2000), ou à dificuldade de

ingestão pelo zooplâncton (devida ao tamanho dos filamentos ou colónias e/ou à produção de mucilagem (Rohrlack *et al.*, 1999; Henning *et al.*, 2001)) ou ainda à produção de toxinas (Laurén-Määttä *et al.*, 1997; Kurmayer and Jüttner, 1999; Lotocka, 2001). No entanto, os cladóceros mostraram ser capazes de ingerir estirpes tóxicas e não tóxicas de *Microcystis* (Rohrlack *et al*., 1999), na ausência de alimento alternativo (clorófitas e diatomáceas), podendo acumular a microcistina (Mohamed, 2001) e potencialmente transferi-la para níveis tróficos superiores através da cadeia alimentar. Mas além de bioacumulada, a microcistina pode provocar efeitos tóxicos sobre várias espécies de *Daphnia* após a ingestão de cianobactérias com microcistinas (Lauren-Määtä *et al.*, 1997; Rohrlack *et al.*, 1999; Rohrlack *et al.*, 2001).

A investigação acerca da microcistina, no que respeita à sua ocorrência, à ecologia das estirpes produtoras e aos processos e genes responsáveis pela sua síntese, torna-se cada vez mais pertinente para permitir o desenvolvimento de estratégias de monitorização, prevenção e controlo da sua produção. A relevância deste tema surge essencialmente devida ao potencial perigo que esta hepatotoxina representa para a Saúde Pública. Com base em alguns estudos epidemiológicos em humanos, mas principalmente nos muitos estudos laboratoriais efectuados em mamíferos, sabe-se que a microcistina é uma toxina selectiva para as células hepáticas, inibindo irreversivelmente as fosfatases PP1 e PP2A (Dawson, 1998), provocando, por exposição aguda, a desintegração da estrutura hepática, apoptose, necrose do fígado e hemorragia interna hepática, podendo levar à morte por choque hemorrágico (Dow and Swoboda, 2000). Os sintomas incluem fraqueza, anorexia, extremidades frias, palidez, apatia, dificuldades respiratórias, gastroenterite, vómitos e diarreia (Codd *et al.*, 1995; Codd, 2000; Dow and Swoboda, 2000). Em 1996, no Brasil, muitas pessoas morreram num centro de hemodiálise devido à exposição directa do sangue, durante o tratamento, a água contaminada por microcistina (Pouria *et al.*, 1998). A toxicidade por inalação da toxina parece ser quase tão elevada quanto a intoxicação por contacto directo com o sangue, mas a toxicidade é muito menor por ingestão oral de água ou de alimentos contaminados (WHO, 1998b). No entanto, a microcistina já provou ser também promotora do desenvolvimento de cancros em humanos, por exposição crónica prolongada das pessoas a baixas concentrações desta toxina, nomeadamente através da água que ingerem diariamente (Ueno *et al.*, 1996; Zhou *et al.*, 2002). Daí, a preocupação da OMS (Organização Mundial de Saúde) em estabelecer 1 μg MC-LR.L^{-1} como o valor recomendado para o nível desta cianotoxina nas águas utilizadas para consumo humano (WHO, 1998b).

Até ao momento, não há ainda tratamentos eficazes comprovados contra as intoxicações provocadas pelas variantes de microcistina, apesar de já existirem alguns resultados interessantes (Dawson, 1998; Fitzgerald, 2001). Assim, apesar do aperfeiçoamento dos métodos de tratamento da água (Morris *et al.*, 2000; Gajdek *et al.*, 2001; Pendleton *et al.*, 2001; Shephard *et al.*, 2002; Yuan *et al.*, 2002), o melhor será ainda evitar a ocorrência de *blooms* de estirpes produtoras de microcistina, de forma a afastar a possibilidade de potenciais intoxicações.

Neste momento, torna-se necessária uma gestão adequada dos sistemas hídricos no que respeita à entrada de nutrientes (especialmente fosfatos e nitratos) e, adicionalmente, o desenvolvimento de uma sensibilização integrada do público, agricultores e industriais relativamente aos potenciais efeitos perigosos para a Saúde Pública, resultantes da introdução excessiva desses nutrientes no meio aquático.

3. Objectivos

Além da monitorização das águas superficiais (especialmente os reservatórios de água para consumo humano) relativamente à formação de *blooms* potencialmente tóxicos, torna-se também necessário desenvolver, em simultâneo, estudos laboratoriais de ecologia, de forma a compreender os processos que conduzem ao desenvolvimento de *blooms* de uma determinada estirpe cianobacteriana e seus efeitos em organismos de diferentes níveis tróficos. Deste modo, poder-se-á contribuir para uma melhor avaliação dos potenciais riscos ecológicos associados a *blooms* dessa estirpe, assim como para uma previsão atempada da sua ocorrência e o desenvolvimento de melhores estratégias de prevenção do seu aparecimento e para o seu controle.

E é neste contexto que se inserem os objectivos do presente trabalho:

- pesquisar bibliografia referente à investigação de *blooms* tóxicos de cianobactérias, no que respeita à sua ocorrência, toxicidade e produção de cianotoxinas, com especial relevo para a microcistina;
- estudar a dinâmica da comunidade fitoplanctónica de uma lagoa eutrofizada (Lagoa da Vela), ao longo de um ciclo anual, relacionando parâmetros físico-químicos com a ocorrência de *blooms* de cianobactérias;
- analisar o crescimento de uma estirpe de *Aphanizomenon flos-aquae*, isolada a partir de um *bloom* de uma lagoa eutrofizada (Lagoa da Vela), quando sujeita a condições de limitação em azoto e fósforo;
- estudar o efeito da mesma estirpe referida acima sobre o crescimento de espécies fitoplanctónicas (*Pseudokirchneriella subcapitata* e *Chlorella*

vulgaris), quando sujeitas a exudatos de culturas da cianobactéria, e a sobrevivência e a reprodução de cladóceros (*Daphnia magna* e *D. longispina*), quando a cianobactéria em estudo é fornecida como fonte de alimento exclusiva.

Em suma, espera-se obter informação adicional que possa auxiliar na previsão e prevenção deste tipo de fenómenos e avaliação de riscos ecológicos inerentes.

4. Estrutura da dissertação

A presente dissertação apresenta sete secções. O capítulo I pretende contextualizar a ocorrência de *blooms* de cianobactérias nos sistemas hídricos superficiais e a produção de cianotoxinas, com especial relevância para a hepatotoxina microcistina, sendo referidos alguns aspectos relativos à sua síntese e à sua toxicidade em organismos, com destaque para o risco que representa para a Saúde Pública. Ainda nesta secção, são apresentados os objectivos do trabalho de investigação conducente à elaboração desta dissertação. Nos capítulos II e III são revistos muitos dos estudos efectuados acerca da temática da ocorrência de *blooms* tóxicos de cianobactérias e a produção de cianotoxinas, em especial a microcistina. O capítulo II é um artigo publicado na revista *Discursos*, da Universidade Aberta, e o capítulo III constitui um artigo submetido a uma revista internacional. O capítulo IV descreve o estudo efectuado sobre o ciclo anual da comunidade fitoplanctónica de uma lagoa eutrofizada (Lagoa da Vela), com particular relevo para as cianobactérias, e é um artigo em preparação para submissão. O capítulo V apresenta os resultados obtidos para a estirpe de *Aphanizomenon flos-aquae* (isolada de um *bloom* na Lagoa da Vela) em ensaios de nutrição, sob limitação de azoto e fósforo, e ainda o efeito dos seus exudatos sobre duas clorófitas (*Pseudokirchneriella subcapitata* e *Chlorella vulgaris*). No capítulo VI são apresentados os resultados relativos aos ensaios de toxicidade da estirpe cianobacteriana acima referida sobre os cladóceros *Daphnia magna* e *D. longispina*. Este capítulo e o anterior pretendem ser esboços de artigos para submeter a revistas da especialidade. No capítulo final, VII, é realizada uma discussão geral dos resultados que foram obtidos na totalidade do trabalho, integrando os resultados físico-químicos e biológicos (fitoplâncton) obtidos para as amostras ambientais, aquando do *bloom* de *A. flos-aquae* na Lagoa da Vela, com os resultados obtidos nos estudos laboratoriais com esta estirpe, relativamente ao crescimento sob limitação em nutrientes (azoto e fósforo) e à toxicidade sobre microalgas e cladóceros.

Capítulo II

Toxic cyanobacterial blooms – occurrence, consequences and control strategies

Toxic cyanobacterial blooms – occurrence, consequences and control strategies

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Discursos *(in press)*

Toxic cyanobacterial blooms – occurrence, consequences and control strategies

Abstract – Cyanoprokaryota, in spite of being a small part of the natural plankton community, play a very important role in the maintenance of a balanced aquatic ecosystem. The intensification of anthropogenic activities such as nutrient loading (enriching the water, in particular with phosphorus and nitrogen from agriculture soil drainage, urban run-off and industrial wastes) and construction of dams (reducing the water flow and leading to temperature increase), along with other factors, have led to the quick increase of cyanobacterial blooms, particularly in warmer months. The biosynthesis of secondary metabolites is current in bacteria but can also occur in cyanobacteria. Many of these metabolites are toxins that have been considered to work as chemical defences (providing competitive advantage over other species and discouraging predation by higher trophic level organisms) in spite the purposes of their synthesis are not yet clear. These cyanotoxins are known to affect many organisms including humans, livestock, cattle, wildlife, food chain for wildlife and fish, and crustaceans and shellfish grown for commercial purposes. Cyanotoxins are complex organic compounds sub classified according to the effects in animals as: dermatotoxins, hepatotoxins and neurotoxins, being these last two more dangerous because the toxic effects are more serious. Hepatotoxins are produced by species of *Anabaena*, *Microcystis*, *Aphanizomenon*, *Cylindrospermopsis and Nodularia*, and are rarely fatal in spite of producing liver damage, general long term debility and promoting liver tumours. Neurotoxins are mainly produced by *Anabaena*, *Planktothrix/Oscillatoria*, *Microcystis*, *Aphanizomenon*, *Lyngbya* and *Cylindrospermopsis*, and these toxins affect the nervous and respiratory systems and death may occur within a short period of time by respiratory arrest. Harmless strains look the same as deadly ones under a microscope, becoming necessary the use of other methods (usually bioassays, using experimental laboratory animals) to assess the toxicity of these organisms. Water management strategies such as reduction of eutrophication must urgently be applied in order to control the occurrence of these toxic blooms in water bodies.
Keywords: pollution, cyanobacterial blooms, cyanotoxins, effects.

Introduction

Cyanoprokaryota organisms are considered an evolutionary link between bacteria and algae because they are prokaryotes but in natural environments they behave like

algae, producing chlorophyll *a* and performing oxygenic photosynthesis, using water as the most common electron donor (Wilmotte, 1994). Besides chlorophyll *a*, they synthesize accessory pigments (Hirschberg and Chamovitz, 1994) with special regard to phycobilins which are responsible for the typical blue-green colour of cyanobacteria (Sidler, 1994). The most ancient cyanobacterial fossil records are from 3.5 billion years ago (Schopf, 2000). Cyanobacterial capabilities like tolerance to low oxygen concentrations, to sulphur and UV radiation, adaptation to large thermal amplitudes and low light and nutrient conditions, have allowed them to survive with success during all the evolution process and to still be found in hot springs at 74º C (Ward and Castenholz, 2000), deserts and polar lakes from Antarctica (Hitzfeld *et al.*, 2000; Vincent, 2000), among many other adverse habitats. There are many cases of symbiosis between cyanobacteria and diverse organisms (Adams, 2000). Cyanobacteria group has an enormous morphological diversity and more than 2000 species have been established until now. They may develop as solitary cells or grouped in colonies or filaments. Presently, five orders are recognized: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales e Stigonematales (Komárek and Anagnostidis, 1999). These organisms can have multiple applications for economically relevant human purposes such as food supplements (*Spirulina/Arthrospira* (Jassby, 1988) and *Aphanizomenon* (Carmichael, 2000)), fertilizers (*Anabaena azollae* in symbiosis with *Azolla*) for rice crops (Metting et *al.*, 1988; Kannaiyan, 1997), waste-water treatment (Tang *et al.*, 1997), synthesis of active metabolites against virus (herpes virus, flu virus or HIV) or antitumoral and antibiotic substances (Ostensvik *et al.*, 1998; Mundt *et al.*, 2001). Nevertheless, in spite of all these and others advantages for humans, some cyanobacteria produce toxins and they may become dangerous by developing massively in water bodies, forming blooms that cause serious ecological and human health problems (Gorham and Carmichael, 1988; Codd *et al.*, 1995; Codd, 2000). Eutrophication is an important cause of the increasing occurrence of toxic cyanobacterial blooms worldwide. Therefore, it is becoming essential a proper water management regarding the nutrient inputs to water systems but also a development of an integrated awareness of public, farmers and industrial owners towards the potentially dangerous effects of those inputs. This is a major Public Health issue to which more attention should be given at a local scale. The present study reviews some recent work made on cyanobacterial blooms and cyanotoxins, regarding its occurrence, control and toxicity on diverse organisms with special regard to humans.

Cyanobacterial blooms

Causes

Water quality depends on a variety of biotic and abiotic factors (Codd, 2000), seasonality (Codd *et al.*, 1995) but also anthropogenic activities such as deviation of water courses, water extraction, drainage, dams construction, human wastes such as sewage and detergents, industrial and intensive farming effluents (Cooperband and Good, 2002), increased soil erosion and run-off of fertilizers and pesticides from agricultural land (Withers and Lord, 2002; Codd, 2000). As a result, eutrophication in water bodies occurs frequently. The development of a bloom is based on the assumption that a species or a species assembling becomes dominant in density by possessing mechanisms that allow a competitive advantage in relation to the other species present in the water body. Cyanobacteria, particularly, have some characteristics that may explain its success under certain conditions. It is known that cyanobacterial growth is affected by many environmental factors such as light (Grossman *et al.*, 1994; Lee and Rhee, 1999), macronutrients (particularly N and P) (Flores and Herrero, 1994; Grossman *et al.*, 1994; Oliver and Ganf, 2000; Bhaya *et al.*, 2000; Reynolds *et al*., 2000) and micronutrients (as Fe and Cu) (Grossman *et al.*, 1994), among others (temperature and pH). In summer and early autumn months, water retention and low turbulence (with no vertical mixing) may lead to a thermal stratification state in deep reservoirs and lakes, with formation of an Epilimnion with light and a dark Hypolimnion, resulting in a nutrient unavailability at surface due to algae development and nutrient settlement (Oliver and Ganf, 2000). Some characteristics allow cyanobacteria to develop successfully under these low nutrient concentrations such as the capability of some cyanobacteria (e.g. *Anabaena* and *Aphanizomenon*) to fix nitrogen (Flores and Herrero, 1994; Wolk, 1994) in specialized cells or in alternation with photosynthesis since nitrogenase is sensitive to oxygen. Planktonic cyanobacteria can also regulate their buoyancy allowing vertical movement in the water column in a way to optimize nutrient availability and light conditions, but this buoyancy regulation depends on environmental conditions (N and C availability, light and water turbulence) (Oliver and Ganf, 2000; Brookes and Ganf, 2001) involving production and collapse of small intracellular cylindrical structures – gas vesicles. Calm conditions may lead to rapid and unexpected development of surface blooms due to massive migration to surface of pre-existing cyanobacteria dispersed in water and not to a rapid population growth. Probably due to the loss of buoyancy regulation (by photo-oxidation, for example) (Oliver and Ganf, 2000; Codd *et al.*, 1995) cells may be densely accumulated at surface forming scum. Hence, cyanobacteria that produce gas-vesicles

are the main responsible for surface blooms or scum. They can be filamentous (*Anabaena*, *Aphanizomenon*, *Anabaenopsis*, *Nodularia*, *Cylindrospermopsis*, *Gloeotrichia*, *Oscillatoria/ Planktothrix*, *Spirulina*) or not, forming globular colonies (*Microcystis*, *Gomphosphaeria*, *Coelosphaerium*) (Oliver and Ganf, 2000). Other characteristics that can present competitive advantage of cyanobacteria over the other algae are the low grazing rate by zooplankton (Kurmayer and Jüttner, 1999; Henning *et al.*, 2001; Laurén-Määttä *et al.*, 1997) or selective rejection in pseudofeces by predators such as mussels (Vanderploeg *et al.*, 2001). Surface blooms are more common in stable waters but can also occur in rivers with high flow rates and turbulence (Codd *et al.*, 1995). In spite planktonic surface blooms with scum formation being the most concerning in terms of animal and human health, some benthic cyanobacteria can homogeneously develop in oligotrophic waters with sunlight reaching the bottom of the lake or reservoir (Oliver and Ganf, 2000).

Consequences

The primary consequence of blooms occurrence is the water quality reduction with economical, ecological and Public Health implications (Codd, 2000). From an ecological point of view, specific biodiversity decreases at all trophic levels and there is a habitat deterioration, with increased turbidity, a decrease in oxygen concentration and production of substances that give a bad taste and odor to water, or toxins noxious to a great variety of organisms (Gorham and Carmichael, 1988; Codd *et al.*, 1995). Cyanobacterial blooms occurrence in drinking water reservoirs have significant economical consequences resulting from deterioration of water quality (with bad taste and odour (Park, 2001)), water treatment filter blockage and requirement for additional and more effective water treatment processes (Rositano *et al.*, 2001; Bláha and Marsálek, 2001; Kruschwitz *et al.*, 2001; Maatouk *et al.*, 2002) to remove cyanotoxins that may endanger consumers health. In recreational waters, surface scum occurrence causes a decrease in local tourism economy due to the loss of water quality and cyanotoxins hazard, hindering the water sports practice and bath contact due to potential outcome of animal and human illness (WHO, 1998a). Cyanobacterial blooms have been recorded in marine, brackish and freshwaters worldwide and a great percentage (50 a 90 %) of them has been considered toxic (Codd *et al.*, 1995; WHO, 1998b; Codd, 2000; Dow and Swoboda, 2000).

Cyanotoxins

Types and structure

Cyanobacterial toxins differ in its chemical structure and toxicity. Generally they are classified as dermatotoxins, neurotoxins and hepatotoxins, according to the toxic effects in animals (Kaebernick and Neilan, 2001). Chemically, dermatotoxins can be lipopolysacharides or alkaloids (lyngbyatoxin-a and aplysiatoxins) and neurotoxins include alkaloids (anatoxin-a, homoanatoxin-a, saxitoxins and neosaxitoxins) and the organophosphate anatoxin-a(s) (Codd, 2000; Dow and Swoboda, 2000; Nicholson and Burch, 2001). Hepatotoxins, the most frequent and dangerous cyanotoxins, include alkaloids (cylindrospermopsin) or cyclic peptides (nodularin and microcystin) (Kaebernick and Neilan, 2001). Nodularin (cyclic pentapeptide) and microcystin (cyclic heptapeptide) both possess the amino acid Adda (*3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid*), considered responsible for the hepatotoxicity. There are more than 60 variants of the microcystin toxin (Codd, 2000; Dow and Swoboda, 2000) and microcystin-LR is the most common and studied variant. Many cyanotoxins not referred in this review are known (Dow and Swoboda, 2000) and much more are to be found but its frequency and toxic effects have not been yet found as relevant for human Public Health.

Producer species

Lyngbyatoxins are mainly produced by marine cyanobacteria belonging to the genera *Lyngbya* (*Lyngbya majuscula*), *Schizothrix* and *Oscillatoria* (Kaebernick and Neilan, 2001; Codd, 2000) and lipopolysacharides are synthesized by many brackish and freshwater species of the genera *Anabaena, Aphanizomenon, Nodularia, Oscillatoria, Gloeotrichia* (WHO, 1998a; Codd, 2000; Gorham and Carmichael, 1988).

Anatoxin-a is mainly produced by *Anabaena flos-aquae*, but it can be also synthesized by *Anabaena circinalis*, *Aphanizomenon flos-aquae* and some species of *Oscillatoria/Planktothrix*, *Cylindrospermum*, *Microcystis* and *Phormidium* (Codd et al., 1995; Codd, 2000). Homoanatoxin-a has been identified in *Oscillatoria formosa* (Dow and Swoboda, 2000) and *Anabaena flos-aquae* is the main producer of anatoxin-a(s) (Codd et al., 1995; Dow and Swoboda, 2000; Kaebernick and Neilan, 2001). Saxitoxins are usually produced by marine dinoflagellates (Daranas *et al.*, 2001) but can also occur in freshwater cyanobacteria such as *Anabaena circinalis*, *Anabaena lemmermanni* and *Aphanizomenon flos-aquae*, as well as in species from the genera *Lyngbya*, *Cylindrospermopsis* and

Planktothrix (Codd *et al.*, 1995; Kaebernick and Neilan, 2001; Codd, 2000; Dow and Swoboda, 2000; Nicholson and Burch, 2001).

The brackish species *Nodularia spumigena* is responsible for nodularins production (Kaebernick and Neilan, 2001; Codd, 2000; Dow and Swoboda, 2000). The main microcystin variants (or microcystins) synthesizers include *Microcystis* spp., *Anabaena* spp., *Planktothrix* (or *Oscillatoria*) *agardhii* and *P. rubescens*, and species of *Anabaenopsis* and *Nostoc*, but microcystins were also found in *Aphanizomenon flos-aquae* and terrestrial *Hapalosiphon* (Hitzfeld *et al.*, 2000; Codd *et al.*, 1995; Dow and Swoboda, 2000; Kaebernick and Neilan, 2001) and the synthesis of these toxins seems to be regulated by several factors (Kaebernick and Neilan, 2001). Cylindrospermopsin is mainly produced by *Cylindrospermopsis raciborskii*, a cyanobacterium that has been increasingly found in tropical and temperate regions (Neilan *et al.*, 2003), but also by *Aphanizomenon ovalisporum* and *Umezakia natans* (Kaebernick and Neilan, 2001; Codd, 2000; Dow and Swoboda, 2000).

Toxicity in mammals

Lyngbyatoxin-a and aplysiotoxins can cause, by direct contact with bloom containing water, eye and skin irritation, rush, sneezing and sore throat and/or gastrointestinal problems if accidentally ingested, but they can also promote cancer (Gorham and Carmichael, 1988; Codd, 2000). Lipopolysacharides are endotoxins that, in spite of not very toxic, contribute to inflammation and fever situations after skin contact with water or gastroenteritis after ingestion (Gorham and Carmichael, 1988; Codd *et al.*, 1995; Codd, 2000).

Neurotoxins are less frequent than hepatotoxins but they act faster. Anatoxin-a and homoanatoxin-a mimic the neurotransmitter acetylcholine, maintaining the sodium channels open and thus blocking the neuromuscular system. Symptoms include staggering, gasping, dizziness, muscle fasciculation (involuntary contractions), abnormal breathing, cyanosis and convulsions, and death may occur by paralysis of respiratory muscles (Gorham and Carmichael, 1988; Fawell *et al.*, 1999b; Codd, 2000; Dow and Swoboda, 2000). In mice, LD_{50} value (dose that results in death of 50% of animals) with intraperitoneal injection (i.p.) is 250 $\mu g.kg^{-1}$ for homoanatoxin-a and 200-250 $\mu g.kg^{-1}$ for anatoxin-a, with death occurring in a few minutes by respiratory arrest (Gorham and Carmichael, 1988; Codd *et al.*, 1995; Dow and Swoboda, 2000). Oral toxicity by LD_{50} is superior to 5000 $\mu g.kg^{-1}$ for anatoxin-a and there is no evidence for chronic toxicity (Fawell *et al.*, 1999b; USEPA, 2001). Anatoxin-a(s) blocks neurotransmission by inhibition of

acetylcholinesterase activity, preventing acetylcholine degradation and so maintaining sodium channels open. Symptoms felt are similar to those for anatoxin-a, adding other symptoms like hipersalivation, ataxia, cramps, diarrhoea, vomiting and tremors. In mice, LD_{50} (i.p.) is only of 20-50 $\mu g.kg^{-1}$ killing in minutes (Gorham and Carmichael, 1988; Codd *et al.*, 1995; Dow and Swoboda, 2000). Saxitoxins and neosaxitoxins are potent sodium channel blockers. Symptoms caused are nausea, vomiting, weakness, twitching, dizziness, irregular breathing and death may occur by cardio-respiratory failure. Using mice, the LD_{50} (i.p.) was 10-30 $\mu g.kg^{-1}$ and death occurred in two minutes (Gorham and Carmichael, 1988; Codd *et al*., 1995; Dow and Swoboda, 2000; WHO, 1998a).

Both nodularin and microcystin variants are selective for hepatic cells, irreversibly inhibiting protein phosphatases 1 and 2A (Honkanen *et al*., 1990) leading to disintegration of hepatocytes structure (Jochimsen *et al*., 1998) or promoting cancer in mammals (Ueno *et al.*, 1996; Ito *et al.*, 1997; Zhou *et al.*, 2002). A recent study indicates that microcystins, chronically administrated, may also induce kidney damage on rats (Milutinovi *et al.*, 2002). Some of the symptoms are weakness, cold extremities, pallor, apathy, respiratory problems, vomiting and diarrhoea (Codd *et al.*, 1995; Codd, 2000). There is necrosis of the liver that may lead to death by hemorrhagic shock or liver failure after some hours or days (Gorham and Carmichael, 1988). LD_{50} (i.p.) value in mice for nodularin ranges between 30 and 50 $\mu g.kg^{-1}$ (WHO, 1998a; Dow and Swoboda, 2000). MC-LR has a value of 50 $\mu g.kg^{-1}$ for LD_{50} (i.p., in mice) (Dow and Swoboda, 2000). In mice, MC-LR is 30-100 times less toxic by oral ingestion than through intraperitoneal injection (Fawell *et al.*, 1999a). Cylindrospermopsin inhibits the protein synthesis and glutathione synthesis, causing cumulative hepatotoxicity by regular ingestion of contaminated drinking water but also damage in kidneys (Falconer *et al.*, 1999), heart, intestine, spleen and thymus (Codd, 2000). Nevertheless, a recent *in vitro* study shows no inhibition of PP2A and considers it unlikely to be a tumour promoter (Chong *et al.*, 2002). LD_{50} values (i.p., in mice) are 200 $\mu g.kg^{-1}$ and 2 $mg.kg^{-1}$, with death occurring in a few days (5-6) or in 24 h, respectively (WHO, 1998a).

Ecological effects (toxicity in organisms other than mammals)

Cyanotoxins ingested along with cyanobacterial toxin-containing cells or dissolved in water after released by cell lysis can be accumulated and/or can cause adverse effects on many aquatic organisms such as bacteria (Ostensvik *et al.*, 1998), phytoplankton (Kearns and Hunter, 2001), macrophytes (Wiegand and Pflugmacher, 2001; Pflugmacher *et al.*, 2001), zooplankton (Metcalf *et al.*, 2002; Claska and Gilbert, 1998; Kurmayer, 2001;

Laurén-Määttä *et al.*,1997; Mohamed, 2001; Henning *et al.*, 2001; Kurmayer and Jüttner, 1999; Rohrlack *et al.*, 2001), mussels (Amorim and Vasconcelos, 1999; Williams *et al.*, 1997), shrimps (Engström *et al.*, 2001), crabs (Monserrat *et al.*, 2001), fish (Oberemm, 2001; Baganz *et al.*, 2001; Kopp and Hetesa, 2000; Fischer *et al.*, 2000.a; Fischer *et al.*, 2000.b; Liu *et al.*, 2002) and amphibians (Prati *et al.*, 2002), but also terrestrial plants (McElhiney *et al.*, 200; Codd *et al.*, 1999) and birds (Matsunaga *et al.,* 1999).

Hazards to Public Health (toxicity in humans)

The symptoms observed for laboratory mammals are thought to be similar to those felt by humans, in spite of the lack of studies in this area. The epidemiological studies are the basis for human poisoning assessment and from the many worldwide cases reported until now, it is proven that cyanotoxins (dissolved in water and in cyanobacterial cells) cause acute and chronic effects on humans (Ueno *et al.*, 1996; Zhou *et al.*, 2002) and even death (Pouria *et al.*, 1998; Jochimsen *et al.*, 1998). The lethal dose of contaminated water depends on factors such as toxin type and its content in cyanobacterial cells, toxin producing cyanobacterial biomass, exposure route and victims' susceptibility to the toxins (age, sex, weight and species) (Dow and Swoboda, 2000). There are also more sensitive groups that require special attention. If microcystins attack the liver, B-hepatitis patients are more susceptible to these cyanotoxins effects. In the same way, a hypersensitive person is more predisposed to an allergenic response by contact with dermatotoxins in recreational waters (Fitzgerald, 2001). Children are another sensitive group to cyanobacterial toxins since the ingested water per body weight is higher and 17 mL of toxic cyanobacterial material is sufficiently lethal for a small child (Chorus and Fastner, 2001). Moreover, the places that children choose to play are shallow waters near the shore were the scum usually accumulates. Presently, there are some experimental studies about attenuation of human intoxication by microcystins (Dawson, 1998; Gehringer *et al.*, 2003) but for neurotoxins that is difficult due to his rapid action, and only procedures such as artificial respiration, lavage and activated carbon are applied to reduce the toxin absorption when dose is not lethal (Fitzgerald, 2001).

Human exposure routes to cyanotoxins

Human intoxications by cyanobacteria can occur through a direct or an indirect route.

Direct exposure

Drinking water

This is the main route for direct human exposure to cyanobacterial toxins and the hazards come from the acute but also chronic effects that they can cause on water consumers. Acute intoxication (with hepatotoxicity and gastroenteritis) usually occurs after bloom degradation or after cyanobacterial lysis by treatment processes (e.g. copper sulphate) when the toxins are released from the cells (Dow and Swoboda, 2000). The long exposure to low levels of the toxins poses great concern due to cancer promotion potential of some cyanotoxins such as microcystins (Ueno *et al.*, 1996; Ito *et al.*, 1997; Zhou *et al.*, 2002). Nodularins are not very common in drinking water, but shouldn't be forgotten because they are also tumour liver promoters. Cylindrospermopsin has already shown to be dangerous through drinking water exposure (Fitzgerald, 2001). Neurotoxins are not very common in drinking water and chronic effects are not sufficiently studied.

Recreational water

Activities like taking a bath, swimming or playing water sports in or on recreational water suffering a cyanobacterial bloom lead to direct exposure of skin, eyes and ears to the water but can also lead to accidental water ingestion, aspiration or inhalation of cyanobacterial cells. There has never been reported a human fatal case due to recreational exposure but usually it results in allergies and irritation of external and internal revestment tissues (gastrointestinal and respiratory organs, eyes, ears, mouth and throat) due to dermatotoxins, in spite hepatotoxic and neurotoxic situations may also occur. The main symptoms felt include headache, nausea, muscular pain, painful diarrhoea, vomiting, flu symptoms, central abdominal pain, fever, mouth ulcers, sore throat, asthma, skin, ear and eye irritation, and even pneumonia (Fitzgerald, 2001; Chorus and Fastner, 2001; Dow and Swoboda, 2000). The chronic effects due to recreational exposure should be also considered because long periods of exposure can occur during summer vacancies with regular swimming in an eutrophic water body with a hepatotoxic bloom, for example.

Haemodialysis

This is an uncommon contamination route in which cyanotoxins come directly in contact with blood. Yet, in 1996 in a haemodialysis unit in Caruaru, Pernambuco, Brazil, the death of 60 patients were associated to microcystins occurrence in the water used in the haemodialysis (Pouria *et al*., 1998; Jochimsen *et al.*, 1998). The contaminated water

was taken from a reservoir with a toxic bloom and treatment was insufficient to eliminate microcystins. All patients suffered acute symptoms of neurotoxicity and hepatotoxicity (Pouria *et al.*, 1998).

Indirect exposure

Food

Food supplements made from natural cyanobacterial blooms can have high microcystin levels (Schaeffer *et al*., 1999) and many organisms accumulate cyanotoxins, endangering the safety of its consumption by humans (Saker and Eaglesham, 1999; Magalhães *et al.*, 2001; Codd *et al.*, 1999; McElhiney *et al.*, 2001; Amorim and Vasconcelos, 1999; Williams *et al.*, 1997; Van Buynder *et al.*, 2001). Yet, there are studies such as the one made by Orr *et al.* (2001).that showed no detectable amounts of this toxin in the milk obtained from lactating dairy cattle exposed to sub-lethal doses of MC-LR.

Guideline values for cyanotoxins

The lethal dose of cyanotoxins contaminated water depends on toxin type, its cellular content, cyanobacterial biomass concentration, exposure route and victim susceptibility (varies with age, sex, weight and species) (Dow and Swoboda, 2000). The existing guidelines are based on bioassays for chronic effects because there are no sufficient and conclusive human studies and for that sensitive differences between laboratory animals and humans should be kept in mind.

Drinking water

With the increasing occurrence of these phenomena all over the world and the cases reported of cyanobacterial poisoning on animals and humans cyanotoxins are already considered part of the emerging pathogens in drinking water (Szewzyk *et al.*, 2000) and international measures are being taken such as definition of guideline values for cyanotoxins as well as monitoring programs implementation in drinking water (Fitzgerald, 2001; WHO, 1998b) but also in recreational waters (WHO, 1998a; Codd, 2000; Nancarrow and Wood, 2000). Presently, there are diverse available methods for detection and quantification of cyanotoxins (Nicholson and Burch, 2001). Other measures include a proper management of water bodies to prevent phytoplankton growth (monitoring and pollution decrease) and adequate treatment processes and for cyanobacteria removal and toxin elimination (Codd, 2000).

As already discussed, microcystin variants are the most common and hazardous cyanotoxins for Public Health due to its hepatotoxicity and tumour promoter potential. Thus, they were the first cyanotoxins to which a guideline was proposed. WHO (*World Health Organization*) has a guideline value of 1 $\mu g.L^{-1}$ for MC-LR (Fitzgerald, 2001; WHO, 1998b) considered as a life time consumption safe level. While many countries (Brazil, New Zealand, United Kingdom) have adopted this value as guideline for drinking water there are some variations for Canada which proposed 1.5 $\mu g.L^{-1}$ and Australia that proposes 1.3 to 10 $\mu g.L^{-1}$ (USEPA, 2001; Fitzgerald *et al.*, 1999). In Canada, there was also proposed a value 10 $\mu g.L^{-1}$ for short-termed exposure (Fitzgerald, 2001). In spite of rare in drinking waters, a life-time drinking-water guideline of 1.0 $\mu g.L^{-1}$ was also proposed for nodularin in Canada (Fitzgerald, 2001) due to its cancer promotion potential and 10 $\mu g.L^{-1}$ as a short-term acute exposure health alert in Canada and Australia (Fitzgerald, 2001; Fitzgerald *et al.*, 1999). There are no international guidelines for cylindrospermopsin in drinking water, in spite of its world distribution and acute toxicity, but Australia has proposed a drinking water guideline value ranging from 1 to 15 $\mu g.L^{-1}$ (USEPA, 2001).

Due to the lack of studies on potential chronic effects of neurotoxins on animals, there is no drinking water guideline value proposed for anatoxin-a and saxitoxins, but there is a health alert of 3 $\mu g.L^{-1}$ (Fitzgerald *et al.*, 1999; Fitzgerald, 2001; USEPA, 2001).

There are also guideline values for cyanobacterial density in drinking waters and they are usually based on water taste and odour. In Australia, 2000 cyanobacterial cells per mL (or 1$\mu g.L^{-1}$ Chl *a*) is the proposed guideline value for drinking water (Fitzgerald, 2001).

Recreational water

WHO has established 3 levels of risk for recreational water with cyanobacterial blooms: 1) mild and/or low adverse health effects to expect (with 20000 cyanobacterial cells per mL (or 10 $\mu g.L^{-1}$ Chl *a* under cyanobacterial dominance conditions)); 2) moderate adverse health effects are likely to occur (with 100000 cells per mL (or 50 $\mu g.L^{-1}$ Chl *a* under cyanobacterial dominance conditions)); and 3) high risk of severe adverse health effects to occur (scum formation or more than 150 $\mu g.L^{-1}$ Chl *a* under cyanobacterial dominance conditions) (WHO, 1998a; Fitzgerald, 2001). Germany adopted these WHO health hazard alert levels but added that sites with microcystin levels superior to 100 $\mu g.L^{-1}$ should be closed until the bloom reduces (Chorus and Fastner, 2001). Specific cell densities for *Microcystis aeruginosa* (50000 $cells.mL^{-1}$), *Nodularia spumigena* (50000 $cells.mL^{-1}$) and *Anabaena circinalis* (20000 $cells.mL^{-1}$) (Fitzgerald *et al.*, 1999) are

provided as health alert indicators to anticipate possible cyanotoxins outbreaks in Australia.

Food

There is a proposed guideline of 10 μg of MC-LR per g of cyanobacterial food supplement (Schaeffer *et al.*, 1999). In Oregon, U.S.A., there has been established a maximum value of 1 $\mu g.g^{-1}$ for food (USEPA, 2001).

Other measures

Public alert and water management

For recreational waters, depending on risk level (WHO, 1998a), some short-term measures include the use of informative material (Henriksen, 2001) for visitors in the bath sites, alerting for possible skin irritations due to the cyanobacterial material accumulation and lysis in bathing thermal suits or gastrointestinal illness due to accidental water ingestion. Regular monitoring of toxic bloom forming species should be made everyday for taking preventive measures on time or even prohibit people contact with the scum (WHO, 1998a; Fitzgerald, 2001). Long-term measures include eutrophication reduction and water management measures such as maintenance of transparency (2m by Secchi disc) and low total phosphorus levels (<0,01 $\mu g.L^{-1}$) (WHO, 1998a) in a way that massive cyanobacterial growth in recreational waters should be prevented.

Cyanobacterial growth control

Artificial mixing of water seems to be a good management measure (van der Veer *et al.*, 1995), due to its effect on cell buoyancy regulation and phytoplanktonic species composition. The reduction of phosphate levels by reducing agricultural effluents and fertilizers as well as protecting soils from erosion is very important for reduction of phytoplanktonic biomass and influences species composition (Oliver and Ganf, 2000). Grazing by copepods, cladocerans and nanoflagellates (Saito *et al.*, 2003) has shown to be another effective mean to control cyanobacterial growth directly by consumption or indirectly by altering light and nutrient conditions, increasing the transparency and nutrient recirculation and reducing primary productivity and pH (Oliver and Ganf, 2000). Another possibility is gas-vesicles collapse by ultrasonic irradiation (Lee *et al.*, 2001; Lee *et al.*, 2002) or UV-radiation (Alam *et al.*, 2001) but the enhancement of cell lysis and toxins release must be considered. Cyanobacterial growth control has been experimentally achieved by plant growth retardants (Romanowska-Duda *et al.*, 2001) but lysis is still a

problem for the immediate use of water due to the release of toxins. Barley straw extract has shown inhibiting effects on growth of *Microcystis* sp. (Ball *et al.*, 2001) and some bacteria (Imamura *et al.*, 2001) such as *Alcaligenes denitrificans* (Manage *et al.*, 2000) and *Vibrio* sp. (Yoshikawa *et al.*, 2000) have algicidal effects on *Microcystis* spp., contributing to bloom control of this genus in freshwater bodies.

Drinking-water treatment processes

This should be the final step and not considered the only one. Conventional water treatment processes like flocculation (Chow *et al.*, 1998) and filtration (Gupta *et al.*, 2001) are effective in removing cyanobacterial cells and cell-bound cyanotoxins but not released cyanotoxins, endangering drinking water consumers due to chronic exposure to low levels of microcystins. Chemicals that lead to cell lysis (like copper sulphate) must thus be avoided, in order to prevent the cyanotoxins release from the cells, which take more than 3 weeks to completely disappear from the water (Gupta *et al.*, 2001). Chlorination appears to be unsuccessful in reducing microcystin levels in water (Gupta *et al.*, 2001; Chorus *et al.*, 2001b), but it seems to degrade effectively cylindrospermopsin (Senogles *et al.*, 2000). To eliminate dissolved cyanotoxins like microcystins additional treatment processes are required such as ozonation and activated carbon filtration (Rositano *et al.*, 2001; Bláha and Marsálek, 2001; Chorus *et al.*, 2001b; Kruschwitz *et al.*, 2001; Maatouk *et al.*, 2002).

Worldwide toxic cyanobacterial blooms occurrence

Since the 19th century that scientific records have been relating the occurrence of toxic blooms of cyanobacteria to animal deaths concerning sheep, horses, pigs, dogs, birds and many others, including humans, in many countries all over the world, as shown in several reviews (Gorham and Carmichael, 1988; Codd, 2000; WHO, 1998a).

Europe

Portugal

In the last 50 years many potentially toxic cyanobacteria have been recorded in portuguese waters with relevance to *Anabaena circinalis* and *Anabaena flos-aquae, Aphanizomenon flos-aquae*, *Lyngbya majuscula*, *Microcystis aeruginosa, M. viridis, M. wesenbergii* and *M flos-aquae*, *Planktothrix/Oscillatoria agardhii*, *P. rubescens*, *Phormidium mucicola*, *Oscillatoria formosa* and *Nostoc* sp. (Vasconcelos, 2001). Some blooms of these species are related to fish kills and also to human intoxications

(Vasconcelos *et al.*, 1996; Vasconcelos, 2001). From 1989 to 1992, various portuguese water bodies used for recreation and as drinking water supplies were found to have hepatotoxic blooms of *Microcystis aeruginosa*, *M. wesenbergii*, *Anabaena flos-aquae* and *Nostoc* sp. with microcystins production (Vasconcelos *et al.*, 1996). In 1996, *Aphanizomenon flos-aquae* was recorded in Crestuma-Lever reservoir, northern Portugal, and Montargil reservoir, centre of Portugal, with PSP toxins production (Ferreira *et al.*, 2001; Pereira *et al.*, 2000). In Montargil reservoir, in late summer of that year, a hepatotoxic *Microcystis aeruginosa* bloom was established (Pereira *et al.*, 2000). Rodrigues *et al.* (2002) reported that in 2001, Bravura Lake, Algarve, observed high cyanobacterial densities of *Microcystis aeruginosa* during all year with high microcystin contents (10-56 $\mu g.L^{-1}$). In the last decade Crestuma reservoir has also suffered toxic blooms of *M. aeruginosa* (Vasconcelos, 2001). After Saker *et al.* (2002), Torrão reservoir suffers regular *Microcystis* spp. blooms. In 1999, a study made on a Wastewater Treatment Plant, in Esmoriz, north Portugal, concluded that cyanobacteria were frequently dominant with *Planktothrix mougeotii*, *Pseudanabaena mucicola* and particularly *Microcystis aeruginosa* as the most common species achieving high levels of total MC-LR equivalents in the WWTP outflow, indicating its cyanotoxins contamination potential to receptor water bodies (Vasconcelos and Pereira, 2001).

France

In summer 1994, Lake Grand-Lieu suffered a bloom that included *Microcystis aeruginosa* strains capable of producing microcystins (Vézie *et al.*, 1998). Saint-Caprais reservoir suffers annually, in autumn, an *Aphanizomenon flos-aquae* bloom with microcystins production (Maatouk *et al.*, 2002).

Belgium

In 1995, near Liège, three adjacent ponds suffered a *Microcystis aeruginosa* bloom and bird deaths were related with microcystins produced in the bloom (Wirsing *et al.*, 1998).

Finland

In 1997 and 1998, a monitoring study in several bank filtration plants and surface waterworks recorded microcystins in only some of the raw water samples with dominance of *Planktothrix agardhii* and there were no significant amounts of microcystins in treated water meaning the processes used in the water treatment plant were effective in microcystin removal (Lahti *et al.*, 2001).

Italy

In the summer of 1997, Lake Varese, Italy, suffered a toxic bloom of *Planktothrix* sp. with the deaths of fish and shellfish by a saxitoxin (Prati *et al*., 2002).

Germany

In a study of Wiedner *et al.* (2001), 133 german water bodies (used for recreational or drinking water purposes) were in their majority dominated by cyanobacteria from genera *Planktothrix*, *Microcystis*, *Anabaena* and *Aphanizomenon*, with microcystins and anatoxin-a production (Chorus *et al*., 2001). In 1998 and 1999, the Bleiloch reservoir, in Thuringia, formerly used as drinking water source (supply), showed the presence of diverse microcystins in water (Hummert *et al*., 2001). Lake Ammersee, southern Germany, has frequently *Planktothrix* sp. blooms and this occurrence has been associated with growth problems of the whitefish *Coregonus lavaretus* from the lake, due to the bloom production of microcystin (Ernst *et al*., 2001). In 1999, many recreational lakes in Baden-Würtemberg, southwestern Germany, were found to have cyanobacteria dominating the phytoplankton and microcystins production (Frank, 2002).

Ireland

Between 1992 and 1994, several dogs died after drinking water from Caragh Lake, County Kerry, exhibiting respiratory problems and convulsions due to the presence of anatoxin-a, produced by a benthic *Oscillatoria* (James *et al.*, 1997). During summer months of 1994 and 1995, in three irish lakes anatoxin-a was detected and associated with planktonic *Anabaena* and benthic *Oscillatoria* species (James *et al.*, 1997). Homoanatoxin-a is rare but has been detected in four (Lough Sillan, Inniscarra Reservoir, Lough Key and Caragh Lake) of twenty Irish lakes studied (Furey *et al*., 2003).

Scotland

Neurotoxin poisoning by anatoxin-a in dogs have been frequently reported in scottish lakes from Scottish Highlands that suffer benthonic blooms of *Oscillatoria-Phormidium*, which accumulate in shores (Codd *et al*., 1995). Symptoms felt by dogs included convulsions, rigors, limb twitching, cyanosis and hypersalivation, and death occurred in 10-30 minutes. In 1998, anatoxin-a was related to a bloom of *Anabaena flos-aquae* in a drinking water reservoir at Loch Muidhe and, for several years (1984, 1991 and 1992), Loch Leven suffered from hepatotoxic blooms of *Microcystis aeruginosa* and *Anabaena flos-aquae* associated with more than 1000 dead fish that accumulated in the

shores and that showed liver necrosis probably due to microcystins released in *Anabaena flos-aquae* bloom senescence (Codd *et al*., 1995).

United Kingdom

In late summer of 1989, a *Microcystis* bloom in Rutland Water reservoir caused the deaths of 20 sheep and 15 dogs that had ingested scum from the reservoir, and caused also skin rushes, mouth blistering and thirst in wind surfers that contacted with the scum. At about the same time, in Rudyard Lake, during a canoeing training exercise soldiers contacted with *Microcystis aeruginosa* scum and suffered from sore throat, headaches, blistered mouth, diarrhoea, vomits and some reported also fever and pneumonia (Dow and Swoboda, 2000; WHO, 1998a).

Norway

Between 1978 and 1998, a study based on dozens of south Norwich water bodies revealed the occurrence of many microcystins producing blooms of *Anabaena* spp., *Microcystis* spp. and *Oscillatoria* (*Planktothrix*) spp., but also anatoxin producing *Anabaena* spp. blooms (Utkilen *et al.*, 2001).

Switzerland

Dense mats of benthic cyanobacteria (mainly *Oscillatoria* and *Phormidium*) have been reported to occur in oligotrophic, cold and turbid alpine waters of south-eastern Switzerland, showing hepatotoxic (by microcystins) and neurotoxic effects in mice and this seems to have been the cause of many cattle deaths in this region during the last two decades (Mez *et al.*, 1997).

Denmark

Between 1993 and 1995, an intensive study on hundreds of freshwater bodies reached the conclusions that the majority of blooms were hepatotoxic (with microcystins production by *Microcystis* spp., *Anabaena* spp., *Planktothrix agardhii* and *Aphanizomenon flos-aquae*) but some neurotoxic blooms also occurred (with synthesis of saxitoxins-like toxins and anatoxin-a by *Anabaena lemmermannii*) (Henriksen, 2001). During this period there have been recorded the deaths of 50000-100000 fish and a cow by hepatotoxic blooms of *Anabaena flos-aquae* and *Planktothrix agardhii*, respectively (Henriksen, 2001). A neurotoxic bloom of *Anabaena lemmermannii* was related to bird deaths (Henriksen, 1997; Onodera *et al.*, 1997). In 1994, in a study involving 96 freshwater ponds and lakes, anatoxin-a(s) and saxitoxins were found in three and eight, respectively, of the studied lakes and associated with the presence of *Anabaena lemmermannii* (Kaas and Henriksen,

2000). In summer of 1996, a small lake in North Sea coast suffered a benthic neurotoxic bloom of *Oscillatoria* causing the death of a dog and convulsion symptoms in another after water lake ingestion (Henriksen, 2001).

Sweden

In 1991, at Lake Vombsjön, South Sweden, there were detected significant MC-LR levels in water from a drinking water treatment plant (Codd *et al.*, 1995). In 1994, there was a river contamination with microcystins produced by a *Planktothrix agardhii* bloom that caused intoxication of pets and 121 persons (WHO, 1998a). In late summer of 1997, Willén *et al.* (2000) studied three Swedish lakes (Lake Mälaren, Lake Lilla Ullfjärden and Lake Storsjön) finding the high microcystin levels associated with dominance of *Microcystis aeruginosa*, *M. viridis* and *Planktothrix prolifica*. In early summer neurotoxicity (not by anatoxin-a) had also been recorded in two of these lakes and coincided with abundance of *Anabaena* spp. and *Aphanizomenon* spp. (Willén *et al.*, 2001).

Czech Republic

During 1993 to 1998, 90 % of samples from dozens of recreational and drinking water reservoirs and fish ponds were found to be hepatotoxic and dominated by *Microcystis* spp., *Planktothrix agardhii* and *Aphanizomenon flos-aquae* (Marsálek *et al.*, 2001). In 1999, a study on samples taken from raw and treated waters from selected Czech drinking-water treatment plants showed that the majority of raw waters and some treated drinking waters had dissolved microcystin contents seven times higher than the WHO guideline value posing possible risks of hepatotoxicity and liver tumour promotion to consumers (Bláha and Marsálek, 2001)

Latvia

During 1995 and 1996, three eutrophic lakes (Lakes Mazais, Lielais Balterzers and Sekitis) had summer blooms of potentially toxic *Microcystis aeruginosa* (with production of microcystins), *Aphanizomenon flos-aquae* and *Anabaena flos-aquae*, leading to a decrease in drinking water quality and health problems resulting from the recreational use of lakes water (Eynard *et al.*, 2000).

Slovenia

In North-Eastern Slovene freshwaters there have been identified many hepatotoxic blooms, most frequently with *M. aeruginosa* dominance and microcystins production (Sedmak and Kosi, 1997).

North America

Canada

Microcystins are the most frequent cyanotoxins in Canada and drinking water is the main exposure route (Gupta *et al.*, 2001). In the summer of 1990, in Alberta, some lakes used as drinking water sources showed the presence of MC-LR and in summer of 1993, at Shoal Lake, Manitoba, a bloom of *Microcystis aeruginosa* produced MC-LR at concentrations higher than WHO guideline, in both raw water and treated tap water (Gupta *et al.*, 2001). In 1995, in southwestern Manitoba MC-LR was detected in the majority of 150 surface water supplies and also in many treated waters (Gupta *et al.*, 2001).

U.S.A.

The first human intoxication by drinking water was reported in 1931 due to the ineffective treatment (by precipitation, filtration and chlorination) of waters taken from Ohio and Potomac rivers that suffered from *Microcystis* blooms at the time, resulting in illness of thousands of people who drank deficiently treated water (WHO, 1998a). Since the 50s, many cases of dermatitis, skin rush, eye irritation and asthma have been connected with *Anabaena*, *Oscillatoria* and *Microcystis* blooms (Gorham and Carmichael, 1988) but also *Lyngbya majuscula* (Dow and Swoboda, 2000). In 1975, at a Sewickley reservoir, Pennsylvania, lipopolysacharides in high concentrations were detected and associated to an outbreak of gastroenteritis affecting about 5000 people (Gorham and Carmichael, 1988). In 1979, in two lakes of Pennsylvania, 2 to 12 h after contact with water blooms of *Anabaena* several cases of gastroenteritis and eye irritation, sore throat, headaches and sneezing were reported (Gorham and Carmichael, 1988). Many intoxications cases by neurotoxins have been documented for dogs, farm animals and ducks with the typical hypersalivation (Codd *et al.*, 1995). In 1977, at Hegben Reservoir, Montana, a neurotoxic bloom caused the deaths of 30 cows and 8 dogs and in 1985, at Richmond Lake, South Dakota, 5 dogs, 8 pups e 2 calves were killed after ingestion of water containing an *Anabaena flos-aquae* bloom (Gorham and Carmichael, 1988). Between 1991 and 1994, majority of samples collected from 10 locations at Guntersville Reservoir, on the Tennessee River, was found toxic with the presence of the mat-forming filamentous cyanobacterium *Lyngbya wollei* and saxitoxins production posing the risk of PSP (Carmichael *et al.*, 1997). In 1994, a prolonged toxic bloom of *Microcystis aeruginosa* occurred in Steilacoom Lake, Washington (Jacoby *et al.*, 2000). In southern Colorado, 24 heifers died from hepatocyte degeneration and liver necrosis after drinking water with a

bloom of a microcystin producing *Microcystis* (Puschner *et al.*, 1998). In Oregon, food supplements made on natural *Aphanizomenon flos-aquae* blooms had high levels of microcystin (Schaeffer *et al*., 1999).

South America

Brazil

In 1996, Caruaru, Pernambuco state, the death of 60 patients from a haemodialysis unit was related to microcystins intoxication because the water used on the process came from a reservoir suffering a bloom of species belonging to the genera *Anabaena* and *Microcystis*, and the water treatment methods used were insufficient to eliminate the toxins (Pouria *et al*., 1998). The state of Paraná has frequent occurrence of microcystins producing *Microcystis* spp. blooms in freshwater lakes and reservoirs used for recreational and animal farming purposes but also in drinking water supplies, as shown in a study made between 1995 and 1996 (Hirooka *et al.*, 1999). The Patos Lagoon estuary, Rio Grande do Sul, southern Brazil, suffers regular blooms of *Microcystis* and recent studies have found microcystins synthesis during its occurrence (Matthiensen *et al.*, 1999; Matthiensen *et al.*, 2000).

Chile

In February 1995 and 1996, *Microcystis* spp. blooms occurred in lake Rocuant, Conception, Chile, and microcystin was detected (Campos *et al.*, 1999). In 1998, Lake Tres Pascualas, also in Conception, suffered a hepatotoxic *Microcystis* sp. bloom with different microcystins production (Neumann *et al.*, 2000).

Africa

Egypt

In July of 1995, in Egypt, River Nile (used as drinking water source but under pressures from agricultural, municipal and industrial effluents) suffered a hepatotoxic bloom of *Oscillatoria tenuis* (with production of microcystins) at Sohag province (Brittain *et al.*, 2000).

Israel

In summer of 1994, in Lake Kinneret, Israel, a bloom of potentially toxic *Aphanizomenon ovalisporum* occurred and caused concern because the lake was a major national source of high-quality water (Hadas *et al.*, 1999).

Morocco

During May-June 1999, Lake Oued Mellah suffered a *Microcystis ichtyoblabe* bloom and microcystins were detected (Sabour *et al.*, 2002). Several cyanobacterial strains belonging to the genera *Microcystis*, *Synechocystis*, *Pseudanabaena* and *Oscillatoria*, that have isolated from ponds, lakes and reservoirs, and showed microcystin synthesis (Oudra *et al.*, 2001; Oudra *et al.*, 2002).

South Africa

Some cattle and sheep death cases were reported in western Cape Province, South Africa, and were related to drinking water contamination with cyanotoxins. The first two poisoning outbreaks were attributed to *Nodularia spumigena* and the third to *Microcystis aeruginosa* (with confirmed MC-LR synthesis) (Van Halderen *et al.*, 1995).

Oceania

Australia

In 1878, Lake Alexandrina, in South Australia was the first spot where a case on poisoning of livestock from drinking water contaminated with cyanobacteria (producing a nodularin) had been reported (Dawson, 1998). In Darling/Barwon River, Australia, in late autumn of 1991, there was an important record of a toxic bloom in a river, with 1000 Km of river suffering from a hepatotoxic and neurotoxic bloom of *Anabaena circinalis*, and more than 1600 sheep and 40 cattle animals died (Codd *et al.*, 1995). At Swan-Canning estuary, Western Australia, in February 2000 there was a dense and severe *Microcystis aeruginosa* bloom (Atkins *et al.*, 2001). In summer of 2001, a toxic bloom of *Nodularia spumigena* occurred in Gippsland Lakes, Southern Victoria, with nodularin production and accumulation of this toxin in mussels and prawns (Van Buynder *et al.*, 2001). In 1979, Palm Island, north Queensland, an human intoxication occurred in an aboriginal community by ingestion of water from a reservoir that has been subjected to a copper sulphate treatment to eliminate a *Cylindrospermopsis raciborskii* bloom causing illness in 139 children and 10 adults, who felt symptoms like headache, vomiting, painful liver enlargement, bloody diarrhoea, anorexia, with hepatoenteritis and renal damage (Fitzgerald, 2001). There are cases of cows and calves death after drinking water from a dam at McKinlay in northwest Queensland, containing a hepatotoxic *Cylindrospermopsis raciborskii* bloom (Saker *et al.*, 1999).

New Zealand

In 1999, Wellington region, New Zealand, the recreational Lake Waitawa suffered the development of an odoriferous scum with synthesis of cylindrospermopsin and microcystins, highlighting the risk of exposure to cyanotoxins by users of recreational lakes (Stirling and Quilliam, 2001).

Asia

South Korea

Between 1992 and 1996, a study on various brackish and freshwater bodies in South Korea, including dams and lagoons used as drinking water sources, showed that every water body had cyanobacteria as dominant phytoplanktonic group with species mainly belonging to *Microcystis* genera, but also *Anabaena* e *Planktothrix/Oscillatoria*, with production of microcystins and anatoxin-a (Park, 2001). The Daechung reservoir was studied from spring to autumn 1999 and *Microcystis* spp. blooms occurred with production of microcystins (Oh *et al.*, 2001).

China

In 1993, in Haimen city and Fusui county, there was found a significant correlation between microcystins producing bloom occurrence in the superficial drinking water sources (ponds and rivers) and primary liver cancer incidence (Ueno *et al*., 1996). Between 1995 and 1996, a study on Donghu Lake and a fish pond in Wuhan, China, revealed the presence of microcystins associated with the presence of species of *Anabaena* and *Oscillatoria* (Xu *et al.*, 2000).

Thailand

Cylindrospermopsin produced by *Cylindrospermopsis raciborskii* has been recently isolated from a fishpond in Thailand (Li *et al.*, 2001).

Japan

Between 1988 and 1992, microcystins were found in various naturally occurring blooms with dominance of cyanobacteria genera, namely *Microcystis* (Park *et al.*, 1993). Between 1992 and 1995, microcystins could be detected in water during cyanobacterial blooms in Lakes Sagami and Tsukui, Kanagawa Prefecture, Japan, used for recreational purposes but also as drinking water sources (Tsuji *et al.*, 1996). Between 1991 and 1994, the hypertrophic Lake Suwa, in central Honshu, suffered *Microcystis* spp. blooms with high production of microcystins (Park *et al.*, 1998). In 1985, at Shin-ike pond, in

Nishinomiya, Hyogo Prefecture, Japan, the death of 20 ducks was related with the occurrence of a toxic *Microcystis aeruginosa* bloom with microcystins production (Matsunaga *et al.*, 1999).

Taiwan

Microcystins have been found in several strains of *Microcystis aeruginosa* isolated from eutrophic aquaculture ponds and water reservoirs in Taiwan (Lee *et al.*, 1998).

Philippines

During 1996, 1998 and 1999, Laguna de Bay suffered periodic blooms of *Microcystis aeruginosa* and many variants of microcystins were detected (Cuvin-Aralar *et al.*, 2002).

Antarctica

In spite of cyanotoxins occurrence being more documented in temperate or tropical populated regions, toxic cyanobacteria also occur in polar regions. Between 1997 and 1999, in a study using melt water ponds on the McMurdo Ice Shelf, Antarctica, cyanobacteria were dominant (*Oscillatoriales*, *Nodularia* sp., *Anabaena* sp. and *Nostoc* sp.) and toxin (nodularin and MC-LR) production was found to occur (Hitzfeld *et al.*, 2000).

Concluding remarks

Cyanobacterial blooms occurrence is an increasing global problem affecting every country in general. Eutrophication and anthropogenic activities associated with it are difficult to control in a way that phytoplanktonic and particularly cyanobacterial growth continues to be enhanced. Thus, the consequences derived from known and yet unknown cyanotoxins production are here to persist. Many international and regional measures have been taken and applied but much more is needed for reaching a solution to control this environmental and Public Health problem. This should start by everyone's awareness concerning causes and consequences of cyanobacterial blooms occurrence and the way to prevent it.

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Capítulo III

Microcystin producing blooms – a serious global Public Health issue

Microcystin producing blooms – a serious global Public Health issue

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Microcystin producing blooms – a serious global Public Health issue

Abstract - All over the world there has been an increasing occurrence of harmful algal blooms, in general due to the eutrophication of water bodies. Under appropriate conditions (especially high nutrient levels, high temperature and light conditions, no wind) some cyanobacteria may develop and form large masses of surface scum (blooms), particularly in freshwaters. Some of these cyanobacterial blooms may become dangerous because certain species are responsible for CTP (Cyanobacteria Toxin Poisoning) by producing toxins such as microcystins (hepatotoxins). The main genera capable of microcystin production are *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc* and *Anabaenopsis* and its biosynthesis seems to be controlled by several environmental factors like temperature, light, nutrients and trace metals, but also genotype diversity. Microcystins can cause serious damages at different trophic levels and it is considered a threat to human public health. The WHO (World Health Organization) has already established 1.0 $\mu g.L^{-1}$ as the maximum level for microcystins in drinking water. In mammals, some symptoms of hepatotoxicity after ingestion of microcystin contaminated water include: weakness, respiratory problems, cold extremities, vomiting and diarrhoea. Microcystins have also been correlated to cancer promotion because they are protein phosphatases 1 and 2A inhibitors. The aims of this study is to review the recent investigations concerning microcystin production, toxicity and occurrence in the last two decades, and alert for the need of a proper local water management regarding the nutrient inputs but also a better understanding of the interactions between the factors influencing these toxins production in each local region in order to control it more efficiently. It would be important that in every country, investigation and higher education focused more on this major Public Health issue in order to understand and adapt control strategies to specific regional characteristics of these cyanobacterial blooms occurrence.

Keywords: eutrophication, hepatotoxic blooms, microcystin synthesis, occurrence and consequences.

Introduction

The increase of human population and the consequent increase in agricultural and industrial activities along with a deficient water management led to eutrophication of

superficial freshwater bodies used for recreational purposes and as drinking water sources. Hence, phytoplanktonic blooms became also more frequent worldwide. More, environmental conditions such as higher temperatures (15-30ºC) and pH (6-9), low turbulence and high nutrient inputs enhance the development of planktonic cyanobacteria in lakes and reservoirs, leading to formation of surface blooms that may accumulate as scum. The dominance of certain cyanobacteria at surface is due to some advantageous characteristics such as less nutrient (particularly nitrogen) requirements and buoyancy regulation in water column for achieving better light and nutrient level conditions (Oliver and Ganf, 2000). The development of cyanobacterial blooms has become a concerning problem because some cyanobacterial species can produce toxins and studies from many countries have concluded that the majority of cyanobacterial blooms are indeed toxic (Codd *et al.*, 1995; WHO, 1998a; Codd, 2000; Dow and Swoboda, 2000). The intoxications caused by cyanobacteria are named CTP (*Cyanobacteria Toxin Poisoning*) and there are many cases documenting the hazardous potential of cyanotoxins for many organisms and also for Public Health (Codd *et al.*, 1995; Gorham and Carmichael, 1988; Codd, 2000). In humans, cyanobacteria may cause irritation of skin and/or mucous membranes or even gastroenteritis by recreational exposure (WHO, 1998a), hepatotoxicity or neurotoxicity by ingestion of contaminated drinking water (Gorham and Carmichael, 1988) or contaminated food (Codd *et al.*, 1999; McElhiney *et al.*, 2001), and even death may occur if blood is directly exposed to the toxins (Pouria *et al.*, 1998). Presently, there are more than 40 known toxic cyanobacteria (Sivonen and Jones, 1998; Dow and Swoboda, 2000) and the most common include: *Microcystis* spp., *Planktothrix/Oscillatoria rubescens* and *P. agardhii*, *Anabaena* spp., *Aphanizomenon* spp., some *Oscillatoria* spp., *Cylindrospermopsis raciborskii*, *Synechococcus* spp., *Gloeotrichia* spp., *Lyngbya* spp., *Nostoc* spp., *Schizothrix* spp., *Synechocystis* spp. and *Nodularia spumigena* (WHO, 1998a). For each species considered toxic there may be toxic and non-toxic strains and in toxic ones toxicity may vary among them (Böttcher *et al.*, 2001; Hesse and Kohl, 2001). Cyanotoxins are very diverse in their chemical structure and toxicity (Dow and Swoboda, 2000; Kaebernick and Neilan, 2001), being usually classified as dermatotoxins (lipopolyssacarides, lyngbyatoxin-a and aplysiatoxins), neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a(s) and saxitoxins) and hepatotoxins (microcystins, nodularin and cylindrospermopsin), according to the toxic effects on animals. Hepatotoxins are the most frequent cyanotoxins (Codd, 2000) and main responsible for CTP in freshwater bodies (Gorham and Carmichael, 1988; Dow and Swoboda, 2000). As exposed above, eutrophication is an important cause of the

increasing occurrence of toxic cyanobacterial blooms worldwide and consequent animal and human illness or death. Therefore, it is becoming essential a proper water management regarding the nutrient inputs to water systems but also a better understanding of the interactions between the factors influencing these toxins production in order to control it. It would be important that in every country, investigation and higher education focused more on this major Public Health issue in order to understand and adapt control strategies to specific regional characteristics of these cyanobacterial blooms occurrence. The present study reviews some recent work made on the microcystin toxicity on diverse organisms (including humans), factors influencing microcystin production and processes to eliminate microcystins from drinking water, as well as the occurrence of microcystin producing blooms worldwide in the last two decades.

Microcystins

Structural characterization

Microcystins are cyclic heptapeptides with the general structure *cyclo(-D-Ala-L-X-erythro-β-methyl-D-Adda-D-isoGlu-N-methyldehydro-ala)*. Of special interest are the variable L-aminoacids X and Z (X is usually leucine (L), arginine (R), tyrosine (Y) or phenylalanine (F), and Z is usually arginine (R), alanine (A) or methionine (M)) and the aminoacid Adda (*3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid*) considered the responsible for the molecule hepatotoxicity (Dawson, 1998). There are more than 60 microcystin isoforms (Codd, 2000; Dow and Swoboda, 2000) in part due to the variable L-aminoacids, but the most frequent and studied variant is microcystin-LR (MC-LR) with the variable aminoacids leucine (L) and arginine (R). Other variants that also occur more frequently are MC-RR, MC-YR and MC-LA.

Function

There are no conclusive studies about the purpose of microcystin (secondary metabolite) synthesis but some results indicate that it may act as a chemical defence against grazing (Laurén-Määttä *et al.*, 1997; Kurmayer and Jüttner, 1999; Henning *et al.*, 2001) or have an allelopathic effect over algal competitors (Kearns and Hunter, 2001) besides regulating endogenous protein phosphatases or being used as nitrogen reserve.

Producer species

These toxins occur in freshwaters worldwide and are mainly produced by colonial *Microcystis* spp. and filamentous *Anabaena* spp., *Planktothrix/Oscillatoria* (*P. agardhii* and

P. rubescens), *Anabaenopsis*, *Nostoc* (*N. rivulare*) and *Aphanizomenon* (*A. flos-aquae*), but also species belonging to the terrestrial genus *Hapalosiphon* (Codd *et al.*, 1995; Dow and Swoboda, 2000; Kaebernick and Neilan, 2001). MC-LR is known to be produced by species belonging to the genera *Anabaena*, *Microcystis*, *Nostoc* and *Anabaenopsis* (WHO,1998a; Dow and Swoboda, 2000) and MC-YR is produced by *Microcystis aeruginosa*, *M. viridis* and *Hapalosiphon* sp. (WHO, 1998a; Dow and Swoboda, 2000). MC-RR has been isolated from *Oscillatoria agardhii*, *Microcystis aeruginosa* and *M. viridis*, and MC-LA from *Microcystis aeruginosa* (Dow and Swoboda, 2000).

Synthesis pathway

Microcystins are secondary metabolites produced non-ribosomally through a microcystin synthetase complex (Kaebernick and Neilan, 2001) and their synthesis seems to be an energy (ATP) dependent process (Bickel and Lyck, 2001). The synthesis enzymatic complex is codified by a *mcy* genes cluster composed by two operons (*mcyA-C* and *mcyD-J*) (Kaebernick and Neilan, 2001) and it is present in toxic strains of the genus *Microcystis* but also in microcystin producing strains of *Anabaena*, *Nostoc* and *Planktothrix* (Neilan *et al*., 1999), allowing the development of rapid and sensitive PCR (*Polymerase Chain Reaction*) methods for its detection directly from environmental samples (Tillet *et al.*, 2001; Pan *et al.*, 2002).

Factors influencing microcystin synthesis

Light

In general, in spite of many contradictory studies, microcystin synthesis seems to increase with light intensity or photosynthetically active radiation (Rapala *et al.*, 1997; Rapala and Sivonen, 1998; Kaebernick *et al.*, 2000; Hesse and Kohl, 2001; Kaebernick and Neilan, 2001; Wiedner *et al.*, 2003) but light quality seems to be also a determinant factor (red light favours toxin production while blue light doesn't) (Kaebernick *et al.*, 2000) and there are maximum irradiance values above which the microcystin production is inhibited (Wiedner *et al.*, 2003). Some recent studies (Böttcher *et al.*, 2001; Hesse and Kohl, 2001) concluded that variations in light intensity in natural environments have little or no significant effect on microcystin cellular content and the differences found in blooms toxicity are probably due to growth rates and toxic characteristics of different strains.

Temperature

Temperature seems to influence the type of toxin produced. High temperatures (> 25ºC) seem to enhance MC-RR production while lower temperatures favour MC-LR synthesis (Rapala *et al.*, 1997; Rapala and Sivonen, 1998).

Macronutrients (N and P)

In the non-nitrogen-fixing cyanobacterium *M. aeruginosa*, microcystin content seems to increase at higher N:P ratios (Utkilen and Gjølme, 1995; Lee *et al.*, 2000) but Long *et al.* (2001) reported that fast cell-growth of *Microcystis aeruginosa* under N-limited conditions is associated with smaller cells and consequent higher intracellular microcystin quota. The growth of *Microcystis* spp. increases with increasing phosphorus concentrations (Utkilen and Gjølme, 1995; Rapala and Sivonen, 1998; Kotak *et al.*, 2000; Oh *et al*., 2000) and microcystin content in *Microcystis aeruginosa* also seems to be higher at high phosphorus concentrations (Jacoby *et al.*, 2000; Kotak *et al.*, 2000). Yet, Oh *et al*. (2000) documented higher values of microcystin content in *M. aeruginosa* under more P-limited conditions. In the N-fixing *Anabaena* spp. nitrogen (nitrates) showed an enhancement on toxin synthesis (Rapala *et al.*, 1997) but in nitrogen-free medium N-fixing cyanobacteria can still produce more microcystin than the non-nitrogen fixing ones. Microcystin content in *Anabaena* spp. seems to have also a tendency to increase with phosphorus concentration (Rapala *et al.*, 1997) probably due to the fact that N-fixing cyanobacteria are less dependent on N concentrations. There are many controversial results concerning the effects of nitrogen and phosphorus concentrations on microcystin content but microcystin production in *Microcystis* strains seems to be influenced by variation in nitrogen and phosphorus concentrations with different responses depending on strain (Vézie *et al.*, 2002).

Micronutrients (Fe and Zn)

Lukac and Aegerter (1993) found that zinc (Zn) enhanced growth and microcystin production in *Microcystis aeruginosa*, and low iron (Fe) concentrations decreased growth but increased the toxin synthesis. Utkilen and Gjølme (1995) had contradictory results (probably due to the use of a different strain) in which a decrease in the iron concentration decreased the microcystin content and microcystin synthetase should be actively controlled by the amount of available free Fe^{2+}.

Energy charge

Phosphorus (constituent of DNA, RNA and ATP), nitrogen (nitrogen metabolism and respiration), iron (Chl *a* synthesis) and light (energetic source) are essential environmental factors for promotion of metabolic energy. Hence, Bickel and Lyck (2001) suggested that if microcystin synthesis requires energy (as ATP), the variation of toxin production should be mostly explained by the energetic state of the cyanobacterial cells, and nutrient limitation (P, N and Fe) and light variation should have only an indirect influence, since cell energetic state changes in stress conditions. In conditions of low levels of energetic charge, available energy in cell is primarily applied in essential protein synthesis and not in microcystin (secondary metabolite) synthesis (Bickel and Lyck, 2001).

Genotype diversity

As shown, there is a considerable number of studies that, is spite of controversial, indicate that the toxicity content of certain cyanobacteria species could be directly influenced by environmental factors but recent approaches pose genotype diversity between strains as the main factor determining the variability in toxicity levels between blooms of the same species (Rohrlack *et al.*, 2001; Kurmayer *et al.*, 2002), with the development and success of strains better adapted to certain environmental conditions. The genotypes may differ in growth strategy, plasmid content, interaction with zooplankton, microcystin content (Hesse and Kohl, 2001) and microcystin synthetase genes cluster, originating different variants of the toxin with different toxicities (Mikalsen *et al.*, 2003).

Toxicity and bioaccumulation

Microorganisms

Some bacteria (Ostensvik *et al.*, 1998) have shown to be sensitive to microcystins and these toxins also showed to inhibit the growth of algal species belonging to the genera *Chlamydomonas*, *Haematococcus*, *Navicula* and *Cryptomonas*. MC-LR is able to paralyze the motile green alga *Chlamydomonas reinhadtii*, enhancing its settlement and creating a lake zone free of competitors for microcystin producer cyanobacteria (Kearns and Hunter, 2001).

Plants

Macrophytes such as *Phragmites australis* (Pflugmacher *et al.*, 2001), *Ceratophyllum demersum* and *Elodea canadensis* (Wiegand and Pflugmacher, 2001)

have shown to absorb MC-LR. In *Phragmites australis* the higher values for absorbed MC-LR were found in the stem and rhizome, with an increase in soluble glutathione S-tranferases (sGST) (Pflugmacher *et al.*, 2001). In the bryophyte *Vesicularia dubyana* MC-LR absorption was higher than in two macrophytes (Wiegand and Pflugmacher, 2001). Microcystins cause also a reduction in the number and mass of fronds in the water plant *Spirodela oligorrhiza* (Romanowska-Duda and Tarczynska, 2002) and MC-LR is known to affect the physiology (including growth) of the white mustard *Sinapis alba* seedlings (McElhiney *et al.*, 2001; Hamvas *et al.*, 2003). Crop plants that are consumed by humans irrigated with microcystin contaminated water may suffer growth and development effects, and may also accumulate the toxins posing the potential risk of toxin transference to humans through the food chain. The salad lettuce (*Lactuca sativa*) grown with spray irrigation of water containing microcystin-producing *Microcystis aeruginosa* retains microcystins (Codd *et al.*, 1999). Under laboratory conditions, microcystins proved to be inhibitors of growth and development in potato shoots and mustard seedlings (McElhiney *et al.*, 2001) as well as plant protein phosphatases inhibitors.

Zooplankton

The lack of alternative phytoplankton for food when cyanobacteria dominate may contribute to unfavourable nutritive conditions for zooplankton (Kurmayer and Jüttner, 1999) but the ingestion of *Microcystis* colonies by zooplankton may also be affected by colonies size and/or mucilage as well as their toxin content (Laurén-Määttä *et al.*, 1997; Henning *et al.*, 2001). While calanoid copepods avoid cyanobacteria that possess microcystins, daphnid cladocerans are less selective (Kurmayer and Jüttner, 1999) being are able to ingest both toxic and non-toxic *Microcystis* (Rohrlack *et al.*, 1999) under depletion of edible food (green algae and diatoms), accumulating the microcystins (Mohamed, 2001) and potentially transferring them to higher trophic levels through the food chain. But also toxic effects have been observed in *Daphnia* spp. after cell-bound microcystins ingestion (Rohrlack *et al.*, 2001) such as inhibition of protein phosphatases PP1 and PP2 (Henning *et al.*, 2001). The brine shrimp *Artemia salina*, a crustacean, as proved to be sensitive to MC-LR (Delaney and Wilkins, 1995), leading to an increasing SGT (detoxification system glutathione S-transferase) activity and conjugation of the toxin to glutathione via this GST as the first step to microcystin detoxification (Beattie *et al.*, 2003).

Molluscs

Mussels used for human consumption can accumulate microcystins posing intoxication hazards to human consumers in such a way that microcystins should be always monitored during and after occurrence of estuarine cyanobacterial blooms. The mussel *Mytilus edulis*, fed on *Microcystis aeruginosa* (with high microcystin content) for 3 days, accumulated microcystins in its tissues (Williams *et al.*, 1997). In another mussel, *Mytilus galloprovincialis*, microcystins were quickly accumulated but its depuration was not a very rapid process with microcystin persistence even after the bloom disappearance, probably due to recontamination by faeces containing the toxins (Amorim and Vasconcelos, 1999). Microcystins seem also to accumulate in some gastropods through grazing activity (Kotak *et al.*, 1996).

Crayfish

The crayfish *Procambarus clarkii* has shown to accumulate microcystins in the intestine and hepatopancreas (Vasconcelos *et al.*, 2001).

Fish

There has been documented that low concentrations of microcystins cause hepatopancreas and kidney damage in european carp (*Cyprinus carpio*) (Fischer and Dietrich, 2000). The rainbow trout (*Oncorhynchus mykiss*) suffers hepatotoxicosis by accumulating MC-LR that leads primarily to changes in cellular morphology, protein phosphatases inhibition and liver necrosis (Fischer *et al.*, 2000). Embryos and larvae of the loach (*Misguruns mizolepis*), a small freshwater fish, have shown to be affected by toxicity of MC-LR which targets their liver and heart (Liu *et al.*, 2002). The young life stages of fish seem to be more sensitive than adults or juveniles to microcystin hepatotoxic effects (Oberemm, 2001a). Microcystins have shown to be also accumulated in fish liver, viscera and muscle tissue, posing risks to humans that consume contaminated fish (Magalhães *et al.*, 2001). The freshwater fish *Oreochromis niloticus* accumulates microcystins in the guts, liver and kidneys (Mohamed *et al.*, 2003). A study using embryos of zebrafish (*Danio rerio*) showed that MC-LR is absorbed by embryos (Wiegand and Pflugmacher, 2001). Probably in order to allow adaptability, this fish species shows changes in its behaviour (such as reduced motility, increased rates of activity at night, reduced activity during the spawning period and reduced reaction on feeding (Baganz *et al.*, 2001)) when exposed to long-term sublethal doses of MC-LR, but these changes can have reproductive effects with substantial ecological consequences

like reducing population growth and changing species composition of the water body (Baganz *et al.*, 2001).

Terrestrial insects

The African locust, *Locusta migratoria migratorioides* has shown to be sensitive to MC-LR (Hiripiri *et al.*, 1998) with a LD_{50} value of 0.2 μg per animal or 130 $mg.kg^{-1}$.

Birds

Microcystins are known to cause liver necrosis in birds (ducks) (Matsunaga *et al.*, 1999).

Mammals

Laboratory studies

Microcystins are selective for hepatic cells, irreversibly inhibiting serine/threonine protein phosphatases PP1 and PP2A (Dawson, 1998) causing disintegration of hepatocytes structure, apoptosis, liver necrosis and internal haemorrhage in liver that may lead to death by hemorrhagic shock (Dow and Swoboda, 2000). MC-LR seems to bind also to ATP synthetase potentially leading to cell apoptosis (Mikhailov *et al.*, 2003). Microcystins orally ingested are transported across the ileum into the bloodstream via a bile-acid transporter that exists in hepatocytes and cells lining the small intestine. Microcystins bound specifically to hepatocytes (reason why they concentrate in the liver) and are actively absorbed to hepatic cells (Dawson, 1998; Dow and Swoboda, 2000). In the hepatocytes, they form adducts with PP1 and PP2A from cytoplasm and nuclei, inhibiting them and leading to disruption of liver cell structures, intrahepatic haemorrhage and death if a high dose is administrated (Fitzgerald, 2001). Microcystins seem not to be hydrolyzed by stomach peptidases and MC-LR appears to be absorbed by the intestine (Dow and Swoboda, 2000). LD_{50} value (i.p., in mice) for MC-LR is usually 50 $\mu g.kg^{-1}$ of body weight (Dow and Swoboda, 2000) but it can range from 25 to 125 $\mu g.kg^{-1}$ (Dawson, 1998; WHO, 1998b) and inhalation toxicity is also high (Dawson, 1998). Yet, MC-LR is much less toxic by oral ingestion with LD_{50} of 5000 $\mu g.kg^{-1}$ in mice (WHO, 1998b). In swine, the lethal (i.p.) dose of MC-LR is 72 $\mu g.kg^{-1}$ and the acute toxicosis results from severe intrahepatic haemorrhage with the blood flow being obstructed through the liver causing hypovolaemic shock, severe hypoglycaemia and/or terminal hyperkalemia (Beasley *et al.*, 2000). MC-YR has a LD_{50} (i.p., in mice) value of 70 $\mu g.kg^{-1}$ and MC-RR 300 to 600 $\mu g.kg^{-1}$ (WHO, 1998a). Some of the symptoms characteristic for this poisoning

are weakness, anorexia, cold extremities, pallor, apathy, respiratory problems, gastroenteritis, vomiting and diarrhoea (Codd *et al.*, 1995; Codd, 2000; Dow and Swoboda, 2000) with necrosis of the liver that may lead to death by hemorrhagic shock or liver failure after some hours or days, depending on species (Gorham and Carmichael, 1988). By inhibiting PP1 and PP2A, two important enzymes involved in tumour suppression, microcystins chronically administered have shown to promote liver cancer in mammals (Ito *et al.*, 1997) by inducing oxidative DNA damage (Zegura *et al.*, 2003). Mice exposed to a sub-lethal dose of MC-LR by intraperitoneal injections developed multiple neoplastic nodules in liver (Ito *et al.*, 1997) in spite oral administration showed no chronic injuries. Yet, chronic effects (increased liver weight and hepatohistological damage) have been detected in rats after a treatment with low concentrations of microcystins in drinking water for 28 days (Heinze, 1999). A recent study indicates that microcystins chronically administrated may also induce kidney damage on rats (Milutinovi *et al.*, 2002).

Humans

Human exposure to microcystins may occur through a direct route such as drinking water (Ueno *et al.*, 1996; WHO, 1998b; Zhou *et al.*, 2002), recreational water (WHO, 1998a) and haemodialysis (Pouria *et al.*, 1998), or through an indirect route such as food (Williams *et al.*, 1997; Amorim and Vasconcelos, 1999; Codd *et al.*, 1999; Schaeffer *et al.*, 1999; Magalhães *et al.*, 2001).

The knowledge about microcystin effects on humans is based on epidemiologic data, reports of intoxications and toxicological studies made on laboratory animals. The symptoms observed for laboratory mammals are thought to be similar to those felt by humans, in spite of the lack of studies in this area. Thus, epidemiological studies are the basis for human poisoning assessment and from the many worldwide cases reported until now, it is proven that microcystins cause acute (WHO, 1998b) and chronic effects on humans (Ueno *et al.*, 1996; Zhou *et al.*, 2002) and even death (Pouria *et al.*, 1998). Acute intoxication by microcystins coincides frequently with the lysis of the bloom forming cells (by natural senescence or water treatment processes) and liberation of toxins to the water. The inhalation of dry cyanobacteria cells or contaminated water is more dangerous than oral ingestion of contaminated water indicating the hazardous potential of practicing aquatic sports in recreational waters that suffers a microcystin producing bloom (WHO, 1998a). As exposed before, MC-LR is a potent cancer promoter in laboratory animals. Thus, chronic exposure to low concentrations of microcystins in drinking water can be a serious problem to Public Health, contributing for promotion of cancer in humans.

Epidemiological studies have already related the presence of microcystins in drinking water to an increase in the incidence of colorectal cancer (Zhou *et al.*, 2002) and primary liver cancer (Ueno *et al.*, 1996). There are more sensitive groups to microcystin poisoning that require special attention such as B-hepatitis patients but also children and old people (Fitzgerald, 2001).

Available treatments

Due to the rapid, irreversible and severe damage that microcystins cause in liver, therapy is difficult to be efficient and prophylaxis is also complicated. In 1988, Gorham and Carmichael referred immediate gastric lavage as the possible treatment if effective antidotes were unavailable. However, in the last fifteen years, several experimental studies were made about attenuation of animal and human intoxication by microcystins, showing interesting results (Dawson, 1998; Fitzgerald, 2001). Some are based on monoclonal antibodies against MC-LR (Nagata *et al.*, 1995) and others on hepatic uptake blockers as the immunossupressant Cyclosporine A and the antibiotic rifampin (Dawson, 1998). Recent studies such as the one from Gehringer *et al.* (2003) show that the membrane active antioxidant vitamin E, taken as a dietary supplement, may protect against toxicity of MC-LR by chronic exposure.

Guidelines for MC-LR

The danger of tumour promotion by chronic exposure of microcystins in drinking water was the main reason for the definition of guidelines for these toxins by WHO (*World Health Organization*). The life time consumption safe level proposed was of 1 $\mu g.L^{-1}$ for MC-LR (WHO, 1998b; Fitzgerald, 2001) and was based on animal studies of MC-LR orally administrated to pigs and mice (Fitzgerald, 2001). Many countries (such as Brazil, New Zealand and U.K.) have adopted this value as guideline for drinking water but Canada proposed the value 1.5 $\mu g.L^{-1}$ and Australia proposes values ranging from 1.3 to 10 $\mu g.L^{-1}$ (USEPA, 2001). In Canada, there was also proposed a value of 10 $\mu g.L^{-1}$ for short-termed exposure (Fitzgerald, 2001). For recreational waters with cyanobacterial blooms WHO has established 3 health hazard alert levels depending on the risk of adverse health effects (WHO, 1998a) and these are based on cyanobacterial densities. For cyanobacterial food supplements there is a proposed guideline for MC-LR of 10 $\mu g.g^{-1}$ (Schaeffer *et al.*, 1999) and, in Oregon, U.S.A., there has been established a maximum value of 1 $\mu g.g^{-1}$ for food (USEPA, 2001).

Monitoring methods

Physico-chemical

Methods based on HPLC (*High Pressure Liquid Chromatography*) (Poon *et al.*, 2001; Spoof *et al.*, 2001) are the most widespread quantitative and sensitive for detection of microcystins and other cyanotoxins, allowing the distinction between microcystin variants but also its isolation. Yet, they are expensive, time consuming, require a considerable sample volume for low concentrations, there aren't many certified standards available for MC variants and usually purification or concentration of the sample is required (Tsutsumi *et al.*, 2000; Nicholson and Burch, 2001). The recently developed method MALDI-TOF-MS (*Matrix Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry*) has been used for the analysis of many peptides, including cyanobacterial secondary metabolites (e.g. antibiotics or toxins like microcystins (Fastner *et al.*, 2001; Welker *et al.*, 2002)). It requires only microgram quantities (not milligram quantities like in HPLC or bioassays) of cell material and the detection is rapidly made, without the need for time consuming extraction or purification processes, allowing the identification of known microcystin variants and other unknown metabolites which can be further characterized (Fastner *et al.*, 2001; Welker *et al.*, 2002).

Immunological and Biochemical

ELISA (*Enzyme Linked Immunosorbent Assays*) are based on mono (Zeck et al., 2001) and polyclonal (Metcalf *et al.*, 2000; Yu *et al.*, 2002) antibodies actions against microcystin structure. They have low equipment requirements and allow a rapid, easy, effective and sensitive detection of microcystins (particularly MC-LR) in water samples (Nicholson and Burch, 2001), microorganisms and animal tissues, but toxicity is not assessed and they can be used only as a semi-quantitative screening tool. The problem of cross-reactivity with non toxic compounds (leading to false positives) has been minimized with competitive ELISA methods which may have detection limits of 0.07 $\mu g.L^{-1}$ (Zeck *et al.,* 2001) or even less, making ELISA suitable for assessing microcystin concentrations below the WHO guideline of 1 $\mu g.L^{-1}$ in drinking water. PPIA (*Protein Phosphatase Inhibition Assays*) are based on immunodetection and the toxic effects of microcystins at a molecular level, i.e. on the ability of microcystins to specifically inhibit the PP1 and PP2A, in spite of toxin transport into the cells is neglected and there is no direct relationship with mammalian toxicity. Many PPIA showed to overestimate the toxin concentrations and, for that, they are presently just used as a screening method. The colorimetric PPIA assay is a rapid, easy and sensitive screening method that doesn't

require much equipment and that is less expensive than ELISA or radiolabelled PPIA (that uses both PP1 and PP2A) in spite of in this assay only PP1 is used. Nevertheless, it is an assay which correlates positively with HPLC (Wirsing *et al.*, 1999; Metcalf *et al.*, 2001) and there are recent options for detecting MC-LR in drinking water with detection limits below the WHO guideline of 1 $\mu g.L^{-1}$ (Bouaícha *et al.*, 2002). Competitive binding assays based on blockage of the active site of PP2A have also been developed for microcystins (Serres *et al.*, 2000), and there are immunoblotting procedures based on anti-microcystin-LR monoclonal antibodies to monitor the formation of microcystin-PP1 adducts *in vitro* and *in vivo* (Liu *et al.*, 2000).

Biological

In vivo bioassays

Bioassays based on *Aeromonas hydrophila, Bacillus cereus* and *B. subtilis* have shown to be sensitive and suitable for assessing toxicity of *Microcystis aeruginosa* extracts (Ostensvik *et al.*, 1998). There are many plants such as *Spirodela oligorrhiza* (Romanowska-Duda and Tarczynska, 2002), *Solanum tuberosum* (McElhiney *et al.*, 2001) and *Sinapis alba* (McElhiney *et al.*, 2001; Hamvas *et al.*, 2003) that have shown to be sensitive to microcystins and may be used to assess toxicity of these toxins. Bioassays using *Daphnia* spp. (Tarczynska *et al.*, 2001; Kim *et al.*, 2003) and *Artemia salina* (Delaney and Wilkins, 1995; Sabour *et al.*, 2002) have become frequently used to assess microcystin toxicity. Test kit bioassays using larvae of the freshwater crustacean *Thamnocephalus platyurus* (Torokne *et al.*, 2000) or using the African locust (*Locusta migratoria migratorioides*) (Hiripi *et al.*, 1998) showed reaction to microcystins in spite the toxic responses were not specific. Fish-embryos tests using the species *Danio rerio* (Zebrafish) have shown to be very sensitive against cyanobacterial metabolites, in relation to adults or juveniles, probably due to their thin epithelia, large ratio of body surface to volume of embryos and vulnerability of developmental processes (Oberemm, 2001b). Mouse bioassays are the most used bioassays for determination of LD_{50} values, symptoms and effects for microcystins in mammals, and allow distinguishing between hepatotoxins and neurotoxins. Adult mice are usually injected intraperitoneally with the sample and according to sample toxicity different intoxication symptoms are observed usually within 24h. There are many studies using this kind of bioassay (Ito *et al.*, 1997; Sedmak and Kosi, 1997; Vasconcelos and Pereira, 2000; Oudra *et al.*, 2002; Sabour *et al.*, 2002). Rat (Sekijima *et al.*, 1999) and swine (Beasley *et al.*, 2000) bioassays have also been used to assess microcystin toxicity. Nevertheless, these bioassays don't detect

low microcystin levels nor distinguish between microcystin variants (Nicholson and Burch, 2001). Besides, *in vivo* mammal bioassays have the inherent ethical questions.

In vitro bioassays

Studies in vitro (Heinze et al., 2001; Zegura *et al.*, 2003) have been adopted as a more ethical and sensitive alternative for toxicity bioassays. The use of freshly prepared rat hepatocyte bioassays as an *in vitro* test system (with semi quantitative microscopic assessment of cell damage) seems to be promising in assessing toxicity of the cyanobacterial bloom samples, showing a strong correlation with the analytical data from HPLC (Heinze *et al.*, 2001) in spite of the operational requirements such as the preparation of cell suspensions (Nicholson and Burch, 2001). Along with the analytical methods like HPLC, bioassays are still an important tool for assessing the toxicity level of the known cyanotoxins or the presence of additional unknown toxic substances. In resume, screening methods such as ELISA, PPIA or bioassays should always be combined with more sophisticated methods like HPLC or MALDI-TOF (Nicholson and Burch, 2001).

Molecular

Methods based on PCR (*Polymerase Chain Reaction*) are a recent approach for detection of pathogen microorganisms in natural environments and they are being proposed also as a mean to rapidly determine if a cyanobacterial bloom or a determined species is potentially toxic or not as well as toxic cyanobacteria quantification by designing primers based on *mcy* genes (Rudi *et al.*, 1998; Tillett *et al.*, 2001; Pan *et al.*, 2002).

Microcystin removal and elimination processes

The removal of cyanobacterial cells by flocculation or filtration methods has proved to be an effective method to reduce toxin levels in water but only if there is no cell lysis and liberation of microcystins to water. If toxins are released, other methods are required such as activated carbon adsorption and ozonation to eliminate effectively dissolved microcystins from drinking water. Hence, methods that lead to cells lysis are not advisable and should be avoided in drinking water treatment plants.

Cell-bound microcystins removal

Flocculation by ferric chloride seems not to cause cyanobacterial lysis nor an increase in dissolved microcystin concentrations for *Microcystis aeruginosa* and *Anabaena circinalis*

(Chow *et al.*, 1998). Slow sand filtration has also shown to remove efficiently cell-bound microcystins from drinking water (Grutzmacher *et al.*, 2002).

Dissolved microcystins elimination

Microcystins show stability in deionised water, in sterilised water and under irradiation by sunlight (Dawson, 1998) or under extreme temperatures (>300ºC) and pH (WHO, 1998b). Thus, in natural environments, microcystins must be instable due to biodegradation and indirect photodegradation. Some of the bacteria known to degrade microcystins are Gram-negative and oxidase positive with low catalase activity (Welker *et al.*, 2001). *Sphingomonas* sp. is a bacterium that degrades MC-LR through microcystinase, a constitutively expressed metallo-protein that is produced even in absence of the toxin (suggesting its hydrolytic activity over other peptides) (Bourne *et al.*, 2001). Other *Sphingomonas*-like bacteria can also degrade MC-YR and -RR besides MC-LR (Park *et al.*, 2001). In natural environments, photodegradation of microcystins occurs indirectly via pigments or humic substances that absorb the sunlight (Welker *et al.*, 2001). Microcystins may be also photodetoxificated by UV irradiation (Kaya and Sano, 1998) and its rapid photocatalytic degradation can be achieved through a reactor with immobilized titanium dioxide catalyst (Shephard *et al.*, 2002). In the environment, microcystins detoxification seems to be enhanced by adsorption on the sediments (Tsuji *et al.*, 2001) but the elimination of microcystins in slow sand filtration filters is probably due to biodegradation rather than adsorption (Grutzmacher *et al.*, 2002). Presently, most drinking water treatment plants have methods like ozonation, activated carbon filtration and chlorination that allow the removal of the majority of microcystins (but not all) in superficial waters (Tsuji *et al*., 1997). Yet, ozonation effectiveness in microcystins destruction has shown to be reduced by high levels of total organic carbon as well as high cyanobacterial densities (cells lyse with ozonation, increasing the dissolved toxins level) (Hoeger *et al.*, 2002). Particularly wood-based activated carbons adsorb efficiently microcystins from aqueous solutions (Pendleton *et al.*, 2001) but clay material also seems to remove effectively microcystin-LR from water by adsorption of the toxin (Morris *et al.*, 2000). Chlorination, using adequate sodium hypochlorite doses after cells removal, seems to be very effective for the elimination of microcystin-LR in raw water with no formation of noxious products from the process (Tsuji *et al.*, 1997). Microcystins may be efficiently decomposed and removed from waters with high total organic carbon by ferrate oxidation-coagulation (Yuan *et al.*, 2002) and Fenton oxidation of MC-LR by Fenton reagent has

shown also to be a promising method for rapid degradation of this kind of hepatotoxins (Gajdek *et al.*, 2001).

Occurrence of microcystin producing blooms

Since the last century many hepatotoxic blooms have been documented and are reviewed by several authors such as Gorham and Carmichael (1988), Codd *et al.*, (1995), WHO (1998a; 1998b) and Codd (2000). The cases reported next have are restricted to the last twenty years.

Europe

In the last decade, several Portuguese freshwater bodies (including lakes, reservoirs and rivers), used for recreational or drinking purposes, have been found to have hepatotoxic blooms with production of diverse microcystins (MC-LR, MC-RR, MC-YR and others) mainly associated with the dominance of *Microcystis aeruginosa* (Vasconcelos *et al.*, 1996; Vasconcelos, 2001; Vasconcelos and Pereira, 2001). In France, microcystins have been detected in Lake Grand-Lieu (Vézie *et al.*, 1998) and Saint-Caprais reservoir (Maatouk *et al.*, 2002) and were produced by *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*, respectively. In 1995, near Liège, Belgium, three adjacent ponds suffered a *Microcystis aeruginosa* bloom with microcystin production related to bird deaths (Wirsing *et al.*, 1998). Dense mats of benthic cyanobacteria (*Oscillatoria* and *Phormidium*) have been reported to occur in oligotrophic, cold and turbid alpine waters of south-eastern Switzerland, showing hepatotoxic (by microcystins) and neurotoxic effects in mice (Mez *et al.*, 1997). Germany has studies from recent years that indicate that many German water bodies used for recreational or drinking water purposes were in their majority dominated by cyanobacteria from genera *Planktothrix*, *Microcystis*, *Anabaena* and *Aphanizomenon*, with microcystin and anatoxin-a production (Hummert *et al.*, 2001; Wiedner *et al.*, 2001; Frank, 2002) and having implications on the growth of fish (Ernst *et al.*, 2001). In the last decade, many Czech recreational and drinking water reservoirs and fish ponds were found to be dominated by *Microcystis* spp., *Planktothrix agardhii* e *Aphanizomenon flos-aquae* that produced microcystins (Marsálek *et al.*, 2001) and high microcystin levels were detected in raw waters and some treated waters from drinking water treatment plants, endangering the consumers health (Bláha and Marsálek, 2001). During 1995 and 1996, three eutrophic Latvian lakes (Lakes Mazais, Lielais Balterzers and Sekitis) had summer blooms of potentially toxic *Microcystis aeruginosa* (with production of microcystins), *Aphanizomenon flos-aquae* and *Anabaena flos-aquae*, leading to a decrease in drinking

water quality and health problems resulting from the recreational use of lakes water (Eynard *et al.*, 2000). North-Eastern Slovene freshwaters have suffered many blooms with *M. aeruginosa* dominance and microcystin production (Sedmak and Kosi, 1997). In the last twenty years the Loch Leven, in Scotland, had several hepatotoxic blooms of *Microcystis aeruginosa* and *Anabaena flos-aquae* associated with more than 1000 dead fish (with liver necrosis) that accumulated in the shores after *Anabaena flos-aquae* bloom senescence (Codd *et al*., 1995). In the United Kingdom, *Microcystis* blooms in lakes and reservoirs have been associated with death of sheep and dogs as well as human illness due to microcystin production (WHO, 1998a; Dow and Swoboda, 2000). In Sweden, between 1991 and 1997, microcystins were detected in some lakes with dominance of *Microcystis aeruginosa*, *M. viridis* and *Planktothrix prolifica* (Willén *et al.*, 2000), in a water treatment plant (Codd *et al.*, 1995) and in a river with a *Planktothrix agardhii* bloom that caused intoxication of pets and 121 persons (WHO, 1998a). A study based on dozens of south Norwich water bodies revealed the occurrence of many microcystin producing blooms of *Anabaena* spp., *Microcystis* spp. and *Oscillatoria* (*Planktothrix*) spp. (Utkilen *et al.*, 2001). In the last decade, an intensive study on hundreds of freshwater bodies from Denmark reached the conclusions that the majority of blooms had microcystin production by *Microcystis* spp., *Anabaena* spp., *Planktothrix agardhii* e *Aphanizomenon flos-aquae* and the deaths of thousands of fish and a cow were related to hepatotoxic blooms of *Anabaena flos-aquae* and *Planktothrix agardhii*, respectively (Henriksen, 2001).

North America

Microcystins are the most frequent cyanotoxins in Canada and drinking water is the main exposure route (Gupta *et al.*, 2001). Lakes used as drinking water sources suffer *Microcystis aeruginosa* blooms and MC-LR is found in concentrations higher than WHO guideline in both raw and treated tap waters (Gupta *et al.*, 2001). In United States of America there have been reported hepatotoxic blooms of *Microcystis aeruginosa* (Puschner *et al.*, 1998; Jacoby *et al.*, 2000) related to animal deaths (24 heifers) (Puschner *et al.*, 1998), and food supplements made on natural *Aphanizomenon flos-aquae* blooms were found to have high levels of microcystin (Schaeffer *et al*., 1999).

South America

In 1996, Caruaru, Pernambuco state, Brazil, the deaths of 60 patients from a haemodialysis unit were related to microcystin intoxication due to the use of water from a reservoir suffering a bloom of *Anabaena* spp. and *Microcystis* spp. and the insufficient

treatment to eliminate the microcystins from that water (Pouria *et al*., 1998). The state of Paraná, Brazil, has frequent occurrence of microcystin producing *Microcystis* spp. blooms in freshwater lakes and reservoirs used for recreational and animal farming purposes but also in some used as drinking water supplies (Hirooka *et al.*, 1999). The Patos Lagoon estuary, Rio Grande do Sul, southern Brazil, suffers regular blooms of *Microcystis* and microcystin is synthesised during their occurrence (Matthiensen *et al.*, 2000). In Conception, Chile, *Microcystis* spp. blooms with the presence of microcystins have been reported in different lakes (Campos *et al.*, 1999; Neumann *et al.*, 2000).

Oceania

In February 2000, at Swan-Canning estuary, in Western Australia, there was a dense and severe *Microcystis aeruginosa* bloom (Atkins *et al.*, 2001). Another hepatotoxin (cylindrospermopsin) occurrence seems to be very frequent in this country (Fitzgerald, 2001).

Asia

Various brackish and freshwater bodies in South Korea including dams and lagoons used as drinking water sources showed dominant species belonging to *Microcystis* genera, but also *Anabaena* e *Planktothrix/Oscillatoria*, with production of microcystins and anatoxin-a (Oh *et al.*, 2001; Park, 2001). In China, lakes suffering from *Anabaena* and *Oscillatoria* blooms revealed the presence of microcystins (Xu *et al.*, 2000) and there has been reported a significant correlation between microcystin producing bloom occurrence in the superficial drinking water sources (ponds and rivers) and primary liver cancer incidence (Ueno *et al*., 1996). Over the last twenty years, in Japan, microcystins were detected in various naturally occurring *Microcystis* blooms (Tsuji *et al.*, 1996; Park *et al.*, 1998; Matsunaga *et al.*, 1999) and in one case they have caused the death of dozens of ducks (Matsunaga *et al.*, 1999). Microcystins have also been found in several strains of *Microcystis aeruginosa* isolated from eutrophic aquaculture ponds and water reservoirs in Taiwan (Lee *et al.*, 1998). In the Philippines, during the last years, Laguna de Bay suffered periodic blooms of *Microcystis aeruginosa* and many variants of microcystins were detected (Cuvin-Aralar *et al.*, 2002).

Africa

In July of 1995, in Egypt, River Nile (used as drinking water source) at Sohag province suffered an *Oscillatoria tenuis* bloom with production of microcystins (Brittain *et*

al., 2000). In Marocco, many ponds, lakes and reservoirs proved to have several cyanobacterial microcystin producing strains belonging to the genera *Microcystis*, *Synechocystis*, *Pseudanabaena* and *Oscillatoria* (Oudra *et al.*, 2002; Sabour *et al.*, 2002). In South Africa, there has been reported a contamination of drinking water by the presence of *Microcystis aeruginosa* (with confirmed MC-LR synthesis) and its relation to a livestock poisoning outbreak (Van Halderen *et al.*, 1995).

Concluding remarks

Cyanobacterial blooms with microcystin synthesis occur worldwide in lakes, rivers and reservoirs used as drinking water sources or for recreation but the most concerning cases are those reporting the detection of these toxins in treated drinking water submitting consumers to a high risk of developing cancer. Thus, blooms that at first sight seemed to be a common ecological problem from eutrophication of water bodies have proved, indeed, to be a serious Human Health problem compromising the safety of the most important resource in which the whole Humanity depends on – drinking water. In spite of all the new approaches for microcystin elimination in drinking water plants and sensitive monitoring methods, water management strategies to reduce these blooms occurrence are urgent. It is necessary to raise a consciousness that the best way to eliminate this kind of problems is to prevent them from happening. Investigation should be also promoted in order to understand the characteristics of a local microcystin producing bloom occurrence as well as the factors influencing the toxin production in those specific local conditions. As a result, efficient local water management strategies could be more effective.

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Capítulo IV

Seasonal dynamics of phytoplankton community in Vela Lake (Portugal)

Seasonal dynamics of phytoplankton community in Vela Lake (Portugal)

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Seasonal dynamics of phytoplankton community in Vela Lake (Portugal)

Abstract – The increasing eutrophication of superficial freshwaters due to the high levels of nutrient inputs (from agriculture, cities and industry), along with summer weather conditions, frequently leads to the occurrence of algal blooms. Cyanobacterial blooms are very common to occur in freshwaters and may become dangerous because some cyanobacteria produce toxins that affect many organisms, causing disease or even death. For humans, cyanobacterial toxic effects usually occur by direct skin contact with contaminated water, by contaminated food consumption or, most frequently, by contaminated water ingestion. In the present work, phytoplankton dynamics of the eutrophied Vela Lake (Figueira da Foz, Portugal), used for recreational purposes and as a water source for agriculture, was monitored in relation to environmental parameters. The collection of water samples for environmental parameters and phytoplankton determinations was made every fortnight during an annual cycle (2000-2001). Vela Lake is a shallow freshwater body surrounded by *Pinus* spp., aquatic macrophytes, sandy soil and agricultural areas. Phytoplankton community showed to be dominated by diatoms and green algae during the winter months (when nutrients were more available) and cyanobacterial blooms were recorded during the warmer months (when nutrients became unavailable) with dominance of the potentially toxic cyanobacteria *Aphanizomenon flos-aquae* and *Microcystis aeruginosa*. The most important bloom occurred in the beginning of May 2001, with the development of a floating green scum characterized by the dominance of an *A. flos-aquae* strain and the scarce presence of *M. aeruginosa*. The development of this bloom was preceded by the lowest nitrogen levels in water but phosphate availability. At the time of bloom senescence, there was a sudden decrease in the dissolved oxygen levels (reaching depletion) and an increase in the ammonium levels (up to 1.8 $mg.L^{-1}$). At the same time, a massive death of ichthyofauna (approximately 8 tons) was recorded in the lake and associated with these severe environmental conditions, although cyanotoxins liberation during the bloom senescence was not assessed. There was also the development of significant *M. aeruginosa* blooms related to high ammonium levels but also to phosphate depletion.

Keywords: eutrophication, phytoplankton dynamics, cyanobacterial blooms, *Aphanizomenon flos-aquae*, *Microcystis aeruginosa*, fish kills.

Introduction

Eutrophication of superficial freshwaters is increasing worldwide mainly due to the pressure of anthropogenic activities on aquatic systems and it is generally related to nutrient inputs from agriculture, livestock production, cities and industry (Hall *et al.*, 1999; Codd, 2000; de Jonge *et al.*, 2000; Cooperband and Good, 2002; Withers and Lord, 2002). These high nutrient concentrations, along with water stability and an increase in water temperature and in pH, frequently lead to the occurrence of algal blooms, particularly cyanobacterial blooms (Hadas *et al.*, 1999; Jacoby *et al.*, 2000; Oliver and Ganf, 2000). Cyanobacteria can dominate due to several characteristics such as their low dependence on nitrogen (by fixing N_2 (Flores and Herrero, 1994)), competition for light and nutrients through buoyancy regulation (Brookes and Ganf, 2001; Porat *et al.*, 2001) and reduced grazing by zooplankton (Kurmayer and Jüttner, 1999; Rohrlack *et al.*, 1999). The primary consequence of blooms occurrence is the water quality reduction with economical, ecological and Public Health implications (Codd, 2000). From an ecological point of view, specific biodiversity decreases at all trophic levels and there is a deterioration of the habitat, with increased turbidity, a decrease in oxygen concentration and production of substances that give a bad taste and odour to the water (Park, 2001). Besides these factors, blooms of cyanobacteria may become dangerous because many cyanobacterial strains produce toxins that affect many organisms, causing disease or even death (Codd *et al.*, 1995; Codd, 2000; Dow and Swoboda, 2000; Briand *et al.*, 2003). In humans, cyanobacterial toxic effects usually occur by direct skin contact with contaminated water (WHO, 1998a), by contaminated food consumption or by contaminated water ingestion (Gorham and Carmichael, 1988; Fitzgerald, 2001; Zhou *et al.*, 2002), but has also happened through haemodialysis with the death of 60 patients (Pouria *et al.*, 1998). According to the effects on animals, cyanotoxins can be classified as dermatotoxins (lipopolyssacarides, lyngbyatoxin-a and aplysiatoxins), neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a(s) and saxitoxins) and hepatotoxins (microcystin, nodularin and cylindrospermopsin) (Kaebernick and Neilan, 2001). Cyanobacterial blooms have been recorded in marine, brackish and freshwaters worldwide and a great percentage (50 to 90 %) of them has been considered toxic (Codd *et al.*, 1995; WHO, 1998b; Codd, 2000; Dow and Swoboda, 2000). Presently, there are more than 40 known toxic cyanobacterial species (Dow and Swoboda, 2000; WHO, 1998a) belonging to the genera *Microcystis*, *Planktothrix/Oscillatoria*, *Anabaena*, *Oscillatoria*, *Aphanizomenon*, *Lyngbya*, *Cylindrospermopsis*, *Synechococcus*, *Gloeotrichia*, *Nostoc*, *Schizothrix*,

Synechocystis and *Nodularia*. Yet, for each toxic species there may be toxic and non-toxic strains and in toxic ones toxicity may vary among them (Hesse and Kohl, 2001). Toxic cyanobacterial blooms have been reported in many Portuguese water bodies (Vasconcelos, 2001). Vela Lake (Figueira da Foz, Portugal) is a shallow eutrophied freshwater body used for recreational purposes and as a water source for agriculture. It is surrounded by *Pinus* spp., aquatic macrophytes, sandy soil and agricultural areas. The water volume is predominantly influenced by the variation of groundwater levels and rainfall. There are not many published studies about this Lake, in spite of being subject of investigation at different levels. Yet, toxic cyanobacterial blooms occurrence in Vela Lake has already been reported by Vasconcelos *et al.* (1993).

In the present work, the phytoplankton dynamics (with special regard towards cyanobacteria) in Vela Lake was monitored during an annual cycle (2000-2001) in relation to chlorophyll *a* and environmental parameters such as pH, water temperature, conductivity, dissolved oxygen, total suspended solids and orthophosphates, ammonium, nitrate and nitrite concentrations.

Material and Methods

Study area and sampling

Vela Lake (44º58'N, 5º18' W) is a shallow eutrophied freshwater body located in Quiaios (Figueira da Foz, Portugal). It has an area of approximately 0.7 km^2 and it is 6 km away from the Atlantic Ocean (Fig.1). This water body is used for recreational and agricultural purposes and organic matter and nutrient inputs come from human activities inside the lake (such as fishing and recreation) and in surrounding areas (such as agriculture). In the East zone of the lake, agriculture is practiced using high amounts of fertilizers and pesticides that are lixiviated into the lake water.

Figure 1 – Vela Lake location in Portugal (adapted from http://earthobservatory.nasa.gov/Newsroom/NewImages/Images/modis_port_20020423.jpg)

The collection of water samples (three replicates of one litre) for environmental parameters and phytoplankton determinations was made every fortnight. For qualitative information about seasonal variation of phytoplankton species it was used a 25 μm mesh size trawl.

Environmental parameters and chlorophyll a

Water temperature, dissolved oxygen, pH and conductivity were analysed *in situ* with a multi-parameter probe. In laboratory, parameters such as total suspended solids, orthophosphates, ammonium, nitrate and nitrite concentrations were determined according to APHA (1992). Chlorophyll *a* determination was also performed according to APHA (1992). Sampling dates distribution was assessed through a Principal Components Analysis (PCA).

Phytoplankton analysis

Phytoplankton material collected with the nylon net 25 μm mesh size was fixed in formol (5% v/v). Identification of phytoplankton species was made by observation with a light microscope using different references for Cyanoprokaryota (Geitler, 1932; Komárek and Anagnostidis, 1989; Komárek and Anagnostidis, 1999), Bacillariophyceae (Germain, 1981; Krammer and Lange-Bertalot, 1986-1991; Lange-Bertalot, 2001) and Chlorococcales (Komárek and Fott, 1983). Quantification samples were fixed in lugol (1% v/v) and the enumeration was performed according to Lund *et al.* (1958) method with at least 400 cells counted. Phytoplanktonic species distribution was assessed through a Principal Components Analysis (PCA).

Results

Physical and chemical parameters

Concerning nutrient concentrations during the study period (from November 2000 to November 2001), there are some interesting observed data (Table 1 and Fig. 2). Orthophosphates, nitrite and nitrate concentrations attained the highest values in late December and beginning of January. Nitrogen sources had higher levels from November until April and the rest of the year levels were low. Nitrate concentration varied from 0.30 to 6.60 $mgNO_3^-.L^{-1}$, corresponding to the sampling dates of 2nd May 2001 and 4th January

2001, respectively. The highest nitrite concentration was 0.205 $mgNO_2^-.L^{-1}$ in 20th December 2000 and generally disappeared from April to November. Ammonium levels were generally low during all year, but showed high levels between November and December (ranging from 1.53 to 2.19 $mgNH_4^-.L^{-1}$) and a sudden increase in 29th May 2001 (up to 1.80 $mgNH_4^-.L^{-1}$).

Table 1 – Environmental data recorded during the one year study period in Vela Lake.

	***In situ* parameters**				**Nutrients**					
Sampling date	**Cond. (□S.cm^{-1})**	**Temp (ºC)**	**pH**	**O_2 (mg.L^{-1})**	**Nitrite (mg.L^{-1})**	**Nitrate (mg.L^{-1})**	**Ammonium (mg.L^{-1})**	**Orthophosphate (mg.L^{-1})**	**Chlorophyll *a* (□g.L^{-1})**	**Total Suspended Solids**
06-10-2000	485	21,0	9,15	8,70	0,000	0,80	0,70	0,00	0,96	
20-10-2000	483	17,4	8,60	6,30	0,000	1,00	0,65	0,08	14,97	0,059
02-11-2000	479	16,3	8,60	7,90	0,000	0,80	0,70	0,24	40,50	0,045
17-11-2000	493	12,2	7,52	5,10	0,003	0,70	1,53	0,53	29,61	0,026
05-12-2000	475	14,6	7,61	7,23	0,064	2,80	2,19	0,75	30,75	0,030
20-12-2000	452	11,9	7,70	7,61	0,205	4,80	1,64	1,65	14,98	0,011
04-01-2001	419	12,7	7,77	7,54	0,086	6,60	0,61	1,06	21,74	0,015
18-01-2001	372	11,5	8,34	7,84	0,009	4,70	0,48	1,22	38,80	0,020
01-02-2001	346	12,3	7,29	24,40	0,026	4,40	0,45	1,27	7,65	0,004
13-02-2001	327	16,6	7,35	7,00	0,038	3,60	0,52	1,27	2,14	0,002
01-03-2001	331	14,5	8,06	9,00	0,032	2,80	0,48	0,93	13,86	0,010
15-03-2001	335	15,1	7,76	7,80	0,042	2,60	0,50	1,01	10,18	0,006
29-03-2001	327	15,3	8,72	11,70	0,023	1,80	0,35	0,67	33,64	0,006
14-04-2001	341	19,0	9,40	9,90	0,000	0,50	0,36	0,16	22,61	0,012
02-05-2001	364	15,2	8,52	10,20	0,000	0,30	0,36	0,19	29,90	0,016
16-05-2001	348	17,5	8,95	10,00	0,000	0,50	0,85	0,24	149,43	0,028
29-05-2001	315	28,7	8,24	0,00	0,010	0,60	1,81	0,24	19,22	0,023
12-06-2001	306	23,4	9,25	8,70	0,000	0,60	0,46	0,00	66,93	0,028
01-07-2001	289	29,4	9,94	13,30	0,000	0,80	0,54	0,00	65,27	0,053
20-07-2001	297	23,5	9,60	11,50	0,000	1,10	0,84	0,00	28,48	0,081
31-07-2001	272	24,9	8,84	6,10	0,000	1,00	0,65	0,00	54,51	0,070
22-08-2001	298	26,4	9,49	10,90	0,000	0,80	0,64	0,00	27,65	0,055
07-09-2001	310	23,8	8,82	9,80	0,000	0,40	0,47	0,00	42,10	0,060
21-09-2001	300	18,6	9,04	9,80	0,000	0,60	0,58	0,00	44,50	0,071
10-10-2001	299	17,9	8,90	8,60	0,000	0,50	0,52	0,00	21,36	0,055
25-10-2001	305	17,0	9,40	11,90	0,000	0,60	0,49	0,00	24,72	0,043
14-11-2001	346	12,0	9,01	10,60	0,000	0,60	0,56	0,00	36,12	0,049
29-11-2001	370	10,3	8,98	10,33	0,000	0,50	0,49	0,00	13,86	0,042

Phosphorus availability was limited to the period between the end of October and the end of May 2001, with the highest value of 1.65 mgPO_4^{3-}.L^{-1} (corresponding to the sampling date of 20th December 2000) and with depletion of this nutrient during the remaining months.

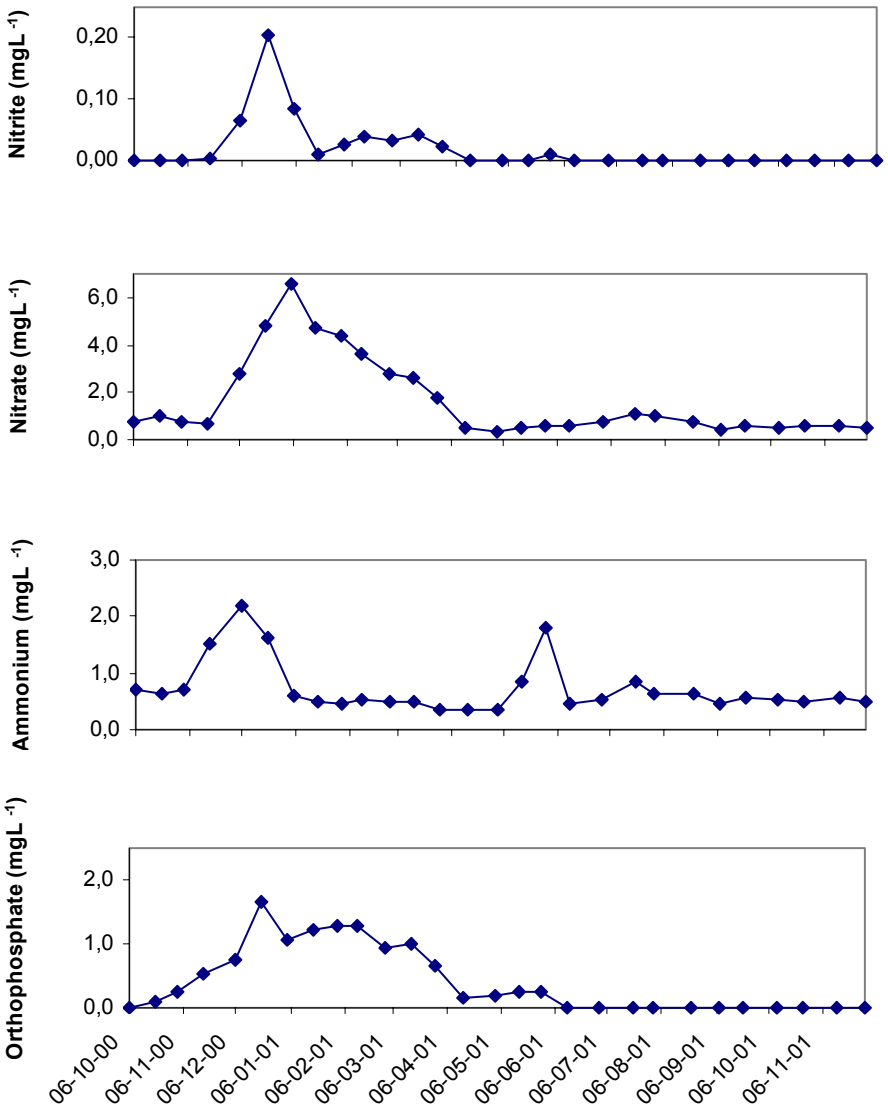

Figure 2 – Nutrient (nitrate, nitrite, ammonium and orthophosphates) concentrations during the study period.

The pH did not show very strong differences during all the year (Table 1 and Fig. 3), but there was a slight increase in its value during the warmer months (from April to November). Temperature showed the highest values in summer months, but also some

increased values in April (Fig.3). Oxygen was available all year, except in 29th May, where there was total oxygen depletion (Fig.3). The highest value for dissolved oxygen was recorded in the beginning of February with 24.40 $mg.L^{-1}$. Total suspended solids showed increased values from June until November, with the two highest values observed in July and October.

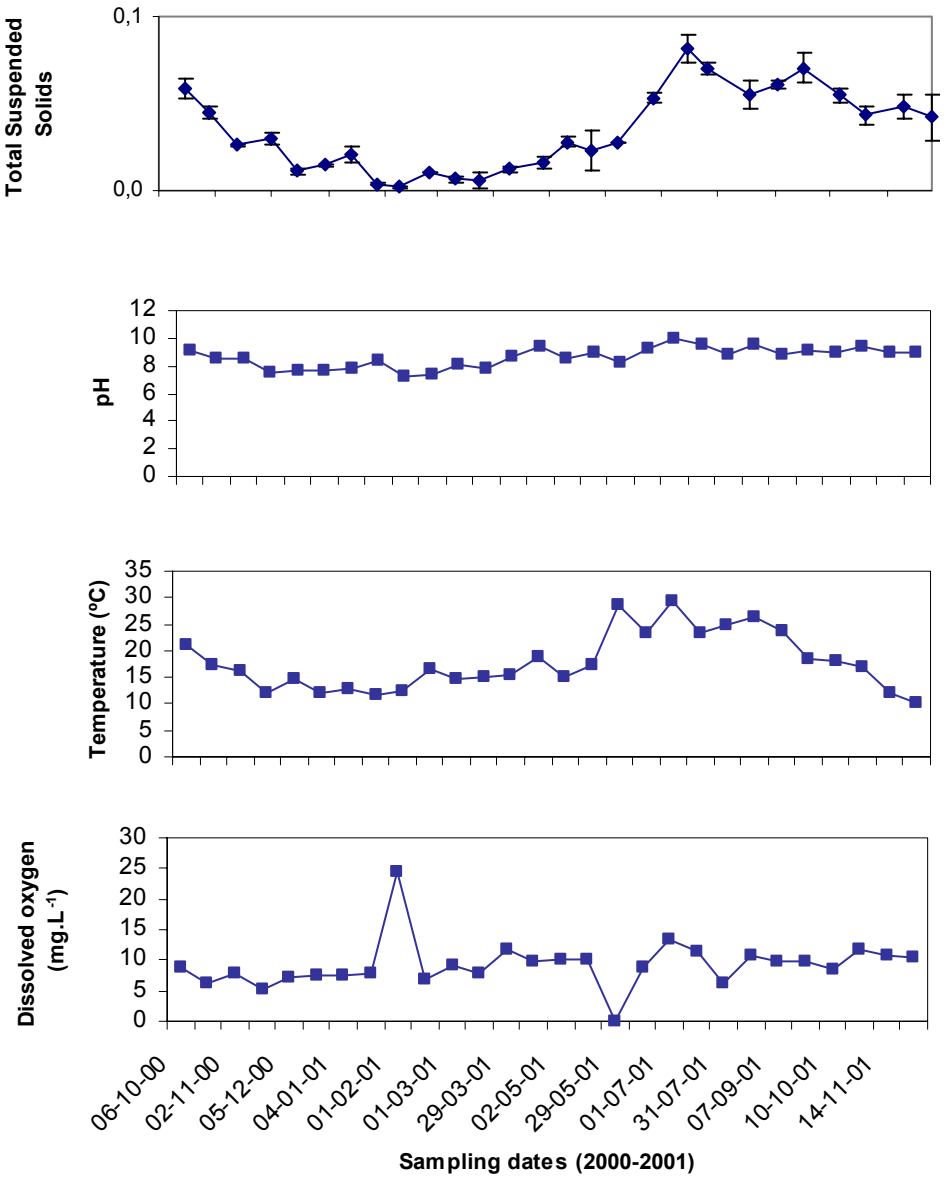

Figure 3 – pH, water temperatures and oxygen concentrations recorded during the study period.

Chlorophyll *a* concentrations suffered many oscillations during the one year study (Fig.4) with the highest value (149.43 μg.L^{-1}) attained for 16th May 2001 (Fig. 4). Of interest there are also the values achieved in 12th June (66.93 μg.L^{-1}) and 1st July 2001 (65.27 μg.L^{-1}).

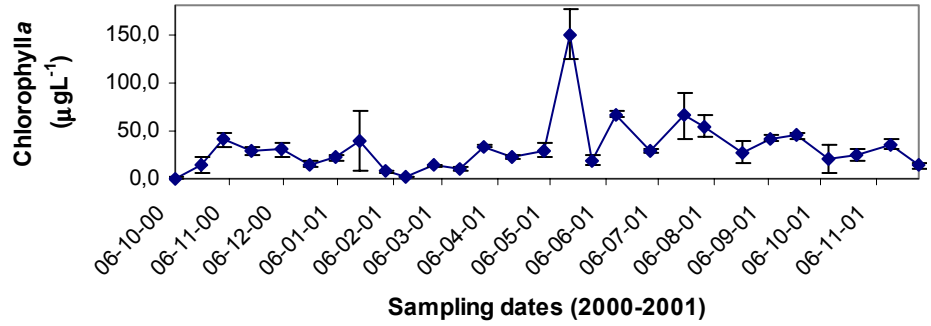

Figure 4 – Chlorophyll *a* concentrations recorded during the study period.

Phytoplankton composition

During the study period, 56 algal *taxa* were identified in Vela Lake. The *taxa* number

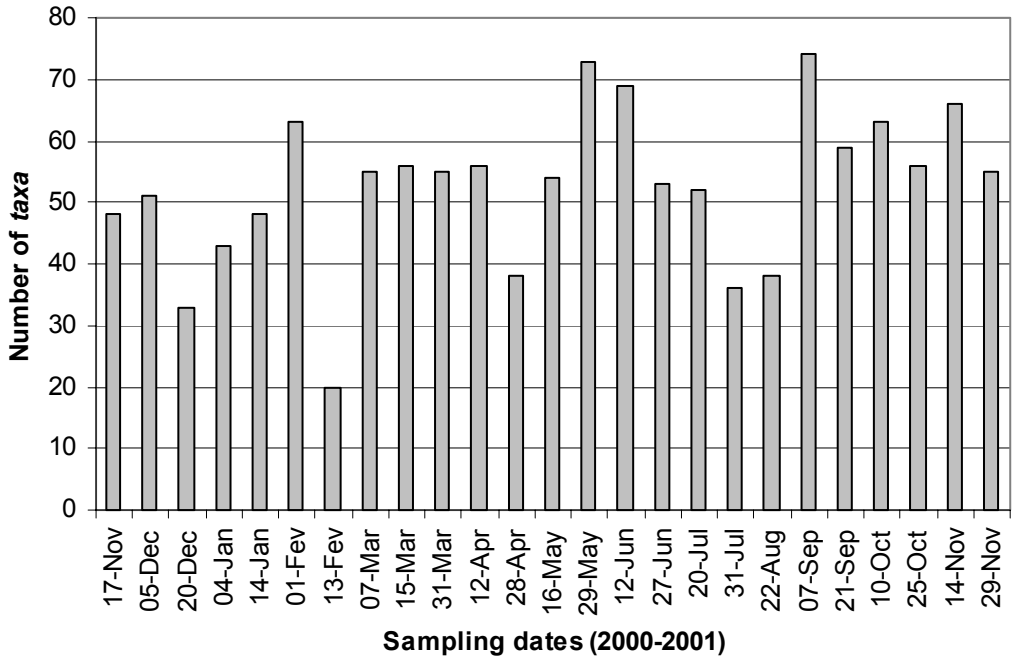

Figure 5 – Number of phytoplanktonic *taxa* in Vela Lake during the one year study period.

showed many variations (Fig. 5) with the lowest values recorded in 13th February and the highest in 29th May and 7th September 2001.

From late April until the beginning of November 2001, cyanobacterial species were the most abundant, and, from November to early April, diatoms and chlorophytes were well established (Fig. 6).

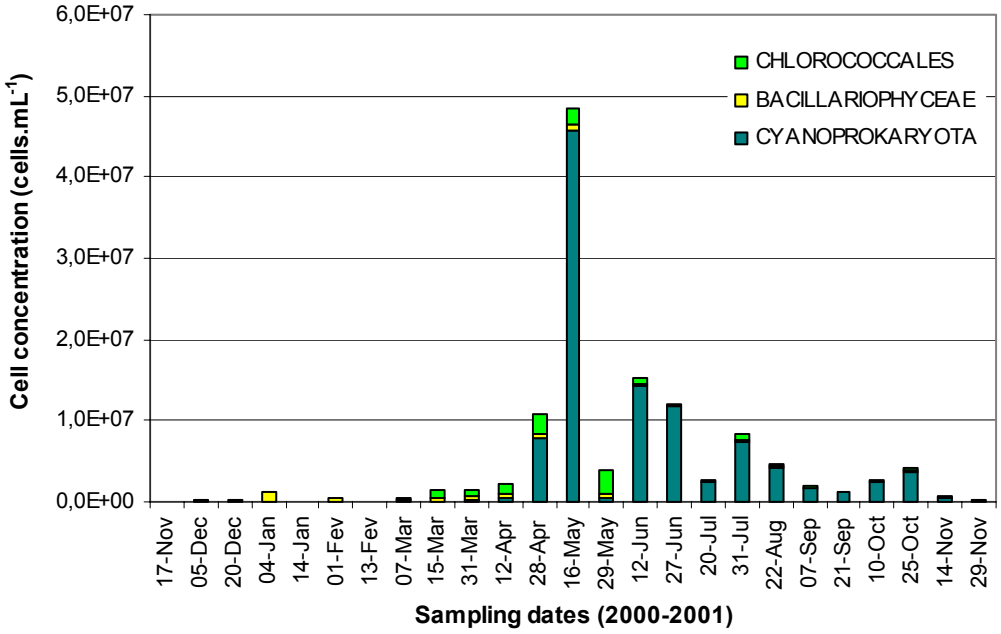

Figure 6 – Seasonal phytoplankton composition in Vela Lake during the one year study period.

The most abundant cyanobacterial species found in this Lake during the study period belonged to the *taxa*: *Aphanizomenon flos-aquae*, *Chroococcus limneticus*, *Microcystis aeruginosa* and *Pseudanabaena* sp. (Fig.6). Regarding the potentially toxic cyanobacteria, *A. flos-aquae* dominated the cyanobacterial community only in mid May 2001 and *M. aeruginosa* dominated in June, late July and from September until November. From the beginning of July until the beginning of September there was a co-dominance of *M. aeruginosa* and *C. limneticus*, the most persisting *taxa* of the cyanobacterial community during the study period.

The main cyanobacterial blooms were observed in 28th April (dominated by *Chroococcus limneticus*), 16th May (dominated by *Aphanizomenon flos-aquae*) and during June and in late July (dominated by *Microcystis aeruginosa*) (Figs. 6 and 7). A

Pseudanabaena sp. showed also a high density only in 12th June, co-dominating the bloom with *M. aeruginosa*.

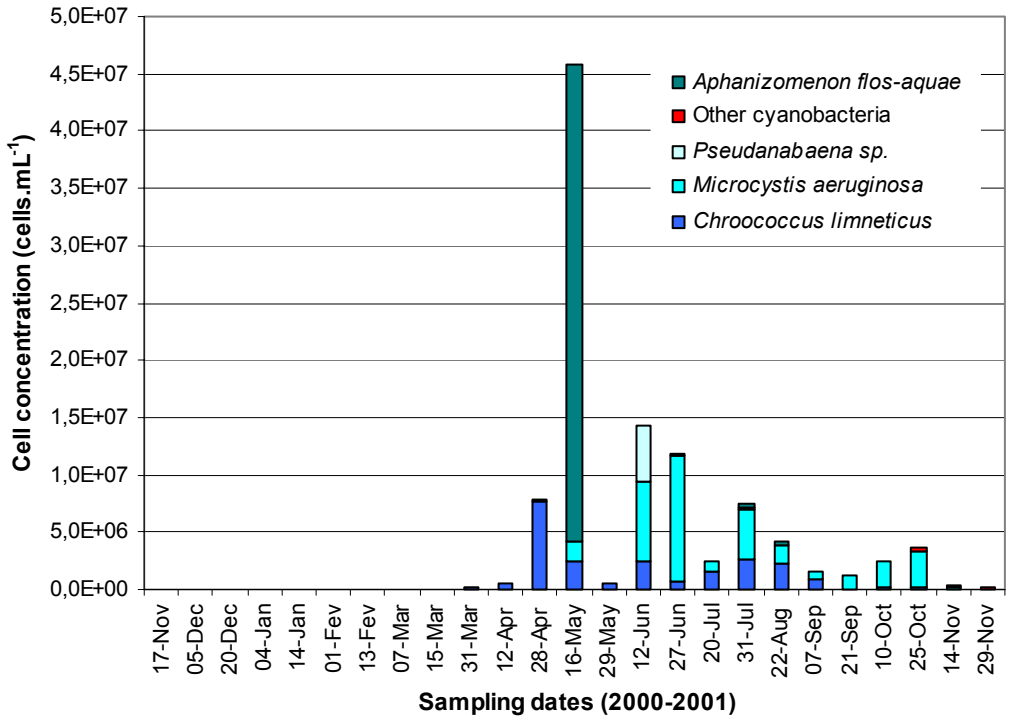

Figure 7 - Seasonal dynamics of the most abundant cyanobacterial *taxa* in Vela Lake during the same period.

The Chlorophyceae community stood persistent from January until November (Fig. 8). The dominant *taxa* included *Coelastrum reticulatum* var. *reticulatum* and *Pediastrum boryanum* var. *boryanum* which were detected from January until November, with higher densities and co-domination of this community between March and May. However, *C. reticulatum* var. *reticulatum* densities were very low in June. In 29th May 2001, the Chlorophyceae community was dominated by the species *Monoraphidium contortum* which presented a high density at this time although its rapid decrease in the following fifteen days (Fig. 8).

Concerning the Bacillariophyceae *taxa* identified, the ones that showed the highest densities included *Fragilaria brevistriata*, *Cyclotella radiosa*, *Cyclotella ocellata*, *Aulacoseira granulata* e *A. granulata* var. *angustissima* (Fig. 9). From November to early

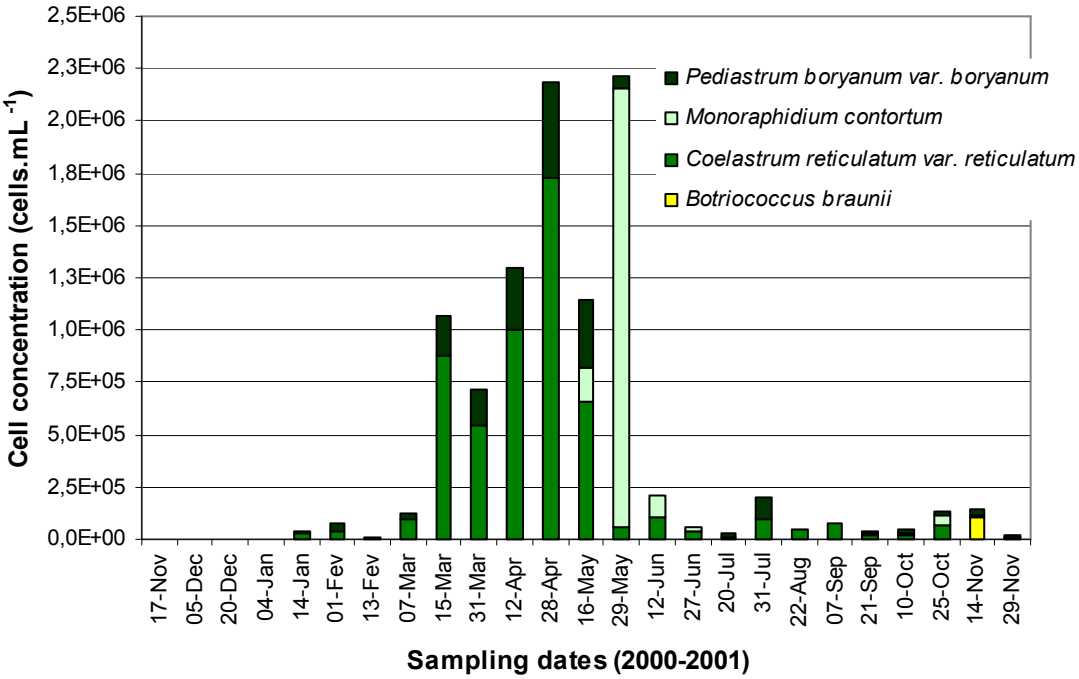

Figure 8 - Seasonal dynamics of the most abundant Chlorococcales *taxa* in Vela Lake during the same period.

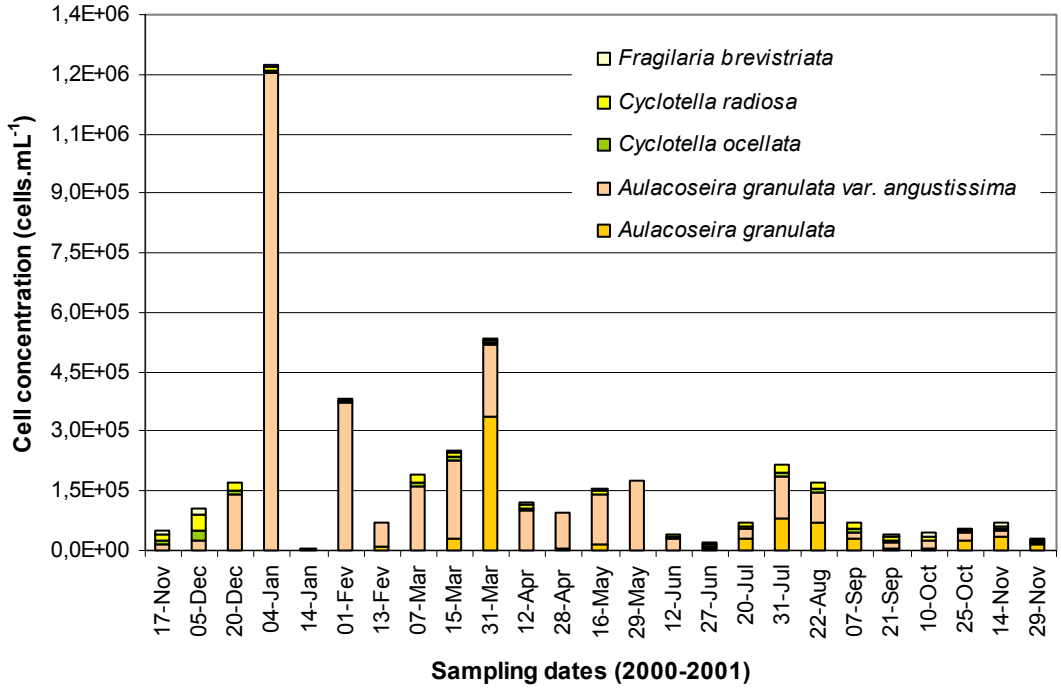

Figure 9 - Seasonal dynamics of the most abundant Bacillariophyceae *taxa* in Vela Lake during the same period.

January 2000, the Bacillariophyceae group almost totally dominated the phytoplankton community in Vela Lake. In 4th January, a small-scale bloom of diatoms was recorded with almost complete dominance of *Aulacoseira granulata* var. *angustissima*.

The 16th May 2001 A. flos-aquae bloom

In 16th May 2001, there was the strongest cyanobacterial bloom with dominance of the potentially toxic *Aphanizomenon flos-aquae* (with 87 % dominance over the total phytoplankton) but there was also detected the potentially toxic *Microcystis aeruginosa* (representing only 3 % of the total phytoplanktonic density) (Fig.10). Yet, during the *A. flos-aquae* bloom, the number of phytoplanktonic *taxa* did not show a strong reduction (Fig. 5).

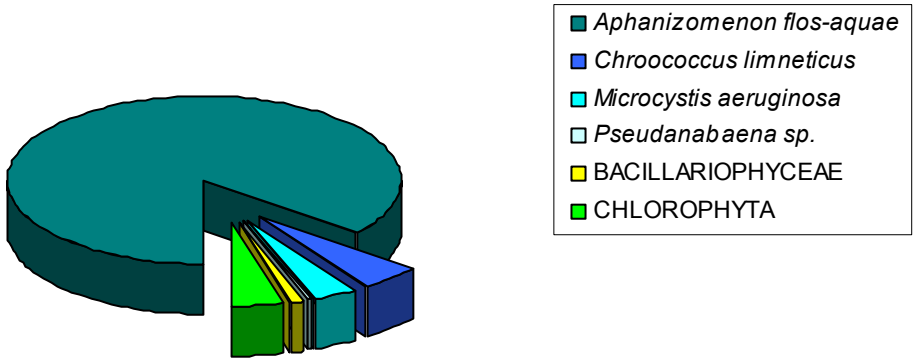

Figure 10 – Phytoplankton composition in Vela Lake at the *Aphanizomenon flos-aquae* bloom (16th May 2001).

The Principal Component Analysis

Results from PCA analysis of phytoplanktonic species occurrence (Fig. 11) indicate that the three first principal components accounted for 56% of total variability. The first axe is defined by the species *Aphanizomenon flos-aquae* (coded as ANSO), among others, on the negative side, and by *Cyclostephanus invisitatus* (CYIN), among numerous species, on the positive side. Along the second axe there is observed a gradient which positive extreme is defined by *Scenedesmus opoliensis* var. *monoensis* (SCOP), among other

species such as *Cyclotella ocellata* (CYOC), and the negative extreme is defined by a cluster in which *M. aeruginosa* (MIAE) is included.

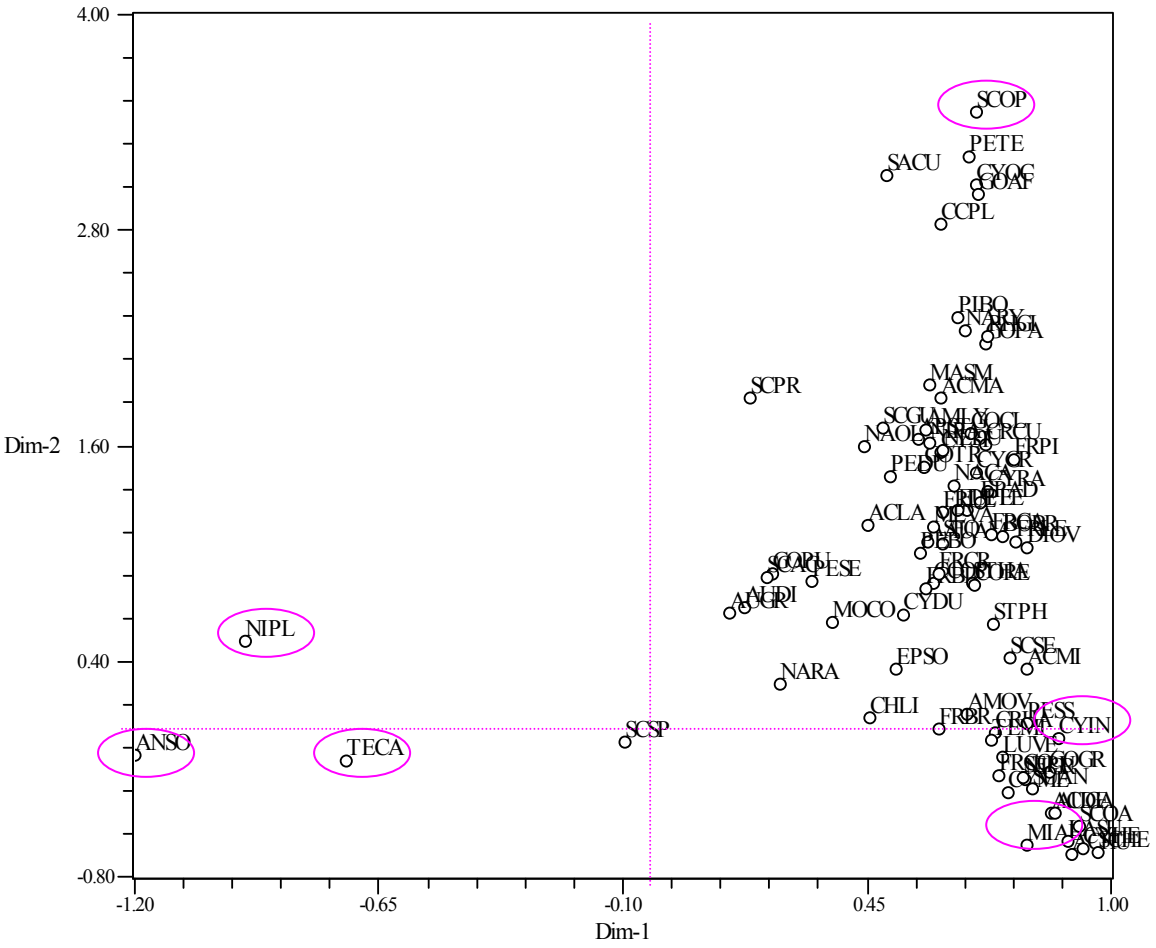

Figure 11 – Phytoplankton species distribution according to PCA analysis.

The distance of *A. flos-aquae* from the other species suggests its occurrence under particular conditions, probably indicating that the first axe gradient is defined by the nitrogen sources levels, particularly nitrate and ammonium, which were the lowest in the 16th May 2001 when the bloom occurred. The second axe should follow a gradient defined by environmental variables such as phosphate levels.

Following this interpretation, the *M. aeruginosa* should have occurred with high nitrate and ammonium levels but with very low phosphate concentrations, which was true.

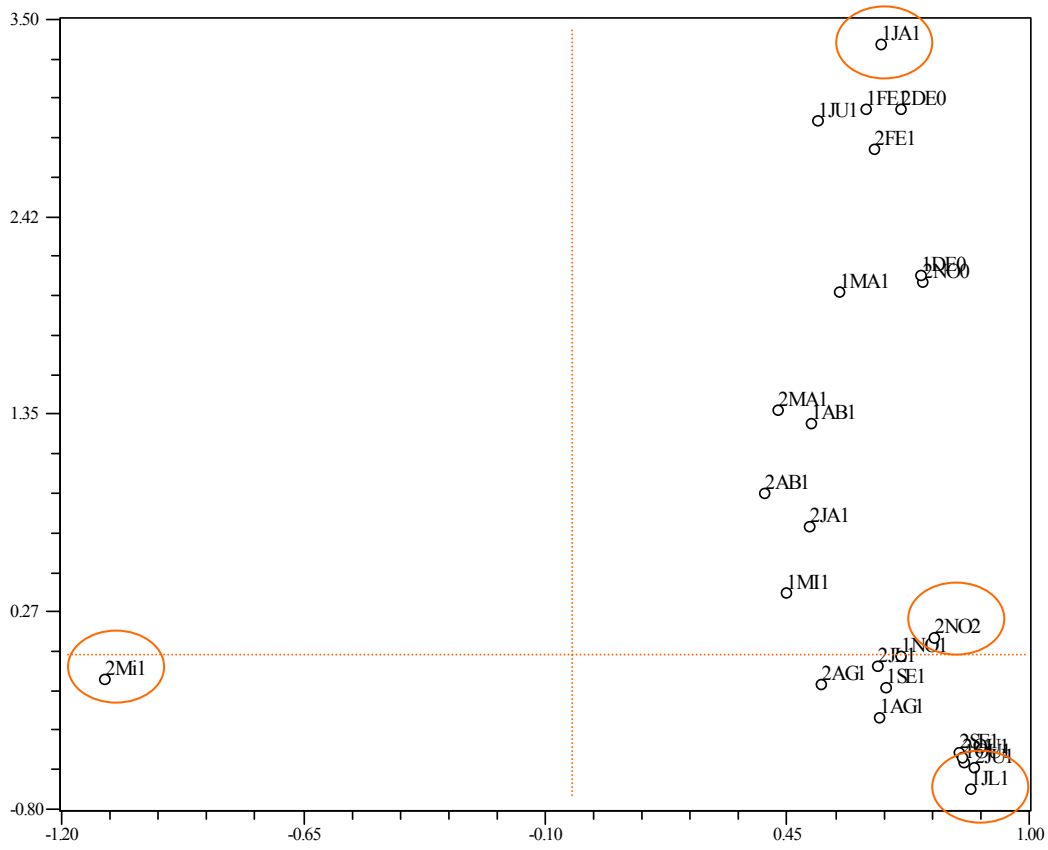

Figure 12 – Distribution of sampling dates according to PCA analysis.

According to the PCA analysis results in figure 12, the sampling dates are distributed through a first axe whose negative extreme is solely defined by the 29th May 2001 (2Mi1) sampling date and the positive extreme by the 29th November 2001 (2NO2), among other sampling dates. The second axe is characterized in its negative extreme by the 1st July 2001 sampling date and the positive extreme of this axe is defined by the 4th January 2001 sampling date, showing in the right positive quadrant the winter and spring sampling dates and in the negative right quadrant the summer and autumn months. This second axe probably follows the water temperature as an important environmental variable. The first axe is most likely defined by the dissolved oxygen level which was depleted in 29th May 2001 but was maintained with low variations for the remaining sampling dates.

Discussion

There is a lack of published investigation concerning Vela Lake, which does not allow an adequate comparative analysis with physico-chemical and phytoplankton dynamics in previous years. Yet, Vasconcelos *et al.* (1993) have reported the occurrence of several toxic strains of *Microcystis aeruginosa* in this lake.

In the present study, a seasonal phytoplankton dynamics could be established in Vela Lake. Orthophosphate, nitrite and nitrate concentrations had the highest values in late December and beginning of January, coinciding with a small-scale bloom of diatoms (with dominance of *Aulacoseira granulata* var. *angustissima*) and the highest dissolved oxygen levels. The results observed in this study are verified in other environmental studies (Eynard *et al.*, 2000; Galvão, 2000 *in* Caetano *et al.*, 2001), in which diatoms dominate under conditions of high levels of N and P and cyanobacterial dominance coincides with the lowest nutrient concentrations. This is probably due to the cyanobacterial capabilities of fixating N and storing P (Oliver and Ganf, 2000).

As observed during the study period, Vela Lake was dominated by potentially toxic cyanobacteria from mid-spring (late April) to late autumn (November). The main potentially toxic blooms were recorded in 16th May (dominated by *Aphanizomenon flos-aquae*) and during June (dominated by *Microcystis aeruginosa*).

By comparing the nutrient data with the cyanobacterial dominant species, the development of the strong *A. flos-aquae* bloom (in 16th May) was preceded by the lowest concentrations of nitrogen sources: nitrate (0.30 mg.L^{-1}), nitrite (0.00 mg.L^{-1}) and ammonium (0.36 mg.L^{-1}) indicating that this cyanobacterial strain is not very dependent on nitrogen availability, probably due to its N-fixing capability. Yet, the availability of phosphate (0.19 mg.L^{-1}) was necessary to the bloom development. The bloom senescence coincided with the oxygen depletion in water. After the restoration of oxygen levels, there was depletion in phosphate levels and this situation remained constant until the end of the year 2001. The *A. flos-aquae* density did not recover and stood low from end of May on, suggesting that this strain is not able to dominate in phosphate depleted conditions. This phosphorus dependence is reported for *Aphanizomenon flos-aquae* (Teubner *et al.*, 1999) and other N-fixing filamentous cyanobacteria (Kahru *et al.*, 2000; Oliver and Ganf, 2000).

During this bloom, the number of *taxa* recorded in the lake did not decrease, indicating there was not a strong alteration of phytoplankton community although the green alga *Coelastrum reticulatum* var *reticulatum* showed to be affected in its density.

The explanations for that fact could remain in the environmental parameters variation but also in a potential allelopathic effect of the dominant cyanobacterium over this algae, as observed for other cyanobacteria and green algae (Kearns and Hunter, 2000; Kearns and Hunter, 2001).

At the time of this *A. flos-aquae* bloom there was recorded a sudden change in the cladocerans community with the disappearance of *Daphnia* spp. (Antunes *et al.*, *in press*).

This *A. flos-aquae* bloom recorded in 16th May had a chlorophyll *a* concentration of 149.43 $\mu g.L^{-1}$ which corresponds to the level 3 of the levels of risk established by WHO (World Health Organization) for recreational waters with cyanobacterial dominance (WHOa). Level 3 is characterized by scum formation or >150 $\mu g.L^{-}1$ Chl *a*, corresponding to a high risk of severe adverse health effects to occur, considering *A. flos-aquae* as potentially toxic, since it has been proved to produce microcystins (Plumley, 1997; Rapala *et al*., 1993 *in* Lehtimäki *et al.*, 1997) and neurotoxins such as anatoxin-a (Rapala *et al*., 1993 *in* Lehtimäki *et al.*, 1997) and saxitoxins (also called PSP (Paralytic Shellfish Poisoning) toxins) (Pereira *et al.*, 2000, Ferreira *et al.*, 2001).

The fish mortality observed during this study coincided with the dissolved oxygen depletion (anoxia) and the highest ammonium levels after the bloom senescence. The determination of cyanotoxins potentially produced by this *A. flos-aquae* strain was not performed but the possibility of a massive liberation of those cyanotoxins during the cells lysis remains also open since *A. flos-aquae* strains isolated from blooms of *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* in Portuguese water bodies, such as Crestuma reservoir, were found to produce several PSP-type toxins (Ferreira, 1994 *in* Vasconcelos, 2001). In Guadiana River, the occurrence of an *Aphanizomenon flos-aquae* bloom was related to a human intoxication episode (Oliveira, 1991 *in* Vasconcelos, 2001) and fish kills during senescence of the bloom. During the oxygen depletion, Chlorococcales organisms dominated the phytoplankton community. Chlorophyll *a* concentration was very low although the number of *taxa* was near the highest (with the development of bacillariophyceae and particularly chlorophyceae species that did not occur during the rest of the year, probably due to the dominant algae competition over them).

The development of this *M. aeruginosa* blooms occurred after the dissolved oxygen depletion and the sudden increase of the ammonium levels to 1.81 $mg.L^{-1}$. The *M. aeruginosa* density remained relatively high until the end of October 2001, even with phosphate depletion in the water, indicating the ability of this strain to resist phosphorus unavailability probably due to its capacity of storing phosphorus (Oliver and Ganf, 2000).

However, non-N-fixing cyanobacteria like *M. aeruginosa* develop in habitats where nitrogen sources must be available (Jacoby *et al.*, 2000;.Oliver and Ganf, 2000) as confirmed in the present study. Total suspended solids showed increased values from June until November (when the rain becomes more intense) probably coinciding to the lower water volume in the lake after the drier months, causing the reduction of light availability in the water column and coinciding with the dominance of *M. aeruginosa* which has low energy requirements and is able to regulate its buoyancy in order to achieve the optimal light intensity (Brookes and Ganf, 2001; Porat *et al.*, 2001). The reduction of this colonial cyanobacterium density in Vela Lake was mainly related to sudden decreases in temperature.

According to the same guidelines mentioned above and established by WHO for recreational waters (WHOa), the *Microcystis aeruginosa* bloom observed in 27th June with a chlorophyll *a* concentration of 65.27 $\mu g.L^{-1}$ corresponded to a level 2 (100000 cells.mL^{-1} or 50 $\mu g.L^{-1}$ under cyanobacterial dominance conditions), representing a moderate risk of occurrence of adverse health effects. Considering the known toxicity of the strains of *Microcystis* worldwide (Oh *et al.*, 2001) and, particularly, in Vela Lake (Vasconcelos *et al.*, 1993), these blooms could have represented a concerning Public Health issue. Further, in Australia, there is the specific cell density of 50000 cells.mL^{-1} for *M. aeruginosa* as health alert indicator to anticipate possible microcystin poisoning outbreaks (Fitzgerald *et al.*, 1999). Therefore, in Vela Lake, health risks were present in 12th and 27th June *M. aeruginosa* blooms.

The control of phosphorus inputs into the water bodies is very important because high phosphate concentrations enhance the growth of *Microcystis aeruginosa* but also its microcystin production (Kotak *et al.*, 2000; Oh *et al.*, 2000). The same is observed for N-fixing cyanobacteria (Rapala *et al.*, 1997). In general, nutrient inputs into the water bodies may arise from losses in land run-off and drainage from agricultural land (Withers and Lord, 2002) due to an inadequate management of nutrient (which exceed production requirements) and land (using farming methods that increase erosion and enhance run-off). Although P losses are higher than N losses, P increase in water is more important for eutrophication. As mentioned by other authors (Withers and Lord, 2002), rather than applying general guidelines, it is becoming imperative the research for site-specific information of each eutrophied water body through the definition of its vulnerability by integrating nutrient sources, nutrient transport and ecological impacts, to achieve more effective strategies in nutrient loss reduction and management. As exposed above, eutrophication is an important cause of the increasing occurrence of toxic cyanobacterial

blooms worldwide and consequent animal and human illness or death. Therefore, it is becoming essential a proper water management regarding the nutrient inputs to water systems but also a better understanding of the interactions between the factors influencing these toxins production in order to control it. It would be important that in every country, investigation focused more on this major Public Health issue in order to understand and adapt control strategies to regional characteristics of these cyanobacterial blooms occurrence and specific local cyanobacterial strains.

Conclusions

After the results obtained during the study period concerning the presence and dominance of potentially toxic cyanobacteria, one should consider future investigation on the monitoring for the presence of cyanotoxins along with these same parameters already performed. If one could establish a direct relation between phytoplanktonic succession and potential for cyanotoxins production, it would be easier to predict, prevent and control intoxication situations for aquatic organisms but also for Vela Lake recreational users. In 16th May 2001, Vela Lake suffered an intense *A. flos-aquae* bloom, and in 12th and 27th June 2001 *M. aeruginosa* blooms also occurred. The development of the *A. flos-aquae* bloom was related to the lowest nitrogen levels recorded in the lake and the *M. aeruginosa* first bloom development was associated with the highest ammonium levels and phosphate depletion. In these blooms, health risks were present, according to the World Health Organization (WHO, 1998a), indicating that some short-term safety measures should have been taken such as the use of informative material for recreational visitors of the lake, referring to possible risks concerning poisoning by cyanotoxins, or/and even the prohibition of people contact with the scum (WHO, 1998a; Fitzgerald, 2001). Long-term measures would include the regular monitoring of potentially toxic bloom forming cyanobacteria in this lake, eutrophication reduction (with phosphorus levels lower than 0.01 $\mu g.L^{-1}$ (WHO, 1998a)), maintenance of transparency, artificial mixing of the water and protection of surrounding soil from erosion.

A proper and effective water management is becoming necessary to prevent these potentially toxic cyanobacterial blooms from happening in order to avoid the possible intoxication situations aroused from their occurrence.

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Capítulo V

Aphanizomenon flos-aquae grown under different nutrient concentrations and its effects over two green algae

Aphanizomenon flos-aquae grown under different nutrient concentrations and its effects over two green algae

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Aphanizomenon flos-aquae grown under different nutrient concentrations and its effects over two green algae

Abstract – Cyanobacterial blooms in freshwaters are becoming more frequent worldwide mainly due to eutrophication of superficial water bodies. These blooms occurrence usually causes oxygen depletion, but sometimes there is also production of toxic compounds. An important cyanotoxin is the hepatotoxin microcystin which is known to cause disease and death at different trophic levels, including humans. This toxin is produced by strains belonging to the genera *Microcystis*, *Anabaena*, *Planktothrix/ Oscillatoria*, *Anabaenopsis*, *Nostoc*, *Aphanizomenon* and *Hapalosiphon*. Toxigenicity variation in a particular species seems to be primarily determined by genotype diversity among strains, in spite of some environmental factors seem also to influence microcystin biosynthesis and toxigenicity. A potentially microcystin producing strain of *Aphanizomenon flos-aquae* was isolated from a bloom in Vela Lake, during May 2001, and the strain was evaluated concerning its growth under different concentrations of nitrate (0.00, 0.85, 8.50 and 85.01 mgNO_3^-.L^{-1}) and phosphate (0.00, 0.55, 2.18, 4.36 and 8.71 mgPO_4^{3-}.L^{-1}), and potential inhibitory effects over the growth of two microalgae (*Chlorella vulgaris* and *Pseudokirchneriella subcapitata*). The growth of the cyanobacterial strain studied showed to be highly dependent on phosphorus rather than on nitrogen, probably due to its nitrogen fixing capability. Concerning the potential effects over other microalgae, the *A. flos-aquae* exudates showed to inhibit, although slightly, the growth of both tested green algae. After the results obtained in laboratory, we may suggest that planktonic blooms of this strain of *A. flos-aquae* are favoured by high phosphate concentrations (from the agriculture effluents, for example) rather than by high nitrate levels. Thus, the control of the phosphate inflow into the superficial water body from which the strain was isolated from would be an important factor to avoid the occurrence of potentially toxic blooms of this strain as well as the inherent potential intoxication problems at the different trophic levels, namely at the phytoplankton level, which zooplankton community depends on.

Keywords: *Aphanizomenon flos-aquae*, nutrient limitation, *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, growth inhibition.

Introduction

The eutrophication of superficial freshwater bodies used as recreational waters or as drinking water supplies has become more frequent worldwide due to the continuous increase of human population and consequent intensification of agricultural and industrial activities, with high nutrient inputs (Hall *et al.*, 1999; Codd, 2000; de Jonge *et al.*, 2000; Cooperband and Good, 2002; Withers and Lord, 2002). Additionally, there is also a deficient water management and environmental factors such as high nutrient levels, low turbulence, higher temperature (15-30ºC) and pH (6-9) values, enhance the development of planktonic cyanobacteria in lakes and reservoirs, leading to formation of surface blooms accumulating as scum (Hadas *et al.*, 1999; Jacoby *et al.*, 2000; Oliver and Ganf, 2000). In laboratory, there is also evidence that factors such as light, temperature, nutrient levels, influence cyanobacterial development (Lee and Rhee, 1999; Saadoun *et al.*, 2001). The main problem about cyanobacterial blooms is that, besides increasing suspended solids, causing oxygen depletion and giving bad odour and taste to water (Saadoun *et al.*, 2001), a high percentage of them has shown to be harmful (Codd *et al.*, 1995; Codd, 2000; Dow and Swoboda, 2000) due to the production of toxic secondary metabolites by many cyanobacteria. Presently, there are more than 40 cyanobacterial species known to have toxic strains (Dow and Swoboda, 2000) because for each species considered toxic there may be toxic and non-toxic strains and in toxic ones toxicity may vary among them (Hesse and Kohl, 2001; Böttcher *et al.*, 2001). Cyanotoxins can be classified as dermatotoxins, neurotoxins or hepatotoxins (Dow and Swoboda, 2000; Kaebernick and Neilan, 2001), according to the effects on animals, and there are many cases reported that show how dangerous they can be for numerous organisms, humans included (Gorham and Carmichael, 1988; Pouria *et al.*, 1998; Codd, 2000; Fitzgerald, 2001; Briand *et al.*, 2003). Microcystin (MC) is a hepatotoxin to which special attention has been given due not only to its effects by acute exposure (resulting in death of animals and humans (Pouria *et al.*, 1998)) but particularly to its potential of promoting cancer in humans after chronic exposure through drinking water (Ueno *et al.*, 1996; Zhou *et al.*, 2002). For this last reason, WHO (*World Health Organization*) has already established a lifetime consumption safe level of 1 $\mu g.L^{-1}$ for the most common microcystin variant (MC-LR), in drinking water (WHO, 1998). Microcystin variants occur in freshwaters worldwide and are mainly produced by colonial *Microcystis* spp. and strains belonging to filamentous *Anabaena*, *Planktothrix/ Oscillatoria*, *Anabaenopsis*, *Nostoc* and *Aphanizomenon* (Dow and Swoboda, 2000; Kaebernick and Neilan, 2001). Microcystin is a secondary metabolite produced non-ribosomally through a microcystin synthetase complex which is codified by

a *mcy* genes cluster (55 kb) composed by two operons (*mcyA-C* and *mcyD-J*) (Tillet *et al.*, 2000) and it is present in toxic strains of the genus *Microcystis* but also in microcystin producing strains of *Anabaena*, *Nostoc* and *Planktothrix* (Neilan *et al*., 1999), allowing the development of rapid and sensitive PCR (*Polymerase Chain Reaction*) methods for detection of toxic strains directly from environmental samples (Tillet *et al.*, 2001; Pan *et al.*, 2002). In spite of many contradictory studies, microcystin synthesis seems to be influenced by environmental factors such as light (Rapala and Sivonen, 1998; Rapala *et al.*, 1997; Kaebernick *et al.*, 2000; Hesse and Kohl, 2001; Wiedner *et al.*, 2003), temperature (Rapala and Sivonen, 1998; Rapala *et al.*, 1997), trace metals (Lukac and Aegerter, 1993; Utkilen and Gjølme, 1995) and nutrients such as phosphorus (Rapala *et al.*, 1997, Kotak *et al.*, 2000; Jacoby *et al.*, 2000; Oh *et al*., 2000; Vézie *et al.*, 2002) and nitrogen (Rapala *et al.*, 1997; Utkilen and Gjølme, 1995; Lee *et al.*, 2000; Long *et al.*, 2001). Bickel and Lyck (2001) have suggested that if microcystin synthesis requires energy (as ATP), the variation of this secondary metabolite production should be primarily explained by the energetic state of the cyanobacterial cells, that decreases in stress conditions (e.g. nutrient limitation and light variation) with available energy being primarily applied in essential protein synthesis. On the other hand, some recent studies (Hesse and Kohl, 2001; Böttcher *et al.*, 2001; Vézie *et al.*, 2002; Rohrlack *et al.*, 2001; Kurmayer *et al.*, 2002) consider that microcystin synthesis depends more on genotype diversity of strains rather than on environmental factors, with genotypes differing in growth strategy, plasmid content, interaction with zooplankton, microcystin content (Hesse and Kohl, 2001) and microcystin synthetase genes cluster, originating different variants of the toxin with different toxicities (Mikalsen *et al.*, 2003). Microcystin variants are known to be accumulated and to cause several toxic effects at different trophic levels (see chapter III of this thesis). For instance, microcystin-LR seems to have an allelopathic function towards other microalgae (Kearns and Hunter, 2000; Kearns and Hunter, 2001), resulting in growth inhibition or in settlement by paralysing them, but having also effects at a higher trophic level by originating unfavourable conditions for zooplankton (Brett and Müller-Navarra, 1997; Brett *et al.*, 2000; DeMott, 1999; Scheuerell *et al.*, 2002).

The laboratory ecological studies of potentially microcystin producing cyanobacterial strains may allow the improvement of water management strategies to control or even avoid the occurrence of such blooms. Many strains of *A. flos-aquae* are able to produce hepatotoxins such as microcystin (Plumley, 1997; Willen and Mattson, 1997 *in* Lotocka, 2001) and neurotoxins such as anatoxin-a (Rapala *et al*., 1993 *in* Lehtimäki *et al.*, 1997) and saxitoxins (Pereira *et al.*, 2000; Ferreira *et al.*, 2001). In Portugal, *Aphanizomenon*

flos-aquae blooms have been recorded, with production of saxitoxins (also named PSP (Paralytic Shellfish Poisoning)-type toxins), in several reservoirs such as Crestuma reservoir (northern Portugal) (Ferreira *et al.*, 2001) and Montargil reservoir (central Portugal) (Pereira *et al.*, 2000).

This report presents the effects of different nutritional conditions (N and P) on the growth of a microcystin potentially producing cyanobacterial strain isolated from a bloom in Vela Lake during May 2001. In this same study there were also assessed the effects of this cyanobacterial strain exudates on the growth of two green algae (*Chlorella vulgaris* and *Pseudokirchneriella subcapitata*) considered highly edible for zooplankton (particularly cladocerans).

Material and Methods

Cyanobacterium cultures

The strain used in this assay was isolated from a bloom occurred in a Portuguese eutrophied shallow lake (Vela Lake, Figueira da Foz, Portugal) in May 2001 and it was identified as belonging to the species *Aphanizomenon flos-aquae* (Komárek and Anagnostidis, 1989). Unialgal cultures were obtained by repeated isolation steps in sterilized liquid Woods Hole Marine Biological Laboratory MBL medium. The cultures media used for the nutritional experiments were based on sterilized MBL medium, but with some modifications concerning nitrogen and orthophosphate concentrations, in order to study the cyanobacterial strain growth under different nutritional conditions.

Two sets of experiments were conducted to assess the *A. flos-aquae* growth under the different nutrient concentrations. In the first one, cyanobacterial cells were grown in 2 L flasks with 1.8 L growth medium for four different concentrations of nitrate and phosphate, ranging from depleted to saturated nutrient conditions (0.00, 0.85, 8.50 and 85.01 $mgNO_3^-.L^{-1}$, and 0.00, 2.18, 4.36 and 8.71 $mgPO_4^{3-}.L^{-1}$, referred as APH0N, APH0.85N, APH8.5N, APH85N, APH0P, APH2P, APH4P and APH8P, respectively). All cultures were grown at 21 ºC under constant illumination using cool white fluorescent lights, positioned vertically, and cultures continuous aeration was assured by a single air pump (through sterile, 0.45-μm-pore-size filters). Initial cell density was 4×10^4 $cells.mL^{-1}$ for all cultures. The growth in each culture was assessed by optical density, chlorophyll *a* and cell density (using a Sedgwick-Rafter counting chamber (APHA, 1995)), and physico-chemical parameters such as temperature, pH, orthophosphates (APHA, 1995), ammonium (data not shown) (APHA, 1995), nitrates and nitrites (Rodier, 1996) were

determined with a 2-4 days interval, corresponding to an aliquot of 250 $mL.day^{-1}$ for analysis.

On the second set of experiments for *A. flos-aquae* growth under different nutrient concentrations, cyanobacterial cells were grown in triplicate for different concentrations of nitrate (0.00, 0.85, 8.50 and 85.01 $mgNO_3^-.L^{-1}$, referred as APH0N', APH0.85N', APH8.5N' and MBL, respectively) and phosphate (0.00, 0.55, 2.18, 4.36 and 8.71 $mgPO_4^{3-}.L^{-1}$, referred as APH0P', APH0.5P, APH2P', APH4P' and MBL, respectively) in 500 mL Erlenmeyer flasks with 500 mL sterilized growth medium (modified MBL). The tests were performed in an incubation chamber under controlled laboratory conditions: temperature was maintained at 24 ºC under a constant illumination (using cool white fluorescent lights) and no bubbling was applied to the cultures, but they were agitated in the same chamber at 40 rpm. Initial cell density was 10^5 $cells.mL^{-1}$ for all cultures. Biomass was assessed by dry weight (filtering 20-50 mL of the cultures, depending on the development stage, through tarred Whatman GF/C filters, which were then dried for 24h at 60 ºC), chlorophyll *a* (filtering 10-50 mL, depending also on cell number, through a Whatman GF/C filter, performing an 90 % acetone treatment and measuring it spectrophotometrically) and optical density at 440, 620 and 750 nm (measured also spectrophotometrically). From day 0 to day 13, optical density was measured with a 2-3 days interval and from that day on, optical density was measured everyday to assess the beginning of the stationary phase in each culture. Chlorophyll *a* and dry weight were determined less frequently in the beginning of the tests due to volume limitation. At the late stationary phase, an aliquot of each culture was taken and frozen for possible measurement of microcystin content.

Green algae growth experiments

The green algae used for the growth inhibition tests were *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and currently used as standard species for algal toxicity tests (OECD, 2002)), due to their importance as highly edible food sources to zooplankton, particularly daphnids. The potential allelopathic effects of released cyanobacterial compounds on the growth of these two green algae were assessed. The tests were conducted, in triplicate, using sterilized MBL medium as the main culture medium. The algae were exposed, during a 96 hours period, to the exudates (growth medium and associated algal products, after filtration through a Whatman GF/C filter) obtained from the stationary phase cultures of *A. flos-aquae* grown in P deficiency, P and N saturation and N depletion conditions, to check if this cyanobacterial strain released some noxious compounds towards the tested green algae. The source culture of each exudate or filtrate (30

mL exudate added to 70 mL MBL) will be referred as 0.5Pf (filtrate of *A. flos-aquae* grown in 0.55 $mgPO_4^{3-}.L^{-1}$ and 85.01 $mgNO_3^{-}.L^{-1}$), MBLf (filtrate of *A. flos-aquae* grown in 8.71 $mgPO_4^{3-}.L^{-1}$ and 85.01 $mgNO_3^{-}.L^{-1}$) and 0Nf (filtrate of *A. flos-aquae* grown in 8.71 $mgPO_4^{3-}.L^{-1}$ and 0.00 $mgNO_3^{-}.L^{-1}$). There were also performed control experiments at the same time. Control 1 corresponds to growth of the green algae in 100mL nutrient saturated MBL medium and control 2 corresponds to the algae growth in 30 mL distilled water added to 70 mL MBL in order to discharge any effect from possible nutrient deficiency due to the dilution caused by the added filtrate volume. The tests were conducted in glass vessels of 250 mL with 100 mL of final test solution and the cultures handling followed aseptic conditions. The vessels were randomly located in the growth chamber and cultures were maintained with a temperature of 24 ºC, continuous light and at 100 rpm. The pH was measured to ensure there were not relevant oscillations. The growth was quantified by measuring the algal biomass density (by optical density) over time and in control cultures there must have been reported at least a 16 fold increase within the test period. The initial cell concentration was approximately $5x10^4$ $cells.mL^{-1}$ for both *C. vulgaris* and *P. subcapitata*. The nomographs used to establish the relationship between absorbance (at 440 nm) and cell density for *C. vulgaris* (1) and *P. subcapitata* (2) were already prepared in our laboratory for daphnids algal feeding procedures:

$$Cells.mL^{-1} = -155820 + Abs \times 13144324 \qquad (1)$$

$$Cells.mL^{-1} = -17107.5 + Abs \times 7.92535x10^6 \qquad (2)$$

where *Abs* is the absorbance value measured at 440 nm.

Data analysis

In the cyanobacterial growth experiments, the Pearson correlation coefficient was used to compare the different methods of biomass determination: cell number, optical density and chlorophyll *a* concentration, in the first set, and dry weight, optical density and chlorophyll *a*, in the second set of experiments. A one-way analysis of variance (ANOVA) was used to assess significant differences among the *A. flos-aquae* densities after the nutritional treatments and also between the green algae cell densities after the control and exudates treatments. This one-way ANOVA was followed by a post hoc multiple comparisons Tukey HSD test, where applicable (Zar, 1996) with statistically significant differences in growth reported for $P<0.05$.

Results

A. flos-aquae growth under N and P limitation

Effect of nitrate limitation

In the first set of nutritional experiments, the limitation of nitrate availability did not show to cause a strong inhibitory effect on the growth of *A. flos-aquae* (Figs. 1 and 2).

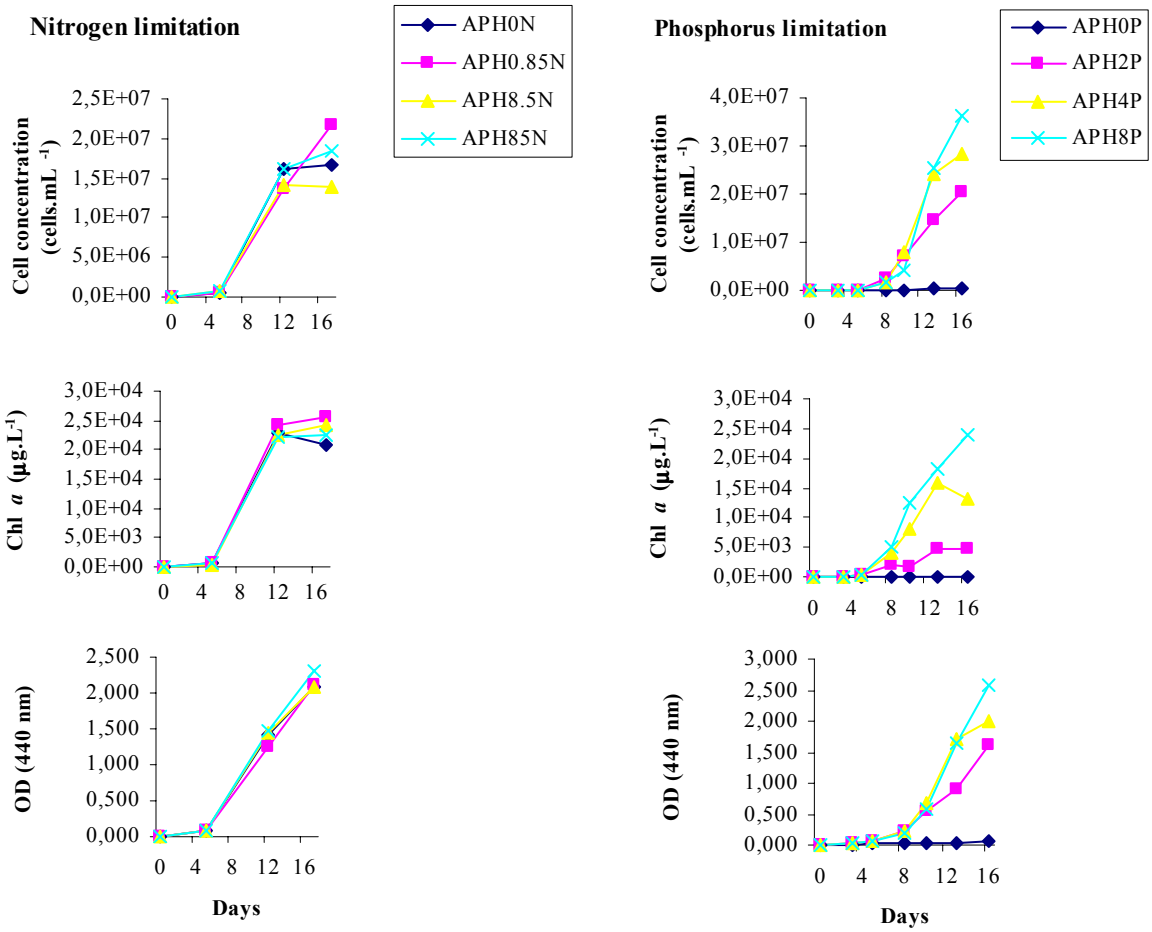

Figure 1 – Effects of different nitrate and phosphate concentrations on the growth of *A. flos-aquae*: APH0N, APH0.85N, APH8.5N and APH85N correspond to the cyanobacterial growth in 0.00, 0.85, 8.50 and 85.01 mgNO_3^-.L^{-1}, respectively; APH0P, APH2P, APH4P and APH8P correspond to 0.00, 2.18, 4.36 and 8.71 mgPO_4^{3-}.L^{-1}, respectively.

There was a similar tendency among the cyanobacterium growth for all the nitrate concentrations tested (Fig. 1). The growth rates obtained during this experiment were very similar among the treatments and ranged from 0.35 to 0.36 d^{-1} for APH8.5N and all the other treatments, respectively. Optical density showed a good positive correlation with the other growth parameters measured: cell counting (r = 0.994, P<0.001, n=27) and

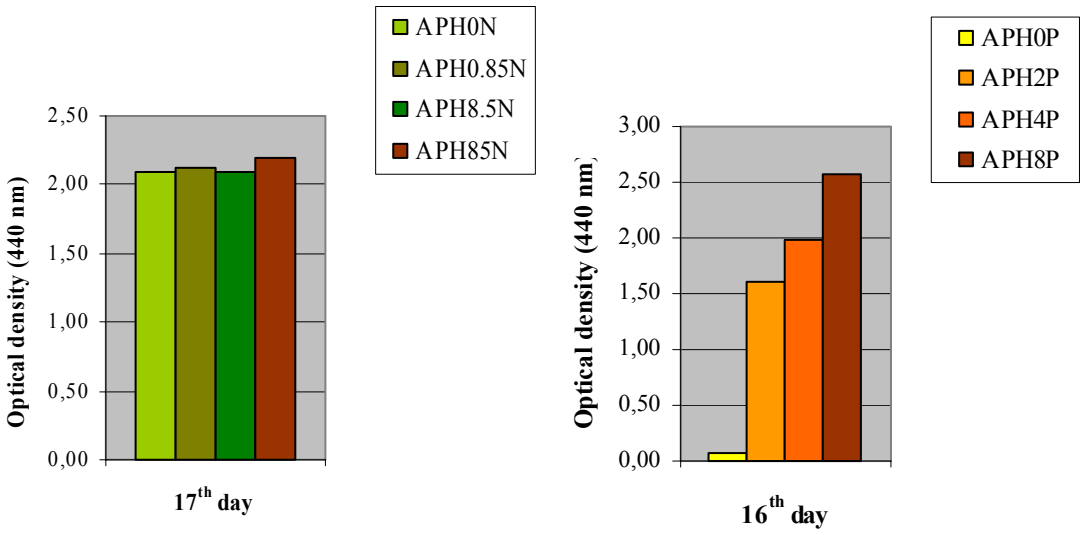

Figure 2 – Optical density measured in the last day of *A. flos-aquae* growth experiment in different concentrations of nitrate (APH0N, APH0.85N, APH8.5N and APH85N correspond to 0.00, 0.85, 8.50 and 85.01 $mgNO_3^-.L^{-1}$, respectively, all with 8.71 $mgPO_4^{3-}.L^{-1}$) and phosphate (0.00, 2.18, 4.36 and 8.71 $mgPO_4^{3-}.L^{-1}$, referred as APH0P, APH2P, APH4P and APH8P, respectively, all with 85.01 $mgNO_3^-.L^{-1}$).

chlorophyll *a* (r = 0.904, P<0.001, n=27). The cultures with different nitrate concentrations on this experiment all presented a strong dark green colour and cells in suspension.

For the second set of nutritional experiments, nitrate limitation also did not seem to decrease the *A. flos-aquae* growth (Figs. 3 and 4). In fact, this cyanobacterial strain grew even better in nitrate depleted medium rather than in the other media with nitrate availability (Figs. 2 and 4), showing statistically significant differences. In this experiment, the optical density showed a positive correlation with dry weight (r = 0.948, P<0.001, n=30) and chlorophyll *a* concentration (r = 0.758, P<0.001, n=30) (data not shown). Between the optical density measurements at different λ, the 440nm absorbance values were highly positively related with the absorbance values for 620nm (r = 0.997, P<0.001, n=154) and 750nm (r = 0.994, P<0.001, n=154) (data not shown).

On this second set of experiments, in a chamber with controlled conditions, all cultures (including the phosphorus treatments) presented a yellowish coloration and the

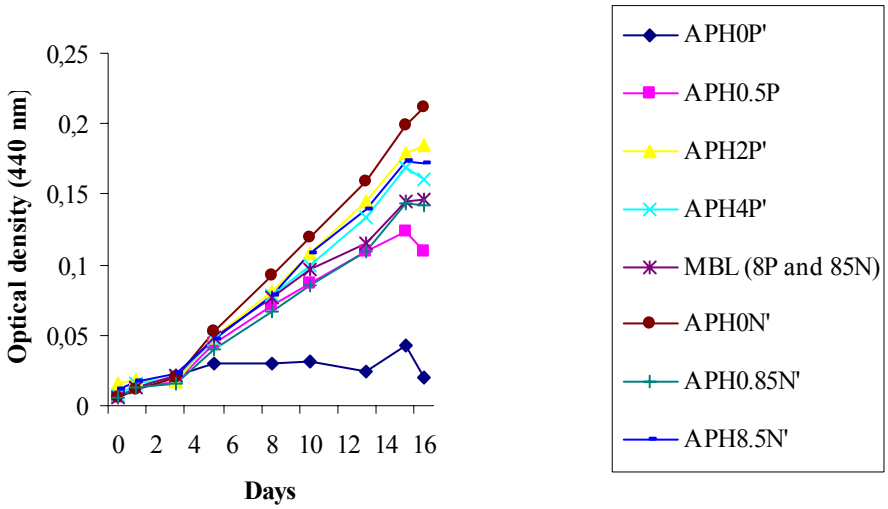

Figure 3 – *A. flos-aquae* growth during the second set of experiments under different concentrations of nitrate (0.00, 0.85, 8.50 and 85.01 $mgNO_3^-.L^{-1}$, referred as APH0N', APH0.85N', APH8.5N' and MBL, respectively, all with 8.71 $mgPO_4^{3-}.L^{-1}$) and phosphate (0.00, 0.55, 2.18, 4.36 and 8.71 $mgPO_4^{3-}.L^{-1}$, referred as APH0P', APH0.5P, APH2P', APH4P' and MBL, respectively, all with 85.01 $mgNO_3^-.L^{-1}$). Each point corresponds to a mean value of three replicates and standard deviations are not presented due to graphics aesthetics.

cyanobacterial cells tended to settle at the bottom of the glass flasks. Besides, the MBL nutrient saturated culture showed a reduced growth output in relation to other cultures with lower nutrient concentrations (such as APH2P', APH4P', APH0N' or APH8.5N'). The highest growth rate value obtained was only of 0.20 d^{-1} for APH0N'.

Effect of phosphate limitation

The strain studied showed to be very sensitive to phosphorus variation (Fig.1), showing a reduced growth under lower phosphorus concentrations and almost no growth in phosphorus depleted medium (Figs.1 and 3). In the first set of nutritional experiments, higher phosphate concentrations enhanced the growth of this *A. flos-aquae* strain (Figs. 1 and 2) following a gradient tendency. Optical density correlated well with cell density and chlorophyll *a* concentration showing *r* values of 0.968 ($P<0.001$, n=16), and 0.951 ($P<0.001$, n=16), respectively. The highest growth rate was recorded for APH8P and the lowest for APH0P with the values of 0.37 and 0.13 d^{-1}, respectively. During the experiment, as the cells had been grown at lower phosphate concentrations the cultures would come more yellow but with cells in suspension.

On the second set of nutritional experiments, the growth gradient observed previously (Figs. 1 and 2) for the phosphate concentrations was not so obvious (Fig. 3 and 4). The APH2P', APH4P' and MBL cultures all showed to have grown better than the APH0.5P and APH0P' cultures. The growth rates obtained for this experiment concerning the different phosphate treatments were 0.19, 0.18 and 0.17 d^{-1} for APH2P', APH4P' and

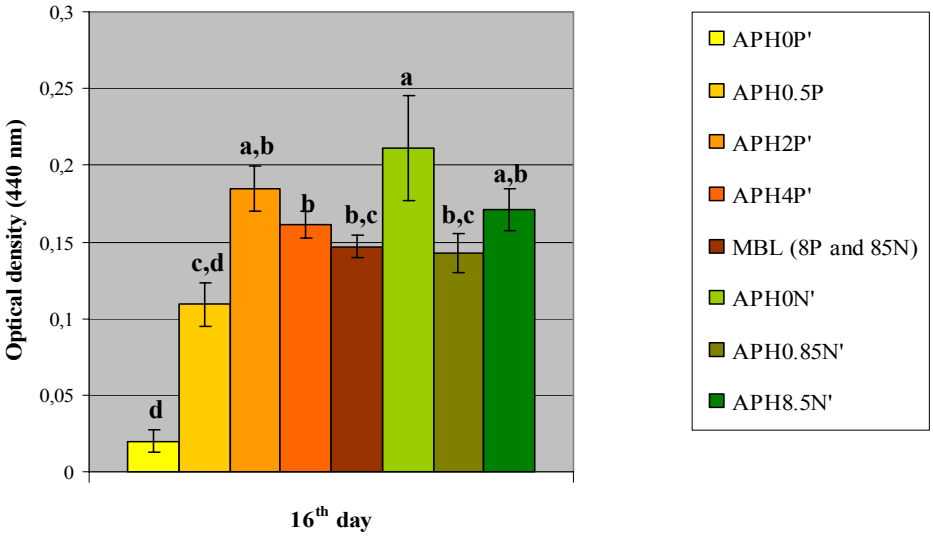

Figure 4 – Optical density measured in the last day of *A. flos-aquae* growth experiment in different concentrations of nitrate (0.00, 0.85, 8.50 and 85.01 $mgNO_3^-.L^{-1}$, referred as APH0N', APH0.85N', APH8.5N' and MBL, respectively, all with 8.71 $mgPO_4^{3-}.L^{-1}$) and phosphate (0.00, 0.55, 2.18, 4.36 and 8.71 $mgPO_4^{3-}.L^{-1}$, referred as APH0P', APH0.5P, APH2P', APH4P' and MBL, respectively, all with 85.01 $mgNO_3^-.L^{-1}$). Each point corresponds to a mean value of three replicates and error bars represent the standard deviation with the different letters representing significant differences between the treatments ($P<0.05$).

MBL, respectively. For APH0.5P', the growth rate was lower (μ = 0.16 d^{-1}) and for APH0P' the cyanobacterial strain almost did not grow at all (μ = 0.05 d^{-1}). After the optical density values obtained for the last day of experiments (Fig. 4), there was a significant difference between the treatments APH2P', APH4P' and MBL, and the treatments APH0.5P and APH0P'. The phosphate concentrations of 2.18 and 4.36 $mg.L^{-1}$ have shown not to be limitating for the *A. flos-aquae* growth. The correlations between the biomass parameters measured were already described in the previous section (different nitrate concentration treatments).

Media physico-chemical parameters during the A. flos-aquae growth

In the first set of nutritional experiments, several physico-chemical parameters were determined during the *A. flos-aquae* growth in the cultures with different nitrate and phosphate concentrations. Those parameters included pH and nitrate, nitrite, ammonium and orthophosphate concentrations. For the nitrogen limitation experiments (Fig. 5), the cultures suffered a rapid decrease in all media phosphate concentration achieving its depletion approximately at the 12th day of growth. The nitrite levels were highest for APH85N and raised from 0.00 to 0.37 mg.L^{-1} in 5 days following a decrease to 0.24 mg.L^{-1} which was maintained. The APH8.5N reached 0.17 mg.L^{-1} in 5 days and approximately maintained that level until the end of the experiment. The nitrate concentration slightly

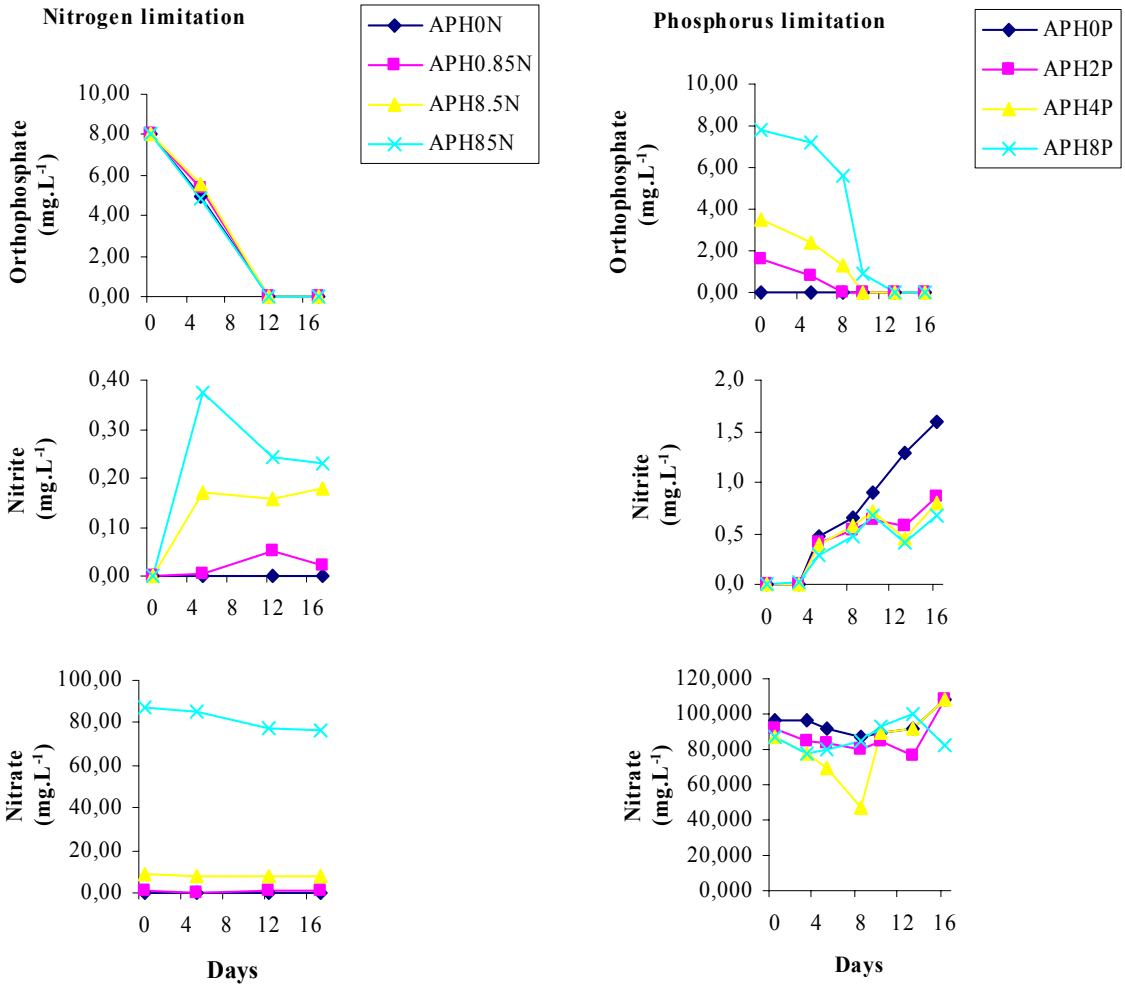

Figure 5 – Medium physico-chemical parameters during the growth of the *A. flos-aquae* strain under different nitrate (APH0N, APH0.85N, APH8.5N and APH85N corresponding to 0.00, 0.85, 8.50 and 85.01 mgNO_3^-.L^{-1}, respectively) and phosphate concentrations (APH0P, APH2P, APH4P and APH8P correspond to 0.00, 2.18, 4.36 and 8.71 mgPO_4^{3-}.L^{-1}, respectively).

decreased in the APH85N during the experiment (from 85 to 76 $mg.L^{-1}$) but in the other cultures it almost did not change.

For the phosphate limitation experiments (Fig. 5), the media nitrite levels showed a gradual increase during the *A. flos-aquae* growth in all different phosphate concentration cultures with the highest nitrite concentration of 1.59 $mg.L^{-1}$ for APH0P (0.00 $mgPO_4^{3-}.L^{-1}$). Nitrate levels did not change much during the experimental period. Phosphate levels decreased until depletion in all cultures but faster in the cultures where the initial phosphate concentration was lower.

For both the nitrate and phosphate limitation experiments, the ammonium levels were undetectable and the temperature and pH values were not significantly altered during the experiments.

Effects of A. flos-aquae cultures exudates on growth of green algae

This strain of *A. flos-aquae* showed to have some influence over the growth of the two green algae tested meaning there is something in the cyanobacterium cultures exudates that, although slightly, inhibits at some extent the growth of both *Chlorella*

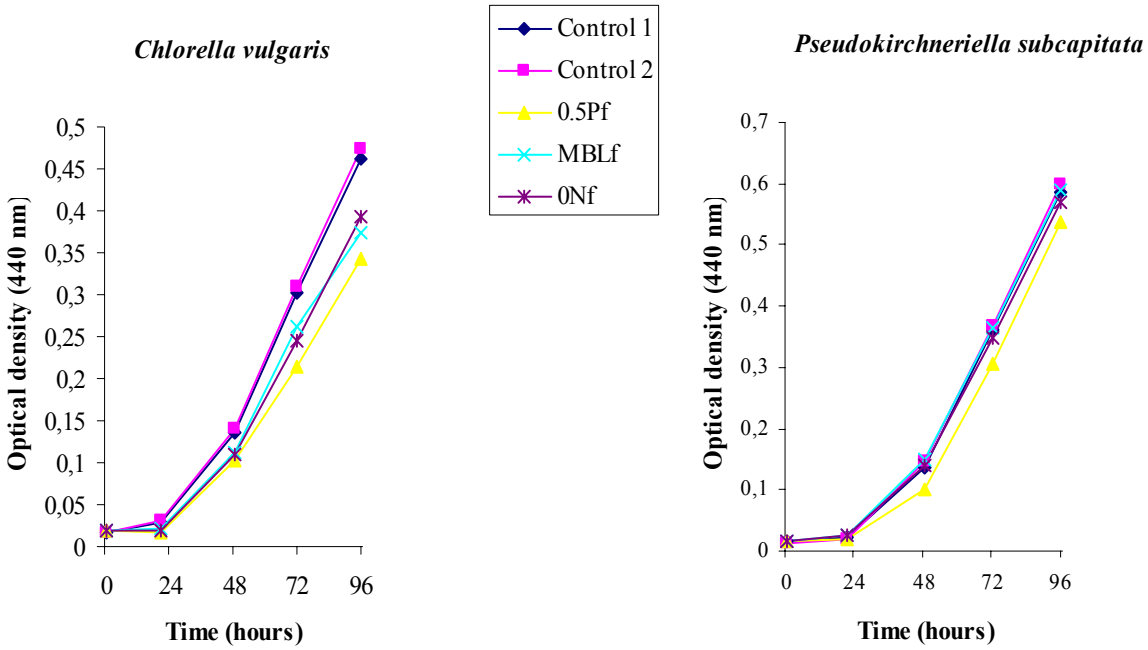

Figure 6 – Effect of the exudates from the *A. flos-aquae* cultures on growth of *C. vulgaris* and *P. subcapitata*. The source culture of each exudate (30 mL added to 70 mL MBL) are referred as 0.5Pf (filtrate of *A. flos-aquae* grown in 0.55 $mgPO_4^{3-}.L^{-1}$ and 85.01 $mgNO_3^{-}.L^{-1}$), MBLf (filtrate of *A. flos-aquae* grown in 8.71 $mgPO_4^{3-}.L^{-1}$ and 85.01 $mgNO_3^{-}.L^{-1}$) and 0Nf (filtrate of *A. flos-aquae* grown in 8.71 $mgPO_4^{3-}.L^{-1}$ and 0.00 $mgNO_3^{-}.L^{-1}$). The control 1 corresponds to 100mL MBL and control 2 corresponds to 30 mL distilled water added to 70 mL MBL. Each point corresponds to a mean value of three replicates.

vulgaris and *Pseudokirchneriella subcapitata*. For *P. subcapitata*, its average growth rate during the test period was not decreased by the dilution effect of the addition of 30 mL distilled water to 70 mL of MBL growth medium and in controls 1 and 2 the μ values were 1.218 and 1.223 d^{-1}, respectively. There was only a slight inhibition of growth (not statistically different from the control treatments) when the *A. flos-aquae* exudates were

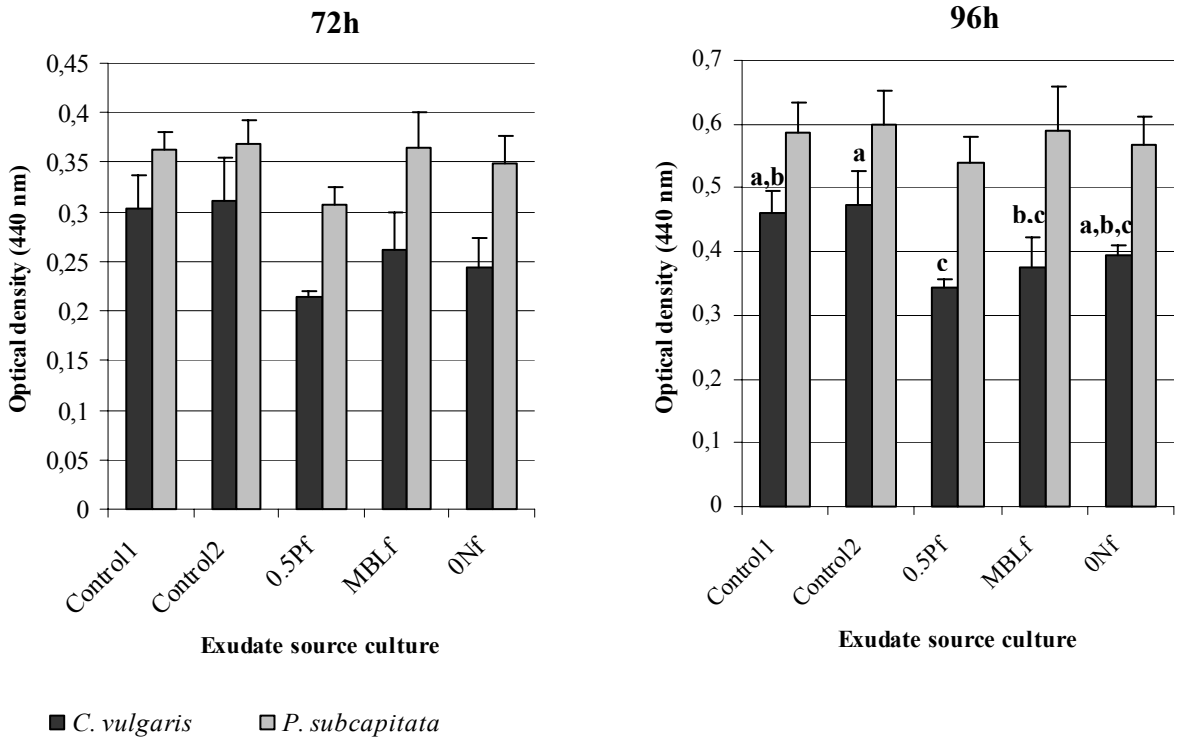

Figure 5 – Effects of exudates from the cyanobacterium cultures on the growth of the two green algae tested (*C. vulgaris* and *P. subcapitata*). The source cultures of each exudate (30 mL added to 70 mL MBL) are referred as 0.5Pf (filtrate of *A. flos-aquae* grown in 0.55 $mgPO_4^{3-}.L^{-1}$ and 85.01 $mgNO_3^{-}.L^{-1}$), MBLf (filtrate of *A. flos-aquae* grown in 8.71 $mgPO_4^{3-}.L^{-1}$ and 85.01 $mgNO_3^{-}.L^{-1}$) and 0Nf (filtrate of *A. flos-aquae* grown in 8.71 $mgPO_4^{3-}.L^{-1}$ and 0.00 $mgNO_3^{-}.L^{-1}$). The control 1 corresponds to 100mL MBL and control 2 corresponds to 30 mL distilled water added to 70 mL MBL. Each is a mean value (3 replicates) and the different letters in error bars represent significant differences between the treatments ($P<0.05$).

present in the growth media. Nevertheless, the exudate from APH0.5P had the most notorious effect over this green alga growth (μ = 1.195 d^{-1}) and MBL culture exudate had the lowest (μ = 1.219 d^{-1}) inhibitory effect of the three cyanobacterial exudates considering the growth rate measured. After 72h, this effect was already visible in the optical density measurements although at both 72h and 96h there were no statistically significant differences between the exudates treatments and the controls. For *C. vulgaris*, the growth

rate at the end of the test did not decrease by the addition of 30 mL distilled water to 70 mL of MBL growth medium achieving average growth rate values of 1.046 and 1.053 d^{-1} for controls 1 and 2, respectively. At 72h, although there was an obvious inhibition of the *A. flos-aquae* exudates on this alga growth in relation to the control treatments, the differences were not statistically significant. However, at 96h, the differences between the control 2 and the 0.5Pf and the MBLf treatments turned statistically significant (Fig. 5). At 96h, the average growth rate of 0.5f treatment was 0.970 d^{-1} and for MBLf treatment the μ value was 0.992 d^{-1}. The exudate from the APH0.5P culture was the most inhibitor for the *C. vulgaris* growth, following the exudates from cyanobacterial cultures MBL and APH0N'.

Discussion

A. flos-aquae growth under N and P limitation

During the one year monitoring period in Vela Lake (see Cap. IV of this thesis), water body from which the *A. flos-aquae* strain was isolated, the nitrate concentration varied from 0.3 to 6.6 $mgNO_3^-.L^{-1}$, corresponding to the sampling dates of 2nd May 2001 and 4th January 2001, respectively. The lowest nitrate concentration preceded the *A. flos-aquae* bloom development which reached its highest cell density in 16th May 2001. But in heavily eutrophied lakes, the nitrate concentration can reach much higher values. Thus, the nitrate concentration range chosen for the nutritional experiments (0.00 $mgNO_3^-.L^{-1}$ to 85.01 $mgNO_3^-.L^{-1}$) included the ranges that may occur in most of the environmental aquatic systems. For the experiments using different phosphate levels, the concentrations ranged from 0.00 to 8.71 $mgPO_4^{3-}.L^{-1}$, including the majority of environmental phosphate concentration ranges (including the fish culture ponds). In Vela Lake the phosphate concentration ranged from 0.00 (corresponding to the sampling dates from 12th June until 29th November 2001) to 1.65 $mgPO_4^{3-}.L^{-1}$ (corresponding to the sampling date of 20th December 2000). The phosphate depletion recorded from the 12th day June on was related with lower densities of this *A. flos-aquae* strain.

The *A. flos-aquae* strain studied is a filamentous cyanobacterium which is capable of fixing atmospheric nitrogen (N_2) in specialized cells called heterocysts (Flores and Herrero, 1994) when the ammonia or nitrate levels are low. This characteristic should be the responsible for the results obtained for different nitrate concentrations where this strain could normally grow even in total nitrate depleted medium. But in the highest TN:TP ratio conditions (APH0.5P) this strain grew worse and almost did not grow in phosphate depleted medium (with nitrate saturation conditions) as observed by Saadoun *et al.* (2001)

for another N-fixing filamentous cyanobacterium. Several studies have reported that the growth of N-fixing filamentous cyanobacteria is highly dependent on phosphorus concentrations (Lehtimäki *et al.*, 1997; Rapala *et al.*, 1997). Environmental studies have also related the development of *A. flos-aquae* strains with low TN:TP (total nitrogen: total phosphorus) ratios (Teubner *et al.*, 1999; Oliver and Ganf, 2000) and the non-occurrence of nitrogen-fixing cyanobacteria blooms in environments with high N:P ratios may be explained by the low competitive advantage of these filamentous cyanobacteria under phosphorus limitation (Kahru *et al.*, 2000; Oliver and Ganf, 2000).

In the second set of nutritional experiments, photooxidation is a possible cause for the observed "yellowish bad look" of the cultures and lower growth rates in relation to the first set of nutritional experiments. Cultures agitation was also probably insufficient (40 rpm showed to be low for the 500 mL Erlenmeyer flasks, by causing the settlement of cells) probably interfering with the availability of CO_2 to the cultures. The higher light intensity, along with higher temperature and lower cultures aeration may have caused the lower grow rate values. Studies using *A. flos-aquae* from the Baltic Sea (Lehtimäki *et al.*, 1997) reported that it grew best at 16 to 22ºC and at low irradiances (25 to 45 $\mu mol.m^{-2}.s^{-1}$). Yet, the 24ºC temperature was chosen because the temperatures between 20 and 25ºC and high light intensities (42 $\mu mol.m^{-2}.s^{-1}$) seem to favour the production of anatoxin-a and microcystin variants in N-fixing filamentous cyanobacteria (Rapala *et al.*, 1997, Rapala and Sivonen, 1998). The MBL nutrient saturated culture showed a lower output than cultures with lower nutrient concentrations (such as APH2P, APH4P, APH0N or APH8.5N), suggesting there may have been some problem with the MBL medium preparation (used in the three replicates), although all cultures were prepared and handled the same way.

During *A. flos-aquae* growth in the different media, the nitrite levels were positively related to the media nitrate concentrations. Nitrate levels were maintained during the growth but phosphate levels rapidly decreased until depletion in all cultures. For both the nitrate and phosphate limitation experiments, the ammonium levels were undetectable probably to the immediate assimilation by the cyanobacteria of the potentially produced ammonia in the media since it is the most preferred nitrogen source used by phytoplanktonic organisms (Bhaya *et al.*, 2000).

Effects of A. flos-aquae cultures exudates on growth of green algae

Cyanobacteria have shown to cause growth inhibition or settlement over other microalgae (Keating, 1978; Kearns and Hunter, 2000; Kearns and Hunter, 2001).

Concerning the present work experiments exposing the green algae to *A. flos-aquae* cultures exudates, in general, it was recorded an inhibition in their growth by the cyanobacterial exudates at some extent. For both green algae, between the controls, their growth rate was not decreased by the dilution factor of the addition of 30 mL distilled water to the growth medium, excluding the nutrient deficiency possibility from the discussion regarding growth inhibition. Hence, it must be the presence of a certain compound in the *A. flos-aquae* exudates that causes the inhibition of the green algae growth. The exudates from *A. flos-aquae* cultures were taken at the late stationary phase when extracellular cyanotoxins levels should be higher as in other filamentous cyanobacteria (Rapala and Sivonen, 1998), due to the beginning of cell lysis.

For both green algae, the greater growth inhibition effects were caused by the exudate of *A. flos-aquae* grown in phosphate limitation (0.5 $mgPO_4^{3-}.L^{-1}$) indicating that the inhibition factor was stronger in this cyanobacterial exudate, although the cyanobacterial material density was 1.35 and 1.93 fold lower than in MBL and aPH0N' cultures, respectively. *C. vulgaris* showed to be more sensitive than *P. subcapitata* to all the *A. flos-aquae* cultures exudates tested suggesting that it could be used as an important species to assess the toxicity of this cyanobacteria rather than *P. subcapitata*. However, *C. vulgaris* showed to have a lower growth rate than *P. subcapitata*, perhaps needing a higher test period (96h) than the 72h test period established for *P. subcapitata* (OECD, 2002). More, the differences between the controls and the 0.5f and MBLf treatments only became statistically significant at 96h for *C. vulgaris*. Generally, the strength of the inhibitory effect was related to the nitrate and, in particular, phosphate depletion in the source culture of the exudate. Thus, the toxic compound seems to be produced by *A. flos-aquae* at higher extent in media with nutrient limitation.

Many strains of *A. flos-aquae* are able to produce microcystin (Plumley, 1997; Willen and Mattson, 1997 *in* Lotocka, 2001), but the synthesis of this hepatotoxin by filamentous cyanobacteria is usually enhanced by high P levels (Rapala *et al.*, 1997). Thus, the production of this toxin should not be the main cause of the green algae growth inhibition. Yet, anatoxin-a production by *Aphanizomenon* does not seem to be altered by phosphate concentration variation, although the cyanobacterial growth is limited at lower phosphate concentrations (Rapala *et al.*, 1993 *in* Lotocka, 2001). Thus, the production of this cyanotoxin could be one of the factors responsible for the green algae growth inhibition under the 0.5f treatment. More, nitrogen has shown to decrease anatoxin-a concentration in *Aphanizomenon* (Rapala *et al.*, 1993) and, thus, in nitrogen depletion, its production should be higher, possibly partially explaining also the growth inhibition by the APH0N'

culture exudate if the anatoxin-a production by this strain is proved. Saxitoxins production by an *Aphanizomenon* sp. has shown to be restricted under phosphate limitation but increased under nitrate limitation (Dias *et al.*, 2002).

Hence, it may be suggested that the higher growth effects obtained for the APH0.5P exudate can be due to anatoxin-a production rather than microcystin or saxitoxin production. An important factor to assess would have been the presence of microcystin and other cyanotoxins in the exudates. Thus, further investigation should be conducted to evaluate if there are cyanotoxins being produced (not only microcystin, but also anatoxin-a and saxitoxins) by the cyanobacterial strain and, by using both toxic and non-toxic *A. flos-aquae* strains exudates, compare and assess if the effects on growth of the green algae tested are indeed due to cyanotoxins production or not. Nevertheless, many other factors rather than the presence of cyanotoxins in the exudate may be responsible for the green algae growth inhibition (Kearns and Hunter, 2000).

Conclusions

After the results obtained for the growth of *Aphanizomenon flos-aquae* under different nutrient concentrations, the cyanobacterium showed to be highly dependent on phosphorus availability rather than on nitrogen availability in the cultures media, which can be explained by its ability to fix nitrogen. These results are in accordance to other studies concerning this filamentous cyanobacterium (Lehtimäki *et al.*, 1997; Teubner *et al.*, 1999) and others (Rapala *et al.*, 1997; Oliver and Ganf, 2000) in which their growth is promoted by high phosphate concentrations and low N:P ratios. The present study shows that this same *A. flos-aquae* strain may inhibit the growth of the green algae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* which are a highly edible food source for zooplankton, namely cladocerans. *C. vulgaris* showed to be more sensitive than *P. subcapitata* to the *A. flos-aquae* exudates and the growth inhibition effects were more strong for the phosphate limiting cyanobacterial culture exudate. The growth inhibition observed is difficult to explain with the available data, but it should be related to the presence of a certain substance which production is promoted by phosphate limitation or inhibited by high phosphate levels. However, further investigation is needed to confirm these suppositions. The assessment of cyanotoxins production potential by this strain should be the immediate step to take subsequently to this study. Then, experiments using toxic and non-toxic strains of *A. flos-aquae* should be conducted to clarify if growth inhibition is indeed caused by cyanobacterial chemical defence (allelopathy).

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Capítulo VI

Chronic effects of *Aphanizomenon flos-aquae* on the survival and reproduction of dapnhids – a preliminary study

Chronic effects of *Aphanizomenon flos-aquae* on the survival and reproduction of dapnhids – a preliminary study

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Paper in draft form

Chronic effects of *Aphanizomenon flos-aquae* on the survival and reproduction of dapnhids – a preliminary study

Abstract – The ingestion of filamentous cyanobacteria by zooplankton may be affected by factors such as the mechanical interference (filaments rigidity or large size) but also the production of cyanobacterial toxins (secondary metabolites). However, among zooplankters, in situations of alternative food source unavailability, daphnid cladocerans are not very selective towards toxic cyanobacteria, being able to ingest both toxic and non-toxic cyanobacterial strains and accumulate the toxins. The aim of this study was to evaluate the effects of a potentially toxic strain of *Aphanizomenon flos-aquae*, given as a food source, on the survival and reproduction of *Daphnia magna* and *D. longispina* (Cladocera; Branchiopoda; Crustacea). Test organisms were fed with *A. flos-aquae* cultured in different concentrations of phosphate. The life history traits of *A. flos-aquae*-fed daphnids were compared to a control, in which the organisms were fed with the green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*). Results showed that *A. flos-aquae* was used as a food source by these daphnids, since they were able to survive and reproduce during the course of the experiment. Nevertheless, the reproductive output of *A. flos-aquae*-fed daphnids was far from reaching the one of organisms fed with the alga *P. subcapitata* (used in routine culture rearing). Additionally, *A. flos-aquae* grown with lower phosphate concentrations revealed a less significant reproductive impairment on *Daphnia* spp. in relation to *A. flos-aquae* grown in phosphate saturated medium. A similar pattern of response was observed in both *D. longispina* and *D. magna,* although the latter daphnid showed to be less susceptible than the former one. Results suggest that food quality may not be the main factor controlling the reproductive output of daphnids and cyanobacterial toxins production may be also involved. However, further research is required to enlighten this point of view.

Keywords: *Aphanizomenon flos-aquae*, phosphorus limitation, *Daphnia magna* and *D. longispina*, survival and reproduction effects.

Introduction

The occurrence of Harmful Algal Blooms is becoming more frequent all over the world, in general due to the increasing eutrophication of superficial water bodies (Codd, 2000). Cyanobacteria, in particular, are able to develop massively with conditions of

eutrophication (high nutrient levels), low turbulence, high temperatures and pH, leading to formation of superficial cyanobacterial blooms that may accumulate as surface scum (Hadas *et al.*, 1999; Jacoby *et al.*, 2000; Oliver and Ganf, 2000). Cyanobacterial blooms may be harmful because many cyanobacteria are able to produce toxic secondary metabolites that can be classified as dermatotoxins, neurotoxins or hepatotoxins (Dow and Swoboda, 2000; Kaebernick and Neilan, 2001), according to the effects on animals. There are many cases reported that show how cyanotoxins can be dangerous for numerous organisms, humans included (Gorham and Carmichael, 1988; Pouria *et al.*, 1998; Codd, 2000; Fitzgerald, 2001). Microcystin (MC) is a hepatotoxin to which special attention has been given due to its potential of promoting cancer in humans after chronic exposure through drinking water (Ueno *et al.*, 1996; Zhou *et al.*, 2002) and WHO (*World Health Organization*) has already established a lifetime consumption safe level of 1 $\mu g.L^{-1}$ for the most common microcystin variant (MC-LR), in drinking water (WHO, 1998). Microcystin variants occur in freshwaters worldwide and are mainly produced by colonial *Microcystis* spp. and strains belonging to filamentous *Anabaena*, *Planktothrix/ Oscillatoria*, *Anabaenopsis*, *Nostoc* and *Aphanizomenon* (Dow and Swoboda, 2000; Kaebernick and Neilan, 2001). Microcystin synthesis seems to be influenced by environmental factors such as light (Kaebernick *et al.*, 2000; Wiedner *et al.*, 2003), temperature (Rapala and Sivonen, 1998), trace metals (Lukac and Aegerter, 1993; Utkilen and Gjølme, 1995) and nutrients such as phosphorus (Rapala *et al.*, 1997, Kotak *et al.*, 2000; Oh *et al*., 2000; Vézie *et al.*, 2002) and nitrogen (Lee *et al.*, 2000; Long *et al.*, 2001), in spite of recent approaches consider the energetic state of the cyanobacterial cells (Bickel and Lyck, 2001) and genotype diversity (Kurmayer *et al.*, 2002; Vézie *et al.*, 2002; Mikalsen *et al.*, 2003) as the main factors that modulate microcystin production. Microcystin variants are known to be accumulated and to cause several toxic effects at different trophic levels (see chapter III of this thesis). For instance, microcystin-LR seems to have an allelopathic function towards other microalgae (Kearns and Hunter, 2001), resulting in growth inhibition or in settlement by paralysing them. The lack of alternative phytoplankton for food when cyanobacteria dominate may contribute to unfavourable nutritive conditions for zooplankton since cyanobacteria are known to be nutritionally poor (Brett and Müller-Navarra, 1997; Brett *et al.*, 2000). Daphnids, in particular, have shown to be affected in their reproduction when cyanobacteria dominate the phytoplankton community (DeMott, 1999; Scheuerell *et al.*, 2002). This could be due to *Daphnia* spp. high requirements for nutrients such as P (phosphorus) to synthesize several cellular constituents (phospholipids, ATP/ADP, nucleic acids) (Scheuerell *et al.*, 2002). RNA:DNA ratio is

highly correlated with somatic growth rate and depending on high food P:C ratios (Vrede *et al.*, 2002). In addition, P-limitation indirectly affects the cladocerans development (by decreasing growth rates and clutch size) through the alteration of algae biochemical composition, by reducing the essential fatty acids content (Ferrão-Filho *et al.*, 2003). Thus, physico-chemical environmental parameters can have a major effect on daphnid cladocerans development through the effect on food quality (Scheuerell *et al.*, 2002). Besides food quality, food limitation in zooplankton may be also due to feeding inhibition. Cyanobacteria may inhibit feeding by mechanical interference with the filtering apparatus (by cyanobacterial filaments/colonies size, shape, filaments rigidity or mucilage production by *Microcystis* spp. (Rohrlack *et al.*, 1999a; Henning *et al.*, 2001)) but cyanotoxins production seems also to work as a direct defence mechanism against grazing by affecting the ingestion (Kurmayer and Jüttner, 1999; Rohrlack *et al*., 1999b; Henning *et al.*, 2001; Lotocka, 2001). Rohrlack *et al.* (2001) suggest that *Microcystis* inhibits the ingestion rate of *Daphnia* by microcystin synthesis rather than by mechanical interference and that this inhibition increases with increasing toxin content. Nevertheless, daphnid cladocerans have shown to be less selective than copepods towards cyanobacteria, being able to ingest both toxic and non-toxic *Microcystis* colonies (Mohamed, 2001) at the same rate (Rohrlack *et al*., 1999b) under depletion of edible food (green algae and diatoms), accumulating the toxin (Mohamed, 2001) and potentially transferring it to higher trophic levels through the food chain. Toxic effects have also been observed in *Daphnia* spp. after cell-bound microcystin ingestion (Laurén-Määttä *et al.*, 1997; Rohrlack *et al*., 1999b; Rohrlack *et al.*, 2001) resulting in reduction of survival (killing in a few days) and population density, and delay of animal maturation. These effects are probably connected to *Daphnia*'s protein phosphatases 1 and 2A activity inhibition (DeMott and Dhawale, 1995 *in* Rohrlack *et al.*, 2001). Most of the recent studies on cyanobacterial toxicity towards daphnids by microcystin production are mainly focused on colonial *Microcystis* spp. (Laurén-Määttä *et al.*, 1997; Rohrlack *et al.*, 1999b; Mohamed, 2001; Rohrlack *et al.*, 2001; Lürling and van der Grinten, 2003) rather than on filamentous cyanobacteria. However, many strains of the filamentous cyanobacterium *A. flos-aquae* are able to produce microcystin (Plumley, 1997; Willen and Mattson, 1997 *in* Lotocka, 2001) but also neurotoxins such as anatoxin-a (Rapala *et al*., 1993 *in* Lehtimäki *et al.*, 1997) and saxitoxins (Pereira *et al.*, 2000, Ferreira *et al.*, 2001). *Aphanizomenon flos-aquae* blooms have been recorded, with production of saxitoxins (also named PSP (Paralytic Shellfish Poisoning)-type toxins), in several Portuguese reservoirs such as Crestuma reservoir

(northern Portugal) (Ferreira *et al.*, 2001) and Montargil reservoir (central Portugal) (Pereira *et al.*, 2000).

This study presents the results of laboratory tests using a strain belonging to the filamentous and potentially toxic cyanobacterium *Aphanizomenon flos-aquae*, isolated from a natural bloom in Vela Lake (May 2001), and two cladocerans (*Daphnia magna* and *D. longispina*). The tests aimed to assess the potential effects of this cyanobacterial strain, grown in different phosphorus concentrations, on the survival and reproduction of these daphnids, when given as an exclusive food source.

Material and Methods

Microalgae cultures

The strain used in this assay was isolated from a bloom occurring in a Portuguese eutrophied shallow lake (Vela Lake, Figueira da Foz, Portugal) in May 2001 and it was identified as belonging to the species *Aphanizomenon flos-aquae* (Komárek and Anagnostidis, 1989). The culture medium was based on sterilized Wood Hole Marine Biological Laboratory MBL medium, but with some modifications concerning orthophosphate concentration. Cyanobacterial cells were grown in 2 L flasks with 1.8 L growth medium with three different concentrations for phosphate (2.18, 4.36 and 8.71 $mgPO_4^{3-}.L^{-1}$, referred as APH2P, aPH4P and APH8P, respectively). The green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) was also cultured in MBL medium, and it was used as a control for food source experiments. All cultures were grown at 21 ºC under constant illumination (40 μmol of photons.$m^{-2}.s^{-1}$) using cool white fluorescent lights, positioned vertically, and cultures continuous aeration was assured by a single air pump (through sterile, 0.45-μm-pore-size filters).

A. flos-aquae feeding table for the daphnids

The correlation between optical density (at 440 nm) and cell number (using a Sedgwick-Rafter counting chamber) was assessed by following *A. flos-aquae* growth in MBL medium. These measurements were performed everyday, in quadruplicate, during 13 days and evaluated by linear regression analysis. After the obtained results, an *A. flos-aquae* feeding table was established for the daphnids tested.

Daphnids cultures

The testing daphnids include a standard species, *Daphnia magna*, frequently used as a test organism in many ecotoxicological studies, and an autochthonous species, *D. longispina* (Antunes *et al.*, 2003), isolated from another eutrophied lake (Mira Lake, central Portugal). Parent cladocerans of both species were reared in ASTM hard water culture medium with an organic additive, as described by Antunes *et al.* (2003). The cultures had no aeration supply and a temperature of 20 ± 2 ºC was maintained with a 16:8 h light:dark photoperiod. Medium renewal was performed every two days and animals were fed everyday with the green alga *Pseudokirchneriella subcapitata* at a concentration of 3.00 x 10^5 cells.mL^{-1} for *D. magna* but only 1.50 x 10^5 cells.mL^{-1} for *D. longispina* as justified on a previous report (Antunes *et al.*, 2003). The cultures were cyclically renewed for individuals by replacement of the progenitor by neonates from the third or forth clutch. The experimental tests were conducted using neonates from the third to the fifth brood of the partenogenic cycle, with all animals ageing less than 24 h old.

Food source experiments

To assess the potential chronic effects of *A. flos-aquae*, grown under different phosphate concentrations and given as an exclusive food source, on the chosen daphnids, preliminary life story experiments were performed during 15 days. The cladoceran clones used were clone A *sensu* Baird *et al.* (1989) and EM7 clone *sensu* Antunes *et al.* (2003) for *D. magna* and *D. longispina*, respectively. *A. flos-aquae* grown in three different phosphate concentrations (2.18, 4.36 and 8.71 mgPO_4^{3-}.L^{-1}, identified as APH2P, APH4P and APH8P, respectively) with a constant nitrogen concentration (85.01 mgNO_3^-.L^{-1}) was given as food source to the test daphnids. Before feeding, the cyanobacterium cultures were centrifuged and resuspended in ASTM medium at the same concentration previously described for the usual food source *Pseudokirchneriella subcapitata* (used in the control test). For the control and for each treatment, ten replicate glass vessels were prepared with one organism in 50 mL of ASTM medium and maintained as already described for daphnids cultures. Everyday, animals' survival and offspring were analysed and juveniles removed and counted. To evaluate reproduction during the test, parameters such as the age at first reproduction, total number of neonates (total offspring) and number of broods were recorded. Survival was assessed by daily observing the mortality of the parent animals. The rate of population increase (r, day^{-1}) was estimated after the Euler-Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-r.x}.l_x.m_x$$

where r is the rate of population increase (d^{-1}), x is the age class (days; 0...n), l_x is the probability of surviving at age x and m_x is the fecundity at age x. Standard deviation for r was determined according to the jack-knifing technique (Meyer *et al.*, 1986).

Data analysis

The relationship between optical density and cell number during *A. flos-aquae* growth in MBL medium was assessed by linear regression analysis.

Daphnids mortality values obtained for the different treatments during the experiment were compared by the Fischer exact test. A one-way analysis of variance (ANOVA) was used to assess significant differences among the food source regimes, for each species, considering the test parameters: age at first reproduction, total number of offspring, number of broods and rate of population increase. This one-way ANOVA was followed by a post hoc multiple comparisons Tukey HSD test, where applicable (Zar, 1996). For all analysis, a statistically significant difference in reproduction or growth is reported for $P<0.05$.

Results

A. flos-aquae feeding table for the daphnids

After the results obtained for the optical density and cell counting during the

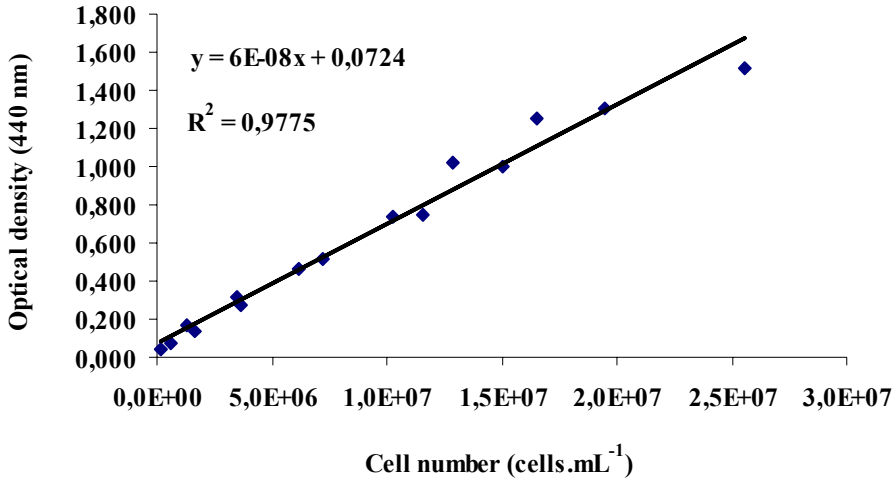

Figure 1 – Linear regression analysis of the growth parameters optical density and cell counting, after measured everyday for 13 days, in quadruplicate.

cyanobacterial strain growth over the 13 days, a high positive correlation (R^2=0.9775) was observed between these two growth parameters (Fig.1) with the equation obtained being

Table 1 – *A. flos-aquae* feeding table for *D. magna* and *D. longispina*, which specifies the *A. flos-aquae* volume (mL) per day for 50 mL *Daphnia* spp. medium.

	A. flos-aquae volume (mL) per day			*A. flos-aquae* volume (mL) per day			*A. flos-aquae* volume (mL) per day			*A. flos-aquae* volume (mL) per day	
Abs	***D. magna***	***D. longispina***	**Abs**	***D. magna***	***D. longispina***	**Abs**	***D. magna***	***D. longispina***	**Abs**	***D. magna***	***D. longispina***
0,900	1,131	0,566	1,050	0,958	0,479	1,200	0,830	0,415	1,350	0,733	0,366
0,905	1,125	0,562	1,055	0,953	0,476	1,205	0,827	0,413	1,355	0,730	0,365
0,910	1,118	0,559	1,060	0,948	0,474	1,210	0,823	0,412	1,360	0,727	0,364
0,915	1,111	0,556	1,065	0,943	0,472	1,215	0,819	0,410	1,365	0,724	0,362
0,920	1,105	0,552	1,070	0,939	0,469	1,220	0,816	0,408	1,370	0,722	0,361
0,925	1,098	0,549	1,075	0,934	0,467	1,225	0,812	0,406	1,375	0,719	0,359
0,930	1,092	0,546	1,080	0,929	0,465	1,230	0,809	0,404	1,380	0,716	0,358
0,935	1,085	0,543	1,085	0,925	0,462	1,235	0,805	0,403	1,385	0,713	0,357
0,940	1,079	0,540	1,090	0,920	0,460	1,240	0,802	0,401	1,390	0,711	0,355
0,945	1,073	0,536	1,095	0,916	0,458	1,245	0,798	0,399	1,395	0,708	0,354
0,950	1,067	0,533	1,100	0,911	0,456	1,250	0,795	0,398	1,400	0,705	0,353
0,955	1,061	0,530	1,105	0,907	0,453	1,255	0,792	0,396	1,405	0,703	0,351
0,960	1,055	0,527	1,110	0,902	0,451	1,260	0,788	0,394	1,410	0,700	0,350
0,965	1,049	0,524	1,115	0,898	0,449	1,265	0,785	0,393	1,415	0,697	0,349
0,970	1,043	0,522	1,120	0,894	0,447	1,270	0,782	0,391	1,420	0,695	0,347
0,975	1,037	0,519	1,125	0,890	0,445	1,275	0,779	0,389	1,425	0,692	0,346
0,980	1,032	0,516	1,130	0,885	0,443	1,280	0,775	0,388	1,430	0,690	0,345
0,985	1,026	0,513	1,135	0,881	0,441	1,285	0,772	0,386	1,435	0,687	0,344
0,990	1,020	0,510	1,140	0,877	0,439	1,290	0,769	0,384	1,440	0,685	0,342
0,995	1,015	0,507	1,145	0,873	0,436	1,295	0,766	0,383	1,445	0,682	0,341
1,000	1,009	0,505	1,150	0,869	0,434	1,300	0,763	0,381	1,450	0,680	0,340
1,005	1,004	0,502	1,155	0,865	0,432	1,305	0,760	0,380	1,455	0,677	0,339
1,010	0,999	0,499	1,160	0,861	0,430	1,310	0,757	0,378	1,460	0,675	0,337
1,015	0,993	0,497	1,165	0,857	0,428	1,315	0,753	0,377	1,465	0,672	0,336
1,020	0,988	0,494	1,170	0,853	0,427	1,320	0,750	0,375	1,470	0,670	0,335
1,025	0,983	0,491	1,175	0,849	0,425	1,325	0,747	0,374	1,475	0,668	0,334
1,030	0,978	0,489	1,180	0,845	0,423	1,330	0,745	0,372	1,480	0,665	0,333
1,035	0,973	0,486	1,185	0,842	0,421	1,335	0,742	0,371	1,485	0,663	0,331
1,040	0,968	0,484	1,190	0,838	0,419	1,340	0,739	0,369	1,490	0,660	0,330
1,045	0,963	0,481	1,195	0,834	0,417	1,345	0,736	0,368	1,495	0,658	0,329
									1,500	0,656	0,328

presented as follows:

$$Cells.mL^{-1} = -1206666.7 + Abs \times 1.6667x10^{7} \quad (1)$$

where *Abs* is the absorbance measured at 440 nm. This equation (1) allowed the construction of a feeding table using this cyanobacterium as food source for the tested daphnids (Table 1), considering the required cell concentrations for each test (3.00×10^5 cells.mL^{-1} for *D. magna* and 1.50×10^5 cells.mL^{-1} for *D. longispina*).

Food source experiments

At the end of the 15 days experiment, after applying the Fischer Exact Test, the mortalities obtained in the food source treatments using *A. flos-aquae* for both daphnid clones were not statistically higher than in the control ($P<0.05$) (Table 2). Nevertheless, *D. longispina* fed on *A. flos-aquae* (APH) grown in higher concentrations of phosphate (APH4P and APH8P) attained the highest mortality percentages (Table 2).

Table 2 – Mortality percentages of *D. magna* and *D. longispina* clones during the 15 days of experiment when fed on a cyanobacterial strain of *A. flos-aquae* grown under different phosphorus concentrations.

Species	Control (*P. subcapitata*)	*A. flos-aquae*		
		APH2P (grown in 2.18 mgPO_4^{3-}.L^{-1})	APH4P (grown in 4.36 mgPO_4^{3-}.L^{-1})	APH8P (grown in 8.71 mgPO_4^{3-}.L^{-1})
D. magna	0.0	20.0	30.0	10.0
D. longispina	0.0	10.0	30.0	30.0

The food source had also a significant effect on the reproduction and rate of population increase (Fig. 2). For *D. magna*, the average age at first reproduction was significantly higher from the control when animals were fed with APH and between APH treatments, the age at first reproduction was significantly higher for APH4P and APH8P food sources. For *D. longispina*, the age at first reproduction was significantly higher for all feeding regimes using APH and, between the APH food source regimes, this same parameter was significantly higher for APH4P and APH8P treatments in relation to APH2P feeding regime.

The total number of offspring (number of neonates produced per female), for both clones, was significantly negatively affected by all the feeding regimes using APH, in relation to the food source used in control (Fig. 2). More, for *D. longispina*, by comparison to APH2P, both APH4P and APH8P regimes significantly decreased the total number of

offspring and, between these last, there is a tendency indicating a gradual decrease in this parameter towards APH8P.

For *D. magna*, there were no significant differences between the number of broods at the control and at APH food source treatments, in spite of evidence indicating a gradual decrease in number of broods from the control to the APH grown in higher phosphate concentration (Fig. 2). However, for *D. longispina*, the number of broods was significantly affected when the animals were fed with APH4P and APH8P, in relation to the food source used in control (Fig. 2). Between the APH food source treatments, APH8P regime caused a significant decrease in the number of broods by comparing with APH2P and APH4P regimes. The number of broods produced in control was 3 and 4 for *D. magna* and *D. longispina*, respectively.

The survival and reproduction effects accumulated during the 15 days experiment are expressed in the population intrinsic growth rate (*r*) for which values are significantly

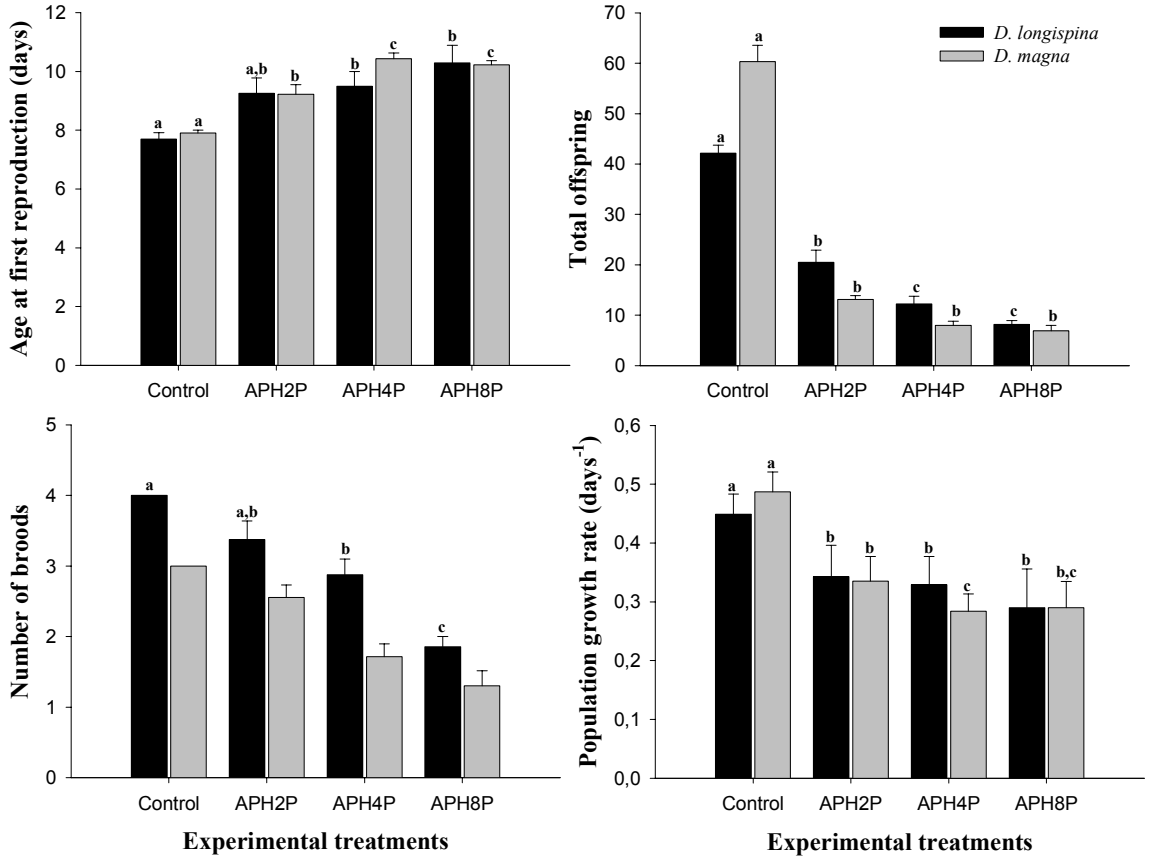

Figure 2 – Age at first reproduction (days), total number of offsring, number of broods and population growth rate during the 15 days experiment using *D. magna* and *D. longispina* fed on a cyanobacterial strain of *A. flos-aquae* grown in different phosphorus concentrations. Error bars represent the standard deviation and the different letters represent significant differences between the food sources (P<0.05).

lower at APH regimes in relation to control, for both species (Fig.2). Among the APH treatments, *D. longispina* did not show significant differences, although there is a decreasing tendency towards the APH grown in higher phosphate concentrations. On the other hand, for *D. magna*, *r* is significantly lower for APH4P and APH8P, by comparison to APH2P treatment.

In general, both species showed to be affected when fed with *A. flos-aquae*, no matter under what conditions of phosphorus availability the cyanobacterium was grown.

Discussion

The exposure of the tested cladocerans to *A. flos-aquae*, in general, increased mortality, delayed maturation of females and decreased offspring production and population growth rate. *D. longispina* showed to be more sensitive for the experiments using the most P-saturated algae (APH8P) as food source. The higher mortalities obtained for all treatments using APH as food source, in relation to control, indicate that these daphnids are not very well adapted to this food source, in spite of ingesting it and achieving considerable survival percentages and reproduction output. The fact that results for average age at first reproduction showed higher values for all feeding treatments using APH (in particular APH4P and APH8P) indicates a delayed maturation of daphnids for these treatments. *A. flos-aquae* strongly reduced the total offspring of both cladocerans, suggesting an important effect of this food source on their reproduction. The number of broods, by comparison to control, is also significantly lower for P-saturated algae (APH4P and APH8P).

As possible hypotheses for the affectation of *A. flos-aquae* treatments on the survival and reproduction of both species, in relation to control treatment with *P. subcapitata*, there are: feeding inhibition, low nutritional value of cyanobacteria and/or the potential toxicity by intracellular cyanotoxins after filaments ingestion. First, feeding inhibition may be caused by mechanical interference due to filaments rigidity, large size or aggregation, reducing the feeding rates or blocking the filtering apparatus, although daphnids have shown to possess a phenotypic plasticity towards filamentous cyanobacteria (Ghadouani and Pinel-Alloul, 2002) by enlarging the area and mesh size of their filtering apparatus. Yet, cyanobacteria have also shown to inhibit feeding through cyanotoxins synthesis (Kurmayer and Jüttner, 1999; Rohrlack *et al*., 1999b; Henning *et al.*, 2001; Lotocka, 2001; Rohrlack *et al.*, 2001), as a chemical defence. Kurmayer and Jüttner (1999) found that, for a microcystin producing strain of the filamentous *Planktothrix rubescens*, grazing resistance (food avoidance) should be mediated by chemical defences

(toxin production) rather than by the large size and rigidity of filaments. A study concerning the grazing of *D. magna* over the potentially toxic cyanobacteria species *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* also showed to have a limiting impact on grazing intensity (Lotocka, 2001). Thus, this factor should be considered in the present discussion because many strains of *A. flos-aquae* are able to produce microcystin (Plumley, 1997; Rapala *et al*., 1993 *in* Lehtimäki *et al.*, 1997). Yet, further investigation, including a set of experiments involving toxic and non-toxic strains of *A. flos-aquae*, should be conducted in order to evaluate if there is a feeding inhibition due to mechanical interference and/or to cyanotoxins production. However, feeding inhibition should not be the only factor behind the differences observed between the control and APH treatments, since the animals survived and reproduced, indicating they ingested and digested enough *A. flos-aquae* filaments to achieve that performance level, as observed by Kurmayer (2001) for *Aphanizomenon flexuosum* (non-toxic), using the alga as a source of energy. This also means that these *Daphnia* clones may feed on *A. flos-aquae* as an alternative food source when an edible food source is lacking. Therefore, taking into consideration that food quantity was approximately the same, food quality should be another important factor to consider, due to the low nutritional value of cyanobacteria (Brett and Müller-Navarra, 1997; Brett *et al.*, 2000), with lack of essential fatty acids used as energy source. As already mentioned, there are several strains of *A. flos-aquae* capable of producing toxins such as microcystin (Plumley, 1997; Willen and Mattson, 1997 *in* Lotocka, 2001) and microcystin as proved to cause toxic effects on *Daphnia* spp., after ingestion (Laurén-Määttä *et al.*, 1997; Rohrlack *et al*., 1999b; Rohrlack *et al.*, 2001), decreasing the survival and population density, and delaying animals maturation. The toxic effects observed in daphnids are thought to be connected with the inhibition of their protein phosphatases 1 and 2A activity by microcystin (DeMott and Dhawale, 1995 *in* Rohrlack *et al.*, 2001). Other filamentous toxic cyanobacteria (*Anabaena* spp.), anatoxin-a producers, have shown to affect both survival and fecundity in *Daphnia pulex* (Claska and Gilbert, 1998), by affecting brood size, brood number, age at first reproduction and interclutch interval.

For differences between APH treatments, neither mechanical interference nor food nutritional value seem to be the cause, since filaments size or rigidity was similar between APH treatments and P-limited algae should lead to negative effects if food quality would be the most important parameter. As observed by several authors (including Scheuerell *et al.*, 2002; Ferrão-Filho *et al.*, 2003), P-limitation indirectly affects the cladocerans development through the reduction of the essential fatty acids content of algae. In the present case, *A. flos-aquae* grown under the lowest P concentration showed the weakest

effects on survival and reproduction of the tested daphnids, suggesting that this should not be one of the factors determining the differences obtained among the APH treatments. The different results recorded among APH treatments in the present study might have been influenced by the possible microcystin (or other cyanotoxins) synthesis, since the most intense effects over daphnids were obtained for animals fed on *A. flos-aquae* grown under the highest P concentrations, which have shown to enhance microcystin production in toxic filamentous cyanobacteria (Rapala *et al.*, 1997). In N-fixing cyanobacteria, microcystin synthesis seems to be considerably more dependent on P rather than on N, due to the referred capability of N fixation. Thus, microcystin (Rapala *et al.*, 1997) content increases with P concentration and if this was a relevant factor modulating our results, APH4P and APH8P should have more microcystin production and could, therefore, induce stronger toxic effects on daphnids such as those observed in this study and also reported by DeMott (1999) and Rohrlack *et al.* (2001) after microcystin ingestion. However, further investigation should also be conducted to evaluate if there are, in fact, cyanotoxins being produced (not only microcystin, but also anatoxin-a and saxitoxins) and compare the effects on daphnids of both toxic and non-toxic *A. flos-aquae* strains. Furthermore, toxicity of a cyanobacterial strain depends on cellular toxin content but also on the rate with which the daphnid is feeding on that strain (Rohrlack *et al.*, 2001). In this case, however, the strongest effects on APH8P can be due to toxic effects but also to a higher inhibition of ingestion as referred by Rohrlack *et al.* (2001) for *Microcystis*. Thus, ingestion rate should also be monitored to check this toxin-mediated feeding inhibition hypothesis for differences observed between the APH treatments. Nevertheless, the inhibition of growth and reproduction may be due to the combined occurrence of more than one inhibition factor.

Conclusions

The present study shows that the strain of *A. flos-aquae* used in this study, when given as an exclusive food source, affects the survival and reproduction of two clones belonging to *Daphnia magna* and *D. longispina*, suggesting that characteristics of this strain as a food source are not the most suitable for these cladocerans development. The differences found, in relation to a control treatment using *Pseudokirchneriella subcapitata* as an edible food source, may be explained by feeding inhibition (through mechanical interference or toxin production), low food quality and/or toxicity after ingestion, but further investigation is needed to confirm these suppositions.

Between the APH treatments, the *A. flos-aquae* grown in higher phosphorus concentration showed stronger effects on reproduction parameters. In general, *D. longispina* showed to be more sensitive than *D. magna* to the gradient of APH phosphorus concentration, with the most significant effects for P-saturated cells (APH8P). The explanation(s) for these differences may rely on toxins production, which may be enhanced at higher P concentrations (Rapala *et al*., 1997) causing feeding inhibition and/or toxic effects after the toxins ingestion, rather than mechanical interference, nutritional value (that could lead to starvation) or food quantity.

However, additional investigation is needed to evaluate these speculated explanations. The assessment of cyanotoxins production potential by this strain should be the immediate prolongation of this study due to the possible risk that, in particular, microcystin may represent to many organisms when accumulated by cladocerans and potentially transferred to higher trophic levels through the food chain (Ferrão-Filho *et al.*, 2002). Then, experiments using toxic and non-toxic strains of *A. flos-aquae* should be conducted to clarify if feeding inhibition is caused by mechanical interference, cyanobacterial chemical defence or both. Another interesting point to check, if cyanotoxins production by this *A. flos-aquae* strain is proved, would be the detoxification potential of these daphnids towards those cyanotoxins, since the clones used in this study could ingest the potentially toxic alga and still survive and reproduce.

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Capítulo VII

Discussão geral

Discussão geral

No presente estudo, foi inicialmente apresentada muita da investigação efectuada internacionalmente acerca das temáticas: ocorrência de *blooms* de cianobactérias, produção de cianotoxinas e sua toxicidade em diversos organismos, incluindo os humanos. Foi dada especial relevância à hepatotoxina microcistina e ao perigo que a presença desta na água pode representar para a Saúde Pública (Gorham and Carmichael, 1988; Codd *et al.*, 1995; Pouria *et al.*, 1998; Codd, 2000). Relativamente aos factores que influem na produção desta, existe muita contrariedade entre os estudos efectuados, principalmente devida à diversidade genotípica entre as diferentes estirpes cianobacterianas (Hesse and Kohl, 2001; Rohrlack *et al.*, 2001; Kurmayer *et al.*, 2002; Mikalsen *et al.*, 2003). Assim, para cada estirpe, existirá um padrão respectivo relativamente à capacidade de produção das variantes de microcistina e à influência dos parâmetros ambientais sobre esta, tornando necessária a identificação, ao nível da estirpe, das cianobactérias que geralmente dominam os *blooms* de um determinado sistema aquático, de forma a conseguir definir estratégias específicas para o controlo do seu desenvolvimento e produção de toxinas.

A segunda fase deste estudo pretendeu ser uma abordagem integrada (de dados ambientais e laboratoriais) ao problema ambiental dos *blooms* de cianobactérias potencialmente tóxicas, destacando os factores que favorecem o seu desenvolvimento, assim como os efeitos em organismos de dois níveis tróficos tróficos (fitoplâncton e zooplâncton). A estirpe utilizada nos estudos laboratoriais pertence à espécie *Aphanizomenon flos-aquae* e foi isolada a partir de um *bloom* ocorrido na Lagoa da Vela, na Primavera de 2001, em que esta estirpe dominava a comunidade fitoplanctónica em cerca de 87% (Cap. IV), com potenciais riscos para a Saúde Pública, de acordo com os níveis propostos pela Organização Mundial de Saúde (WHO, 1998a). O desenvolvimento deste *bloom* foi precedido de condições de indisponibilidade em azoto. Após o pico do *bloom*, verificou-se um período de senescência deste, provavelmente devido às condições de anoxia verificadas, seguindo-se um pico nos níveis de amónia (possivelmente resultante da degradação da massa fitoplanctónica remanescente), favorecendo o desenvolvimento de um *bloom* de *Microcystis aeruginosa*.

Uma vez que os estudos incidem normalmente sobre estirpes de *Microcystis aeruginosa* e que a sua produção de cianotoxinas já foi estudada nesta lagoa (Vasconcelos, 1993), a escolha da estirpe de *A. flos-aquae* teve o carácter de aprofundar o conhecimento acerca das estirpes potencialmente tóxicas que co-dominam com estirpes de *M. aeruginosa* provavelmente tóxicas (Vasconcelos, 1993), aquando de

blooms na Lagoa da Vela. E é neste contexto que se inseriram os ensaios laboratoriais de crescimento desta estirpe de *A. flos-aquae* em meio com diferentes concentrações de fósforo e azoto (Cap. V) e os seus efeitos em clorófitas (*Chlorella vulgaris* e *Pseudokirchneriella subcapitata*) (Cap. V) e cladóceros (*Daphnia magna* e *D. longispina*) (Cap. VI). A estirpe cianobacteriana estudada mostrou ter um crescimento mais sensível ao teor em fósforo relativamente aos níveis de azoto (Cap. V), presumivelmente devido à sua capacidade de fixação de azoto, que a torna mais independente da concentração deste nutriente no meio, como confirmado em estudos semelhantes efectuados para outras cianobactérias fixadoras de azoto (Lehtimäki *et al.*, 1997; Rapala *et al.*, 1997).

Integrando os dados ambientais e laboratoriais, o desenvolvimento desta estirpe de *A. flos-aquae* na Lagoa da Vela foi precedido dos níveis mais baixos de nitratos no meio (0.30 $mgNO_3^-.L^{-1}$), mas pode ainda crescer perfeitamente num meio com depleção total de nitratos (0.00 $mgNO_3^-.L^{-1}$) devido à sua capacidade de fixação do azoto atmosférico, sugerindo que esta seja uma das vantagens competitivas mais determinantes no seu sucesso sobre as restantes espécies fitoplanctónicas aquando do seu desenvolvimento num *bloom*. No entanto, a depleção em fosfatos (0.00 $mgPO_4^{3-}.L^-1$) condiciona em larga escala o desenvolvimento desta estirpe, tal como verificado no ambiente e no laboratório. Aquando da ocorrência do *bloom*, a concentração de fosfatos era de 0.24 $mgPO_4^{3-}.L^{-1}$. Em laboratório, a concentração de fosfatos 0.55 $mgPO_4^{3-}.L^{-1}$ provou ter resultados significativamente inferiores relativamente aos obtidos para concentrações superiores de fosfatos (de 2.18 $mgPO_4^{3-}.L^{-1}$ a 8.71 $mgPO_4^{3-}.L^{-1}$), indicando que o crescimento desta estirpe pode ser estimulado pela disponibilidade de maiores concentrações de fosfatos. Assim, a combinação de baixas concentrações de nitratos (ou sua depleção) e elevadas concentrações de fosfatos (provenientes da actividade agro-pecuária, por exemplo) num sistema aquático como a Lagoa da Vela pode tornar-se "explosiva", levando ao desenvolvimento de grandes *blooms* desta estirpe de *A. flos-aquae*, superiores ao ocorrido em Maio 2001.

Os resultados obtidos nos testes utilizando as espécies fitoplanctónicas *C. vulgaris* e *P. subcapitata* mostraram uma ligeira inibição do crescimento das mesmas, especialmente em *C. vulgaris*, provocada pelos compostos presentes nos exudatos da estirpe de *A. flos-aquae* estudada (Cap. V). Tal efeito sugere a possibilidade de alelopatia desta estirpe relativamente a espécies fitoplanctónicas potencialmente competidoras. Existem outros estudos que demonstraram efeitos alelopáticos das cianobactérias sobre outros grupos fitoplanctónicos (Keating, 1978; Kearns and Hunter, 2000; Kearns and Hunter, 2001). As espécies testadas no presente estudo são fontes de alimento

altamente edíveis pelo zooplâncton, sugerindo que a sua redução no meio ambiente, devida ao efeito alelopático mencionado, pode também levar ao decréscimo da comunidade zooplanctónica e da sua diversidade específica.

Além deste efeito indirecto sobre o zooplâncton, a estirpe de *A. flos-aquae* utilizada mostrou também provocar efeitos significativos na sobrevivência e reprodução dos cladóceros testados (*D. magna* e *D. longispina*), ao ser fornecida como alimento exclusivo, relativamente a um controlo utilizando *Pseudokirchneriella subcapitata* (Cap. VI). Os efeitos negativos acompanharam uma tendência gradual de aumento, à medida que a estirpe de *A. flos-aquae* utilizada tivesse sido cultivada num meio com concentrações cada vez maiores de fosfatos. A produção de toxinas pode ser um importante factor responsável pelos efeitos registados, pois os níveis mais elevados de microcistina são normalmente verificados em condições de elevadas concentrações em fósforo (Rapala *et al.*, 1997). No entanto, a produção de outras cianotoxinas poderá também ter determinado o decorrer dos ensaios, visto *A. flos-aquae* possuir estirpes capazes de sintetizar também anatoxina-a (Rapala *et al*., 1993 *in* Lehtimäki *et al.*, 1997) e saxitoxinas (Pereira *et al.*, 2000; Ferreira *et al*., 2001). Em próximos ensaios, o despiste da produção destas outras toxinas deverá ser tomado em consideração. No entanto, os efeitos negativos desta estirpe de *A. flos-aquae* sobre a comunidade de cladóceros confirmam o declínio acentuado registado para as espécies de *Daphnia* aquando do *bloom* da cianobactéria na Lagoa da Vela (Antunes *et al.*, *in press*).

Concluindo, os resultados obtidos em laboratório parecem estar em conformidade com os dados ambientais. Assim, em resumo, a estirpe de *A. flos-aquae* isolada do *bloom* na Lagoa da Vela desenvolve-se bem em condições de disponibilidade de fósforo, resistindo à indisponibilidade de azoto pela sua capacidade de fixação do azoto atmosférico e apresentando, assim, uma vantagem competitiva relativamente às restantes espécies fitoplanctónicas existentes na Lagoa quando os níveis de azoto no meio são muito reduzidos. Mais, os estudos laboratoriais de ecotoxicidade sugerem uma capacidade alelopática desta cianobactéria sobre outras microalgas, nomeadamente clorófitas. Os cladóceros também mostraram ser afectados na sua sobrevivência e na sua reprodução, ao serem sujeitos a um regime alimentar utilizando esta estirpe de *A. flos-aquae* como fonte de alimento exclusiva. Além disso, a falta de alimento edível, provocada pelo efeito alelopático acima referido, pode também condicionar a manutenção da comunidade zooplanctónica. Produza ou não cianotoxinas, esta estirpe cianobacteriana provou ter efeitos aos níveis das comunidades fitoplanctónica e zooplanctónica.

Se a produção de microcistina por esta estirpe de *A. flos-aquae* vier a ser confirmada por estudos posteriores, é necessário focar a atenção no potencial perigo de contaminação dos produtos agrícolas (particularmente os utilizados para consumo humano) por bioacumulação da toxina (Codd *et al.*, 1999), ao irrigar as culturas com água da lagoa. Além disso, já foram provados os efeitos que esta toxina pode provocar no desenvolvimento de muitas culturas agrícolas (McElhiney *et al.*, 2001; Hamvas *et al.*, 2003).

Actualmente, é necessária uma gestão adequada e eficaz dos sistemas hídricos relativamente à entrada de nutrientes (especialmente fosfatos e nitratos) nos sistemas aquáticos, de forma a evitar a ocorrência destes *blooms* de cianobactérias potencialmente tóxicas e os possíveis riscos de intoxicações por elas provocados. Adicionalmente, é imperativo o desenvolvimento de uma sensibilização integrada do público, agricultores e industriais relativamente aos potenciais efeitos perigosos deste tipo de fenómenos para a Saúde Pública, enfatizando a responsabilidade de cada interveniente na introdução excessiva desses nutrientes no meio aquático. Através de uma melhor compreensão da dinâmica ecológica dos sistemas aquáticos, por todos os intervenientes, poder-se-á atingir uma gestão integrada, responsável e mais eficiente e efectiva desses mesmos sistemas aquáticos.

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