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COSTA MARTINS OSSWALD

OCORRÊNCIA DE CIANOBACTÉRIAS PRODUTORAS DE ANATOXINA-A E AVALIAÇÃO DO RISCO PARA A SAÚDE PÚBLICA, EM PORTUGAL

OCCURRENCE OF ANATOXIN-A PRODUCING CYANOBACTERIA AND EVALUATION OF ITS PUBLIC HEALTH RISK, IN PORTUGAL

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Dissertação de Doutoramento em Biologia de
Ph. D. Dissertation in Biology by
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Orientada por Supervised by
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ÍNDICE TABLE OF CONTENTS

RESUMO

A anatoxina-a é uma neurotoxina produzida por alguns géneros cianobacterianos de água doce. A sua ocorrência já foi descrita por vários autores, tendo sido detectada em alguns países europeus e americanos. Os estudos científicos mostram que não é a cianotoxina que ocorre com mais frequência mas o facto de ser uma neurotoxina potente (*i.p.* LD_{min} em ratinho 0,25 $mg.kg^{-1}$) torna-a importante em termos de risco para a saúde humana e para os ecossistemas aquáticos e terrestres. A ocorrência de anatoxina-a em água doce, já foi a causa de morte de gado e de animais selvagens em diversos países. Em Portugal estava descrita a ocorrência de géneros cianobacterianos produtores de anatoxina-a mas não existiam trabalhos que visassem a detecção desta neurotoxina. Esta falta de informação devia-se em parte, à falta de laboratórios portugueses capazes de detectarem esta neurotoxina.

A colaboração com a Universidade de Vigo durante esta dissertação, permitiu implementar a técnica de HPLC-FLD para quantificação da anatoxina-a na água, em células cianobacterianas e em tecidos animais. Isto permitiu-nos executar os trabalhos subsequentes que envolveram a detecção e quantificação da anatoxina-a, nomeadamente o estudo da ocorrência da anatoxina-a em amostras naturais de água e de estirpes cianobacterianas portuguesas e a execução de bioensaios com anatoxina-a em organismos aquáticos.

Primeiro colheram-se amostras de água em locais de recreio e de captação de água de consumo para detecção e quantificação de anatoxina-a por HPLC-FLD. Após a análise de 13 amostras de água que provinham de vários locais geográficos de Portugal continental, verificou-se a inexistência de anatoxina-a (LOD=25 $ng.l^{-1}$). No entanto, isolaram-se dessas mesmas amostras 22 estirpes cianobacterianas, das quais 13 produziram anatoxina-a em laboratório (LOD=3 $ng.g^{-1}$ dw). A elevada proporção de estirpes produtoras de anatoxina-a encontrada neste trabalho (59.1%) é discutida colocando-se a hipótese da anatoxina-a constituir um metabolito cianobacteriano mais frequente do que se julgava.

Para se estudar o efeito da anatoxina-a em organismos aquáticos, fizeram-se três bioensaios com concentrações de anatoxina-a ou de células produtoras de anatoxina-a ecologicamente relevantes (640-80 $\mu g.l^{-1}$ e 10^4–10^7 $cell.ml^{-1}$). Os organismos testados foram o mexilhão (*Mytilus galloprovincialis*) e a carpa (*Cyprinus carpio*) em diferentes estádios de desenvolvimento (ovo, fase larvar e juvenil). Constatou-se que a toxicidade desta neurotoxina dependeu do organismo em estudo.

Para os mexilhões, a concentração de 10^5 $cell.ml^{-1}$ durante 15 d não causou a morte de nenhum exemplar. O estudo da dinâmica de acumulação/depuração ao longo de um mês (15 dias de acumulação seguidos de 15 d de depuração) mostrou uma máxima efi-

ciência de acumulação de 10,92%. Passado 1 d após a entrada no período de depuração já não era possivel detectar anatoxina-a nas partes comestíveis do mexilhão.

Submetendo juvenis de carpa a 10^7 cel/ml de cianobactérias tóxicas, verificou-se uma letalidade de 100% às 29 h; para os ovos de carpa, a concentração de $5{,}46 \times 10^6$ cel.ml^{-1} provocou a morte de toda a população testada em 4 d. No bioensaio com juvenis de carpa, verificou-se uma concentração de anatoxina-a total nos peixes capaz de pôr em risco a saúde humana por corresponder a cerca de duas vezes o TDI de uma criança de 10 kg. No bioensaio com ovos de carpa comparou-se o efeito da anatoxina-a pura com o efeito de extractos celulares de uma estirpe cianobacteriana produtora de anatoxina-a. Embora ambos os tratamentos tenham sido tóxicos, os extractos cianobacterianos revelaram um efeito muito mais acentuado. Para além da mortalidade, registaram-se também deformações esqueléticas nas larvas após a eclosão. A comparação entre estes dois tipos de tratamento reforçou a necessidade de se efectuar bioensaios com extractos cianobacterianos tóxicos uma vez que estes se comportam de modo diferente quando comparados com bioensaios com toxina pura. Por outro lado, os extractos celulares são ecologicamente mais representativos.

Esta dissertação cumpriu os objectivos inicialmente propostos: analisaram-se amostras naturais de massas de água portuguesas e suas estirpes cianobacterianas, tornando-se evidente a provável ocorrência de anatoxina-a em Portugal, e estudou-se o efeito tóxico da anatoxina-a em organismos aquáticos, aspecto que pensamos ser essencial para a avaliação do risco desta neurotoxina para a saúde pública e ecológica.

SUMMARY

Anatoxin-a is a neurotoxin which is produced by several cyanobacterial genera from freshwaters. Some authors have described its occurrence in European and American countries. Research has shown that anatoxin-a is not the most frequent cyanotoxin, although due to its high toxicity *(i.p.* LD_{min} in mice 0.25 $mg.kg^{-1}$) it is considered very important in terms of human as well as aquatic and terrestrial health. The occurrence of anatoxin-a in different countries was already the cause of several deaths of cattle and wild animals. In Portugal it was known that cyanobacterial genera potentially producers of anatoxin-a were present in some freshwater bodies. Nevertheless, in part due to the lack of national laboratories capable to detect anatoxin-a, there were no studies about the occurrence of this neurotoxin.

Our cooperation with the University of Vigo during this dissertation, made it possible to implement the HPLC-FLD technique for the quantification of anatoxin-a in water, cyanobacterial cells and animal tissues. This gave us the possibility to perform the work that followed, namely the study about the occurrence of anatoxin-a in natural water samples and cyanobacterial strains as well as the bioassays with aquatic organisms.

First, several water samples were collected from water systems for recreational and human consumption usage. No anatoxin-a was detected in 13 natural water samples (LOD=25 $ng.l^{-1}$) obtained from different geographic locals. Nevertheless we isolated 22 cyanobacterial strains and analyzed them for anatoxin-a production. Thirteen strains gave positive results (LOD=3 $ng.g^{-1}$ dw). This high proportion of anatoxin-a producing strains (59.1%) is discussed here, considering the hypothesis that anatoxin-a is a more frequent metabolite of cyanobacteria than it was thought before.

In order to study the effects of anatoxin-a in aquatic organisms, three bioassays were carried out using ecologically relevant concentrations of anatoxin-a or of anatoxin-a producing strain (640-80 $\mu g.l^{-1}$ and 10^4 -10^6 $cell.ml^{-1}$). The test organisms were the mussel *(Mytilus galloprovincialis)* and the carp *(Cyprinus carpio)* at different stages of development (egg, larval stage and juvenile). Results demonstrated different sensibilities to the toxin depending on the tested organism.

In the study about the dynamics of accumulation/depuration of anatoxin-a by the mussels, they were submitted to a cell density of 10^5 $cell.ml^{-1}$ during one month (15 d of accumulation phase followed by 15 days of depuration). No deaths were registered and the mussels attained a maximum accumulation efficiency of 10.92%. After being one day in the depuration period, no anatoxin-a could be detected in the edible parts of the mussels.

Exposing juveniles of carp to a cellular concentration of 10^7 $cel.ml^{-1}$ caused 100% of

deaths at 29 h after exposure. For the carp eggs, a cell density of $5.46x10^6$ cel.ml^{-1} was lethal for 100% of the tested population in 4 d.

In the bioassay with juveniles of carp it was observed that the total concentration of anatoxin-a in the whole fish reached levels indicative of health risk to humans (2 times TDI of anatoxin-a for a 10 kg child). In the case of the bioassay with carp eggs, the effect of toxic cellular extracts was compared with pure toxin at 4 different concentrations each. Besides mortality, skeletal malformations were also observed in the larvae stage. Both treatments caused alterations in early developmental stages of carp but the extracts were more toxic. These data reinforced the need to perform bioassays using cyanobacterial extracts and not only pure cyanotoxins. This way the natural conditions are better simulated and the obtained data are more ecologically relevant.

This dissertation achieved its proposed objectives: Anatoxin-a was detected in Portuguese cyanobacterial strains, making its occurrence in natural freshwaters almost certain. The toxic effects of anatoxin-a were studied in aquatic organisms thus obtaining data that we believe will be important for the evaluation of health and environmental risk associated with this neurotoxin.

RÉSUMÉ

L'anatoxine-a est une neurotoxine produite par quelques genres cyanobacteriens d'eau douce. Sa présence a déjà été décrit par plusieurs auteurs, ayant étai détectée dans quelques pays européens et américains. Les études scientifiques indiquent que ce n'est pas la cyanotoxine qui apparaît plus fréquemment mais le fait d'être une neurotoxine puissante (*i.p.* LD_{min} en souris 0,25 $mg.kg^{-1}$) la rendent importante en ce qui concerne les risques pour la santé humaine et pour les écosystèmes aquatiques et terrestres. L'apparition de l'anatoxine-a dans l'eau douce, a déjà été la cause de mort de bétail et d'animaux sauvages dans divers pays. Au Portugal était décrite l'apparition de genres cyanobactéries producteurs d'anatoxine-a mais il n'existait pas des travaux qui visaient la détection de cette neurotoxine. Ce manque d'information était dû surtout, à l'absence de laboratoires portugais capables de détecter cette neurotoxine.

La collaboration avec l'Université de Vigo pendant cette dissertation, a permit d'implanter la technique de HPLC-FLD pour quantification de l'anatoxine-a dans l'eau, dans des cellules cyanobacterienes et dans des tissus animaux. Ceci nous a permis exécuter les travaux subséquents qui comprennaient la détection et quantification de l'anatoxine-a. Notamment l'étude de l'apparition de l'anatoxine-a dans les échantillons naturels d'eau et de souches cyanobacterienes portugaises et l'exécution de bio essais avec anatoxine-a dans des organismes aquatiques.

Avant tout on a prit des échantillons d'eau dans des locaux de récréation e de captation d'eau de consommation pour détection et quantification d'anatoxine-a par HPLC-FLD. Après l'analyse de 13 échantillons d'eau qui parvenaient de plusieurs locaux géographiques de Portugal continental, o a vérifié l'absence d'anatoxine-a (LOD=25 $ng.l^{-1}$). Néanmoins, on a isolé de ces mêmes échantillons 22 souches cyanobacterienes, dont 13 ont produit anatoxine-a en laboratoire (LOD=3 $ng.g^{-1}$ dw). L'haute proportion de souches productrices de anatoxine-a trouvée dans ce travail (59.1%) est discutée en colloquant l'hypothèse de l'anatoxine-a constituer un métabolite cyanobactérien plus fréquent de ce qu'on pensait.

Pour étudier l'effet de l'anatoxine-a dans des organismes aquatiques, on a fait 3 bioessais avec des concentrations d'anatoxine-a ou des cellules productrices d'anatoxine-a écologiquement importantes (640-80 $\mu g.l^{-1}$ et 10^4-10^6 $cell.ml^{-1}$). Les organismes testés ont été le moule (*Mytilus. galloprovincialis*) et la carpe (*Cyprinus carpio*) en différents degré de développement (oeuf, larve et juvenile). On a constaté que la toxicité de cette neurotoxine a dépendu de l'organisme en étude. Pour les moules, la concentration de 10^7 $cell.ml^{-1}$ pendant 15 jours n'a pas causé la mort d'aucun exemplaire. L'étude de la dynamique de

l'accumulation/dépuration pendant un mois (15 jours d'accumulation suivis de 15 jours de dépuration) a montré un maximum d'efficacité d'accumulation de 10,92%. Un jour après l'entrée dans la période de dépuration ce n'était plus possible de détecter anatoxine-a dans les parties comestibles du moule. Cependant, en soumettant juveniles de carpe à la même concentration de cyanobactéries toxiques, on a observé une mortalité de 100% aux 29 h; pour les oeufs de carpe, la concentration de $5,46x10^6$ cel.ml^{-1} a provoqué la mort de toute la population testée en 4 jours.

Au bioessai avec les juveniles de carpe, on a vérifié une concentration de anatoxine-a total dans les poissons, capable de mettre en risque la santé humaine car elle correspond a environ deux fois le TDI d'un enfant de 10 kg. Au bio essai avec des œufs de carpe on a comparé l'effet de l'anatoxine-a pure avec l'effet d'extraits cellulaires d'une extirpe cyanobacteriene productrice d'anatoxine-a. Même si les deux traitements ont été toxiques, les extraits cyanobacteriens ont révélé un effet beaucoup plus nette. Par ailleurs de la mortalité, on a aussi registré déformations squelettiques dans les larves après l'éclosion. La comparaison entre ces deux types de traitement a renforcé la nécessité d'effectuer bio essais avec des extraits cyanobactériens toxiques une fois que ceux-ci se conduisent de façon différente quand comparés avec toxine pure. D'autre part, les extraits cellulaires sont écologiquement plus représentatifs.

Cette dissertation a atteint les objectives initialement proposés: on a analysé échantillons naturels de masses d'eau portugaises et ses souches cyanobacterienes, revenant évident la probable apparition d'anatoxine-a au Portugal, et on a étudié l'effet toxique da l'anatoxine-a en organismes aquatiques qui était un aspect indispensable pour l'évaluation du risque de cette neurotoxine pour la santé publique.

NOTA PREAMBULAR PREAMBLE

Findos quatro anos, aprendi muito, descobri algumas coisas e despertaram-se dúvidas que espero abram caminho para outras pesquisas. Perspectivas diferentes e novas ideias dão-me agora redobrado alento para continuar.

After four years new things have revealed but also new doubts have emerged, hoping that new thesis will come and new researches will start. Different perspectives and new ideas can now give me the will to continue.

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Aos meus colegas, aos meus amigos e à minha família.

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DECLARAÇÃO

A candidata planeou a execução experimental e foi responsável pela análise e conclusão dos resultados apresentados nesta dissertação. De referir que todos os trabalhos que envolveram a quantificação da anatoxina-a na água, em cianobactérias, em carpas e em mexilhões foram parcialmente executados na Universidade de Vigo, Espanha, em estreita colaboração com o grupo da Prof. Doutora Ana Gago-Martínez do Departamento de Química Analítica e Alimentar.

ORGANIZAÇÃO DA DISSERTAÇÃO

Seguidamente expõe-se a dissertação da tese através da compilação de artigos científicos publicados ou em processo de submissão a revistas internacionais especializadas.

Inicia-se esta dissertação fazendo uma breve introdução geral, justificando a escolha do tema e explicando os objectivos propostos. A seguir apresenta-se uma revisão bibliográfica sobre a anatoxina-a (Artigo I) à qual se segue a descrição e explicação dos métodos químicos utilizados na detecção e quantificação da anatoxina-a em várias matrizes (Artigo II). No ponto seguinte relata-se o estudo sobre a presença de anatoxina-a em Portugal (Artigo III). Depois apresentam-se os três bioensaios efectuados em carpas e em mexilhões (incluindo Artigos IV, V) e finalmente é apresentada a conclusão e considerações finais desta dissertação.

STATEMENT

The candidate is responsible for the experimental design, for analysing the results and conclusions presented in this dissertation. Most of the scientific work related to anatoxin-a quantification in different matrices, was performed at University of Vigo, Spain, in straight cooperation with the group of Prof. Doutora Ana Gago-Martínez from the Department of Analytical and Food Chemistry, Faculty of Sciences.

ORGANIZATION OF THE DISSERTATION

This thesis was made by compilation of scientific papers that were published or are under the process of publication in international specialized scientific journals.

A general introduction is presented with the rational of the thesis followed by a bibliographic review about anatoxin-a (Article I). Secondly there is a description of the chemical methodology used in anatoxin-a analyses in different matrices (Article II). In the following chapter it is decribed the study about anatoxin-a occurrence in Portugal (Article III). Then three bioassays in carp and mussels are reported (including Articles IV, V) and, finally, overall conclusion and final remarks of this dissertation are presented.

LISTA DOS ARTIGOS EM PUBLICAÇÃO
LIST OF ARTICLES IN PRESS

Rellán, S., Osswald, J., Vasconcelos, V., Gago-Martínez, A. 2007. Analysis of anatoxin-a in biological samples using liquid chromatography with fluorescence detection after solid phase extraction and solid phase microextraction. *Journal of Chromatography A.*

Osswald, J., Rellán, S., Carvalho, A.P., Gago, A. and Vasconcelos, V. 2007. Acute effects of an anatoxin-a producing cyanobacterium on juvenile fish – *Cyprinus carpio* L. *Toxicon.*

LISTA DOS ARTIGOS SUBMETIDOS PARA PUBLICAÇÃO
LIST OF ARTICLES SUBMITTED FOR PUBLICATION

Osswald, J., Rellán, S., Gago, A. and Vasconcelos, V. Uptake and depuration of anatoxin-a by the mussel *M. galloprovincialis* under laboratory conditions.

Osswald, J., Rellán, S., Gago, A. and Vasconcelos, V. Toxicology and detection methods of anatoxin-a, a neurotoxic alkaloid produced by cyanobacteria.

Osswald, J., Rellán, S., Gago, A. and Vasconcelos, V. Anatoxin-a occurrence in cyanobacteria strains isolated from Portuguese fresh water systems.

ABREVIATURAS ABREVIATIONS

Organizadas por ordem alfabética Listed alphabetically

Abreviaturas e siglas de nomes de estirpes bem como de unidades não são consideradas.

Abreviations and acronyms of name of strains as well as of units were not considered.

APx. . . . . Ascorbate peroxidase.
BAF . . . . . Bioaccumulation factor.
bw or b.w. . . . . Body weight.
CAT. . . . . Catalase.
CIMAR . . . . . Interdisciplinary Centre of Marine and Environmental Research.
CNS . . . . . Central nervous system.
DNA . . . . . Deoxyribonucleic acid or deoxyribose nucleic acid.
dw or d.w. . . . . Dry weight.
ECD . . . . . Electron capture detection.
EI-QTOF . . . . . Electrospray ionization with quadrupole-time-of-flight mass spectrometer.
ESI . . . . . Electrospray ionization.
FCT. . . . . Portuguese Foundation for Science and Technology.
FLD . . . . . Fluorescence detection.
fw or f.w. . . . . Fresh weight.
GC . . . . . Gas chromatography.
GC/MS . . . . . Gas chromatography coupled with mass spectrometry.
GF/C . . . . . Glass microfibre filters from Whatman®.
GST . . . . . Glutathione S transferase.
HILIC-MS . . . . . Hydrophilic interaction liquid chromatography with electrospray mass spectrometry.
hpf . . . . . Hours post fertilization.
HPLC . . . . . High performance liquid chromatography.
HPLC-FLD or HPLC-FL . . . . . High performance liquid chromatography with fluorescence detection.
HPLC-MS . . . . . High performance liquid chromatography coupled with mass spectrometry.
i.d. . . . . Internal diameter.
i.n. . . . . Intra nasal.
i.p. . . . . Intraperitoneal injection.
LC. . . . . Liquid chromatography.
LC/DAD . . . . . Liquid chromatography with diode array detector.
LC-MS . . . . . Liquid chromatography coupled with mass spectrometry.
LC-MS/MS . . . . . Liquid chromatography coupled with double mass spectrometry.
LC-UV. . . . . Liquid chromatography with ultaviolet detection.

LC-WCX . . . . . Liquid chromatography-weak cation exchange.
LD_x . . . . . Dose that is lethal to x percentage of the assayed population.
LLE . . . . . Liquid-liquid extraction.
LOD . . . . . Lower limit of detection.
LPS . . . . . Lipopolyssacarides.
mAChR . . . . . Muscarinic acetylcholine receptors.
MAV . . . . . Maximum acceptable value.
MLD 100 . . . . . Necessary dose to kill 100% of the population.
MLT . . . . . Median lethal time, which is the time necessary to kill half of the assayed population.
MS . . . . . Mass spectrometry.
MS/MS . . . . . Double mass spectrometry.
MSPD . . . . . Matrix solid phase dispersion.
MW . . . . . Molecular weight.
nAChR . . . . . Nicotinic acetylcholine receptors.
NBD-F . . . . . 4-Fluoro-7-nitrobenzofurazan. For fluorescence.
NMR . . . . . Nuclear magnetic resonance.
NOAEL . . . . . No observed adverse effect level.
ODS . . . . . Octadecyl siloxane.
OPA . . . . . o-phthaldialdehyde.
PAR . . . . . Photosynthetically active radiation (400-700 nm).
pf . . . . . post fertilization.
Phe . . . . . Phenylalanine.
PKa . . . . . Acid-base Ionization/Dissolution constant
PNS . . . . . Periferic nervous system.
POD . . . . . Peroxidase activity.
PSP . . . . . Paralytic Shellfish Poisoning.
PST . . . . . Paralytic Shellfish Toxin.
QIT . . . . . Quadrupole ion trap mass spectrometry.
QTOF . . . . . Quadrupole time of flight mass spectrometry.
ROS . . . . . Reactive oxygen species.
RSD . . . . . Relative standard deviation [(Standard deviation/Average)*100].
SL . . . . . Standard length refers to the length of a fish measured from the tip of the snout to the posterior end of the last vertebra.
SOD . . . . . Superoxide dismutase.
SPE . . . . . Solid phase extraction.
SPME . . . . . Solid phase microextraction.
TDI . . . . . Total daily intake.
TFA . . . . . Trifluoracetic acid.
TLC . . . . . Thin-layer chromatography.
u.m.a . . . . . units of atomic mass.
U.S.A . . . . . United States of America.
UV or U.V . . . . . Ultra violet radiation (1-380 nm).
UV-DAD . . . . . Ultraviolet detection coupled with diode array detector.
VFDF . . . . . Very fast death factor.
WCX . . . . . Weak Cation Exchange.
WHO . . . . . World health organization.
Z8 . . . . . Denomination of the growing media for cyanobacteria described by Kotai (1972).

1. INTRODUÇÃO

É bem conhecido mundialmente que algumas cianobactérias (algas azuis–verdes) são produtoras de toxinas – cianotoxinas – potencialmente tóxicas para o meio aquático e indirectamente para todos os seres vivos, incluindo o homem. A toxicidade das cianobactérias tem sido alvo de inúmeros estudos, demonstrando-se o aumento da ocorrência de florescências tóxicas que podem pôr em risco a saúde pública e a dos ecossistemas aquáticos (Bartram *et al.*, 1999; Falconer, 2005).

Estão decritas e classificadas várias cianotoxinas (Tabela I), no entanto, com o desenvolvimento de técnicas de detecção e análise, surgem frequentemente novas variantes.

Tabela I – Principais tipos de cianotoxinas (*adaptado de* Sivonen e Jones, 1999).

Estrutura química	*Toxina*	*Estrutura alvo nos mamíferos*
Peptídeos cíclicos	Microcistina (mais de 60 variantes)	Fígado
	Nodularina (2 variantes)	Fígado
Alcalóides	Anatoxina-a(s)	Sinapses nervosas
	Anatoxin-a	Sinapses nervosas
	Homoanatoxina-a	Sinapses nervosas
	Aplisiotoxina	Pele
	Cilindrospermopsina	Fígado e outros tecidos
	Lyngbiatoxina-a	Pele e intestino
	Saxitoxinas (PSP) (16 variantes)	Axónios nervosos
Lipopolissacarídeos (LPS)	LPS	Qualquer tecido exposto. Efeito irritante.

Quanto ao modo de acção, as cianotoxinas são denominadas neurotoxinas, hepatotoxinas, citotoxinas, e irritantes/gastrointestinais (Codd *et al.*, 2005).

As microcistinas e a sua hepatotoxicidade, foram alvo da maior parte dos estudos so-

bre toxicidade de cianobactérias (Codd e Roberts, 1991; Chorus, 2001). Isto sucede não só a nível mundial como também nacional (Vasconcelos, 1993; 1994; 1995; 1999; 2001; Vasconcelos *et al.*, 1995; 1996). Várias publicações científicas que descrevem outras cianotoxinas, nomeadamente as do tipo neurotóxico, demonstram que elas ocorrem em sistemas dulciaquícolas mas não desenvolvem o seu estudo de modo a ser possível fazer uma avaliação de risco para a saúde pública, tal como acontece com as hepatotoxinas.

A anatoxina-a, o objecto de estudo desta tese, é uma neurotoxina potente, produzida por vários géneros cianobacterianos que podem ocorrer em diferentes zonas do mundo (Bumke, *et al.*, 1999; Park *et al.*, 1993; Viaggiu, *et al.*, 2004; Namikoshi, *et al.*, 2003; Ballot, *et al.*, 2005; Gugger, *et al.*, 2005; Araóz, *et al.*, 2005).

JUSTIFICAÇÃO

Os aspectos referidos nos pontos indicados abaixo constituiram a razão principal para a proposta da tese que seguidamente se discute.

1. Num estudo efectuado na Alemanha (Chorus *et al.*, 2001) verificou-se a presença de anatoxina-a em 22% de 391 amostras efectuadas em 78 locais diferentes. Esta percentagem foi superior à encontrada para a neurotoxina PSP (13% em 114 amostras de 29 locais diferentes). Levantou-se assim a dúvida relativamente à ocorrência de anatoxina-a em Portugal.
2. A eutrofização das massas de água doce em Portugal – 37,4% dos sistema lênticos apresentam eutrofização elevada com o subsequente aumento de florescências cianobacterianas (Barros, 1996) – bem como a ocorrência de vários géneros cianobacterianos potencialmente produtores de anatoxina-a: *Oscillatoria (Planktothrix), Anabaena, Cylindrospermopsis* e *Aphanizomenon* (Vasconcelos, 1994; Pereira *et al.*, 2000; Ferreira *et al.*, 2001 e dados registados no nosso laboratório mas não publicados) constituem motivos adicionais para justificação de estudos do tipo aqui apresentado.
3. O facto da captação de água para consumo humano em Portugal ser cada vez mais superficial, aumenta o risco de intoxicações por cianobactérias.
4. A falta de informação sobre os efeitos e acumulação da anatoxina-a em organismos aquáticos não permite uma avaliação do risco ecológico desta neurotoxina.

OBJECTIVOS

Após a elaboração e conclusão desta dissertação cumpriram-se os objectivos propostos:

1. De um modo geral, pretendia-se alargar o conhecimento sobre cianobactérias produtoras de anatoxina-a em Portugal.
2. Para a execução dos vários trabalhos experimentais com a anatoxina-a, foi necessário aferir uma técnica de detecção e quantificação desta toxina. Em colaboração com a Prof. Doutora Ana Gago-Martínez e a Dra. Sandra Rellán da Universidade de Vigo, foi possível implemetar e aferir a técnica de detecção e quantificação por HPLC-FLD da anatoxina-a na água e em tecidos biológicos.
3. Através do isolamento de estirpes cianobacterianas de águas nacionais e de bioensaios com organismos aquáticos.
4. Obtiveram-se dados importantes que permitirão efectuar a avaliação do risco da anatoxina-a para a saúde pública e para o ambiente.

INTRODUCTION

It is well known worldwide that some cyanobacteria (blue-green algae) may produce toxins – cyanotoxins – which may be toxic to aquatic environment thus to all organisms, including men. There are many studies demonstrating that toxic cyanobacterial blooms have been increasing and may pose public health and ecosystems at risk (Bartram *et al.*, 1999; Falconer, 2005).

Several cyanotoxins have been described and classified (Table I) but with the development of new detection techniques, new toxin variants are found.

Table I - Main types of cyanotoxins (*adapted from* Sivonen and Jones, 1999).

Chemical structure	*Toxin*	*Target structure in mammals*
Ciclic peptides	Microcystin (more than 60 variants)	Liver
	Nodularin (2 variants)	Liver
Alcaloids	Anatoxin-a(s)	Nervous synapses
	Anatoxin-a	Nervous synapses
	Homoanatoxin-a	Nervous synapses
	Aplysiotoxin	Skin
	Cylindrospermopsin	Liver and other tissues
	Lyngbiatoxin-a	Skin and liver
	Saxitoxins (PSP) (16 variants)	Nevous axons
Lipopolyssacarides (LPS)	LPS	Any exposed tissue. Irritant effect

Atending to mode of action, cyanotoxins are denominated neurotoxins, hepatotoxins, cytotoxins and irritants/gastrointestinal toxins (Codd *et al.*, 2005).

Microcystins are the most and the better well studied cyanotoxins (Codd & Roberts, 1991; Chorus, 2001). This happens at international and national scenarios (Vasconcelos, 1993; 1994; 1995; 1999; 2001; Vasconcelos *et al.*, 1995; 1996). Several scientific publications refer the occurrence of other cyanotoxins as the neurotoxins in freshwater systems. Although on the contrary to what happens with microcystins, those works do not enclose

enough data to make health risks evaluation.

Anatoxin-a, the object of this study, is a potent neurotoxin that is produced by various cyabnobacterial genera that occur in different parts of the world (Bumke, et al., 1999; Park *et al.,* 1993; Viaggiu, *et al.,* 2004; Namikoshi, *et al.,* 2003; Ballot, *et al.,* 2005; Gugger, *et al.,* 2005; Araóz, *et al.,* 2005).

RATIONALE

The rationale of this thesis can be divided in the next three items.

1. The occurrence of anatoxin-a in Germany was studied by Chorus *et al.* (2001) who found 22% of positive samples, out of 391 from 78 locations. This percentage was higher than the one found for PSP (13% were positive in 114 sample from 29 locations). These data incited our curiosity about the Portuguese situation.
2. Eutrophication in Portuguese freshwater bodies is high - 37.4% of the lentic systems are eutrophic and cyanobacterial blooms have increased (Barros, 1996). The occurrence of several cyanobacterial genera that are potential producers of anatoxin-a: *Oscillatoria (Planktothrix), Anabaena, Cylindrospermopsis* and *Aphanizomenon* have been refered (Ferreira *et al.,* 2001; Pereira *et al.,* 2000; Vasconcelos, 1994 and unpublished data from our laboratory) was also one important causative factor.
3. The fact that more frequently, surface water is being abstracted for human consumption increases the risk of cyanobacterial intoxication.
4. The lack of information about the effects of anatoxin-a in aquatic organisms does not allow the assessment of ecological risk evaluation.

OBJECTIVES

The proposed objectives for this thesis have been accomplished:

1. To broader the knowledge about anatoxin-a producing strains of cyanobacteria in Portugal.
2. Implementation of the detection technique for anatoxin-a in colaboration with Prof. Doutora Ana Gago-Martínez and Dª. Sandra Réllan from University of Vigo, Dpt. of Analytical and Food Chemistry, Faculty of Sciencies.
3. Several pure cultures of Portuguese strains that produce anatoxin-a were attained.
4. The obtained knowledge about anatoxin-a effects on aquatic organisms will contribute to public health risk evaluation of this neurotoxin.

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submitted

TOXICOLOGY AND DETECTION METHODS OF ANATOXIN-A, A NEUROTOXIC ALKALOID PRODUCED BY CYANOBACTERIA

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SUMMARY

Freshwater resources are under stress due to naturally occurring conditions and human impacts. One of the consequences is the proliferation of cyanobacteria, microphytoplankton organisms that are capable to produce toxins called cyanotoxins. Anatoxin-a is one of the main cyanotoxins. It is a very potent neurotoxin that was already responsible for some animal fatalities. In this review we endeavour to divulgate much of the internationally published information about toxicology, occurrence and detection methods of anatoxin-a. Cyanobacteria generalities, anatoxin-a occurrence and production as well as anatoxin-a toxicology and its methods of detection are the aspects focused in this review. Remediation of anatoxin-a occurrence will be addressed with a public health perspective. Final remarks call the attention for some important gaps in the knowledge about this neurotoxin and its implication to public health. Alterations of aquatic ecosystems caused by anatoxin-a are also addressed. Although anatoxin-a is not the most frequent cyanotoxin worldwide, it has to be regarded as a health risk that can be fatal to terrestrial and aquatic organisms because of its high toxicity.

1. INTRODUCTION

Freshwater resources are under stress due to naturally occurring conditions and human impacts. In order to invert or stop the tendency of degradation of this vital resource we ought to understand how it functions and what are the factors involved in its dynamics, biology and ecology.

Cyanobacteria are versatile microorganisms that live in terrestrial and aquatic environments. In freshwater they are part of the phytoplankton and include many different genera. In unpolluted water-systems, cyanobacteria are a component of the phytoplankton seasonal cycle, but in eutrophic situations, they may become dominant for short or long periods causing health and environmental problems. They can cause alterations of the water odour and colour (green and bluish colour, scum formation) as well as changes in the water organoleptic properties and may produce toxic secondary metabolites - cyanotoxins. The production of cyanotoxins has been the object of scientific attention and concern because of associated public health and environmental hazards, including economical losses and environmental impairment.

The purpose of this review is to summarize the scientific knowledge of one of the main neuro-cyanotoxins: anatoxin-a. We will focus on anatoxin-a occurrence and toxicity associated with its chemical characteristics. Detection methods, environmental and public health concerns, and water treatment aspects will also be addressed.

2. CYANOBACTERIA

Cyanobacteria also called blue-green algae are very ancient Gram-negative prokaryotes with fossil records of thousand million years (3.5 Gyr) of Earth's history (Wilmotte, 1994; Falconer, 2005). Their oxygenic photosynthetic capabilities seem to be responsible for providing primitive oxygen to Earth's atmosphere, being very important in studies about the origin of life (Cavalier-Smith, 2006). Some Cyanobacteria (diazotrophic) can use atmospheric nitrogen (N2) transforming it to the bioavailable form nitrite (NO_2^-). This capability associated with low demands for light and nutrients as well as the presence of gas vesicles that permitted them to migrate along the water column, give them ecological advantages in freshwater ecosystems. Whenever cyanobacteria reach high cell densities and dominate over other phytoplankton groups, a cyanobacterial bloom occurs. Usually these blooms become apparent on the water surface and margins with blue-green colour caused by the photosynthetic pigments (phycobiliproteins-blue and chlorophyll a/b-green). These pigments absorb light of a wider spectrum than terrestrial plants, making Cyanobacteria important primary producers, which play crucial role in ecosystems (Dittman and Wiegand, 2006).

Morphologically, Cyanobacteria are prokaryotes attaining 2 μm to 40 μm in diameter being colonial or unicellular with filamentous or coccoid shapes (Kaebernick and Neilan, 2001). Due to its dual characteristics of plant and bacteria, taxonomical classification has been polemical among botanists and bacteriologists (Wilmotte, 1994). The classical taxonomy was based on morphological features of cyanobacteria but these organisms may change their morphology depending on environmental conditions causing erroneous classification: Drouet (1968) called the attention to genetic variability in the same phenotypes. As it happens for other bacteria, nowadays a polyphasic approach to taxonomical classification is the most desirable (Wilmotte and Herdman, 2001). According to last edition of The Bergey's Manual® of Systematic Bacteriology (2001), Cyanobacteria is a Phylum ascribed to the Bacteria Domain. Their nomenclature of taxa is governed by the Botanical Code of Nomenclature, rather than the Bacteriological Code, and it includes five major groups (Sections) proposed by Rippka *et al.* (1979):

I- Unicellular cyanobacteria that reproduce by binary fission or budding,

II- Unicellular cyanobacteria that reproduce by multiple fission,

III- Filamentous non-heterocystous cyanobacteria that divide in only one plane,

IV- Filamentous heterocystous cyanobacteria that divide in only one plane,

V- Filamentous heterocystous cyanobacteria that divide in more than one plane (Fig. 1, page 30) (Boone *et al.*, 2001).

Although some cyanobacterial blooms may happen naturally (*e.g.* crater lakes in Kenya (Ballot *et al.*, 2005), hot springs (Falconer, 2005)) their frequency is higher in eutrophic water bodies. These blooms are influenced by pollutants such as fertilizers and nutrients (P:N), by light, salinity and water flow (Ballot *et al.*, 2004; Bartram *et al.*, 1999, Falconer, 2005). Their specific composition varies very much, being multi or monospecific, with one

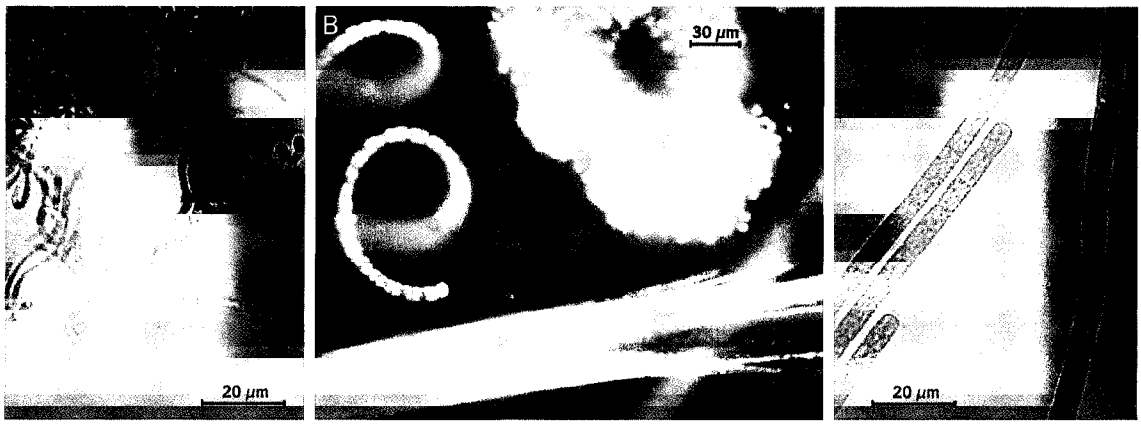

Fig. 1 Microscopic photographs showing examples of cyanobacteria (photographs were kindly provided by Micaela Vale and Viviana Lopes from CIMAR, Portugal).

or more genus present.

Cyanobacteria present some special adaptations to the environment: some are able to use atmospheric nitrogen, to regulate buoyancy along the water column, to harvest light in different wavelengths and to reproduce either through differentiated cells (called hormogonia) or through resting cells (called akinets). These adaptations permit cyanobacteria to outcompete other microalgae, thus becoming dominant, even when there is lack of nitrates or ammonia or at low light intensities (Kaebernick and Neilan, 2001).

Cyanobacterial blooms have been registered worldwide: In temperate regions, usually the cyanobacterial diversity decreases along the warm periods and at the end of summer we assist to mass development of only one cyanobacterial genus (frequently *Microcystis*, *Planktothrix* and *Anabaena*) (Dittmann and Wiegand, 2006). In Africa, *Arthrospira* can form intense blooms due to the high tolerance of this genus to different salinities (Ballot *et al.*, 2005). In the Baltic Sea and Australia, the genus *Nodularia* persists in high densities during long periods (Sivonen and Jones, 1999).

The main issue about cyanobacteria in freshwater is their ability to produce potent cyanotoxins, presenting waterborne hazards to human and ecosystems health. This has led scientific research to reach a better understanding of Cyanobacteria and their toxins, although it is still not known why and in what circumstances Cyanobacteria produce toxins. In fact, in the same species there are toxic and non-toxic strains living in the same habitat. This makes impossible to predict toxic blooms and raises the question: what triggers toxin production by Cyanobacteria? Cyanobacterial toxins represent a major source of poisonings due to the drinking and recreational use, in haemodialysis and to consumption by animals of contaminated food. There are different types of cyanotoxins, which differ in their chemical nature as well as in their toxic action. Regarding their mode of action, cyanotoxins are classified into four major groups: neurotoxins, hepatotoxins, cytotoxins/irritants and gastrointestinal toxins (Codd *et al.*, 2005). Anatoxin-a is a potent neurotoxin produced by several cyanobacterial genera, namely *Anabaena, Aphanizomenon, Microcystis,*

Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium and *Oscillatoria* (Park *et al.*, 1993; Bumke-Vogt *et al.*, 1999; Namikoshi *et al.*, 2003; Viaggiu *et al.*, 2004; Ballot *et al.*, 2005; Araóz *et al.*, 2005; Gugger *et al.*, 2005).

After all the scientific efforts to understand and prevent nuisance blooms of cyanobacteria worldwide, much has been already unveiled: distribution, occurrences, ecostrategies, physiology and molecular biology, but scientists and responsible authorities are still far from predicting and controlling toxic cyanobacteria events (Chorus and Bartram, 1999; Chorus, 2001; Falconer, 2005; Meriluoto and Codd, 2005). Although anatoxin-a is not the most frequent cyanotoxin worldwide, it has to be regarded as a health risk that can be fatal to terrestrial and aquatic organisms due to its high toxicity.

3. OCCURRENCE AND PRODUCTION OF ANATOXIN-A

The occurrence, chemical properties and toxic effects of cyanotoxins, have been largely studied but their physiological functions, regulation and the reasons for their production are still unknown. Kaebernick and Neilan (2001) and Wiegand and Pflugmacher (2005) have outlined some putative functions of cyanotoxins: (1) avoidance of grazing on cyanobacteria by other organisms such as zooplankton and higher animals, (2) inducing alteration of population structures to gain ecological advantage, (3) mediating cell signalling allelopathy and chemotaxy to establish trophic relationships with other cyanobacteria or other organisms.

The first report of occurrence of anatoxin-a dates back to 1951 in USA (Olson, 1951). At that time there were no chemical and toxicological skills to identify this toxin. But later a similar toxin was identified in Canada, with identical toxicological symptoms and it was suspected that they were identical (Gorham *et al.*, 1964). A neurotoxic cyanobacterial strain from a bloom in Burton Lake in Canada was identified and its toxin named Very Fast Death Factor (VFDF) because of its high lethality: mice died on 1-4min after intraperitonial injection *(i.p.)*. After the isolation of a toxic strain from Canada (NRC-44) in 1966, Stavric and Gorham were able to characterize chemically and toxicology the VFDF. Later, Devlin and collaborators (1977) defined the structure of this VFDF by x-ray crystallographic analysis and denominated it anatoxin-a: 2-acetyl-9-azabycyclo[4.2.1.]non-2-ene (MW=165) (Fig. 2).

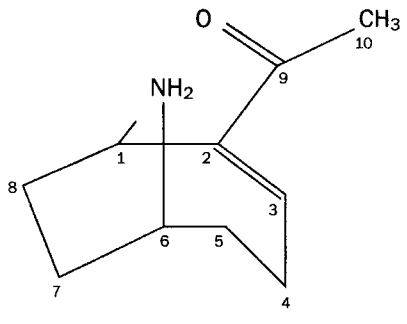

Fig. 2 Structure of anatoxin-a (adapted from James *et al.*, 1998).

The unique pharmacological potential of this toxin as a post synaptic depolarizing agent incited the same Canadian research group to develop the synthesis of anatoxin-a from cocaine, which has the same absolute stereochemistry as anatoxin-a (Campbell *et al.*, 1977). Research proceeded and this group attained the total synthesis of racemic anatoxin-a (Campbell *et al.*, 1979). In the same year, Bates and Rapoport (1979) also published the synthesis of anatoxin-a but through another chemical process. Since then, other authors have achieved the synthesis of this alkaloid, all having in mind its use in neurological investigations and as a pharmacological tool (Petersen *et al.*, 1984; Tufariello *et al.*, 1985; Danheiser *et al.*, 1985; Parsons *et al.*, 1996; Aggarwal *et al.*, 1999; Trost and Oslob, 1999; Brenneman and Martin, 2004; Hjelmgaard *et al.*, 2005). In 1992, after scientific advances in the knowledge of the neurological effects and mode of action of anatoxin-a, Wonnacott and colleagues synthesized a homologue of anatoxin-a that had the advantage of being radiolabeled, stable and with the same potency as exhibited by anatoxin-a itself (Wonnacott *et al.*, 1992). This homologue was designated homoanatoxin-a (2-(propan-1-oxo-1-yl)-9-azabicyclo[4.2.1]non-2-ene. MW=179) and differs from *anatoxin*-a by having an additional methylene unit on the side chain (Fig. 3).

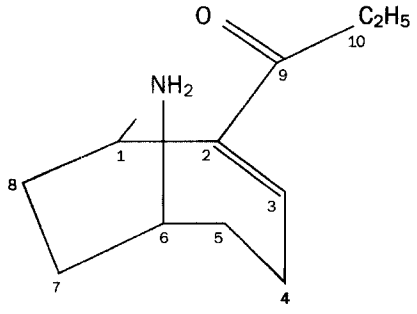

Fig. 3 Structure of homoanatoxin-a (adapted from James *et al.*, 1998).

Anatoxin-a chemistry is now well known due to the chemical and pharmacology research on this neurotoxin and it was possible to develop accurate detection and quantification techniques (Huby *et al.*, 1991; Mansell, 1996). Much of the knowledge on the occurrence of anatoxin-a was obtained following monitoring programs of cyanobacteria or after animal intoxication episodes. The fact that one strain may produce anatoxin-a simultaneously with other cyanotoxins (*e.g.* hepatotoxins) (Sivonen *et al.*, 1989; Park *et al*, 1993) could have masked the presence of one or the other toxin, depending on the predominant effects. This associated with the need of specialized techniques of detection (see chapter 4of this article), might give a biased view of the occurrence of anatoxin-a according to the countries where monitoring programs and techniques of detection are available. The occurrences of anatoxin-a published so far are listed in Table 1, page 33.

Table 1 Published occurrence of anatoxin-a and homoanatoxin-a in water bodies.

n.r. - not reported. Values expressed in µg/g refer to blooms material, and expressed in µg/l refer to dissolved toxin in water. L. - lake, Rs. - reservoir, E. - estuary, R. - river.

Country	Location	Year	Intoxication episodes	Method of detection	Anatoxin-a concentration	Reference
Canada	L. Burton	1961	2 cows died	*i.p.* mice	n.r.	Gorham *et al.*, 1964
USA	Hebgen Rs.	1977	9 dogs and 30 heads of cattle died	*i.p.* mice	n.r.	Juday *et al.*, 1981
	Idaho	1981	11 cows died, 2 dogs died	GC/ECD	2.5 µg/mg dw	Stevens and Krieger, 1988
Finland	7 lakes	1985/87	5 cows	*i.p.* mice HPLC/GC/MS	12 – 4360 µg/g dw	Sivonen *et al.*, 1989, 1990
Scotland	Loch Insh	1990/91	4 dogs died and one intoxicated	*i.p.* mice HPLC/DAD and GC/MS	n.r.	Edwards *et al.*, 1992
Japan	L. Kasumi	1991	n.r.	TSP/LC/MS	0.4 µg/g dw	Harada *et al.*, 1993
Finland	n.r.	n.r.	n.r.	TSP/LC/MS	2642 µg/g dw	
Japan	L. Barato	1988/92	n.r	TSP/LC/MS	0.3 – 16 µg/g dw	Park *et al.*, 1993
Italy	L. Mulargia	1987/90	n.r.	HPLC, GC/MS	100 µg/g dw	Bruno *et al.*, 1994
Ireland	L. Caragh	1992/94	6 dog died	HPLC/FL, GC/MS	112 – 444 µg/l, 10 –16 µg/g dw	James *et al.*, 1997
	L. Inniscarra	1995	n.r.	HPLC/FL, GC/MS	2 – 390 µg/l	
	L.Corbally	1995	n.r.	HPLC/FL, GC/MS	60 - 100 µg/g dw	
S. Korea	L.Choongiu	1992	n.r.	HPLC/DAD	1190 µg/g dw	Park *et al.*, 1998
	L. Jangsong	1992	n.r.	HPLC/DAD	1444 µg/g dw	
	Youngsan E.	1992	n.r.	HPLC/DAD	570 µg/g dw	
	Younglang R.	1993	n.r.	HPLC/DAD	417 µg/g dw	
Germany	20 water bodies	1995/97	n.r.	GC/ECD, GC/MS	0.01 – 6.4 µg/l intracellular, 0.39 – 6.7 µg/l extracellular	Bumke-Vogt *et al.*, 1999
Canada	L. Steele	1985	1000 bats, 24 mallards and American wigeons	GC/MS	n.r.	Sivonen, 2000
	n.r.	n.r.	16 cows affected	GC/MS	n.r.	
N. Zealand	Waikanae R	1998	5 dogs died	*i.p.* mouse, HPLC	n.r.	Hamill, 2001
	Mataura R	1999/00	7 dogs died	*i.p.* mouse	n.r.	
Kenya	L. Bogoria	2001	mass mortalities of Lesser Flamingos	HPLC/PDA, MALDI/TOF	10 – 18 µg/g dw	Krienitz *et al.*, 2003
	L. Baringo	2001/02	n.r.	HPLC/PDA	273 – 1256 µg/g dw, 0.05 – 0.21 µg/l	Ballot *et al.*, 2003
Ireland	L. Lough Sillan	2003	n.r.	LC/MS/MS, HPLC/FL	24 µg/l of homoanatoxin-a	Furey *et al.*, 2003[a], Furey *et al.*, 2003[b]
	Inniscarra Rs.	2003	n.r.	LC/MS/MS	34 µg/l of homoanatoxin-a	Furey *et al.*, 2003[b]
	L.Lough Key	2003	n.r.	LC/MS/MS	12 µg/l of homoanatoxin-a	
	L.Caragh	2003	n.r.	LC/UV, LC/FLD, LC/MS/MS	1.4 µg/l of homoanatoxin-a	
Poland	Coast of Gdynia	2001/02	n.r.	n.r.	n.r.	Mazur and Plinski, 2003
Italy	L. Spino	2000/01	n.r.	*i.p.* mice, Microtox, HPLC-DAD, LC/MS	12.13 ng/mg fw of fresh cells	Viaggiu *et al.*, 2004
Kenya	L. Bogoria	2001/02	mass mortalities of Lesser Flamingos	HPLC-PDA, MALDI-TOF	0.3 – 9 µg/g dw	Ballot *et al.*, 2004
	L. Nakuru	2001/02	mass mortalities of Lesser Flamingos	HPLC-PDA, MALDI-TOF	5 – 223 µg/g dw	
	L. Simbi	n.r.	n.r.	HPLC-PDA, MALDI-TOF	1.4 µg/g dw	Ballot *et al.*, 2005
	L. Sonachi	n.r.	n.r.	HPLC-PDA, MALDI-TOF	0.5 – 2 µg/g dw	
Spain	n.r.	n.r.	n.r.	n.r.	n.r.	Quesada *et al.*, 2005
France	La Loue	2003	2 dogs died	HPLC/UV/MS, ESI/MS/MS	8000 µg/g dw	Gugger *et al.*, 2005

Table 2 Isolated cyanobacteria strains producing anatoxin-*a* according to country of origin. Taxonomy and code of the strains was transcribed as in the respective bibliographical reference.
(n.d. – not determined, n.i.- not indicated). *homoanatoxin-a producer. ** Axenized 193 strain (Sivonen *et al.*, 1989). *** Axenized NIVA-CYA 92 strain (Skulberg *et al.*, 1992).

Taxa (strain code)	Planktonic (P) or benthic (B) sample	Country	anatoxin-a concentration (μg/g dw)	Method	Reference
Anabaena flos-aquae (NRC-44h and NRC-44h-1)	not explicit	Canada	1119 and 13013	TSP/LC/MS	Stavric and Gorham, 1966; Devlin *et al.*, 1977; Carmichael *et al.*, 1975; Harada *et al.*, 1993
Aphanizomenon sp. (3)	P	Finland	6700	GC/MS	Sivonen *et al.*, 1989
Anabaena flos-aquae (14)			10000		
Anabaena flos-aquae (37)			13000		
Anabaena circinalis (54)			8200		
Anabaena circinalis (86)			4400		
Anabaena circinalis (123)			3500		
Cylindrospermum sp.(191)			92		
Oscillatoria sp. (193)			13000		
Oscillatoria agardhii. (226)			7		
Oscillatoria formosa *(NIVA-CYA 92)	n.i.	Sweden	600		Skulberg *et al.*, 1992, Aas *et al.*, 1996
Oscillatoria sp.	B	Scotland	n.d.		Edwards *et al.*, 1992
Anabaena flos-aquae (Finland - 1)	n.i.	Finland	1017	TSP-LC/MS	Harada *et al.*, 1993
Anabaena circinalis (Finland - 2)	n.i.		1396		
Aphanizomenon sp. (Finland - 3)			1562		
Oscillatoria sp. (Finland-4)			2713		
Anabaena sp. (TAC210)		Japan	0.6 - 2.1		
Microcystis aeruginosa (TAC121)			0.4 - 52.4		
Microcystis aeruginosa (TAC80)			0.2		
Anabaena mendotae (130)		Finland	<17000	HPLC/PDA/UV	Rapala *et al.*, 1993
Anabaena flos-aquae (C2)		U.S.A	n.d.	TLC and *i.p.* mouse	Kangatharalingam and Priscu, 1993
Anabaena flos-aquae (C18)					
Anabaena flos-aquae (IC-1)		n.i.	13000	TLC	Gallon *et al.*, 1994
Anabaena planctonica		Italy	100	GC/MS	Bruno *et al.*, 1994
Raphidiopsis mediterranea (LBRI 48)*	P	Japan	5700	HPLC/UV and FAB	Watanabe *et al.*, 2003, Namikoshi *et al.*, 2003
Planktothrix rubescens		Italy	12.13	LC/MS	Viaggiu *et al.*, 2004
Arthrospira fusiformes (AB2002/10)		Kenya	10.38	HPLC/PDA and MALDI-TOF	Ballot *et al.*, 2004
Arthrospira fusiformes (AB2002/04)			0.14		
Arthrospira fusiformes (AB2002/02)			0.3		Ballot *et al.*, 2005
Nostoc carneum (Fremy and Geitler)	n.i.	Iran	156	SPME/GC/MS	Ghassempour *et al.*, 2005
Anabaena sp.(SP2)			248		
Phormidium favosum (PMC 240.04)	B	France	n.d.	ESI/MS/MS	Gugger *et al.*, 2005
Oscillatoria sp. (PCC 9240)**	P	Finland	4000	GC/MS	Araóz *et al.*, 2005
Oscillatoria formosa (PCC10111)***	n.i.	Sweden	n.d.		
Oscillatoria sp. (PCC6407)		U.S.A.			
Oscillatoria sp. (PCC6412)					
Oscillatoria sp. (PCC6506)	unknown	unknown			
Oscillatoria sp. (PCC9029)					

Several strains producing anatoxin-a have been isolated and maintained under laboratory conditions (Table 2, page 34). This permitted to study the toxicology and production of anatoxin-a.

Anatoxin-a has a vast occurrence, with a worldwide distribution (Table 1, page 33). From Canada to New Zealand, it comprises temperate, tropical and cold climatic regions. Although it is not reported in all geographical areas in the world, it is expected that anatoxin-a may be found in many other water bodies. More surveys are needed to evaluate and characterize the occurrence of anatoxin-a in fresh and brackish water bodies. That would permit an evaluation of health and environmental risks associated to this potent neurotoxin.

Ten different benthic and planktonic cyanobacteria genera – *Anabaena, Aphanizomenon, Cylindrospermum, Oscillatoria, Microcystis, Raphidiopsis, Planktothrix, Arthrospira, Nostoc* and *Phormidium* are found to be anatoxin-a producers (Table 2). Since 1992, when anatoxin-a was detected for the first time in a benthic strain after causing canine fatalities, benthic cyanobacteria may be regarded more seriously as potential toxin producers (Edwards *et al.*, 1992).

It is also important to understand why and in what situations the production of anatoxin-a is reduced or increased. Unfortunately, there are no concluding results about the biochemical or physiological role of anatoxin-a for the cyanobacteria itself. Few investigations in laboratory and in the field tried to answer those questions but results did not reach any definitive conclusion (Table 3, page 36).

Table 3 work accomplished to study the production of anatoxin-a by cyanobacteria.

Aim of the Study	Conclusions	Reference
Evaluate toxicity of one *Anabaena* strain under different conditions.	Highest toxicity was observed at 14 d of growth, 22 °C and 2000 - 7500 lx	Peary and Gorham, 1966
Evaluate the production and biodegradation of anatoxin-a in batch cultures.	Highest cellular anatoxin-a registered at 3 weeks of growth. Highest dissolved anatoxin-a registered at 4 weeks, atributed to cell lysis. *Pseudomonas sp.* was found to biodegrade dissolved anatoxin-a.	Kiviranta *et al.*, 1991
Determine cellular and dissolved anatoxin-a under different conditions in batch cultures (42 d) of *Anabaena* and *Aphanizomenon*.	Two first weeks of growth corresponded to the highest levels of cellular anatoxin-a. *Aphanizomenon* released 3.6-19% *per* volume into the growth media. *Anabaena* did not release any anatoxin-a into the growth media. Highest production was registered at 20 °C. Lowest production was registered at 30 °C. *Aphanizomenon* produced more anatoxin-a with increasing light flux. *Anabaena* produced maximum levels of anatoxin-a at 26 and 44 $\mu mol.m^{-2}.s.$ of irradiance. Different PO_4^- levels did not alter anatoxin-a production. The highest the NO_3^- was, the less toxin was produced. Toxin production was higher when source of nitrogen was N_2 or NH_4 rather than NO_3^- or urea.	Rapala *et al.*, 1993
Study of anatoxin-a biosynthesis.	The amount of total anatoxin-a produced, increased steadily during the exponential phase of growth until day 12, when it reached a plateau.	Gallon *et al.*, 1994
Study of anatoxin-a production and release during batch cultures of *Anabaena* flos-aquae.	Anatoxin-a production followed growth curves up to 6–7 weeks but reached a plateau and then even declined before culture reached the stationary phase. At the beginning of exponential growth, at least half of the total anatoxin-a was in dissolved phase. Dihidro-anatoxin-a was found only in the media and not in freeze-dried cells.	Bumke-Vogt *et al.*, 1996
Study of how the production of toxin varies with temperature under light-limited growth conditions.	Total anatoxin-a production varied differently between the two tested strains and no pattern could be established for the growth rate. Decreasing light increased extracellular concentration of anatoxin-a in both strains. In the natural samples, anatoxin-a presence coincided with high concentrations of PO_4^- (17–65 µg/l).and median water temperature of 19.8 °C.	Rapala and Sivonen, 1998
Survey to identify anatoxin-a in German water bodies.	Anatoxin-a was found in the water (extracellular fraction) and in the seston (intracellular fraction). 40% of the samples analysed for extra and intracellular had higher levels of extracellular fraction.	Bumke-Vogt *et al.*, 1999

The optimal temperature for anatoxin-a production either in laboratory or in natural samples is between 19.8 and 22 °C and the maximum concentrations of total anatoxin-a occurred at the first two-three weeks of culture, corresponding to exponential phase. A considerable amount of this toxin can be dissolved in the water during a bloom and can be degraded by micro-organisms present there. Explanation for it might rely on the size of the molecule: anatoxin-a is small and it possibly passes easily through the cell membrane, into the medium or due to cell lyses. Further investigations should be achieved in order to understand how anatoxin-a is kept intracellularly, if it can be accumulated in cyanobacteria and in what conditions, if there is a maximum limit of accumulation and if anatoxin-a is liberated through cell lyses only or through any passive or active mechanism. The evaluation of production and occurrence of anatoxin-a implies the understanding of the partioning of anatoxin-a between cellular and water fractions. Hence, its synthesis by the cells and its stability and fate in water are to be considered.

3.1. BIOSYNTHESIS OF ANATOXIN-A

Gallon and colleagues were the first to investigate the biosynthesis of anatoxin-a using the strain NRC-44-1 *(Anabaena flos-aquae)* but due to the loss of its capacity to produce anatoxin-a, their studies had to proceed with another strain: IC-*1 (Aphanizomenon flos-aquae)* (Gallon *et al.*, 1990; 1994). The loss of anatoxin-a producing capacity was associated with the replacement of one 10 kb DNA plasmid by a 6.5 kb in strain NRC-44-1. After incubating one non-toxic strain (CCAP 1403/13f) with the total DNA from NRC-44-1 (producing anatoxin-a), they found that it started to produce anatoxin-a. These findings suggested that anatoxin-a production could be coded in the 10 kb DNA plasmid.

Establishing a parallelism with the alkaloids from plants, Gallon and colleagues (1994) confirmed the involvement of putrescine via pyrroline in the biosynthesis of anatoxin-a.

With the aim to elucidate the biosynthesis of anatoxin-a and its homologous homoanatoxin-a by cyanobacteria, Hemscheidt *et al.* (1995) concluded that the carbon atoms of anatoxin-a were derived from the precursor glutamic acid. This was incompatible with the opinion that cyanobacterial anatoxin-a is produced in the same way as tropane alkaloids in higher plants because carbon atoms in the tropane alkaloids derive from acetate (Gallon *et al.*, 1990). The putative biosynthesis of anatoxin-a in cyanobacteria is shown in figure 4.

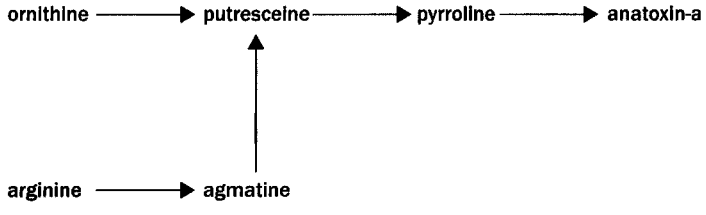

Fig. 4 Proposed biosynthetic pathways for production of anatoxin—a in cyanobacteria (Gallon *et al.*, 1990, 1994; Hemscheidt *et al.*, 1995).

The biochemical production of (+)anatoxin-a is still not well understood and there are no studies about the natural production of the negative enantiomer *(-)anatoxin*-a. These issues would be very helpful to develop molecular probes of detection of anatoxin-a and would contribute to a better understanding about the role of anatoxin-a in cyanobacteria and aquatic ecosystems.

3.2.STABILITY OF ANATOXIN-A

Stevens and Krieger (1991) were the first to study the stability of anatoxin-a in order to learn how this neurotoxin could be degraded. They considered four natural routes of degradation: dilution, adsorption, photolysis and non-photochemical degradation. These factors are related with stability of anatoxin-a in water and influence the rates of degrada-

tion. Later, Rapala and colleagues (1994) also evaluated the biodegradation of anatoxin-a by micro-organisms. This was done after one study by Kiviranta and colleagues, who isolated a *Pseudomonas sp.* capable to degrade anatoxin-a at a rate of 2-10 µg/ml per day (Kiviranta *et al.*, 1991).

Due to its chemistry, anatoxin-a is unstable under natural conditions being partially or totally degraded and converted to the non-toxic products dihydroanatoxin-a and epoxyanatoxin-a, depending on environmental conditions (Stevens and Krieger, 1991; James *et al.*, 1998) (fig. 5).

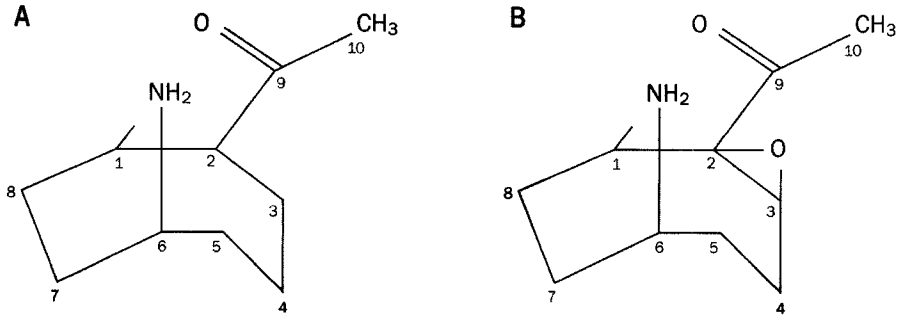

Fig. 5 Structure of the two non-toxic degradation products of anatoxin-a: A-dihydroanatoxin-a and B-epoxyanatoxin-a (adapted from James *et al.*, 1998).

Anatoxin-a degrades readily, especially in sunlight and at high pH, to non toxic degradation products (Stevens and Krieger, 1991), such as the stable alkaloid dihydroanatoxin-a identified from an ageing bloom of *Anabaena flos-aquae* (Smith and Lewis, 1987) and the anatoxin-a epoxide, another non toxic metabolite of anatoxin-a which has also been identified from various blooms (Harada *et al.*, 1993).

Photodegradation of anatoxin-a is independent of oxygen, indicating that degradation by light is not photo-oxidation, but depends on pH and sunlight intensity. At pH=2 there is no photolysis (Stevens and Krieger, 1991). Stevens and Krieger (1991) examined the effect of sunlight, pH, oxygen, copper and iron on the stability of anatoxin-a and found that light and pH are the tested factors that influence more the degradation (Table 4).

Table 4 Half-life of anatoxin-a (minutes or days) under different pH, light and atmosphere conditions (from Stevens and Krieger, 1991).

	Sunlight photolysis at lower intensity	Sunlight photolysis at higher intensity	Nitrogen atmosphere	Oxygen atmosphere	Oxygen atmosphere and 10 µM Cu^{2+} in solution
aqueous anatoxin-a	∞ at pH 2 151 min at pH 6 96 min at pH 9	330 min at pH 9 270 min at pH 12	10 d at pH 9	5 d at pH 9	3.8 d at pH 9
anatoxin-a in algae lysate	690 min at pH 9	—	—	—	—

Degradation of anatoxin-a, was also evaluated in another experiment by Rapala and colleagues (1994). Comparing the effect of sterilized sediments with non-sterilized on anatoxin-a degradation along 22 d, they observed that the degradation was remarkably different between the two situations. In vials with sterilized sediments, anatoxin-a was detectable throughout the experiment, whereas in the vials with non-sterilized sediment anatoxin-a decreased 25-48% after 22 d. This supports the idea that anatoxin-a is also degraded by micro-organisms that can be present in natural sediments, affecting microbial populations of freshwater bodies (Rapala et al., 1994).

Smith and Sutton (1993) reported that anatoxin-a is not particularly persistent in reservoir water and has a half-life of 5 d under normal pH conditions (Smith and Sutton, 1993).

Considering the data on the occurrence, partioning and production of anatoxin-a we may conclude that when production of anatoxin-a exceeds the degradation capacity of the surrounding media, proportional levels of cellular anatoxin-a raise. The knowledge that anatoxin-a is not stable in water and its concentration can be reduced to 50% after several hours to days indicates that this toxin may not have a high health and environmental risk.

Even though there are only a few studies in this area, the complexness of anatoxin-a production and occurrences is evident. It is still unknown which factors stimulate and control anatoxin-a production, even in the same genera no patterns could be established. Production of anatoxin-a appears to be strain specific and varies very much with environmental factors such as light, temperature and nitrogen. These factors in turn vary according to water bodies and environmental conditions. In terms of health and environmental issues the unpredictable production of this potent neurotoxin should lead to the use of the precautionary principles.

4. CHEMISTRY AND DETECTION METHODS OF ANATOXIN-A

Anatoxin-a is a low molecular weight bicyclic secondary amine (MW=165 u.m.a.) (Devlin *et al.*, 1977). It is a homotropane alkaloid derivative with an enlarged ring (2-acetyl-9-azabicyclo[4,2,1]non-2-ene). Anatoxin-a has a pK_a value of 9.6, therefore it exists mainly in cationic form.

Anatoxin-a was the first toxin from cyanobacteria to be structurally elucidated, therefore the development of analytical strategies for its detection was relatively easy.

The anatoxin-a homologue, homoanatoxin-a, was synthesized prior to its first discovery in nature (Wonnacott *et al*, 1992) and it was supposed to be a natural neurotoxin, which was confirmed by its subsequent isolation from *Oscillatoria formosa* (Skulberg *et al.*, 1992). Homoanatoxin-a differs from anatoxin-a because of its additional methylene unit on the side chain.

Although standard materials of the degradation products of anatoxin-a are not commercially available, some products have been synthesized and further characterised together with anatoxin-a.

4.1 BIOLOGICAL METHODS

Due to its high toxicity to mammals, mouse bioassay via *i.p.* administration, was used in the early studies to assess anatoxin-a (determining the LD_{50} or MLD_{100}) (Falconer, 1993). Symptoms of poisoning as rapid death after convulsions and respiratory distress reveal the presence of neurotoxins, however it is very difficult to determine the toxin responsible for the toxicity. Alternative biological assays using different test organisms commonly used in ecotoxicological testing, such as brine shrimp (*Artemia salina*) (Lahti *et al.*, 1995), larvae of the freshwater crustacean *Thamnocephalus platyurus* (Torokne *et al.*, 2000), or even different insect larvae tests (Kiviranta *et al.*, 1993) have already been performed and applied for the evaluation of the toxicity of samples with anatoxin-a. Sensitivity of the *Artemia salina* test is similar to the one obtained with mouse bioassay. The main disadvantages of both tests are the lack of selectivity at high doses (Henriksen *et al.*, 1997) and the need of high amounts of test material for such evaluation. Recently, the high affinity of anatoxin-a and homoanatoxin-a for the nicotinic cholinergic receptors was exploited to adapt the radioligand-binding assay for routine detection of these neurotoxins directly in low-molecular-mass cell extracts of cyanobacteria (Aráoz *et al.*, 2005).

4.2. CHROMATOGRAPHIC METHODS

4.2.1. SAMPLE PREPARATION

Apart from the bioassay, most reported methods for the detection of anatoxin-a are based on chromatography.

The complexity of the matrices in which anatoxin-a is present, causing important toxicity at very low levels, makes sample preparation a critical step that must be carefully carried out not only to obtain reliable analytical data, but also for an efficient chromatographic performance. Sample extraction and cleanup are the most critical steps in the sample pretreatment protocol.

A selective extraction is highly recommended, because it can speed up all further operations with the extract, therefore simplifying sample preparation and allowing direct chromatographic analysis in some cases (Dräger, 2002). However, in most cases, several interferences are present in the extracts the mentioned purification step being necessary. Pre-concentration must also be carried out when increased sensitivity is required.

Anatoxin-a has been successfully extracted from cells with a number of different solvents (Chorus and Bartram, 1999). The most commonly used are water (Stevens and Krieger, 1988; Edwards *et al.*, 1992; Bruno *et al.*, 1994), acidified water (Himberg *et al.*, 1989; Harada *et al.*, 1989; Poon *et al.*, 1993), acidified methanol (James *et al.*, 1998) or a mixture of methanol/water (Dahlmann *et al.*, 2003; Namikoshi *et al.*, 2003). Rapala and collaborators evaluated the efficiency of different solvents for the extraction of anatoxin-a from freeze-dried cyanobacteria. Water proved to be the most efficient solvent; however water/methanol (50/50) resulted in a decrease in the amount of interfering peaks and gives good extraction efficiency (87%). Moreover, methanol has the added advantage of allowing rapid sample concentration through evaporation (Rapala *et al.*, 1993). Extraction of in-

tracellular toxins requires rupture of the cell wall. For this purpose, the methodologies require cells to be freeze-dried or freeze-thawed in combination with mechanical methods such as shaking or ultrasonication. This last one followed by centrifugation to remove cellular debris is the most commonly applied (Zotou *et al.*, 1993; Dahlmann *et al.*, 2003; Viaggiu *et al.*, 2004). Different reports showed that for cyanobacterial cells ultrasonication using a probe was more efficient than using an ultrasonication bath (Rapala *et al.*, 2002; Spoof *et al.*, 2003). Aranda-Rodríguez *et al.* (Aranda-Rodríguez *et al.*, 2005), evaluated the suitability of pressurized liquid extraction for the extraction of toxins from cyanobacterial cells as an alternative to ultrasonication, however anatoxin-a was labile under experimental conditions and only 50% of recovery was obtained.

Anatoxin-a was also extracted from other complex matrices. For fish muscle samples, a mixture of methanol, water and acetone was used and hexane was added to remove the lipids from the extracts. Ethanol and water were also added to aqueous residue prior purification (Hormazabal *et al.*, 2000). Food supplements containing *Spirulina* were extracted by sonication with methanol in an ultrasonic bath (Draisci *et al.*, 2001). After increasing the pH value of water or algae extracts to pH 11, anatoxin-a can be extracted by Liquid-liquid extraction (LLE) with dichloromethane, which is further evaporated prior to analysis (Bruno *et al.*, 1994; Bumke-Vogt *et al.*, 1996; Fromme *et al.*, 2000).

Most of the purification and concentration methods for anatoxin-a involve Solid Phase Extraction (SPE) procedures. Previously adjusting samples to pH 10, octadecyl siloxane (ODS) silica gel cartridge can be used and an efficient elution can be carried out with methanol (Poon *et al.*, 1993; Rapala *et al.*, 1994; Namikoshi *et al.*, 2003). SPE using a weak cation-exchange material, can also be used for the clean-up of water samples or cyanobacterial extracts at pH 7 and the elution must be carried out with methanol containing 0.2% trifluoroacetic acid (James *et al.*, 1998). This procedure, based on the one initially developed by Harada *et al.* (Harada *et al.*, 1993), can be applied to the simultaneous extraction of anatoxin-a, homoanatoxin-a as well as to their degradation products.

More recently, Solid phase microextraction (SPME) was applied to the simultaneous extraction, purification and concentration of anatoxin-a. This technique allows automatization and on-line extraction and analysis. SPME has been efficiently coupled with HPLC and fluorescence detection (Namera *et al.*, 2002) however regarding to extraction efficiency and detection limits (20 ng/ml), no satisfactory results were obtained. Improvements on the application of SPME for the HPLC/FLD analysis of anatoxin-a in different matrices were reported by Rellán *et al.* (Rellán, *et al.* 2007). A linearity of 10-2000 ng and improved limit of detection (LOD) of 0.47 ng were obtained. Moreover, the experimental design used in this work avoids the rapid damage of the fibre coating. A SPME method, coupled to GC/MS has been developed by Ghassempour *et al.* (Ghassempour *et al.*, 2005) for the analysis of anatoxin-a. A linear range of 50-10000 ng/ml and a detection limit of 11.2 ng, using a polyaniline coated fibre were reported. Rodríguez *et al.* (Rodríguez *et al.*, 2006) have also developed a SPME strategy to be coupled to GC/MS for the analysis of anatoxin-a in water samples. A linear range of 2.5-200 ng/ml with a LOD of 2ng/ml was ob-

tained.

Matrix solid phase dispersion (MSPD), has been recently applied by Bogially *et al.* (Bogially *et al.*, 2006) for the anatoxin-a extraction from fish muscle tissue. Although the use of MSPD is not widely extended in the sample pre-treatment protocols of phycotoxins, this novel procedure offers advantages such as simplicity, reduction of the use of toxic and expensive solvents and the elimination of the possibility of emulsion formation. Extraction efficiency is also enhanced as the entire sample is exposed to the extractant.

4.2.2. CHROMATOGRAPHIC ANALYSIS

The high toxicity of anatoxin-a at very low levels and the regulations worldwide imposed for these particular compounds led to intensive work mainly focused in the development of improved analytical methodologies for increasing the sensitivity of the analysis, High Performance Liquid Chromatography (HPLC) coupled with UV or FL detection, Gas Chromatography (GC) coupled with Mass Spectrometry (MS) or HPLC-MS being the most commonly used techniques.

The detection of anatoxin-a in biological and environmental matrices has shown certain problems related to the rapid degradation of the toxin (half-life less than 1 day). Another drawback in the investigation of suspected anatoxin-a contamination is the unavailability of standards or reference materials commercially available for anatoxin-a, its analogue homoanatoxin-a and their degradation products.

Earlier studies on the determination of anatoxin-a as freshwaters contaminant, have been carried out using LC-UV (Wong and Hindin, 1982; Harada *et al.*, 1989), but this method suffers from limitations such as sensitivity and a large number of interferences in complex matrices. Moreover, LC-UV does not allow the detection of the degradation products of anatoxin-a. Derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and fluorimetric detection greatly enhances sensitivity. This HPLC-FL method has been developed for the simultaneous determination of anatoxin-a, its analogue homoanatoxin-a and their degradation products (James *et al.*, 1997; James *et al.*, 1998). LOD of 10 ng/l can be obtained, for all the analyzed anatoxin in water samples. An improvement to this method, was reported by Rawn and collaborators for the analysis of anatoxin-a in blue-green algae (Rawn *et al.*, 2005). Addition of o-phthaldialdehyde (OPA) and mercaptoethanol to the extract before addition of the NBD-F resulted in the successful removal of primary amines from the background noise, leading to lower detection limits.

GC has been also applied for the determination of anatoxin-a in freshwaters, using both, MS or Electron capture detection (ECD). Derivatizaton is a prerequisite to enhanced sensitivity for the Gas chromatographic analysis of anatoxin-a. Different approaches, mainly based on acetylation and alkylation of the anatoxin-a have been developed (Ross, *et al.*, 1989). GC coupled with ECD detection allows a sensitive determination of anatoxin-a at ng level and was successfully applied after derivatization with pentafluorobencyl bromide (Bumke-Vogt *et al.*, 1996) or trichloroacetic anhydride (Stevens and Krieger, 1988).

The coupling of HPLC or GC with mass spectrometry has greatly improved the identification of anatoxin-a, homoanatoxin-a and made possible the identification of the degradation products of these compounds (dihydro and epoxy anatoxins and homoanatoxins). Edwards *et al.*, (Edwards *et al.*, 1992) applied CG-MS as a confirmatory technique for the analysis of anatoxin-a, previous acetylation of the amine group with pyridine and anhydride acetic. As an acetyl derivative, anatoxin-a has been successfully analysed by a number of GC/MS methods (Himberg *et al.*, 1989; Zotou *et al.*, 1993; Bruno *et al.*, 1994). The sensitivity found for most GC/MS methods, is similar to that obtained for ECD detection.

As with most applications, the development of sophisticated LC-MS and LC-MS/MS methods has led to important advances regarding sensitivity and rapidity of the anatoxin-a analysis, eliminating the need for derivatization. A number of LC-MS methods have been used for the determination of anatoxin-a in water (Takino *et al.*, 1999; Hormazábal *et al.*, 2000; Pietsch *et al.*, 2001; Maizels and Budde, 2004), cyanobacteria (Harada *et al.*, 1993; Poon *et al.*, 1993; Furey *et al.*, 2003[a]), fish muscle (Hormazábal *et al.*, 2000; Bogialli *et al.*, 2006) and food supplements (Draisci *et al.*, 2001). Dell'Aversano and collaborators, examined the suitability of hydrophilic interaction LC with electrospray mass spectrometry (HILIC-MS) for the analysis of assorted toxins produced by cyanobacteria, including anatoxin-a in field and cultured samples of *Anabaena circinalis* and *Cylindrospermopsis raciborskii* (Dell'Aversano *et al.*, 2004). No sample clean-up or pre-concentration step was required with this method.

However, LC/MS has shown certain problems in forensic or in-vivo investigations of suspected anatoxin-a poisonings, related with the presence of the amino acid phenylalanine (Phe). The death of a young man in USA in 2002, after the exposure to lake water was attributed to anatoxin-a poisoning regarding to the evidence of the identification of this toxin using LC-single quadruple MS (James *et al.*, 2005). This identification resulted to be incorrect and the error was associated to the incapability of LC/MS to distinguish between Phe and anatoxin-a, which show similar chromatographic retention characteristics, having the same molecular mass and also producing isobaric fragment ions in their MS/MS spectra (Carmichael *et al.*, 2004). This fact reflects the necessity for complimentary or additional methods with the aim to discriminate between anatoxin-a and Phe. A UV-DAD detector would be able to differentiate both molecules since they show different UV spectra. Recently, the intoxication of two dogs in a lake in France was associated with the presence of anatoxin-a in benthic cyanobacteria. However, the analysis of the complex animal matrices containing the amino acid Phe required in fact, both LC/DAD and LC-MS (EI-QTOF) methods to ascertain the presence of anatoxin-a in the stomach contents of the dogs (Gugger *et al.*, 2005).

James *et al.* (2005) have focused their investigations on the elucidation of mass fragmentation pathways for anatoxin-a, homoanatoxin-a and degradation products, for their detection in forensic samples. Quadruple ion trap (QIT) and Quadruple time of flight (QTOF) MS data were used for this approach. Research was also carried out in the way to develop LC/MS/MS methodologies to avoid de miss-identification of anatoxin-a due to

the presence of Phe in forensic investigations of acute neurotoxic poisonings (Furey *et al.*, 2005; Bogialli *et al.*, 2006).

Recently, a proton nuclear magnetic resonance (NMR) method has been developed for the accurate quantification of microgram quantities of anatoxin-a in much diluted samples (Dagnino and Schripsema, 2005).

In short term, Thin-layer chromatography (TLC) has been used for toxin purification and as a low cost and rapid screening method (Ojanperä *et al.*, 1991). This method has a LOD of 10 µg/g, thus it could be applied to the determination of anatoxin-a in cyanobacterial cells.

Table 5 shows the most relevant methods developed up to date for the determination of anatoxins.

Table 5 Methods developed for sample pre-treatment and analysis of anatoxin-a.
LOD - limit of detection.

Method	Type of matrix	Sample pre-treatment	LOD	Comments	Bibliographic reference
TLC	Cyanobacteria	Sonication of the sample with acidic water (pH=2.5) and LLE with CH_2Cl_2	10 µg/g	*In situ* colour reaction with Fast Black K. Inexpensive screening method. Semi-quantitative.	Ojanperä *et al.*, 1991
HPLC/UV	Cyanobacteria	Extraction with 0.05M acetic acid, reversed phase ODS and purification with cation exchange (COOH)	Not specified	Time consuming sample pre-treatment. Low sensitivity. Not useful for complex samples and not applicable to degradation products.	Harada *et al.*, 1989
HPLC/FLD	Water and Cyanobacteria	Extraction with methanol (pH 4), purification with weak cation exchange SPE	10 ng/l	Derivatization is required. High sensitivity. Determination of anatoxin-a, homoanatoxin-a and degradation products.	James *et al.*, 1998
HPLC/FLD	Water	SPME with CW/TPR	20 ng/ml	Poor sensitivity. Derivatization on-fibre. Complicated experimental design.	Namera *et al.*, 2002
HPLC/FLD	Cyanobacteria	Extraction with methanol-0.2 M HCl and SPE-C18 clean-up	10-50 µg/kg	High sensitivity. Addition of o-phthaldialdehyde (OPA) and mercaptoethanol before NBD-F to remove primary amines.	Rawn *et al.*, 2005
GC/ECD	Water and cyanobacteria	Utrasonication of the samples. Two steps SPE-C18 clean-up are necessary	5 ng injected	Derivatization with trichloroacetic anhydride. Time consuming sample pre-treatment.	Stevens *et al.*, 1988
GC/MS	Water and cyanobacteria	Cyanobacteria extraction with acidic methanol and clean-up with Sep-Pak silica cartridge. LLE for water samples.	0.1 ng injected	Derivatization to N-acetyl anatoxin-a. Long derivatization time (16h).	Himberg, 1989
LC-ESI-MS	Water	Extraction using disk type SPE after pH adjustment to 10	2 ng/l	Pre-column derivatization with Fluorenyl methylchloroformate to increase retention of the anatoxin-a on the LC column.	Takino *et al.*, 1999
LC-QTOF/QIT-MS	Water and cyanobacteria	Extraction with methanol (pH 4), purification with weak cation exchange SPE	2 µg/l	Detailed mass fragmentation pathways for anatoxin-a, homoanatoxin-a and degradation products. Low sensitivity.	James *et al.*, 2005
LC-ESI-MS/MS	Water and fish tissue	Filtration of the water. Fish samples extraction by MSPD using sand for matrix dispersion and water	8 ng/l; 0.2 ng/g	Highly sensitive. Expensive equipment.	Bogialli *et al.*, 2006

5. MODE OF ACTION OF ANATOXIN-A

Anatoxin-a is a neurotoxin that interferes with transmission of nervous impulses in the chemical synapses of the vertebrates. This called the attention of chemists and pharmacologists, in particular of the group of Albuquerque during the 1980 and 1990 decades, who highly contributed to the present knowledge about mode of action of this cyanotoxin (Aracava *et al.*, 1987; Aracava *et al.*, 1988; Wonnacott *et al.*, 1992; Thomas *et al.*, 1993). In fact, (+)-*anatoxin*-a effects are related to acetylcholine, a neurotransmitter whose impairment can lead to severe consequences such as Parkinson's disease and Myasthenia Gravis (Koskinen and Rapoport, 1985).

The system of nerve cells that uses acetylcholine as its neurotransmitter is termed the cholinergic system being present in CNS (central nervous system) as well as PNS (peripheral nervous system). There are two types of acetylcholine receptors: nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). The nAChR receptors are present mainly in PNS on the neuromuscular junction, in autonomic ganglia and in some neurons of CNS. mAChR are found in both the CNS and the PNS, in the heart, smooth muscle glands and neurons (Sá, 2005).

Acetylcholine is synthesized by the neurones and is released into the synapses after an action potential (nervous impulse) has reached the cell. Once released in the synapses, acetylcholine is captured by the receptors exerting its effect on the target organ, acetylcholine then is removed rapidly in order to allow re-polarization to take place. This last step, hydrolysis of the acetylcholine, is carried out by the enzyme acetylcholinesterase (Fig 6).

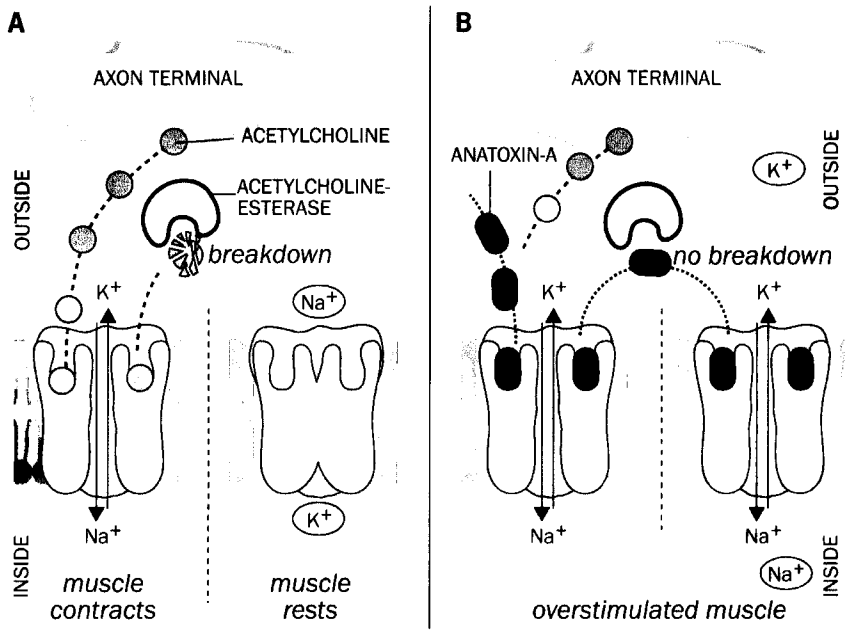

Fig. 6 Scheme showing the anatoxin-a effect on acetylcholine receptors at a neuromuscular junction.
A – normal event
B – intoxication by (+)anatoxin-a
(adapted from Carmichael, 1994).

Anatoxin-a is a potent cholinergic agonist that has the ability to compete with acetylcholine for nicotinic and muscarinic receptors (Aronstam and Witkop, 1981; Dar and Zinder, 1998; Campos *et al.*, 2006). In fact (+)-anatoxin-a is more potent than acetylcholine in stimulating muscle contracture due to a greater affinity for the agonist recognition site of the nicotinic receptors and to a slower rate of desensitization than acetylcholine itself (Koskinen and Rapoport, 1985; Swanson *et al.*, 1986; Aracava *et al.*, 1988).

The action of (+)anatoxin-a at the level of the muscarinic receptors has been studied by few authors. Carmichael and colleagues found a cholinergic involvement at the muscarinic receptors of Guinea pig ileum preparation (Carmichael *et al.*, 1979). Aronstam and Witkop (1981) found that anatoxin-a has 100-fold selectivity for nicotinic compared to muscarinic receptors. Aas and colleagues (1996) investigated the effect of the natural homologue of anatoxin-a, homoanatoxin-a, on the peripheral cholinergic system, including both types of receptors. They concluded that homoanatoxin-a enhances the influx of Ca^{2+} ions in the cholinergic nerve terminals in rat bronchi, rat brain and GH_4C_1 cells.

At the nicotinic receptors, (+)-Anatoxin-a mode of action is compared to a "key" which opens the cationic channels leading to an influx of Na^+ into the cell and an efflux of K^+. This ionic trade results in a depolarization of the neuron initiating a new action potential (nerve impulse). Differently from acetylcholine, (+)anatoxin-a is not hydrolyzed by acetylcholinesterase and the receptors end up over stimulated (Fig. 6B). Over stimulation of nicotinic receptors abolishes the answer of the effectors organ, which can be a ganglion, a neuron or a motor plate (so that sustained contraction occurs). At the level of the adrenergic system, (+)anatoxin-a acts in the nicotinic receptors and stimulates the secretion of catecholamines (dopamine, adrenaline and noradrenalin) that are chemical compounds with numerous effects in the CNS and PNS (*i.e.* cardiac functions, metabolism of carbohydrates) (Soliakov *et al.*, 1995; Molloy *et al.*, 1995; Dar and Zinder, 1998; Guimarães, 2005).

The most evident and lethal effect of (+)anatoxin-a is death from respiratory arrest due to over stimulation of the muscles (Spivak *et al.*, 1980; Aronstam and Witkop, 1981; Swanson *et al.*, 1986; Sá, 2005). In CNS, both (+)anatoxin-a and acetylcholine are nicotinic agonists but with less potency than in neuromuscular junctions. In CNS the agonist effect of (+)-anatoxin-a showed relatively low affinity for muscarinic acetylcholine receptors and, compared to the peripheral nicotine receptors, 5 to 10 times greater concentration of (+)-anatoxin-a were necessary to activate channels on hippocampal and brain stem neurons (Aronstam and Witkop, 1981; Aracava et al., 1987). According to several authors, this might be related to the recognized existence of different subtypes of receptors that are present in CNS and in peripheral nervous system (PNS) (Aracava *et al.*, 1987; 1988; Wonnacott *et al.*, 1992; Thomas *et al.*, 1993; Campos *et al.*, 2006).

The negative enantiomer (-)anatoxin-a (synthetic) was also investigated in relation to its potency but it was concluded that it is far less potent (150 fold) than (+)anatoxin-a, evidencing the stereoselectivity of its receptors (Spivak *et al.*, 1983; Koskinen and Rapoport 1985; Swanson *et al.*, 1986). Production of *(-)-anatoxin*-a by cyanobacteria has never been

reported.

The mode of action of anatoxin-a has been described solely in animals but toxic effects have been described in other organisms as it is described in the following chapter.

6. TOXICOLOGY OF ANATOXIN-A

Carmichael and Gorham contributed largely to the knowledge on the toxicity of anatoxin-a, publishing their first work in 1964 (Gorham *et al.*, 1964), followed by other investigations (*e.g.* Stavric and Gorham, 1966; Carmichael *et al.*, 1975; 1977; Carmichael and Gorham, 1978).

In the first study by Gorham *et al.* (1964), several toxic and non-toxic cyanobacterial strains were isolated from a bloom in Canada (Saskatchewan Lake) and injected intraperitoneally in mice in order to determine its lethal effects. Mice died in 2-5 min, death being preceded by gasps and tremors. This evident high toxicity plus several wildlife fatalities caused by cyanobacterial blooms (Carmichael, 1981), incited the curiosity and concern about the toxicology of anatoxin-a in animals, especially in mammals as models for human health risk evaluation and pharmacological purposes.

Before the 1980's, the lack of chemical detection methods for anatoxin-a, associated with the ease and rapidity of the mouse bioassay to evaluate total toxicity of natural samples, made this latter method widely used by researchers and authorities that were responsible for the surveillance of freshwater bodies (Harada *et al.*, 1999). Therefore, the study of anatoxin-a toxicology was then investigated mainly in mice and tissue preparations (*e.g.* Carmichael *et al.*, 1975; 1979; Carmichael and Biggs, 1978; Spivak *et al.*, 1980; 1983; Astrachan *et al.*, 1980; Aronstam and Witkop, 1981). Afterwards, the development of chemical detection methods (Stevens and Krieger, 1988; Harada *et al*, 1989), the rising of ethical concerns about animal experimentation (European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (86/C 331/01)) and the accumulation of evidences about cyanobacterial effects on aquatic biota (Carmichael, 1981; Jungmann and Bennford, 1994; Wiegand and Pflugmacher, 2005), directed the toxicological studies of anatoxin-a towards other organisms such as plants and zooplankton (Gilbert, 1994; Claska and Gilbert, 1998; Rogers *et al.*, 2005; Pflugmacher *et al.*, 2006).

Before describing toxic doses and effects of anatoxin-a, it is important to know how organisms may be exposed to the toxin. Routes of exposure of anatoxin-a for aquatic organisms are oral, dermal and respiratory organs, while for human we should add intravenous exposure during haemodialysis (Kuiper-Goodman *et al.*, 1999; Carmichael *et al.*, 2001; Draisci *et al.*, 2001).

It was observed in calves and mice that anatoxin-a is rapidly absorbed after oral administration and can enter the brain from the systemic circulation, which possibly contributes to its rapid lethal effect (Carmichael *et al.*, 1977; Stolerman *et al.*, 1992).

Considering the possible exposure routes of all organisms, it is rather curious to verify

that most of the experiments about toxicology of anatoxin-a were done using intraperitonial route in rodents. Subcutaneous injection was administered only in one work where 0.2 mg/kg was lethal to one rat only (Stolerman *et al.*, 1992). In aquatic organisms, including plants and micro-organisms, the route of exposure should be studied according to the target organism.

Lethal doses and sublethal effects of anatoxin-a are described in Table 6 and 7 (page 49), respectively.

Table 6 Lethal doses of cyanobacterial extracts with anatoxin-*a* in different vertebrates administered by different routes (mg of cyanobacteria biomass per kg of body weight).
*NRC-44-1 is the axenized NRC-44h strain. MLD or LDmin - minimum lethal dose, i.p. - intraperitoneal administration, LDx - dose required to kill x% of the tested animals, NOAEL- no observed adverse effect level, i.v. - intravenous administration. n.r. – not referred.

Species/toxin origin	Test animal	Toxicity assessment			Reference
		Parameter	Biomass (mg/kg)	Survival time	
Anabaena flos-aquae / NRC-44-1	mouse	*i.p.* LD_{min}	0.25	2-7 min	Gorham *et al.*, 1964, Stavric and Gorham, 1966
Anabaena flos-aquae / NRC-44-1*			0.3	n.r.	Carmichael *et al.*, 1975
Anabaena flos-aquae / lyophilized cells NRC-44-1*		*i.p.* MLD	60	4 - 5 min	
	rat		60	14-16 min	
Anabaena flos-aquae / lyophilized cells NRC-44-1*	calf	Oral MLD	420	n.r.	Carmichael *et al.*, 1977
Anabaena flos-aquae / toxic fraction NRC-44h	mouse	*i.p.* LD_{min}	0.25		Devlin *et al.*, 1977
Anabaena flos-aquae / lyophilized cells NRC-44-1*		Oral LD_{90}	1800		Carmichael and Biggs, 1978
		i.p. LD_{90}	60		
	rat	Oral LD_{90}	1500		
		i.p. LD_{90}	60		
	chick	*i.p.* LD_{90}	50		
	mallard duck	Oral LD_{90}	350		
		i.p. LD_{90}	50		
	ring-necked pheasant	Oral LD_{90}	850		
		i.p. LD_{90}	120		
	goldfish	Oral LD_{90}	120		
		i.p. LD_{90}	60		
Lyophilized bloom containing anatoxin-a	mouse	*i.p.*MLD_{100}	125-2250	1-57 min	Sivonen *et al.*, 1989
Aphanizomenon sp. / strain 3		*i.p.* MLD_{100}	125	5-14 min	
Anabaena flos aquae / strains 14 and 37		*i.p.* MLD_{100}	125	2-22 min	
Anabaena circinalis / strains 54, 86 and 123		*i.p.* MLD_{100}	125	2-12 min	
Cylindrospermum sp. / strain 191		*i.p.* MLD_{100}	500	12-127 min	
Oscillatoria sp. / strain 193		*i.p.* MLD_{100}	50	2-11 min	
Oscillatoria agardhii / strain 226		*i.p.* MLD_{100}	250	43-163 min	
Oscillatoria formosa / strain NYVA-CYA92 (homoanatoxin-a fraction)		*i.p.* LD_{50}	0.25	n.r.	Skulberg *et al.*, 1992
purified anatoxin-a / Calbiochem Corporation		Oral LD_{50}	>5		Fitzgeorge *et al.*, 1994
		i.p. LD_{50}	0.375		
		i.n. LD_{50}	2		
NYVA-CYA92 / lyophilized cells (homoanatoxin-a)		Oral LD	**2890		Lilleheil *et al.*, 1997
		i.p. LD	**288		
Anabaena flos-aquae / lyophilized cells NRC-44-1*		Oral LD	*5580		
		i.p. LD	**558		
(+)anatoxin-a / Calbiochem Corporation		*i.v.* LD	≤ 0.06		Fawell *et al.*, 1999
		Oral NOAEL	0.098 day^{-1}		
		Oral NOAEL for teratogenicity over organogenesis: (28 day)	**2.46 day^{-1}		
pure anatoxin-a / Sigma Chemical Company		*i.p.* LD_{50}	0.250		Rogers *et al.* 2005

Table 7 Sublethal and pre-death clinical symptoms and onset time of different animal species intoxicated with anatoxin-a.
*homoanatoxin-a effects

Test animal	Time post-treatment (min)	Effects and symptoms	Reference
mouse	2	Gasps, tremors, mild convulsions, paralysis, no detectable changes upon autopsy	Gorham *et al.*, 1964
calf	4	Staggers, convulsions, muscle fasciculation (shoulder and limbs), loss of muscle coordination, breathing abdominal (animal down), ptosis, collapse from respiratory arrest, no detectable changes upon autopsy	Carmichael *et al.*, 1975, 1977
rat	14	latency followed by twitching, gasping and convulsion	
mouse	4		
goldfish	2	latency followed by muscle rigidity, mouth contracted open	
mallard duck	15	opisthotonus (appearance of fowl botulism "limberneck"), muscle rigidity	
mouse	60	decrease locomotor's activity	Stolerman, *et al.*, 1992
*mouse	-	flanks withdrawing violently, back arching, heavy breathing, ataxia, violent spasms	Lilleheil *et al.*, 1997
mouse	1	increased respiration, salivation, micturation, hyperactivity, Straub tail	Fawell *et al.*, 1999
mouse	5-6	decreased motor activity, altered gait, difficult breathing, convulsions	Rogers *et al.*, 2005
mouse	-	decrease in pup weight on post-natal day 1	MacPhail *et al.*, 2005

The difference of sensitivity to the toxin among different animals is important, particularly in avian species, e.g. the ring-necked pheasant demonstrated a higher resistance to anatoxin-a than the Mallard duck (Carmichael and Biggs, 1978). These differences in the same group have to be taken into account in environmental risk evaluation of anatoxin-a, and should reinforce the need to study the toxicity in different species even from the same taxonomic group. Gilbert (1994) corroborated this idea, after showing that two species of Rotifera reacted differentially to anatoxin-a (survivorship and fecundity).

Besides testing anatoxin-a alone, Fitzgeorge and colleagues (1994) also investigated the co-effect of anatoxin-a toxicity with microcystin-LR, the most common cyano-hepatotoxin. They found a significant synergistic effect between anatoxin-a and microcystin-LR: the administration of a sub lethal dose of microcystin-LR (30 µg/kg bw, *i.n.)* to mice, before dosing with anatoxin-a, reduced the intranasal LD_{50} of the latter from 2000 µg/kg to 500 µg/kg bw. This called the attention to possible interactions among cyanotoxins and should be considered in risk evaluations. In nature and in the same water body, several strains and therefore several cyanotoxins may be found simultaneously (Krienitz *et al.*, 2003; Ballot *et al.*, 2005). Moreover, Sivonen *et al.* (1989) found simultaneous production of anatoxin-a and hepatotoxins in *Cylindrospermum sp.* and *Oscillatoria agardhii.* This finding was supported by a previous work that described the simultaneous production of anatoxin-a and hepatotoxins by the strain NRC-44-1 (Al-Layl *et al.*, 1988). Thus, the risk evaluation of cyanotoxins becomes more complex and difficult to attain.

In 2005, Rogers and colleagues studied the synergism between microcystin-LR and anatoxin-a. They gavaged these toxins to mice and found no lethality and no pre- to post-mortem alterations in weights after exposure to 1000 µg/kg bw of microcystin-LR fol-

lowed by 2500 µg/kg bw of anatoxin-a (Rogers *et al.*, 2005).

After death by anatoxin-a intoxication, the autopsy did not reveal any physical sign capable to be used in the diagnosis of the death cause. According to Fawell and co-authors (1999), if lethal dose was not reached, mice recovered rapidly and completely from a single dose. These two aspects could have contributed to the under evaluation of the number of fatalities caused by anatoxin-a.

Anatoxin-a causes paralysis in contaminated organisms (Adams and Swanson, 1994). The effects and symptoms referred in Table 7, are very important but should not deserve more attention than others less evident and more spectacular such as alterations of animal behaviour. Stolerman and colleagues (1992) achieved the first behavioural study about the effects of (+)anatoxin-a in locomotor activity in rats. Their results showed that (+)anatoxin-a has powerful behavioural effects, namely decreasing locomotor activity.

Because anatoxin-a is a neurotoxin, a behavioural approach of the toxicity of anatoxin-a to aquatic wildlife is essential in order to understand the effect of this cyanotoxin in aquatic environment. In terms of natural impact of the occurrence of anatoxin-a, sub-lethal toxicity should be studied. Although animals may recover from a sub-lethal dose, behavioural alterations might have relevant effects in mating and predatory interactions that could cause alterations on the structure of aquatic communities.

Astrachan *et al.* (1980), Fawell *et al.* (1999) and MacPhail *et al.* (2005), studied the effect of parenteral exposure in rodents. Sublethal doses of anatoxin-a (*i.p.*), produced stunted foetuses with weight significantly different from control group in all treated groups and hydrocephaly in only one treatment. Nevertheless, the authors considered anatoxin-a not a potent teratogenic in hamsters because the toxin did not cause significant effects on the nervous system in amounts less than those causing acute signs. No effects were registered besides its specific neuromuscular depolarizing activity (Astrachan *et al.*, 1980).

Fawell and colleagues submitted female mice to anatoxin-a (gavage, 2.46 mg/kg of anatoxin-a per day) from day 6 to 15 of pregnancy and found no external abnormalities of the foetuses, nor any adverse effects on maternal organism, as shown by necropsy and bodyweights (Fawell *et al.*, 1999).

Recently, Rogers and colleagues evaluated post-natal viability, growth and neurodevelopment maturation in pup mice submitted to anatoxin-a by parenteral i.p. route and found no significant alterations with 200 µg/kg bw except for some. Nevertheless, maternal toxicity was evidenced by decreased motor activity immediately after treatment (Rogers *et al.*, 2005)

Besides studies in animals and neuronal cells (see chapter 5 of this article), anatoxin-a effects have also been investigated in zooplankton, cultivated animal cells and plants. Rotifers are susceptible to anatoxin-a, their fertility and survival being decreased (Gilbert, 1994). Later it was found that toxicity of anatoxin-a to rotifers was enhanced by increasing water temperature or lowering food availability (Gilbert, 1996[a,b]).

Rao and collaborators submitted rat thymocytes and Vero cells (monkey kidney cells)

to anatoxin-a (cell extracts and pure toxin) and found cytotoxic effects: loss of viability and mitochondrial function, lactate dehydrogenase leakage, caspase-3 activation and apoptosis. Anatoxin-a apoptosis was attributed to generation of reactive oxygen species and caspase activation (Rao *et al.*, 2002).

Exposure of the amphibian *Bufo arenarum* to different concentrations of anatoxin-a (0.03-30 mg/l) during stages 17 and 25, showed a dose-dependant transient necrosis, edema and loss of equilibrium. No survivors were found at the highest dose (Rogers *et al.*, 2005).

Kearns and Hunter (2000) studied the interaction between a green alga (*Chlamydomonas reinhardtii*) and one anatoxin-a producing cyanobacterium (*Aphanizomenon flos-aquae*) and found that some extra cellular products of the green alga induced anatoxin-a production by the cyanobacterium. Nevertheless, the toxin did not reduce growth of the green alga.

Later, oxidative stress and detoxication processes caused by anatoxin-a were studied. Mitrovic and colleagues (2004) investigated for the first time, the effects of anatoxin-a in the aquatic plants *Lemna minor* and *Chladophora fracta*. They found that exposure of the plants to 25 µg anatoxin-a /ml increased peroxidase activity (POD) and soluble glutathione S-transferase (GST). Exposure to 5-20 µg anatoxin-a /ml lowered photosynthetic oxygen production, increased catalase activity (CAT) and caused formation of reactive oxygen species (ROS). These results were the first evidence about anatoxin-a negative effects on plants. Recently, Pflugmacher and colleagues (2006) showed that the exposure to 5 µg/l of anatoxin-a strongly inhibited primary root development and altered enzymatic activities of alfalfa (*Medicago sativa*). Increased SOD (superoxide dismutase), APx (ascorbate peroxidase) and glutathione reductase activities, suppressed catalase and GST (glutathione S-transferase) activities and slightly increased lipid peroxidation (quantified by malondialdehyde variation) were also registered. These results are ecologically significant because terrestrial plants can be exposed to cyanotoxins through irrigation events and in hydroponics cultures (Pflugmacher *et al.*, 2006).

7. ENVIRONMENTAL AND PUBLIC HEALTH CONCERNS

Evidences of adverse health effects of anatoxin-a in animals have been accumulating through time in many regions of the globe. Contrasting with the most studied cyanotoxin, microcystin-LR, the harm posed by anatoxin-a was not yet translated into any guideline value (WHO, 1986). It is evident that exposure and effects of this cyanotoxin have not been fully determined in humans or aquatic biota; therefore no risk evaluation could be done. Nevertheless, Fawell and collaborators suggested that a 1µg/l of anatoxin-a would provide a significant margin of safety of around three orders of magnitude with regard to drinking water (Fawell *et al.*, 1999). New Zealand Ministry of Health calculated a maximum acceptable value (MAV) for anatoxin-a in drinking water of 6 µg/l. The MAV is determined

on the basis of protection to avoid adverse health effects from chronic exposures (Ministry of Health of New Zealand, 2005).

No doubt the hazard is identified and it is possible to enumerate its concerns in a health and environmental perspectives. That is evidenced by the reports of animal fatalities by several authors.

The first concern about possible intoxication of domestic animals by anatoxin-a was reported by Gorham and colleagues in 1964, after the death of two cows that drank water from a water body with a cyanobacterial bloom. Since then, many other reports followed after animal fatalities associated with anatoxin-a (see chapter 3 of this article).

Any organism that contacts with water, algae or food, contaminated with anatoxin-a, may suffer from intoxication. This has to be taken into account for any organism, aquatic or not, that is directly or indirectly in contact with contaminated water. Until now there are no studies determining transference or bio amplification of anatoxin-a through food webs, although this situation should be considered.

Degradation products of homoanatoxin-a and anatoxin-a (dihydrohomoanatoxin-a and epoxyanatoxin-a) in health food samples for human consumption that contained cyanobacteria were found by Draisci *et al.* (2001).

It is mandatory that storage facilities and tanks for water storage are monitored for the presence of cyanobacteria. Some species, including *Oscillatoria sp.*, a potential anatoxin-a producer, can proliferate in these systems.

Environmental risk of anatoxin-a for aquatic ecosystems has not been studied but according to our observation of juvenile carps placed in water contaminated with extracts of an anatoxin-a producing strain, swimming was altered (Osswald *et al.*, 2007). This concern is corroborated with other work that found negative effects on *Bufo arenarum* (Rogers *et al.*, 2005) (see previous chapter of this article).

Considering the high toxicity of anatoxin-a to humans and vertebrates, besides the potential harm to ecosystems, the presence of cyanobacteria should always be considered as a potential health hazard.

Acute toxicity is the most obvious concern of anatoxin-a although a long-term risk may also be a problem. Further investigation in this area is required.

8. PREVENTATIVE AND REMEDIAL MEASURES

Anatoxin-a production by cyanobacteria is not yet fully understood. Why, when and by what strains anatoxin-a is produced, are still unanswered questions. Solving this puzzle would maybe permit to control toxin production. The only way to hinder anatoxin-a intoxications is avoiding cyanobacterial blooms formation, by preventing and decreasing eutrophication, or eliminating the toxin itself, through water treatment processes. Together with these two aspects, monitoring of water bodies, public awareness about cyanobacterial blooms and its risks, plus efficient warning measures are essential.

Measures and approaches to control eutrophication of water bodies should start by the evaluation of carrying capacity of the water system. This will permit determining which is the resource limiting cyanobacterial growth, in order to achieve adequate measures. Characterization of light and hydrophysical situation may also be important issues because of the cyanobacterial capacity to migrate vertically along the water column (Chorus and Mur, 1999). Measures to alter favourable conditions for cyanobacterial dominance include flow regulation and flushing by controlled discharges and destratification of water column by artificial mixing (bubble plume aerator) (Falconer, 2005). Nutrient inflow contributes largely to eutrophication thus favouring cyanobacterial blooms. Phosphorous reduction will ultimately be the most successful approach in reducing cyanobacterial occurrence (Chorus and Mur, 1999; Falconer, 2005).

Integrated cachment management maintains ecological equilibrium and sustainable development. The maintenance of a homeostatic equilibrium within the ecosystem would diminish eutrophication and thus lessen cyanobacterial hazards (Zalewski, 2002).

The preferred approach to manage and control cyanobacteria in water is the prevention of blooms. The next level of response to cyanobacterial occurrence is water treatment. Preferentially, water treatment should be considered only after other preventative measures are taken, including selection of the right depth of water intake (Haider *et al.*, 2003).

Once the toxic bloom is established, much care should be taken in order to assure cyanobacterial cell integrity. Bank filtration, flocculation, coagulation, precipitation and dissolved air flotation are processes that may disrupt the cells and then cause liberation of the toxin to water. In case of toxin production, uncontrolled cell disruption would represent high amounts of toxin in the dissolved form. Dissolved cyanobacterial toxins are not visible and thus are more prone to contaminate terrestrial animals, including humans.

Elimination of cyanotoxins in the water must involve chemical or physical procedures able to completely reduce toxicity. Chlorine has low efficiency and U.V. radiation is ineffective in the removal of dissolved anatoxin-a. Ozonation and nanofiltration, through electrostatic interactions and steric hindrance, were found to be effective (Teixeira and Rosa, 2006). Data on elimination of anatoxin-a from water is limited, therefore more scientific effort is needed in this area.

9.CONCLUDING REMARKS

Natural products such as anatoxin-a, may be promising substances for medicine and this accentuates the idea that efforts should be done to preserve aquatic biodiversity.

Anatoxin-a toxicity should never be disregarded. Responsible authorities have to be aware of this toxin and consider it as a potential cause of animal and human fatalities.

Risk of intoxication with anatoxin-a is, in our point of view, of significant concern to wildlife and aquatic species. Its high toxicity, associated with the increasing cyanobacterial occurrence, should be taken into account and a serious evaluation of the health and en-

vironmental risks has to be achieved.

The implications respecting to aquatic and terrestrial ecosystems have yet to be fully understood. Chronic effects of anatoxin-a on reproduction in humans and wildlife have not been investigated. There are many gaps about the knowledge of this cyanotoxin that should be discovered in order to achieve a proper risk evaluation for environmental and human health. Efforts should be made to conduct a better evaluation of its effects in aquatic ecosystems. This should be done using acute and chronic exposures, having in mind the exposure routes and behavioural alteration of organisms.

To have a more conscious and responsible protection of public and wildlife health, other cyanobacterial genera, besides *Anabaena* and *Aphanizomenon*, should also be seen as potential producers of anatoxin-a. A good surveillance and monitoring of anatoxin-a in water-bodies should be undertaken.

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Analysis of anatoxin-a in biological samples using liquid chromatography with fluorescence detection after solid phase extraction and solid phase microextraction

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Abstract

Anatoxin-a is a naturally occurring, potent neurotoxin produced by some species of cyanobacteria in freshwaters. This toxin, which is a potential health hazard, especially to animals, has been determined in different biological matrices such as several cyanobacterial cultures and water samples and carps and mussels tissue using a sensitive High Performance Liquid Chromatography with Fluorescence detection method. Sonication was the technique selected for the extraction of intracellular anatoxin-a and solid phase extraction using weak cation exchange was used for the concentration and purification of the samples. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was used to convert anatoxin into a highly fluorescent derivative. Recovery experiments were performed for each type of matrix used in this work, and adequate values were obtained (71–87%). Limits of detection for anatoxin-a were estimated to be in the ng/L and ng/g level for water and cyanobacterial samples, respectively. The results obtained were also compared with those obtained after using solid phase microextraction, as an alternative for the extraction and purification of the samples. Advantages and disadvantages regarding to the efficiency for impurities removal, simplicity and rapidity and the potential for concentration enhancement of using both methodologies have been also discussed.

Keywords: Anatoxin-a; Solid phase extraction; Solid phase microextraction; High Performance Liquid Chromatography; Fluorescence detection

1. Introduction

The frequency of cyanobacterial blooms has dramatically increased through the world in freshwaters lakes and brackish water environments presenting a hazard to animal and human health [1–4]. It is known that under certain environmental conditions, cyanobacteria can produce in addition to volatile odour compounds and bioactive chemicals [5], toxic compounds of more concern including both hepatotoxins and neurotoxins [6–8]. Three types of cyanobacterial neurotoxins are known; (homo)anatoxin-a, anatoxin-a(s) and saxitoxins. Neurotoxins block neurotransmission, but unlike the hepatotoxins, do not cause any apparent organ damage. In mouse bioassays, death by respiratory arrest occurs within minutes. Symptoms include respiratory distress, convulsions, fasciculations and in some cases, salivation [9,10]. The anatoxin-a containing blooms and isolated cyanobacterial strains have been reported mostly from North America and Europe and recent chemical analysis has shown anatoxin-a also to occur in freshwater in Asia (North Korea and Japan) [11–13]. Occurrences of all three types of neurotoxic blooms have been reported from America and Europe whereas in Australia only saxitoxins are common [14,15].

Anatoxin-a is a low molecular weight secondary amine and was the first neurotoxin, unique to freshwater cyanobacteria to have its chemical structure elucidated. It is the most potent neurotoxin and it has been found to be produced by *Anabaena flos aquae*, *Anabaena planktonica*, *Aphanizomenon flos aquae*, *Cylindrospermum*, benthic *Oscillatoria* and more

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recently in *Planktothrix rubescens* and *Arthrospira fusiformis* [16–18]. Small amounts of anatoxin-a have been also produced by a Japanese *Microcystis* sp. [11].

This small alkaloid acts as a post-synaptic cholinergic agonist which binds to the nicotinic receptor with a higher affinity than acetylcholine. Toxicity studies with intraperitoneal injection into mice have determined the LD_{50} to be 200 μg/kg. Oral toxicity determinations using suspensions of cells containing anatoxin-a have resulted in LD_{50} values 100–1000 times lower than that obtained with i.p. injection [19,20].

Anatoxin-a is considered one of the most toxic freshwater toxin, even when is present at very low levels, being one of the toxins included within the highest priority group for further research. The presence of the anatoxin-a in biological samples (fish, algae, water, etc.) after a toxic cyanobacterial bloom is actually common. This fact together with the need to strict control at the specific levels imposed by the worldwide regulations, justifies the search for sensitive and selective analytical techniques. Regarding to this, the development of analytical methods for anatoxin-a has been rather easy, since the structure of the toxin had already been solved in the late 1970s and chemical synthesis yielded the pure compound to be used as a standard. The method selected depends on the samples to be analyzed and on the availability of equipment. Currently, several chromatographic methods are available for the analysis of this toxin. Thin layer chromatography (TLC) has been used for purification of the toxin and as an inexpensive screening method [21]. High performance liquid chromatography (HPLC) with UV and with more sensitive fluorescence (FL) detection have been widely used to quantify anatoxin-a [22,23]. Gas chromatography (GC) based methods have been also developed using different derivatization reagents [24,25]. Recently, the combination of both liquid chromatography (LC) and Gas chromatography with mass spectrometric (MS) detection has represented an important advance in the analysis of anatoxin-a, mainly in terms of confirmation [26–30].

On the other hand, the complexity of the biological matrices makes sample pre-treatment a critical step for an efficient and reliable analysis and for this reason improvements in the strategies for the extraction and purification have been also carried out including solid phase extraction (SPE) and more recently solid phase microextraction (SPME) [31].

The versatility of HPLC, its ability to separate complex molecules, even when they are present in complex matrices as well as its potential to be coupled to several detection modes, makes this technique a powerful tool for the analysis of these kind of compounds, even at trace levels.

The aim of the present study was to asses the applicability of the SPE using weak cation exchange (WCX) cartridges prior to HPLC with fluorescence detection for the analysis of anatoxin-a present in different matrices. For this purpose, the present method, based on the one initially developed by James et al. [23] has been applied to different samples such as mussels and carps tissue, cyanobacterial cultures and water. SPE was evaluated in all cases in terms of recovery and repeatability. Once the applicability was assessed, the method was applied to the analysis of several anatoxin-a producing cyanobacterial cultures and water samples potentially contaminated with this toxin. This method has been also applied for the evaluation of the acute effect of anatoxin-a producing cyanobacteria on juvenile fish *Cyprinus carpio* and mussels. Moreover, the extraction efficiency of both SPE and SPME with on-fiber derivatization was compared.

2. Experimental

2.1. Chemicals and standards

Anatoxin-a fumarate was purchased from Tocris (Bristol, UK). NBD-F was purchased from Sigma–Aldrich (Gillingham, UK). All solvents used were HPLC grade and were supplied from Panreac (Barcelona, Spain). Deionized water was prepared by passing glass-distilled water through a Milli-Q water purification system.

Stock standard solution (1 mg/mL) of anatoxin-a fumarate was prepared by using Milli-Q water and stored in an amber glass vial at −20 °C. Working solutions were prepared daily and stored in the dark at 4 °C.

2.2. Instrumentation

The HPLC system consisted of a quaternary pump, Agilent 1100 Series QuatPump, a degasser Agilent 1100 Series Degasser and a Fluorescence detector Agilent 1100 FLD (Agilent Technologies, Waldbronn, Germany). Separation was performed on a Luna C18 column, 5 μm, 250 mm × 4.6 mm i.d. (Phenomenex, Macclesfield, UK).

For the SPME–HPLC-FL approach the HPLC system used was a Jasco PU-980 HPLC Pump with a Jasco LG-980-02 Ternary gradient unit (Jasco Analitica Spain, Madrid, Spain) coupled with a Fluorescence detector Hitachi F-1000 Fluorescence Spectrophotometer (Merk, Darmstadt, Germany). SPME–HPLC interface from Supelco (Supelco, Bellefonte, PA, USA) were used. The interface consists of a six-port injection valve and a 70 μL desorption chamber, which replaced the injection loop in the HPLC system.

SPE extractions were carried out in a Vacuum Manifold (Visiprep™) from Supelco using Supelclean LC-WCX, 3 mL tubes (Supelco, Bellefonte, PA, USA).

Solvent evaporation under nitrogen was carried out using a Turvo Vap® II, (Concentration Workstation, Zymark, Warrington, UK). For sample preparation it was used an Ultraturrax T25 (Ika-Labortechnik, Staufen, Germany), an ultrasonic processor (Vibra cell™, Sonics & Materials Inc., Danbury, CT, USA), centrifuges (Digicen, Orto Alresa ad a Hermle Z 231M, BHG). The 0.45 μm filter disks and Ultrafree®-MC, 0.45 μm filter (Millipore, Bedford, MA, USA) were used for filter extracts and samples, respectively.

The SPME manual holder for HPLC analysis and 60 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers were obtained from Supelco (Supelco, Bellefonte, PA, USA). Before the first use, fibers were conditioned, according to instructions provided by the manufacturer. A magnetic stirrer/heater RCT basic (IKA, Werke, Germany) provided with

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a temperature sensor controller was used to perform the SPME extraction under magnetic stirring and controlled temperature. A Tem Bloc (P-Selecta, Spain) was used to control the temperature during derivatization reaction.

Chromatography data analysis was performed using a Chem-Station (Agilent Technologies) and a Jasco-Borwin LC-Net II (Jasco Analitica Spain).

2.3. Biological samples

2.3.1. Water samples

The presence of anatoxin-a in fresh water bodies in Portugal was evaluated in several samples of freshwater from different sites. Samples were selected from sites with human usage (recreational and/or consumption usage) presenting cyanobacterial cells. Water samples (100 mL) were collected directly from the water surface and transported to the laboratory conditioned on ice in the dark. Samples were maintained at −22 °C prior analysis.

2.3.2. Cyanobacterial cultures

In the same survey referred above, some strains of Portuguese cyanobacteria were established for investigating anatoxin-a production. Cyanobacterial cells obtained from pure laboratory batch cultures (25 ± 1 °C, light intensity of 10 μmol m^2 s^{-1} (14-h light:10-h dark cycle)) were freeze-dried before analysis.

2.3.3. Carps and mussels

With the aim of evaluating the toxicity of anatoxin-a in aquatic organisms, juvenile carps (*C. carpio* L.) and mussels (*Mytilus* sp.) were exposed, separately, to a cyanobacterial strain that produces anatoxin-a. This anatoxin-a producing strain of *Anabaena* sp. (ANA 37) was isolated from the Baltic Sea and kindly supplied by Professor Kaarina Sivonen (University of Helsinki, Department of Applied Chemistry and Microbiology). Carps 3 months old (0.19 ± 0.1 g and 18.43 ± 2.75 mm) were exposed to freeze-dried cells of ANA37 (equivalent to 10^5 cell mL^{-1}) in freshwater aquaria during 96 h. Mussels were submitted to live cells of ANA37 (3.27×10^5 cell mL^{-1}) during 15 days in a marine water aquarium. Results and a more detailed description of these two bioassays are being prepared for publication elsewhere.

2.4. Sample preparation

2.4.1. Extraction of anatoxin-a from matrix

Freeze-dried material (cyanobacterial cells, carps and mussels, 100 mg approximately) was previously homogenized with an Ultraturrax for 5 min with 10 mL of methanol (1% HCl, 1 M). Suspension was then ultrasonicated for 2 min at ca. 80 amplitude. Finally, solution was centrifuged at 3200 rpm for 15 min and the supernatant was collected. This procedure was repeated twice and the combined extracts were evaporated in a Turbo Vap at 40 °C under N_2 stream. Samples were re-dissolved in water (5 mL) and then filtered using 0.45 μm disc filters.

2.4.2. Solid phase extraction clean-up

A filtered water sample (10 mL) or mussel, carp or cyanobacterial extract (5 mL) was adjusted to pH 7 with NaOH 0.1M before SPE clean-up using weak cation exchange. The cartridge was conditioned with methanol (6 mL) and water (6 mL). The samples were loaded into the cartridge and then 3 mL of methanol–water (1:1) were used in the wash step for water samples, whereas 3 mL methanol 100% were used for carp, mussel and cells extracts, due to the high level of organic impurities present in these matrices. After air drying the cartridges for 30 min, the anatoxin-a was eluted using 10 mL of methanol containing 0.2% trifluoroacetic acid and then the solvent was evaporated under nitrogen stream at 40 °C. Extracts thus obtained were re-dissolved in methanol and kept at −22 °C in amber vials until HPLC-FL analysis.

2.4.3. Solid phase microextraction

Optimization and evaluation of the SPME–HPLC-FL method for the analysis of anatoxin-a is reported in [32]. Aliquots of 1600 μL of aqueous standard solutions or water samples with 20% of sodium chloride and pH 10 with sodium hydroxide 1 M, were placed into a 2 mL amber vials sealed with aluminium caps. Extractions were carried out by immersing the fiber in sample solution. Magnetic stirring (1000 rpm) was used and temperature of sample solution was set at 45 °C. An equilibration time of 2 min was established for bringing the sample temperature. After extraction for 20 min, the fiber with the anatoxin-a adsorbed, was transferred to the headspace of a vial containing 5 μL of dried NBD-F (1 mg/mL in acetonitrile). The vial was heated to 60 °C for 20 min for the reaction takes place on the surface of the fiber. After reaction, the fiber is placed in the desorption chamber of the SPME/HPLC interface, previously filled with desorption solvent (60:40, acetonitrile:water) for static desorption. Anatoxin-a derivative was desorbed by exposing the fiber for 12 min in the interface. Separations were carried out in a Luna C18 column (5 μm, 250 mm × 4.6 mm i.d., Phenomenex) at room temperature using acetonitrile–water (50:50) as mobile phase with a flow rate of 0.8 mL/min. The excitation and emission wavelengths were set at 470 and 530 nm, respectively.

Previous dilution of the sample with deionized water is recommended for very dirty samples to avoid the irreversible saturation of the fiber.

For solid samples, 20 μL of methanolic extract were dried under N_2 stream and re-dissolved in 1600 μL of water (20% NaCl, pH 10). Then, SPME was performed following the procedure above described.

2.5. HPLC-FL analysis

2.5.1. Derivatization of standards and SPE extracts prior HPLC-FL analysis

Standards or SPE extracts were evaporated under nitrogen stream and then reconstituted with 100 μL of sodium borate 0.1 M (pH 10). Fifty microlitres of NBD-F (1 mg/mL in acetonitrile) were added and the vial was kept in dark for 10 min at room temperature for the anatoxin derivatization.

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Table 1
Recovery and repeatability studies for anatoxin-a from spiked samples of cyanobacteria, mussel and fish tissue

Fortification (μg/g)	Type of matrix					
	Cyanobacteria		Mussel		Fish	
	Recovery (%)	% RSD ($n = 3$)	Recovery (%)	% RSD ($n = 3$)	Recovery (%)	% RSD ($n = 3$)
2	84	0.6	75	2.8	78	3.2
0.1	85	2.8	71	2.4	–	–

100 mg of a spiked sample was homogenized for 5 min with 10 mL of methanol (1% HCl, 1 M). Suspension was ultrasonicated for 2 min and solution was centrifuged at 3200 rpm for 15 min. Procedure was repeated twice. The combined extracts were evaporated at 40 °C under N_2 stream. Samples were re-dissolved in water (5 mL) and filtered. Extracts were purified by SPE–WCX (conditioning: 6 mL methanol, 6 mL water; load: 5 mL extract (pH 7); wash: 3 mL methanol 100%; elution: 10 mL methanol with 0.2% trifluoroacetic acid).

Fifty microlitres of 1M hydrochloric acid were added to stop reaction.

2.5.2. HPLC-FL conditions

Separations were carried out using a LC reversed phase, Luna C18 column (5 μm, 250 mm × 4.6 mm i.d., Phenomenex) at room temperature. The mobile phase was acetonitrile–water (50:50) with a flow rate of 0.8 mL/min. The excitation and emission wavelengths for fluorimetric detection were set at 470 and 530 nm, respectively.

3. Results and discussion

3.1. SPE–HPLC-FL evaluation

3.1.1. Linearity, repeatability and limit of detection

Standard solutions containing anatoxin-a fumarate in the range 1–20 μg/mL were prepared in ultrapure water. Ten microlitres of each standard solution were derivatized with NBD-F following the procedure above described and 20 μL of each derivative were injected into the instrument, resulting calibration standards ranging from 0.05 to 1 μg/mL (1–20 ng anatoxin-a fumarate injected on column). Triplicate HPLC-FL analyses of the prepared calibration levels were performed. Good linearity was achieved with a correlation coefficient $R^2 = 0.9992$.

For routine use in sample monitoring by SPE and HPLC-FL analysis, concentration of anatoxin-a was corrected for recovery by using the mean recovery for the corresponding type of matrix tested at the adequate spiking level. The fact of using anatoxin-a fumarate instead of pure anatoxin-a was also taken into account for quantitative results. Samples derivatives containing anatoxin-a at concentrations above the calibrated range were diluted with water.

Instrument and method detection and quantitation limits (LOD and LOQ) based on a signal-to-noise ratio of 3 and 10, respectively, were estimated. Instrument limits were determined by using five replicates of 0.05 ng injected on column. According to the used criteria detection limit was estimated to be 0.02 ng of anatoxin-a injected on C18 column, which corresponds to method detection limits close to 25 ng/L for water and 3 ng/g for cyanobacterial samples, mussels and fish tissue, respectively. These method detection limits were further checked by spiking uncontaminated water and a freeze-dried samples with the appropriate amount of anatoxin-a standard. However, for mussels and fish tissue real detection limits resulted to be higher (15 ng/g). Resulting method quantification limits were 70 ng/L for water and 10 and 50 ng/g for cyanobacteria and mussel/fish tissue, respectively.

Instrument within-day repeatability was determined for a set of 10 injections in the same day whereas between-day repeatability was studied with the results of three injections per day on 3 different days. Both parameters were evaluated in terms of RSD using 20 ng of anatoxin-a standard injected on column. Acceptable values for within-day (1.1%) and between-day repeatability (1.5%) were obtained.

Repeatability of the method was evaluated as part of the recovery studies, by using two different fortification levels for each type of matrix (water, cyanobacteria, mussel and carp tissue). Study was made in triplicate, on the same day, using three different samples for each case. Results are presented in Tables 1 and 2. Adequate repeatability values in terms of RSD were obtained (0.6–5.7%).

3.1.2. Recovery studies

In order to asses the recovery of anatoxin-a from different types of matrix using weak cation exchange SPE, uncontaminated river water sample and freeze-dried mussel, fish tissue and cyanobacterial algae were spiked with two different concentrations of anatoxin-a standard (20 and 1 ng/mL for water samples and 2 and 0.1 μg/g for solid samples). Study was made in triplicate for each type of matrix and for each spiking level. The recovery rates were determined by comparing the peak areas using HPLC-FL of the spiked samples with those obtained for equivalent concentration of standard solutions that were directly derivatized without SPE.

Table 2
Recovery and repeatability studies for anatoxin-a from spiked samples of water

Fortification (ng/mL)	Water matrix	
	Recovery (%)	% RSD ($n = 3$)
20	85	5.7
1	87	4.4

Spiked water samples (10 mL, pH 7) were extracted by SPE–WCX (conditioning: 6 mL methanol, 6 mL water; load: 10 mL sample (pH 7); wash: 3 mL of methanol–water (1:1); elution: 10 mL methanol with 0.2% trifluoroacetic acid).

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The recovery and corresponding RSD (%) values are summarized in Tables 1 and 2. All recoveries were obtained in the range 71–87%. No significant difference was observed between both concentration levels. The higher recoveries were obtained for water and cyanobateria matrices. Lower values were observed for mussel and fish tissue probably due to the complexity of the matrix.

3.2. Application of SPE–HPLC-FL method to real samples

SPE–HPLC-FL determination was applied to 18 cyanobacteria cultures. Anatoxin-a was found in most of analyzed cultures in concentrations ranging 0.057–967 μg/g. The higher concentrations were observed in strains of *Anabaena* sp. and *A. flos aquae*. An example is shown in Fig. 1(A).

The presented SPE–HPLC-FL method was also applied to the analysis of 18 water samples potentially contaminated with anatoxin-a. Results obtained showed no presence of the toxin

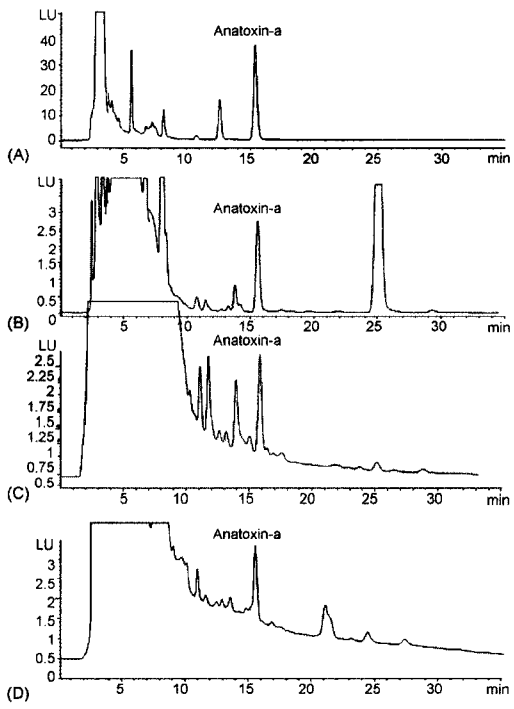

Fig. 1. Examples of the application of the SPE–HPLC-FL method to the analysis of anatoxin-a (RT = 15.6 min) in different samples. (A) Analysis of an anatoxin-a producing strain of *Anabaena* sp. (B) 10 mL of river water sample spiked with 200 ng of anatoxin-a standard. (C) Group of carps sample exposed in vivo to freeze-dried cells of ANA37 (equivalent to 10^5 cell mL^{-1}) during 96 h. (D) Group of mussels sample exposed in vivo to live cells of an anatoxin-a producing strain of *Anabaena* sp. (3.27×10^5 cell mL^{-1}) during 15 days. HPLC-FL conditions: 5 μm C18 column (250 mm × 4.6 mm); mobile phase, acetonitrile–water (50:50, v/v); flow rate, 0.8 mL/min; excitation and emission wavelengths were 470 and 530 nm, respectively.

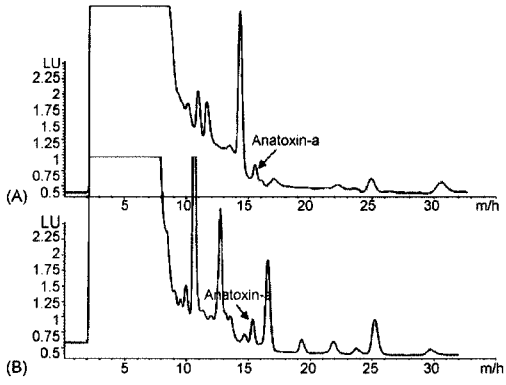

Fig. 2. Chromatograms corresponding to the analysis by SPE–HPLC/FL of a carp sample (A) and a mussel sample (B) previously spiked with anatoxin-a at the quantification limit (50 ng/g). HPLC-FL conditions: 5 μm C18 column (250 mm × 4.6 mm); mobile phase, acetonitrile–water (50:50, v/v); flow rate, 0.8 mL/min; excitation and emission wavelengths were 470 and 530 nm, respectively.

in any sample. An explanation of this can be related with the instability of anatoxin-a which could be decomposed during sample handling. This fact has already been mentioned by Stevens and Kriegger [33]. In Fig. 1(B) is shown a water sample spiked with anatoxin-a. High sensitivity and efficiency was obtained.

Carps and mussels previously exposed to anatoxin-a as was referred before, were homogenized, extracted and derivatized prior HPLC-FL analysis. Fig. 1(C and D) shows examples of the application of the method to carp and mussel samples. Good sensitivity was obtained although the background noise was high in both cases due to the presence of co-extractives. The analysis of a carp and a mussel sample previously spiked with a concentration of anatoxin-a corresponding to the quantitation limit (50 ng/g) are shown in Fig. 2.

3.3. Application of SPME–HPLC-FL method to real samples

The SPME–HPLC-FL method was tested with different samples. The analysis of a river water sample spiked with anatoxin-a and a cyanobacteria algae naturally contaminated with anatoxin-a are shown in Fig. 3(A and B), respectively. In both cases no additional peaks associated with the presence of interferences were found. Method was also applied to a fish sample spiked with anatoxin-a and good efficiency was obtained despite the high complexity of the matrix. Resultant chromatogram is shown in Fig. 3(C).

3.4. SPE and SPME comparison

A comparison between both SPE and SPME to be applied for sample preparation of the anatoxin-a has been carried out taking into account the main parameters affecting to their performance (Table 3). Regarding to sensitivity when fluorescence detection

Table 3
SPE vs. SPME

	LOD (ng)	LOD (ng/L)	LOQ (ng)	LOQ (ng/L)	RSD (%)	Extracted sample amount (mL)
SPE	0.24	24	0.70	70	0.6–5.7	10
SPME	0.47	290	1.70	1050	11.5–13.9	1.6

Comparison was established for the analysis of a river water sample.

is used, both techniques showed comparable LOD in terms of mass of anatoxin-a present in the sample. However, SPE offered lower LOD in terms of concentration due to that for SPE treatment higher sample volumes are required. This last fact can be an important advantage in SPME when high sample amount are not available. SPME has shown poor precision, although values were obtained within the accepted range in SPME (<15%). In terms of time per sample SPE has resulted in a tedious and time-consuming technique in contrast to SPME approach. Whereas 4 h are required for SPE pre-treatment less than 1 h are consumed for SPME which allowed integrating extraction, purification and concentration in a single step, therefore simplifying the analytical protocol and the required materials. SPME also reduces sample manipulation avoiding the risk of contamination. On the other hand, SPE includes several evaporation steps which can give rise to analyte losses. When solid matrices were treated with SPME, previous extraction was required as for SPE, therefore increasing the time for analysis.

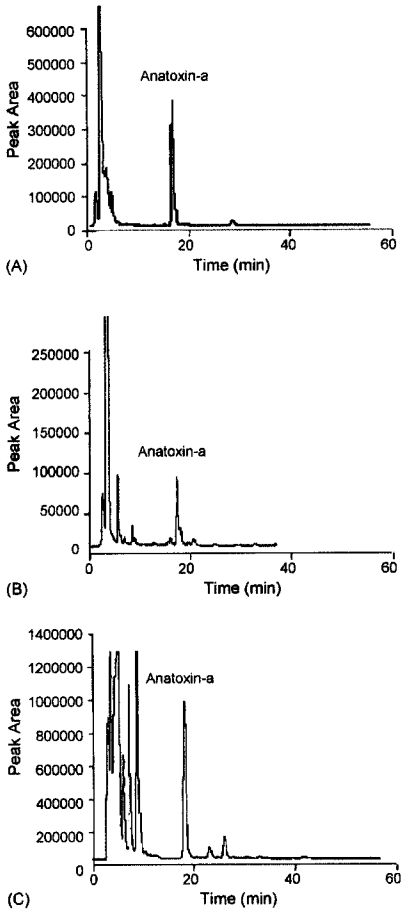

Fig. 3. Examples of the application of the SPME method using PDMS/DVB, 60 μm fiber with on-fiber derivatization for the HPLC-FL analysis of anatoxin-a. (A) River water sample spiked with 1000 ng of anatoxin-a. (B) Cyanobacterial algae naturally contaminated with anatoxin-a. (C) Fish sample spiked with 2 μg of anatoxin-a. HPLC-FL conditions: static desorption (60:40, acetonitrile:water, 12 min); 5 μm Luna C18 column (250 mm × 4.6 mm); mobile phase, acetonitrile–water (50:50, v/v); flow rate, 0.8 mL/min; λ_{ex} = 470 nm, λ_{em} = 530 nm.

SPME can result a cost-saving methodology regarding the cost of the fibers and cartridges and considering that a SPME fiber can be used in approximately a hundred of extractions while a SPE cartridge is for an only use. Approximately, cost per sample for SPE is four times higher than for SPME. However, the necessity of an interface to coupling SPME to HPLC and the fragility of the fibers could mean a disadvantage necessary to be taken into account.

4. Conclusions

HPLC with fluorescence detection using previous derivatization with NBD-F resulted in a very sensitive technique for this particular application. Weak cation exchange solid phase extraction step coupled to HPLC-FL determination was applied to the analysis of different type of samples. A high degree of sensitivity was achieved and appropriate recoveries were obtained for all studied matrices. High extraction efficiency was also obtained for both water and cyanobacteria samples. However, high background noise was obtained in mussels and fish matrices due to the presence of interferences.

SPME has been presented as an alternative for the HPLC-FL analysis of anatoxin-a. Achieved sensitivity with this method in terms of mass of anatoxin-a in sample was comparable to that obtained with the SPE–HPLC-FL method, however in terms of concentration poor limits were obtained for SPME because lower sample volumes are extracted. Poorer precision was obtained for SPME in comparison with SPE.

Regarding to interferences removal, improved chromatograms were obtained using SPME for more complex matrices. Thus, SPME combined with on-fiber derivatization with NBD-F and fluorescence detection has contributed to increase the selectivity for the HPLC determination of anatoxin-a.

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submitted

ANATOXIN-A OCCURRENCE IN CYANOBACTERIA STRAINS ISOLATED FROM PORTUGUESE FRESH WATER SYSTEMS

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Ana Gago[3]
Vitor Vasconcelos[1,2]

INTRODUCTION

Aquatic cyanobacterial blooms have been described in freshwater habitats worldwide. Cyanobacteria may produce several types of toxins (cyanotoxins) including neurotoxins such as anatoxin-a which is a potent neurotoxin produced by several genera (*i.e. Anabaena, Aphanizomenon, Microcystis, Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium* and *Oscillatoria* (Bumke-Vogt *et al.*, 1999; Park *et al.*, 1993; Viaggiu *et al.*, 2004; Namikoshi *et al.*, 2003; Ballot *et al.*, 2005; Gugger *et al.*, 2005; Araóz *et al.*, 2005)).

International scientific research has described the widespread occurrence and distribution of anatoxin-a in many geographic regions as, for example Canada (Gorham *et al.*, 1964), Scotland (Stevens and Krieger, 1988), Japan (Park *et al.*, 1993) Kenya (Ballot *et al.*, 2005) and Italy (Viaggiu *et al.*, 2004). The harmful effects of anatoxin-a have been evidenced by several animal fatalities as cattle and dogs in USA and Finland (Juday *et al.*, 1981; Sivonen *et al.*, 1989, 1990; Edwards *et al.*, 1992). This has called attention for the putative public health risk that might be associated with this neurotoxin. Although anatoxin-a is not the most frequent cyanotoxin worldwide, it is a potent neurotoxin that

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should be regarded as a health risk fatal to terrestrial and aquatic organisms. Moreover the occurrences of anatoxin-a might be negatively biased by the lack of surveys about this neurotoxin reinforcing the need to study of its occurrence.

Portuguese scientific evidences show that potential anatoxin-a producing cyanobacteria occur in Portuguese water bodies (Ferreira *et al.*, 2001; Pereira *et al.*, 2000; Vasconcelos, 1994) but never refer any occurrence of anatoxin-a. Our results are the first evidence that anatoxin-a may occurr in Portuguese freshwater. Although it was not found anatoxin-a in natural water bodies, several Portuguese cyanobacterial strains were capable to produce anatoxin-a after cultivation in laboratory.

B. MATERIALS AND METHODS

The presence of anatoxin-a in Portuguese fresh water bodies was evaluated in sites used for recreational and/or production of drinking water (Fig. 1).

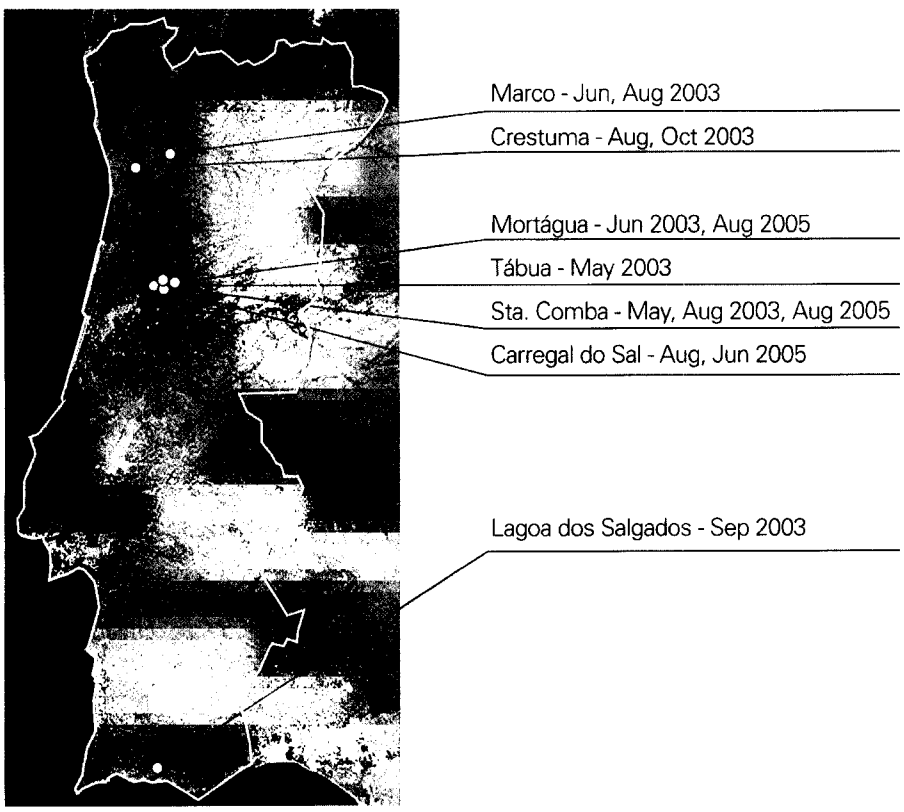

Figure 1 Location and date of sampling sites of anatoxin-a producing cyanobacteria in Portugal.

Sites that presented cyanobacteria were selected. Surface water samples were collected and immediately transported to the laboratory where pH was measured assuring values ≤ 7 (anatoxin-a degrades in pH above 9). Samples were then preserved at –22 °C

until solid phase extraction (SPE).

The production of anatoxin-a by Portuguese cyanobacteria from various fresh water bodies, was evaluated in 22 isolated strains cultivated in laboratory. Alive phytoplankton samples from various water bodies were taken for isolation of the cyanobacterial strains. We tried to isolate several strains from each sample. The isolation of one cyanobacterial trichome was achieved under optical microscope. Each trichome was washed in sterile Z8 culture medium (Kotai, 1972) prior to its inoculation in polystyrene tubes with 10 ml of sterile Z8 medium. Success of the isolation was confirmed after 1-2 months and the monocultures were then bulked up in 6 litre containers aerated with filtered air (0.2 µm) at 25±1 °C, light intensity of 10 $\mu mol \cdot m^2 \cdot s^{-1}$ (14:10 light/dark cycle). After reaching exponential phase of growth, cyanobacterial cells were collected either by centrifugation or filtration with a plankton net (mesh of 40 µm) and immediately freeze-dried.

Defrosted water samples were ultrasonicated to assure the breakage of the cyanobacterial cells and liberation of anatoxin-a. Finally, cell debris and particles were removed by filtration trough membrane glass fiber filters before SPE.

Freeze-dried strains of cyanobacteria and water samples were extracted following the method initially described by James *et al.* (1998). The obtained extracts were evaporated at 40 °C under N_2 stream and were re-dissolved in water (5 ml) prior to SPE. These extracts were adjusted to pH 7 with NaOH 0.1M before SPE. SPE was carried using weak cation exchange (Supelclean LC-WCX, 3 ml tubes Supelco). The cartridge was conditioned with 6 ml of methanol and 6 ml of water. The samples were loaded into the cartridge and then 3 ml of methanol–water (1:1) were used in the washing step for water samples, whereas 3 ml methanol 100% were used for the cell extracts, due to the high level of organic impurities present in these matrices. After air-drying the cartridges for 30 min, the anatoxin-a was eluted using 10 ml of methanol containing 0.2% trifluoroacetic acid.Then the solvent was evaporated under nitrogen stream at 40 °C and the extracts thus obtained were re-dissolved in methanol and kept at -22 °C in amber vials until analyses by HPLC-FLD.

Before HPLC-FLD, the SPE extracts were evaporated under nitrogen stream and then reconstituted with 100 µL of sodium borate 0.1 M (pH=10). Derivatization was done by adding 50 µL of NBD-F (4-Fluoro-7-nitro-2,1,3-benzoxadiazole) (1 mg/ml in acetonitrile) to the vial that were kept in dark for 10 min at room temperature. Finally, 50 µL of 1M hydrochloric acid were added to stop reaction and 20 µL of the derivative were injected to perform the HPLC-FL analysis. The HPLC system consisted of a quaternary pump (Agilent 1100 Series QuatPump), a degasser (Agilent 1100 Series Degasser) and a fluorescence detector (Agilent 1100 FLD). Separations were carried out using a LC reversed phase, Luna C18 column (5 mm, 250–4.6 mm i.d., Phenomenex) at room temperature. The mobile phase was acetonitrile-water (50:50) with a flow rate of 0.8 ml/min. The excitation and emission wavelengths for fluorimetric detection were set at 470 and 530 nm respectively. For system calibration, standard solutions containing anatoxin-a fumarate (Tocris, Bristol, UK) in the range 1-20 µg/ml were prepared in ultrapure water. 10 µL of each standard so-

lution were derivatized with NBD-F following the procedure described before. 20 µl of each derivative were injected into the instrument, resulting calibration standards ranging from 1 to 20 ng anatoxin-a fumarate injected on column.

C. RESULTS AND DISCUSSION

A correlation coefficient of R^2=0.9992 was obtained for the calibration line (Y(area)=2.8947x(concentration)–0.7173). Concentration of anatoxin-a was corrected for extraction recovery (84 and 85% for cyanobacterial and water samples respectively). The fact of using anatoxin-a fumarate instead of pure anatoxin-a was also taken into account for quantitative results.

Detection limit was estimated to be 0.02 ng of anatoxin-a injected on column, which corresponds to method detection limits of 25 ng.l^{-1} for water and of 3 ng.g^{-1} for cyanobacterial samples.

Anatoxin-a detection in water samples was always negative but some cyanobacterial isolated strains produced anatoxin-a under laboratory conditions (Table 1).

Table 1 Quantification of anatoxin-a by HPLC-FL in Portuguese cyanobacterial strains. LOD was 3 ng per gram of lyophilized cells.

Taxa	Strain code	Origin	Date	Anatoxin-a concentration (µg.g^{-1} dw)	RSD (%)
Anabaena sp.	LEANJ.14	B. Maranhão (Clube Náutico)	7-Jul-2000	0.06	8.62
Microcystis sp.	LEANJ.11	B. Maranhão (Clube Náutico)	7-Jul-2000	0.12	2.08
Anabaena sp.	LEANJ.12	B. Maranhão (Clube Náutico)	7-Jul-2000	0.14	3.01
Anabaena sp.	LEANJ.37	Chaves espelho de água	9-Jul-2000	0.16	4.43
Anabaena sp.	LEANJ.83	Marco (near water treatment station)	13-Jul-2004	0.36	3.81
Oscillatoria sp.	LEANJ.69	Lagoa dos Salgados	26-Sep-2003	0.53	5.68
Aphahnizomenon sp.	LEANJ.50	Marco (near water treatment station)	31-Aug-2000	1.30	4.93
Aphanizomenon sp.	LEANJ.29	Tábua	Jul-2000	1.39	6.14
Oscillatoria limnetica	LEANJ.7	Mortágua (inlet of water treatment station)	18-Jul-2000	1.50	6.43
Anabaena sp.	LEANJ.18	B. Maranhão Benavila Pontes	7-Jul-2000	2.14	1.06
Anabaena sp.	LEANJ.20	B. Maranhão Benavila Pontes	7-Jul-2000	21.29	0.09
Anabaena sp.	LEANJ.46	B. Maranhão Benavila Pontes	7-Jul-2000	21.90	3.15
Aphanizomenon flos-aquae	LEANJ.81	Marco (near water treatment station)	26-Jun-2005	24.62	1.26

The fact that the majority of cyanobacterial strains were isolated from different sampling sites of the water samples does not permit to conclude about the natural concentration of total anatoxin-a at the time of sampling. Two exceptions were the water samples from L. dos Salgados in september-2003 and Marco in august-2000. In spite of the negative results for anatoxin-a in these two water bodies, they were the origin of two anatoxin-a producing strains (LEANJ50 and LEANJ69). This indicates that the strains in natural habitat did not have the proper environmental conditions to produce detectable amounts of total toxin in natural environment. The explanation could be either because of low cell densities or because of physiological condition of the cells.

According to the few studies about anatoxin-a production and environmental conditions, the optimal temperature for anatoxin-a production either in laboratory or in natural samples is between 19.8 and 22 °C and the maximum concentrations of total anatoxin-a occurred at the first two to three weeks of culture, corresponding to the exponential phase (Rapala *et al.*, 1993; Rapala and Sivonen, 1998). Because data about physical parameters and phytoplankton density at the time of sampling at L. dos Salgados (september, 2003) and Marco (august, 2000) were not registered, we can not know about what determined the absence of anatoxin-a in the water. The factors that influence anatoxin-a production by cyanobacteria are not established yet. The same happens for other cyanotoxins in spite of several scientific efforts. There are only a few studies about the production of anatoxin-a where the complexness of anatoxin-a production is evident, even in the same genera no patterns could be established. Production of anatoxin-a appears to be strain specific and varies much with environmental factors such as light, temperature and nitrogen source (Peary and Gorham, 1966; Kiviranta *et al.*, 1991; Rapala *et al.*, 1993; Gallon *et al.*, 1994; Bumke-Vogt *et al.*, 1996; Rapala and Sivonen, 1998; Bumke-Vogt et al., 1999). Understanding these issues would be very helpful in the prediction of toxic cyanobacterial blooms. Cyanobacterial researchers should put more effort in the study of anatoxin-a biosynthesis to determine in what conditions the toxin production is maximized. Gallon and colleagues (1990; 1994) and Hemscheidt (1995) studied the anatoxin-a bio-pathway but this issue is still far from being comprehended.

Anatoxin-a production was found in 13 strains (59.1 % of the analysed strains) with different geographic origins from north and centre Portugal (Table 1). Figure 2 (next page) shows the chromatograms obtained for some of the positive strains: LEANJ20, LEANJ46, LEANJ81, LEANJ7 and LEANJ29. The amounts of anatoxin-a, varied between 24.62 $\mu g.g^{-1}$ dw and 0.06 $\mu g.g^{-1}$dw. Comparing to the Finish *Anabaena sp.* strain that produces 967 $\mu g.g^{-1}$ dw (Osswald *et al.*, 2007), these values are low. Because other studies evidenced that anatoxin-a production by the same strain can vary, it has to be considered that the amounts produced by Portuguese strains detected herein could also vary in nature in a way reaching toxic levels for wildlife and humans. It would be rather interesting and also needed to study the physical conditions that could maximize the anatoxin-a production by the Portuguese producing strains found herein.

Portuguese anatoxin-a producing genera (Table 1) include *Anabaena* (8 strains), *Apha-*

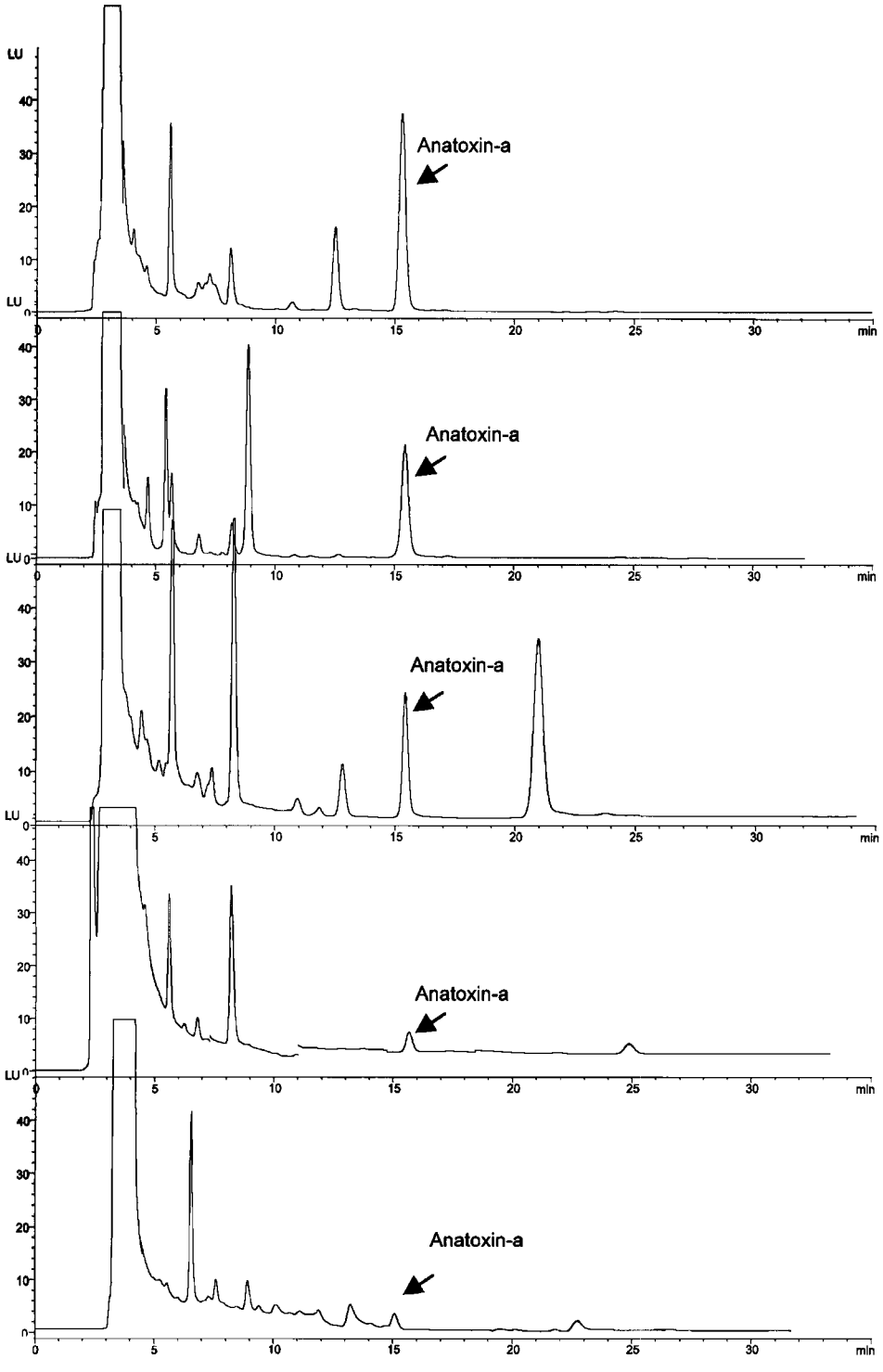

Figure 2 Chromatograms corresponding to the SPE-HPLC–FLD analysis of some of the anatoxin-a producing cyanobacterial strains from Portuguese freshwaters. From top to bottom: LEANJ20, LEANJ46, LEANJ81, LEANJ7 and LEANJ29.

nizomenon (8 strains), *Microcystis* (1 strain) and *Oscillatoria* (2 strains). *Aphanizomenon flos-aquae*, strain LEANJ.81 from Marco (near water treatment station) was the most toxic.

The high proportion found in the analysed strains of anatoxin-a producers (59.1 %) but in low concentration, may indicate that the production of this alkaloid in cyanobacteria is quite common. This data is in accordance with the situation found in Germany by Bumke-Vogt *et al.* (1999) where 22% of the natural water samples were positive for anatoxin-a but always in low concentrations (intracellular median concentration was 0.037 $\mu g.l^{-1}$) comparing to microcystins. These data supports the idea that anatoxin-a is a frequent metabolite of cyanobacteria, as happens with other alkaloids in plants (Herbert, 2003) but due to environmental conditions and to its chemical instability, usually it is not found in high concentration in nature. Nevertheless if conditions are appropriate, anatoxin-a can reach high concentrations enough to causes health and environmental concerns. In Finland anatoxin-a levels of 12 to 4360 $\mu g.g^{-1}$ dw were found in total bloom material (Sivonen *et al.*, 1989, 1990).

This work is a strong evidence on the ubiquosity of anatoxin-a produced by cyanobacteria in Portuguese freshwaters.

D. ACKNOWLEDGMENTS

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submitted

UPTAKE AND DEPURATION OF ANATOXIN-A BY THE MUSSEL *M. GALLOPROVINCIALIS* UNDER LABORATORY CONDITIONS

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ABSTRACT

Cyanobacterial blooms tend to be more common in warm and nutrient-enriched waters and are increasing in many aquatic water bodies due to eutrophication. The aim of this work is to address the accumulation and depuration of anatoxin-a by *Mytilus galloprovincialis*, which is a widespread mussel in estuarine and coastal waters and has been worldwide recognized as bioindicator (*e.g.* Mussel Watch programs). Besides the ecological concerns about the effect and distribution of anatoxin-a in *M. galloprovincialis*, it should be also considered the risk of human intoxications due to the consumption of these bivalves. The understanding on the way *M. galloprovincialis* accumulates and depurates anatoxin-a from a toxic cyanobacterial bloom is one of the important aims of this work. A toxic bloom was simulated in an aquarium with $5x10^5$ cell.ml^{-1} of an anatoxin-a producing strain of *Anabaena sp.* (ANA 37). Mussels were exposed for 15 days and then 15 days of depuration were followed. Three or more animals were sampled every 24 h for total toxin quantification and distribution in soft tissues (edible parts). Water samples were also taken every 24 h in order to follow dissolved and particulate anatoxin-a concentrations. Anatoxin-a quantifications were carried out by HPLC with fluorescence detection. Data have shown that anatoxin-a is probably actively detoxified and it is mostly distributed in the mussels and digestive tract. One day after beginning of depuration, toxin could not be detected in the animals.

This was the first study describing the dynamics of the intake and liberation of the cyanobacterial neurotoxin, anatoxin-a, by a filter feeder, namely the bivalve *Mytilus galloprovincialis*.

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1. INTRODUCTION

Aquatic cyanobacteria can dominate fresh and brackish water systems due to certain physiological characteristics: minimal need of nutrients and capacity to form gas vacuoles. Cyanobacterial blooms tend to be more common in warm and nutrient-enriched waters and are increasing in many aquatic water bodies along with eutrophication (Chorus, 2001). When high concentrations of these photosynthetic organisms occur (denominated cyanobacterial bloom), water may show alterations of odour and colour (green and bluish colour, scum formation) with effects on its organoleptic properties. Besides these consequences, cyanobacteria can produce toxic metabolites - cyanotoxins - that pose environmental and public health concerns.

Cyanobacterial toxins may be of different types (neurotoxins, hepatotoxins, cytotoxins and irritants and gastrointestinal toxins (Codd *et al.*, 2005) and were already responsible for several human and animal poisoning including deaths (Falconer, 2005). Anatoxin-a was the first cyanotoxin to be chemically characterized (Devlin *et al.*, 1977). It is an alkaloid and a potent neurotoxin (mice LD_{50} of 250 $\mu g.kg^{-1}$ *i.p.* (Rogers *et al.* 2005)), which can be produced by several cyanobacterial genera: *Anabaena, Aphanizomenon, Microcystis, Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium* and *Oscillatoria* (Park *et al.*, 1993; Bumke-Vogt *et al.*, 1999; Namikoshi, *et al.*, 2003; Viaggiu, *et al.*, 2004; Ballot *et al.*, 2005; Gugger *et al.*, 2005; Araóz *et al.*, 2005).

As some other cyanotoxins, anatoxin-a has been reported mainly in freshwaters but also in brackish waters (Mazur *et al.*, 2003). Although this toxin is very toxic, it has received less scientific attention comparing to other cyanotoxins such as microcystin and cylindrospermopsin, which have already caused serious human intoxications including deaths in Brazil (Carmichael *et al.*, 2001; Byth, 1980). This is probably due to its chemical characteristics that make it very unstable and labile in the water turning it difficult to detect and to study (Stevens and Krieger, 1991). Because no chronic effects have been associated with anatoxin-a, human health aspects of this toxin have been disregarded. Nevertheless, some animal fatalities have occurred, pointing out the need to investigate anatoxin-a effects on aquatic organisms and communities. In another study (Osswald *et al.*, 2007), we found that anatoxin-a may be bioaccumulated by carps in significant levels (0.768 µg of anatoxin-a *per* gram of dry weight of carp). Whether this could alter aquatic food webs and communities, it is not yet known.

In this experiment, we wanted to address the accumulation and depuration of anatoxin-a by *M. galloprovincialis*, which is a very widespread mussel in estuarine and coastal waters and has been worldwide recognized as bioindicator (*e.g.* Mussel Watch programs) (Izquierdo *et al.*, 2003; Catsiki and Florou, 2006). This mussel is an important component of estuarine and marine food webs and because it is a sessile filter feeder, it may be exposed to high density of cyanobacteria and their toxins. Several studies with cyanotoxins have shown that filter feeders are able to accumulate cyanotoxins as microcystin-LR (Pires *et al.*, 2004), paralytic shellfish toxins (Pereira *et al.*, 2004), nodularin (Karlsson, 2003) and

cylindrospermopsin (Saker *et al.*, 2004). This later toxin is also an alkaloid as anatoxin-a but it is very stable. As far as we know there is no scientific literature about the effects of anatoxin-a in mussels. Besides the ecological concerns about the effect and distribution of anatoxin-a in *M. galloprovincialis*, we may also consider the risk of human intoxications due to the consumption of these bivalves or others as happens with toxins from dinoflagelates. Adding to dinoflagelates and diatoms, responsible health authorities should also be aware of cyanobacteria as toxin producers and agents of intoxication.

In this work, we wanted to understand how *M. galloprovincialis* accumulates and depurates anatoxin-a from a putative toxic cyanobacterial bloom. A toxic bloom was simulated in an aquarium with 10^5 cell.ml^{-1} of an anatoxin-a producing strain of *Anabaena* sp. and the mussels.

2. MATERIAL AND METHODS

2.1. CYANOBACTERIAL CULTURE AND PREPARATION OF INOCULUMS

To simulate the toxic bloom of cyanobacteria, an anatoxin-a producing strain was cultivated in batch cultures. The cyanobacteria *Anabaena* sp. (strain ANA 37) was isolated from the Baltic Sea by Professor Kaarina Sivonen from the University of Helsinki (Dpt. of Applied Chemistry and Microbiology) who kindly supplied us one culture of that organism in solid medium (Sivonen *et al.*, 1989). In our laboratory, we cultivated ANA 37 in liquid Z8 media (Kotai, 1972) in aerated batch cultures (monocyanobacterial, non-axenic) (20±1 °C, photoperiod of 14 h light – PAR of 10 µmol.m^{-2}.s^{-1}). After 1 month of growth (in exponential phase of growth), the number of cells *per* ml, of lugol fixed samples of the culture, was determined in a Sedgewick-rafter counting cell. In order to obtain number of cells *per* counting transect and to convert it to number of cells *per* ml, total length of trichomes was measured and divided by average cell diameter (5.68 µm±0.67, n=81) (Lawton *et al.*, 1999). Measurements were attained with the aid of software Leica QWin®, version 1, connected to an optical microscope. Based on the counting, a suspension in Z8 of ANA 37 was prepared and kept in the dark at 4 °C. During the experiment, this procedure was achieved 5 times at days 1, 3, 6, 10 and 15.

2.2. EXPERIMENTAL SET UP

Specimens of *M. galloprovincialis* were obtained from a commercial depuration plant: Âncoramar – mariscos Lda. Situated at Viana do Castelo, Portugal. 82 animals were used in this experiment: average valve length was 7.63 ± 0.52 cm and average total fresh weight, with shell, was 33 ±7.05 g. Before the experiment, the molluscs were acclimatized for one week to the experimental conditions: they were all maintained in one glass aquarium with natural seawater (filtered through 0.2 µm), aerated, at constant temperature of 18 °C, photoperiod with 14 h light (PAR 1.9x10^{-6} E.m^{-2}.s^{-1}). Loading was 6.3-7.9 animals *per* litter in accumulation phase and 5.4-9.8 animals *per* litter in depuration phase.

The experiment consisted of two phases:

Accumulation phase (I) – during 15 days, exposure of *M. galloprovincialis* to live cells of the cyanobacterial strain ANA 37 ($3.27x10^5$ cell.ml^{-1} ± $1.63x10^5$ cell.ml^{-1}). This density was found to be adequate to this bioassay where we want to simulate natural and ecological relevant concentrations of cyanobacteria (Bartram *et al.*, 1999; Pereira *et al.*, 2004; Briand *et al.*, 2002). During phase I, the sea-water was replaced every 24 h, the volume readjusted according to the number of animals in the tank and new inoculate of cyanobacterial culture was added. In that way, we assured a better simulation of a natural bloom.

Depuration phase (II)- during 15 days, the mussels were maintained in the same conditions as in the phase I but without cyanobacteria.

2.3. SAMPLING

WATER

During phase I, the water from the aquariums was sub-sampled every 24 hours immediately before and after adding new inoccula of cyanobacterial cells.

A volume of 100 ml of total water was filtered through GF/C filters (Whatman®) and frozen (-22 °C) - for quantification of dissolved anatoxin-a. The corresponding GF/C filters were frozen to quantify anatoxin-a in suspended matter. 10 ml of water were fixed with lugol solution to quantify cell concentration and thus to determine filtering rate.

During phase II, sampling of the total water was carried only before changing the water daily and because expected anatoxin-a concentration was lower than in phase I, 250 ml were sampled instead of 100 ml. The filtered water (GF/C), as well as its respective filter, was kept frozen until anatoxin-a extraction.

MUSSELS

Mussels were collected from the tank every 24 hours for anatoxin-a quantification, maximum shell length and weight were recorded and after freezing, animals were freeze-dried and kept frozen until further analysis. At the day 10 of the accumulation phase, 10 mussels were dissected, being separated the foot plus other muscles, gills, digestive tract plus heart and mantle plus rest, for toxin quantification.

2.4. ANATOXIN-A EXTRACTION AND PURIFICATION

All procedures of extraction and purification of anatoxin-a described below were based on a method described by Piñeiro *et al.* (2006).

Filtrated water samples, previously adjusted to pH 7, were extracted by Solid phase extraction (SPE) using weak cation exchange (WCX) in a vacuum manifold of Millipore (Waters™, Sep-Pak®). Supelco's Supelclean LC-WCX cartridges (3 ml) were conditioned with 6 ml of methanol followed by 6 ml of ultra-pure water (Milli-Q®, Millipore). Samples were then loaded into the cartridge and washed with methanol-water (1:1, 3 ml). After air-drying the cartridges, the anatoxin-a was eluted with 10 ml of methanol (0.2% TFA). The solvent of the eluates was removed by evaporation in a sample concentrator with nitrogen

flux (Tecnhne Dri-block® DB.3) at 40 °C. Extracts thus obtained were reconstituted in methanol and kept at -22 °C in amber vials until HPLC/FL analysis.

Lyophilized mussels and dissected parts were weighed in order to register dry weight. After grounding, they were triturated and homogenized with an Ultraturrax homogenizer for 5 min with 10 ml of methanol (1% HCl, 1 M). The suspension was then sonicated with an ultrasonic processor for 2 min at *ca.* 80 amplitude (Sonics Materials, Vibra Cell 50). Finally the suspension was clarified by centrifugation at 3,000 g for 10 min. This procedure was repeated twice and the three supernatants were pooled together before evaporation and dissolution in 5 ml of ultra pure water (pH=7). The aqueous extracts were adjusted to pH 7 and then subsequently purified by SPE as described before for water samples. Washing step was done with 3 ml of 100% methanol instead of methanol-water (1:1) due to higher complexity of the matrix of these samples comparing to the water ones.

2.5. HPLC-FL ANALYSES

SPE extracts were evaporated under nitrogen stream and then reconstituted with 100 µl of sodium borate 0,1 M (pH=10). 50 µl of NBD-F (1 $mg.ml^{-1}$ in acetonitrile) was added and the vial was put aside in the dark for 10 min at room temperature for the derivatization of the anatoxin-a to take place. Hydrochloric acid (50 µl, 1M) was added to finish reaction. 20 µl of the derivative were injected in the HPLC system to perform the analysis (James *et al.*, 1998). The instrument consisted of a HPLC-FL a Lachrom by Merck Hittachi (Interface D-7000, Fl detector L-7480, Auto sampler L-7200 and Pump L-7100) with a LC reversed phase, Luna C_{18} column (5 µm, 250 mm x 4.6 mm i.d., Phenomenex®). Analysis was carried out at room temperature; the mobile phase was acetonitrile-water (50:50) with a flow rate of 0.8 $ml.min^{-1}$. The excitation and emission wavelengths for fluorimetric detection were set at 470 and 530 nm respectively.

3. RESULTS

There were no significant behavioural alterations between the two phases of the experiment, except that pseudo faeces production decreased markedly during the last 8 days of the experiment. This was expected since the last 15 days corresponded to depuration phase (Phase II) when there was no food supplied to the aquarium. At the tested cell density, no deaths were observed during the 30 days of the experiment and there was no evidence of any adverse effects on *M. galloprovencialis* subjected to anatoxin-a producing cyanobacteria.

Evolution of ingestion of toxic cyanobacteria by *M. galloprovincialis* in phase I is shown in Fig.1.

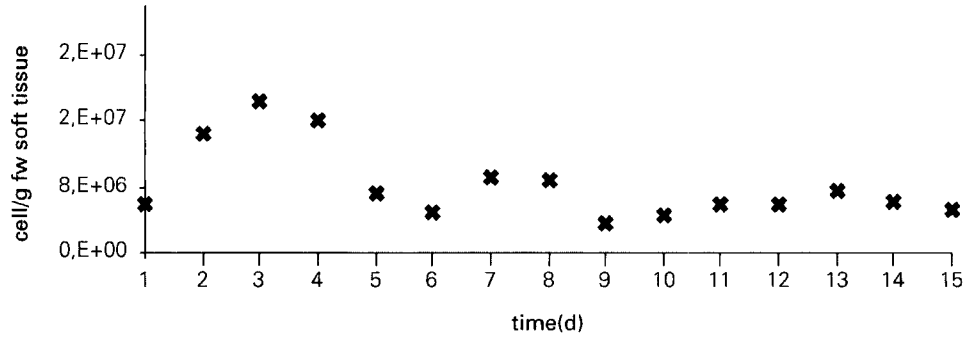

Fig. 1 Evolution of cyanobacterial cell ingestion by *M. galloprovincialis.* Removed cells at 24 hours interval during the 15 days of accumulation phase.

The ingestion rate reached its maximum at the 3rd day of Phase I ($2x10^7$ cell.g^{-1} f.w. *per* day) then decreased reaching a dynamic equilibrium with an average of $7x10^6$ cell.g^{-1} f.w. soft tissue *per* day (Fig. 1). Daily clearance rate (100(removed cells)/added cells) of the cells was always 100%. Anatoxin-a concentration in the particulate matter (GF/C filters) was always under the lower limit of detection (0.96 ng.l^{-1}).

Dissolved anatoxin-a in water was monitored during both phases. Phases I and II are represented in Fig.2 and table I, respectively.

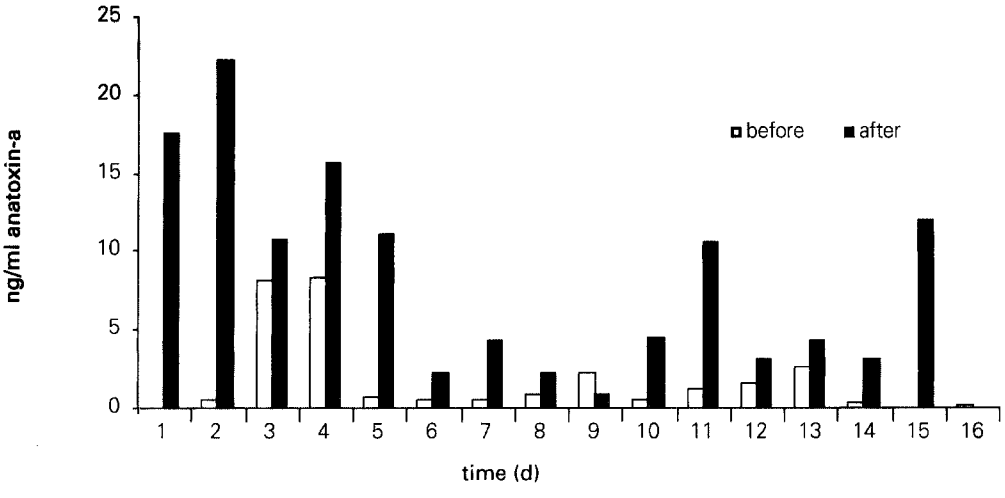

Fig. 2 Dissolved anatoxin-a (ng.ml^{-1}) in the aquarium during phase I. "Before" and "after" corresponds to samples taken immediately before and after inoculation of cyanobacteria every 24 hours, along the 15 days of accumulation. Time is given in days after beginning of phase I. New inoculate suspension was prepared on days 1, 3, 6, 10 and 15.

Table I
Dissolved anatoxin-a in the aquarium during phase II. Inferior limit of detection was $9.6x10^{-4}$ ng.ml^{-1}. nd - no data due to technical problems or no sampling session. Time is given in days after beginning of phase I.

Time (d)	Dissolved antx-a (ng/ml)
17	0.053
18	0.043
19	0.143
20	undetectable
21	nd
22	0.042
23	nd
24	nd
25	undetectable
26	undetectable
27	nd
28	undetectable
29	nd.

Because different ANA 37 inoccula had to be used throughout the experiment, there are fluctuations of dissolved anatoxin-a that can be observed in Fig. 2. During the accumulation phase, concentration of dissolved anatoxin-a expresses the variation of anatoxin-a concentration provided by the different inoccula. During the depuration, very low amounts of anatoxin-a were found in the water (table I).

Anatoxin-a concentration in the soft tissue of mussels and corresponding accumulated cells of ANA 37 are represented in Fig. 3.

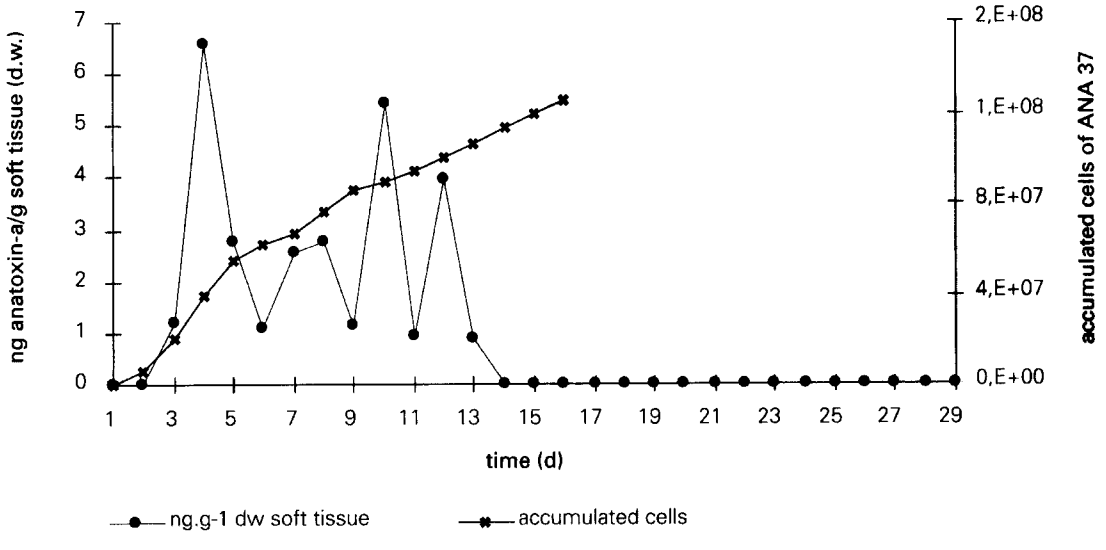

Fig. 3 Detected anatoxin-a concentration in the soft tissue of mussels and corresponding accumulated cells of ANA 37. The last adding of ANA 37 inoculate occurred on day 15.

This graph demonstrates that anatoxin-a level in the mussels did not increase steadily until a maximum value along the accumulation period as happened with other toxins in other bivalves. On the contrary, it is observed a dynamic decreasing of anatoxin-a con-

centration in the soft tissue of *M. galloprovencialis* with a maximum concentration of 6.6 ng of anatoxin-a *per* g (d.w.) at day 4, after day 3 when the maximum of cell removal was observed (Fig. 1). This rhythmic oscillation of anatoxin-a concentration in soft tissue, follows the variation of removed anatoxin-a during phase I (Fig. 4), except for the last days when mussels did not accumulate any toxin.

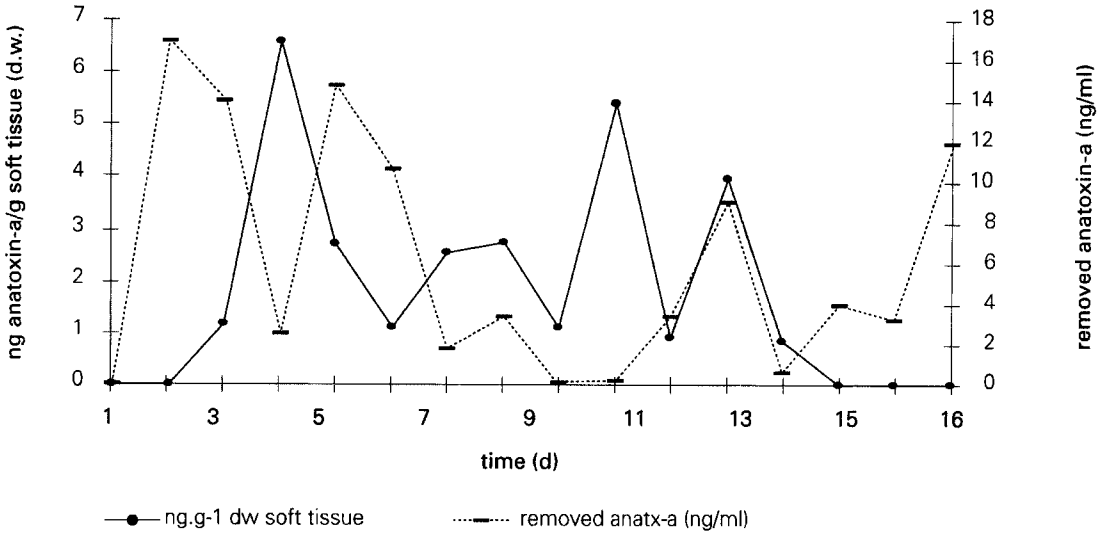

Fig. 4 Detected anatoxin-a concentration in the soft tissue of mussels and corresponding removed anatoxin-a from water.

The fact that anatoxin-a concentration in the soft tissues did not follow the same profile of the number of ingested cells (Fig. 3) might be explained by degradation of the toxin through a biochemical process undertaken by the mussels or by the short half-life of anatoxin-a due to its chemical instability, photodegradation and microbial degradation.

The distribution of the toxin in the soft tissue (edible parts) shown that half of the toxin (53% of total) is accumulated in the digestive tract and heart and the other half is distributed mostly in foot, muscles and gills (Fig. 5).

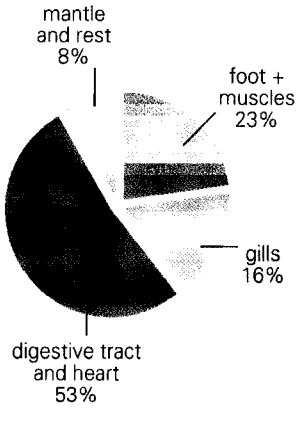

Fig. 5 Concentration of anatoxin-a and its distribution in soft tissue of *M. galloprovincialis* at day 10 of the accumulation experiment.

4. DISCUSSION

No death occurred during the accumulation and depuration experiments, showing that mussels are very resistant to cyanotoxins, being good toxins vectors, as shown in other previous experiments using other cyanotoxins (Vasconcelos, 1995; Amorim and Vasconcelos, 1999).

The fact that 100% of the cells were filtered by the mussels everyday demonstrates that maybe the mussels could have filtered more cyanobacteria and probably would have accumulated higher concentration of anatoxin-a, if a higher cell concentration was provided. In other experiments with cyanobacteria and *M. galloprovincialis* (Vasconcelos, 1995; Amorim and Vasconcelos, 1999) no clearance rate was determined that would permit us to compare with the present values.

Anatoxin-a level in the mussels did not increase steadily until a maximum value along the accumulation period as happened with other toxins in other bivalves such as PSP in *Hiatula dophos* L. (Chou *et al.*, 2005), PST in *Anodonta cygnea* (Pereira *et al.*, 2004) and Microcystin-LR in *Unio douglasiae* (Yokoyama and Park, 2003) and *Mytilus galloprovincialis* (Vasconcelos, 1995). This might be explained by degradation of the toxin through a biochemical process undertaken by the mussels or by the short half-life of anatoxin-a due to its chemical instability, photodegradation and microbial degradation (Kiviranta *et al.*, 1991; Stevens and Krieger, 1991; James *et al.*, 1998; Rapala *et al.*, 1994; Smith and Sutton, 1993). Further investigations should be considered in order to know if there is any detoxication physiological process of this neurotoxic alkaloid, anatoxin-a, by *M. galloprovincialis*.

The low values of dissolved anatoxin-a observed during our work, could be explained by the anatoxin-a instability in water (Stevens and Krieger, 1991) or due to some physiological process that was actively metabolizing and eliminating the toxin. Similar situation happened with *Anodonta cygnea* and cylindrospermopsin that is also a cyanobacterial alkaloid but more stable than anatoxin-a (Saker *et al.*, 2004). Maybe these two mussels have a similar metabolism for elimination of the two alkaloids, anatoxin-a and cylindrospermopsin.

The maximum concentration of anatoxin-a detected in soft tissue (6.6 ng of anatoxin-a *per* g of soft tissue d.w.) is low when compared to other accumulation experiments of cyanotoxins by bivalves (Negri and Jones, 1995; Vasconcelos, 1995; Yokohama and Park, 2003; Pereira *et al.*, 2004, Pires *et al.*, 2004; Saker *et al.*, 2004), crayfish (Vasconcelos *et al.*, 2001) and fish (Osswald *et al.*, 2007). Our results evidence very low bioaccumulation of anatoxin-a by *M. galloprovencialis* that was rapidly remediate by the depuration process in phase II. During Phase I, accumulation efficiency (total toxin accumulated by the mussels/total dissolved toxin removed from the aquarium) x 100) varied between 0 (at days 2, 14 and 15) and 10.92 (at day 10).

The pattern of anatoxin-a accumulation in the various mussels organs is similar to what happens to other cyanotoxins such as PST (Paralytic Shellfish Toxin) in *Anodonta cygnea,* that accumulated most of the toxin (between more or less 50% and 78%) in the viscera

(Pereira *et al.*, 2004). Other two experiments had more evident results showing the importance of the viscera: Negri and Jones (1995) detected 96% of PSP (Paralytic Shellfish Poisoning) toxin in the viscera of *Alathyria condola* and Vasconcelos (1995) showed that 95% of the microcystin was present in the viscera. Different results were reported by Saker *et al.* (2004) who was the first author to evaluate the toxin content in the haemolymph of *Anodonta cygnea* and found that most of cylindrospermopsin was accumulated in this fluid that accounts for *ca.* 50% of the animal bodyweight. Optimization for the detection of anatoxin-a in the haemolymph is being study in our laboratory.

5. CONCLUSIONS

At ecologically relevant density of an anatoxin-a producer cyanobacteria (10^5 cell·ml^{-1}), *M. galloprincialis* did not show any behaviour alteration and no deaths were registered. Because clearance rates were always near 100%, maybe this mussel is able to filter at higher cell densities and thus to accumulate higher amounts of anatoxin-a. The maximum value of anatoxin-a accumulated by *M. galloprovincialis* (6.6 ng per gram of dry weight) was lower than other toxins. Half of the toxin was accumulated in the digestive tract. After 15 days of an accumulation phase followed by other 15 d of depuration, demonstrated the existence of a dynamic of the toxin that could be explained either by some physiological remediation of anatoxin-a by the mussel and/or due to chemical instability of this alkaloid in water.

Although *M. galloprovincialis* is able to intake anatoxin-a it does not accumulate it in very high rates (maximum accumulation efficiency=10.92%) and depurates it fast. These two aspects highly reduce the possible concerns about health risk due to consumption of the edible parts of *M. galloprovincialis*.

This work left two issues that need further investigation: quantification of accumulation of anatoxin-a by the haemolimph and what is the role of the shells. Although the later are not edible parts, in terms of aquatic ecosystem and interaction with other organisms, namely parasites, this issue should be explored.

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ACUTE EFFECTS OF AN ANATOXIN-A PRODUCING CYANOBACTERIUM ON JUVENILE FISH – *CYPRINUS CARPIO* L.

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Acute effects of an anatoxin-a producing cyanobacterium on juvenile fish—*Cyprinus carpio* L.

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Abstract

The worldwide increase of eutrophication in fresh water bodies has caused cyanobacterial blooms to be more frequent. Anatoxin-a is a potent neurotoxin known to be produced by several genera of cyanobacteria including *Anabaena*. In this work, we exposed juvenile carps to freeze-dried cells of a toxic strain of the cyanobacterium *Anabaena* sp. during a 4-day period. Two different cell density—10^5 and 10^7 cell ml^{-1}—were assayed. Lethality and anatoxin-a concentration in the whole fish were determined. In the higher cell density, all fish died between 26 and 29 h after exposure to toxin, whereas in the 10^5 cell ml^{-1} density no deaths were observed. Levels of anatoxin-a in the whole fish ranged between 0.031 $\mu g\,g^{-1}$ d.w. at the 10^5 cell ml^{-1} concentration and 0.768 $\mu g\,g^{-1}$ d.w. at 10^7 cell ml^{-1}. Minor uptake of anatoxin-a occurred.

Keywords: Cyanobacteria; Anatoxin-a; *Cyprinus carpio*; Lethal effect; Toxin uptake

1. Introduction

Cyanobacteria can produce several potent toxins as secondary metabolites presenting hazard to human and ecosystem health. These organisms can proliferate in freshwater systems forming blooms, dominating freshwater habitats such as rivers, lakes and reservoirs. Toxicity of these blooms has concerned health authorities and the scientific community due to their health risk and ecotoxicological implications. Regarding their mode of action, cyanotoxins are classified into four major groups: neurotoxins, hepatotoxins, cytotoxins and irritants and gastrointestinal toxins (Codd et al., 2005). Among the neurotoxins, anatoxin-a is one of the most common worldwide, being reported in countries such as Canada (Devlin et al., 1977), USA (Stevens and Krieger, 1991), Scotland (Edwards et al., 1992), Germany (Bumke-Vogt et al., 1999) as well as in Africa (Ballot et al., 2005). This dispersion stresses out the need to evaluate its ecotoxicity and human intoxication risk. This neurotoxin is produced by several cyanobacterial genera namely

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Anabaena, *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Raphidiopsis*, *Arthrospira*, *Cylindrospermum*, *Phormidium* and *Oscillatoria* (Park et al., 1993; Bumke-Vogt et al., 1999; Namikoshi et al., 2003; Viaggiu et al., 2004; Ballot et al., 2005; Gugger et al., 2005; Aráos et al., 2005). Anatoxin-a is an alkaloid and it is a low molecular weight (mw = 165) secondary amine (Devlin et al., 1977). It is a postsynaptic depolarizing neuromuscular blocking agent that binds to the nicotinic acetylcholine receptors of the nervous system of animals but it is not hydrolyzed by cholinesterase (Thomas et al., 1993). Acute effects in vertebrates include a rapid paralysis of the peripheral skeletal and respiratory muscles, causing symptoms such as loss of co-ordination, twitching, irregular breathing, tremors, altered gait and convulsions before death by respiratory arrest (McBarron et al., 1975; Carmichael and Gorham, 1978; Stolerman et al., 1992; Fitzgeorge et al., 1994; Rogers et al, 2005). Death can occur from 2 min, with an LD_{50} in mice of 375 μg/kg i.p. (Fitzgeorge et al., 1994) to 10 min, with an LD_{50} of 250 $\mu g\,kg^{-1}$ in mice (i.p.) (Rogers et al., 2005). Because anatoxin-a is very unstable and labile in the water (Stevens and Krieger, 1991) and no chronic effects have been described in mammals, this toxin has been considered of less concern comparing to other cyanotoxins. In this study, we wanted to study the possibility of accumulation of this toxin in fish. The tested species was the common carp—*Cyprinus carpio* that belongs to the largest and most successful family of primary freshwater fish in Eurasian waters as well as Northern American and African (Zardoya and Doadrio, 1999). Their ecological importance and biogeographic distribution in Eurasian waters as well as North America and Africa makes this group of aquatic organisms ecologically representative for the study of cyanotoxins impact in the aquatic environment.

2. Material and methods

2.1. Cyanobacterial culture

The anatoxin-a producing strain of *Anabaena* sp. (ANA 37) isolated from the Baltic Sea was kindly supplied by Professor Kaarina Sivonen (University of Helsinki, Department of Applied Chemistry and Microbiology) (Hemscheidt et al., 1995). This *Anabaena* strain, ANA 37 was cultivated in liquid Z8 media (Kotai, 1972) in aerated batch cultures (monocyanobacterial, non-axenic) (20 ± 1 °C, photoperiod of 14 h light-PAR of 10 $\mu mol\,m^2\,s^{-1}$). At the end of the exponential phase of growth, cells were collected by filtration with a nylon-net of 20 μm mesh. The cells were then washed with distilled water, frozen at −22 °C and freeze dried. An equivalent of biomass per dry weight was calculated after counting a lugol fixed culture with the aid of software Leica QWin®, version 1, connected to an optical microscope.

2.2. Fish and experimental set up

The experimental design was decided upon a preliminary assay done with two concentrations of live cells (1.1×10^6 and 4.4×10^6 cell ml^{-1}), sonicated and intact. These concentrations of *Anabaena* in nature are considered of high risk and may cause adverse health effects in recreational waters (Falconer et al., 1999). This preliminary test demonstrated behavioural alterations in all treatments and at the higher concentration, sonicated cells caused death in 75% of the exposed animals within 24 h. Deleterious effects were more pronounced with the sonicated cyanobacteria compared to the exposure to intact cells. In this present experiment, juvenile common carps—*Cyprinus carpio*—3 months old (0.19 ± 0.1 g and 1.843 ± 0.275 cm) were used. They were obtained from a hatchery of the Faculty of Sciences, University of Porto. The increasing of water temperature up to 19 °C induced the natural egg position. The experiments were carried out in accordance with the current guidelines for the care of laboratory animals and ethical guidelines for investigation in conscious animals set by the General Directorate of Veterinary of Portugal and it was approved by the Bioethical Institute of Portuguese Catholic University. Before the experiment, juvenile carps were pre-acclimatized during one week to the experimental conditions: 20 °C, 16 h light (PAR of 1.9×10^{-6} $\mu mol\,m^{-2}\,s^{-1}$). Six groups of 10 carps were held separately in glass aquariums with 400 ml dechlorinated tap water. Experimental treatments were done in duplicates: two control aquariums with no cyanobacteria, two aquariums with 10^5 cell ml^{-1} (12.1×10^{-3} mg ml^{-1} d.w.) and the other two with 10^7 cell ml^{-1} (1.21 mg ml^{-1} d.w.). These concentrations are environmentally relevant and may occur in natural cyanobacterial blooms, e.g. Portugal, 9.9×10^5 cyanobacterial cells ml^{-1} in 1996 (Pereira et al., 2000); France, 3.3×10^8 cells ml^{-1} in 1999 (Briand et al., 2002). The inoculum was obtained by preparing

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a cell suspension of freeze-dried cells in ultra-pure water. This suspension was kept at 4 °C during the 96 h of the experiment. The water of the aquariums was renovated before re-inoculation of the cyanobacterial suspension every 24 h. Fish were not fed during the 96 h and were observed continuously during the first 8 h; from then after observations were done every 12 h until the end of the experiment (4 d).

2.3. Anatoxin-a extraction and purification

Fish were euthanized by concussion and after death carps were weighted and measured prior to freezing and lyophilisation. The fish from the same aquarium were pooled together and each group was treated as one sample. Following the extraction procedure described by James et al. (1998), freeze dried carps were grounded and homogenized with an Ultraturrax homogenizer for 5 min with 10 ml of methanol (1% HCl, 1 M). The suspension was then sonicated with an ultrasonic processor for 2 min at ca. 80 amplitude (Sonics Materials, Vibra Cell 50). Finally the suspension was clarified by centrifugation at 3000*g* for 10 min. This procedure was repeated twice and the three supernatants were pooled together before evaporation and dissolution in 5 ml of ultra pure water (pH = 7). The aqueous extracts were subsequently purified by solid-phase extraction (SPE) following the procedure described by James et al. (1998). SPE was carried out in a Vacuum Manifold of Millipore (Waters™, Sep-Pak®) using Supelco's Supelclean LC-WCX cartridges (3 ml). The cartridges were conditioned with 6 ml of methanol followed by 6 ml of ultra-pure water (Milli-Q®, Millipore). Samples were then loaded into the cartridge and washed with 3 ml of 100% methanol. After air-drying the cartridges for 30 min, the anatoxin-a was eluted with 10 ml of acidified methanol (0.2% TFA). The solvent of the eluate was removed by evaporation in a sample concentrator with nitrogen flux (Tecnhne Dri-block® DB.3) at 40 °C. Extracts thus obtained were reconstituted in methanol and kept at −22 °C in amber vials until HPLC analysis. The extraction of anatoxin-a from 22.3 mg of freeze-dried cells of ANA37 was carried out in a similar way as the carps.

2.4. HPLC analyses

Carps and cyanobacterial SPE extracts were evaporated under nitrogen stream and then reconstituted with 100 µl of sodium borate 0.1 M (pH = 10). Fifty µl of NBD-F (1 mg ml^{-1} in acetonitrile) was added and the vial was put aside in the dark for 10 min at room temperature for the derivatization of the anatoxin-a to take place. Hydrochloric acid (50 µl, 1 M) was added to finish reaction. Twenty µL of the derivative were injected in the HPLC system to perform the analysis (James et al., 1998). The instrument consisted of a quaternary pump (Agilent 1100 Series QuatPump), a degasser (Agilent 1100 Series Degasser) and a fluorescence detector (Agilent 1100 FLD). The analytical LC column was Luna C18 column (5 µm, 250 × 4.6 mm i.d., Phenomenex), operated at room temperature. The mobile phase was acetonitrile–water (50:50) with a flow rate of 0.8 ml min^{-1}. The excitation and emission wavelengths for fluorimetric detection were set at 470 and 530 nm, respectively.

3. Results and discussion

The anatoxin-a content of the freeze-dried cyanobacterial cells was 967 ± 33 µg of anatoxin-a per gram. This gave an estimated anatoxin-a quota of 116.92 fg cell^{-1}, equivalent to a biovolume quota of 1.37 fg µm^{-3}. After exposing the juvenile carps to the cyanobacterial suspensions (10^5 and 10^7 cell ml^{-1}), we registered two behavioural alterations in both treatments, compared to the controls: rapid opercular movement and abnormal swimming. This interference with fish behaviour caused by anatoxin-a may have negative consequences on fish populations due to changes in reproductive and predator–prey interactions.

At the higher cell concentration all the fish died between 24 h and 29:30 h—medium lethal time (MLT) was 26:39 h (Fig. 1). The slope of the curve shows that this is a very fast acting toxin in carps. At this cell density (10^7 cells ml^{-1}) the detoxication and depuration mechanisms seem not to be efficient enough to avoid rapid lethal effects. The maximum concentration of anatoxin-a in the whole fish was 0.768 µg g^{-1} dw (Table 1). No deaths were registered in the controls or at the lower concentration of cells (10^5 cell ml^{-1}) but the fish were all sacrificed at the end of the exposure period to determine anatoxin-a concentration in the whole fish. To our knowledge, there is no published data about MLT produced by cyanotoxins in juvenile fish but comparing to other toxic substances, anatoxin-a may be considered a very fast toxin (Fig. 2).

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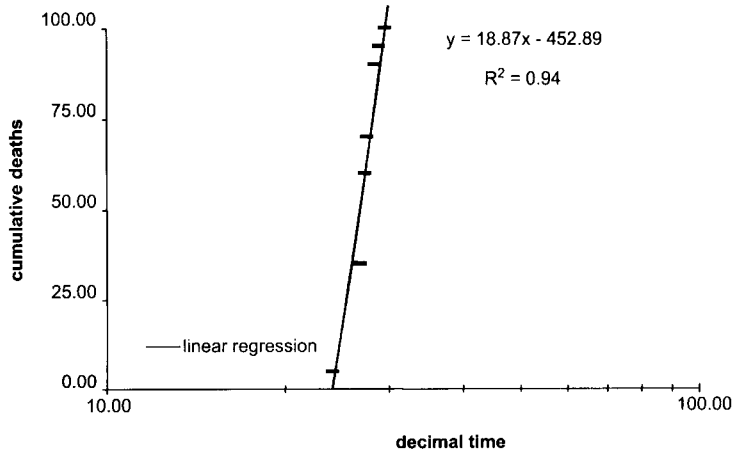

Fig. 1. Average cumulative death (%) of carps exposed to 10^7 cell ml^{-1} of freeze dried cyanobacterial cells. Average of two replicates ($n = 10$/replica).

Table 1
Total anatoxin-a ($\mu g\,g^{-1}$) in the juvenile fish exposed to suspensions of freeze-dried cyanobacteria (10^5 and 10^7 cell ml^{-1}) $n = 10$/replica

Cell density (cell ml^{-1})	Toxin content ($\mu g\,g^{-1}$ dry weight)	Toxin content ($\mu g\,g^{-1}$ fresh weight)
10^5	0.031 ± 0.16	0.005 ± 0.002
10^7	0.768 ± 0.23	0.073 ± 0.071

Anatoxin-a concentration in the whole carps demonstrates the uptake of this cyanotoxin when the fish are exposed to a freeze-dried cell suspension similar to a natural cyanobacterial bloom. Concentration of 10^5 and 10^7 cell ml^{-1} are possible in natural conditions, normally near lake shore areas after drifting and accumulation of live cells by wind action on the water surface. These results clearly show that decaying blooms containing anatoxin-a may be lethal to juvenile carps. Areas with extensive cyanobacterial scum may be avoided by people because of the obviously unpleasant appearance. Nevertheless, this behaviour is not yet described for fish and their juveniles may look for shelter and protection against predators near lake shores where toxic cyanobacteria concentrate. In this situation, carp may up take anatoxin-a and this could have negative ecological impact because of its possible transference to the higher levels of food chains. On the other side, due to its instability and hydrophilicity we believe that this might be a minor problem.

To our knowledge this is the first study on the effects of a cyanobacterial strain producing anatoxin-a in freshwater fish. Two previous related studies were published: Carmichael et al. (1975) reported death of goldfish after intraperitonial injection or oral administration of pure toxin (by the time named VFDF, before being characterized and nominated anatoxin-a). No effects were reported when goldfish were exposed to a freeze-dried culture or to an aqueous toxin extract. Oberemm et al. (1999) concluded that $400\,\mu g\,l^{-1}$ of anatoxin-a altered temporarily the heart rate in zebrafish.

The uptake of anatoxin-a by carps exposed to the two different cell densities followed its concentration in solution (Table 1) but was not proportional to the theoretical values of toxin in the water. Anatoxin-a concentration in the fish exposed to 10^7 cell ml^{-1} was only 25 times greater than in the fish exposed to 10^5 cell ml^{-1} (in theory, should be 100 times). This might be explained by the toxic and rapid lethal effect in the higher concentration that reduced the uptake of anatoxin-a by carp. Fish accumulated 0.31% and 0.30% of the total toxin provided by the 10^5 and 10^7 cells ml^{-1} treatments, respectively (Table 2). This suggests diffusion of the toxin present in the medium to the fish body via external surface, gills and/or digestive tract. We may assume that at intermediate cyanobacterial

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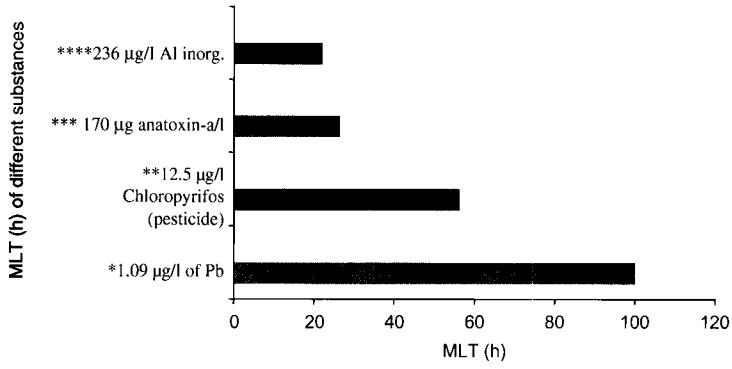

Fig. 2. Values of MLT described in other studies. *2 g trout (Chindah et al., 2004), **3 g tilapia (Macdonald et al., 2002), *** 3 g carp in the present study, ****salmon smolt (Rosseland et al., 1992).

Table 2
Maximum theoretical anatoxin-a accumulation in carps during exposure periods, percentage of the toxin accumulated and bioaccumulation factor (BAF) for the two treatments

Treatment (exposure period)	Maximum theoretical anatoxin-a in fish ($\mu g\,g^{-1}$)	% accumulated	BAF
10^5 cell ml^{-1} (96 h)	10.12	0.05	2.65
10^7 cell ml^{-1} (26:39 h)	252.63	0.26	0.65

densities, if they would not be lethal, more toxin would be accumulated. The bioaccumulation factor (BAF, is the ratio of chemical concentration in the organism to that in the water/food) was higher for the lowest toxin concentration because carps probably because they were exposed for a longer period (4 d) than at 10^7 cells ml^{-1} (24–27 h). The differences in the BAF between the two treatments, led us to believe that cyanobacterial blooms of lower cell density may represent higher risk for toxin accumulation. Due to high instability of anatoxin-a in the water (Stevens and Krieger, 1991) and to the lack of human casualties caused by this toxin, it has been considered of less concern than other cyanotoxins. Nevertheless, this work draws the attention for anatoxin-a and its risks, considering the possibility of human exposures to this toxin through contaminated fish ingestion as well as the accumulation of anatoxin-a in aquatic ecosystems.

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ACUTE EFFECTS OF CYANOBACTERIAL ANATOXIN-A ON CARP (*CYPRINUS CARPIO* L.) EARLY STAGES OF DEVELOPMENT

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1. INTRODUCTION

Cyanobacteria (blue-green algae) can easily proliferate competing and excluding other phytoplanktonic organisms in freshwater habitats, thus forming the cyanobacterial blooms. The main issue about Cyanobacteria has to do with their capacity to produce toxic secondary metabolites that are potent toxins hazardous to human and health ecosystems. There are different kinds of cyanotoxins classified into four major groups: neurotoxins, hepatotoxins, cytotoxins, irritants and gastrointestinal toxins (Codd *et al.*, 2005).

The neuro-cyanotoxin anatoxin-a has a wide distribution and was the first to be chemically characterized by Devlin and collaborators (1977). Furthermore, its mode of action on the nervous system of mammals has been the object of many studies in the pharmacological area (Aggarwal *et al.*, 1999; Trost and Oslob, 1999; Brenneman and Martin, 2004; Hjelmgaard *et al.*, 2005).

Several cyanobacterial genera have been described as anatoxin-a producers: *Anabaena, Aphanizomenon, Microcystis, Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium* and *Oscillatoria* (Bumke-Vogt *et al.*, 1999; Park *et al.*, 1993; Viaggiu *et al.*, 2004; Namikoshi *et al.*, 2003; Ballot *et al.*, 2005; Gugger *et al.*, 2005; Araóz *et al.*, 2005). These genera have been reported around the globe, sometimes with drastic consequences, as deaths of animals (e.g. Gorham *et al.*, 1964; Edwards *et al.*, 1992; James *et al.*, 1997; Hamill, 2001).

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Anatoxin-a is a postsynaptic depolarizing neuromuscular blocking agent that binds to the nicotinic acetylcholine receptors of the nervous system of animals but it is not hydrolyzed by cholinesterase (Thomas *et al.*, 1993). Acute effects in vertebrates include a rapid paralysis of the skeletal and respiratory muscles, causing symptoms such as loss of co-ordination, twitching, irregular breathing, tremors, altered gait and convulsions before death due to respiratory arrest (Carmichael and Gorham, 1978; Stolerman *et al.*, 1992; Fitzgeorge *et al.*, 1994; McBarron *et al*, 1975; Rogers *et al*, 2005). Death can occur within 2 minutes, the LD_{50} in mice being 375 µg/kg *i.p.* (Fitzgeorge *et al.*, 1994). Rogers *et al.* (2005) referred an LD_{50} of 250 µg/kg in 10 min (mice, *i.p.*).

To our knowledge, the only experiment that tested anatoxin-a on first development stages of fish was the one achieved by Oberemm and collegues (1999). These authors described temporary alterations of heart-rate in zebrafish (*Danio rerio*) embryos (55 h and 80 h of development) after exposure to 400 $\mu g.l^{-1}$ of pure anatoxin-a. The assessment of possible ecological risk caused by anatoxin-a has not been fully evaluated yet because of the lack of scientific data about the effects of this neurotoxin on aquatic organisms. Moreover, under natural conditions, as in a cyanobacterial toxic bloom, it is difficult to attribute the toxic effects only to the cyanotoxin itself. With this laboratorial work it is endeavoured to differentiate between the toxic effects caused by the cyanotoxin anatoxin-a and by the total aqueous cyanobacterial extracts from an anatoxin-a producing strain (ANA 37), on an important aquatic organism, the carp. Cellular extracts more closely mimic the real environmental situation and pure toxin returns data about anatoxin-a toxicity (Kuiper-Goodman *et al.*, 1999).

This work describes the effects of aqueous cell extracts of anatoxin-a producing cyanobacteria as well as of pure anatoxin-a on the first stages of development of the common carp *Cyprinus carpio* L. Carp has been chosen because it has a significant ecological importance due to its wide bio-geographic distribution in Eurasian waters as well as in North America and Africa (Zardoya and Doadrio, 1999).

Observations of mortality, larvae standard length (SL) and skeletal malformations were registered with the two types of treatment and the comparison between the two is also discussed.

The present study adds paramount scientific data for the assessment of ecological risk evaluation of anatoxin-a.

2. MATERIAL AND METHODS

The experiments were carried out in accordance with the current guidelines for the care of laboratory animals and ethical guidelines for investigation in conscious animals set by the General Directorate of Veterinary of Portugal.

2.1. CARP SPAWNING AND EGG FERTILIZATION

Spawning of a common carp (*Cyprinus carpio* L.) brood stock maintained at CIIMAR facilities was induced by increasing water temperature up to 25 °C and keeping that temperature during two weeks followed by intra-peritoneal injection of a carp pituitary extract. Eggs and sperm were obtained by abdominal compression of females and males, and mixed in order to fertilize the eggs. Egg adhesiveness was removed by immersion and shaking of the egg mass in a tannic acid solution (1 $g.l^{-1}$) during 15 seconds.

2.2. PREPARATION OF TREATMENTS

The concentration of anatoxin-a (640-80 $\mu g.l^{-1}$) and cell density ($6.6x10^5$ to $8.3x10^4$ $cell.l^{-1}$) in the various treatments were selected in a way to simulate natural occurrence of toxic cyanobacteria thus are ecologically relevant.

2.2.1. PURE ANATOXIN-A

Pure anatoxin-a was purchased from TOCRIS bioscience, USA ((±)-Anatoxin-a fumarate, cat. no: 0789). A stock solution of 1 $mg.ml^{-1}$ in MilliQ® water was prepared and divided in aliquots of 111 µl at -22 °C. Every 24 hours a new solution of anatoxin-a was prepared and diluted to the final concentrations of 1) 640 $\mu g.l^{-1}$, 2) 320 $\mu g.l^{-1}$, 3) 160 $\mu g.l^{-1}$ and 4) 80 $\mu g.l^{-1}$ (Table I).

Table I Anatoxin-a concentration ($\mu g.l^{-1}$) in the different treatments: pure anatoxin-a (numbers) and cyanobacterial extracts (letters), and equivalent cellular concentration ($cell.ml^{-1}$).

	control	1	2	3	4	A	B	C	D
Anatoxin-a concentration ($\mu g.l^{-1}$)	0	640	320	160	80	666	333	165	83
Cellular concentration ($cell.ml^{-1}$)	0	0	0	0	0	$5.46x10^6$	$2.73x10^6$	$1.36x10^6$	$6.9x10^5$

These solutions were then distributed in the incubation wells. This procedure was done under dim light to avoid photo-degradation of the toxin.

2.2.2. CYANOBACTERIAL CELL EXTRACTS

The anatoxin-a producing strain of *Anabaena* sp. (ANA 37) was kindly supplied by Professor Kaarina Sivonen (University of Helsinki, Department of Applied Chemistry and Microbiology) who isolated it from the Baltic Sea (Sivonen *et al.*, 1989). ANA 37 was cultivated in liquid Z8 media (Kotai, 1972) in aerated batch cultures (monocyanobacterial, non-axenic) (20 ±1 °C, photoperiod of 14 h light-PAR of 10 $\mu mol.m^2.s^{-1}$). After 1 month of growth (in the exponential phase of growth), cells were collected by filtration with a nylon-net of 20 µm mesh. The cells were then washed with distilled water and freeze-dried. The anatoxin-a content of ANA 37 at the given culture conditions was estimated to be 0.1% dw (Osswald *et al.*, 2007).

The cyanobacterial aqueous cell extracts were prepared from freeze-dried ANA 37

cells. In order to liberate cell constituents to the aqueous suspension, the cells were frozen/thawed twice and then sonicated for 3 min at *ca.* 80 amplitude (Sonics Materials, Vibra Cell 50). The suspension was then filtered through a 0.45 µm sterile membrane, ME 25/21 ST Whatman® Schlleicher&Schuell, to exclude cell debris. This procedure was done under dim light to avoid photo-degradation of the toxin. At the beginning of the experiment, one stock of cell extract (0.02 $g.ml^{-1}$ dw) was prepared and kept a 4 °C in the dark. Every 24 h part of this stock was diluted to obtain four different concentrations equivalent to the pure treatment ones (A-666 $\mu g.l^{-1}$, B-333 $\mu g.l^{-1}$, C-165.5 $\mu g.l^{-1}$ and D-83.3 µg. $^{-1}$) (Table I).

2.3. EXPOSURE PROCEDURE

Fertilized eggs with 2:30 hours post fertilization (hpf) were incubated in six-well cell culture plates (TC plates for suspension cells, sold by Sarstedt) during 96 hours in the dark at 19 °C. Each well contained 5 ml of the test substance - cyanobacterial cell extract or pure anatoxin-a – diluted in dechlorinated tap water (0.45 µm sterile membrane, ME 25/21 ST Whatman® Schlleicher&Schuell). As a control, four replicates with fertilized eggs (2:30 hpf) were incubated in the same conditions as the treatments but without anatoxin-a or cell extracts. Every 24 hours the medium was renewed to ensure constant conditions during the four days of incubation. Oxygen and pH were monitored daily to ensure acceptable levels in the treatments and the controls.

The test substances were assayed at different concentrations as described in table I. Each concentration of both treatments and the control was done in four replicates (*ca.* 20 eggs per well, n = *ca.* 20 x 4 = *ca.* 80 eggs *per* concentration). After the 4 d of incubation, it was decided to aggregate the larvae of the 4 replicates in nine separated cages and in aerated de-chlorinated tap water (0.45 µm sterile membrane, ME 25/21 ST Whatman® Schlleicher&Schuell) until the end of the experiment - 8 d after beginning of exposure, before feeding stage. This reduced all handling procedures to a minimum, allowed a higher volume of water per treatment (100 ml) and thus permitted good levels of oxygen and pH.

2.4. OBSERVATIONS

During the first day of experiment, mortality was registered at 4, 9 and 24 h. From then on counting of dead eggs (that included non-viable hatchments) was done every 24 hours until the 8th day post fertilization (pf) inclusive.

Viable hatchings were observed every 24 hours and photographs of each newly hatched viable larvae were taken within 24 h period, using a stereomicroscope (Zeiss-Stemi DV4) connected through an adaptor (Canon LA-DC 58F) to a digital camera (Canon PowerShot A620). Standard length (SL) of the viable larvae and its skeletal malformations were evaluated based on the photographs with the free software UTHSCSA Image Tool program, developed at the University of Texas Health Science Centre at San Antonio, Texas and available from the Internet by anonymous FTP from maxrad6.uthscsa.edu.

2.5. STATISTICAL ANALYSES

Statistical analysis was performed on mortality (calculated as the proportion of dead eggs and non-viable hatchments) and SL of viable larvae (within 24 h after hatchment). One-way ANOVA followed by Newman-Keuls test, was used to detect differences among treatments and controls of the larvae SL. In this case, the computer software Stat Graphics v.7.0, for MS-DOS was used.

Differences on mortality and skeletal malformations among treatments and the controls were performed using the statistical Kruskal-Wallis test (Kruskal and Wallis, 1952) with the computer software SPSS 14.0 for Windows (SPSS Inc., 2005)

3. RESULTS

During the whole experiment, water in the wells presented an oxygen concentration □5.6 $mg.l^{-1}$ (62.15%) and pH between 7 and 8. These values are within the limits for carp (Michaels, 1989).

During the 4 d incubation period, percentage of accumulated mortality (relative to the total number of eggs) showed no significant differences among replicates in all concentrations (Table II).

Table II Percentage of accumulated mortality of eggs (including non-viable hatchments) during the 8 d of experiment. Values until 96 h inclusive correspond to average of 4 replicates (except treatment 4 due to technical problems where only three replicates were considered). Shadowed cells correspond to exposure period.

Time	control	1	2	3	4	A	B	C	D
4 h	0	0	0	0	0	0	0	0	1.28
9 h	4.88	5.00	1.20	4.88	3.23	4.60	6.10	4.76	6.41
1 d	14.63	12.50	16.87	14.63	12.90	12.64	14.63	15.48	17.95
2 d	14.63	15.00	18.07	15.85	12.90	13.79	14.63	15.48	20.51
3 d	14.63	15.00	19.28	15.85	12.90	13.79	18.29	16.67	21.79
4 d	14.63	16.25	19.28	17.07	12.90	100	18.29	16.67	21.79
5 d	17.07	18.75	20.48	19.51	16.13	_	18.29	20.24	26.92
6 d	19.51	21.25	25.30	21.95	17.74	_	25.61	25.00	26.92
7 d	20.73	21.25	26.51	30.49	17.74	_	90.24	52.38	30.77
8 d	20.73	21.25	26.51	30.49	17.74	_	90.24	52.38	35.90

No significant differences of mortality were observed among the 4 concentrations with pure toxin and the controls. Therefore, pure anatoxin-a was not lethal to carp eggs at the tested concentrations.

All treatments with ANA 37 extracts were toxic. Egg mortality increased with increasing concentration reaching 100% at the highest one, treatment A, where all eggs died dur-

ing exposure period. Hatchment (Table III) was also affected in the cell extracts treatment in a concentration dependant way.

Table III Cumulative hatchments (% of total eggs) of carp submitted to different concentrations of pure anatoxin-a and cellular extracts of ANA37. Shadowed cells correspond to exposure period.

Time (d)	control	1	2	3	4	A	B	C	D
4	4	3	1	0	1	_	0	0	0
5	40	36	29	30	27	_	0	12	24
6	73	71	69	68	72	_	10	48	58
7	79	76	73	70	77	_	10	48	60
8	79	79	73	70	77	_	10	48	64

Larvae SL measured within 24 h after hatching is presented in table IV.

Table IV Average standard length of all newly hatched carp *per* treatment. Means sharing a common superscript letter are not statistically different (ANOVA Newman-Keuls test. p=0.05).

	control	1	2	3	4	B	C	D
Average lenght	5.05 ^{a} ±0.41	4.69 ^{bc} ±0.50	5 ^{ab} ±0.57	4.77 ^{ab} ±0.56	4.98 ^{ab} ±0.47	4.46 ^{c} ±0.37	4.89 ^{ab} ±0.47	4.99 ^{ab} ±0.36
n	62	62	62	57	61	8	40	50

All viable hatched larvae were measured in all treatments. Treatment A has no data due to 100% mortality before hatchment.

At the higher concentrations of pure toxin (1) or of cell extracts (B), average SL of the larvae was smaller than the control. For the other treatments, apparently no differences in standard length were observed, relative to control.

Skeletal malformations were photographically documented within 24 hours after hatchment. The main skeletal deformity that was observed in higher concentrations in both treatments (Table V) was bent tails/body axes shown in figure 1.

Table V Percentage of bent tail/body axes registered in all the hatched larvae within 24 hours after hatching.

	control	1	2	3	4	B	C	D
%	20.3	31.7	27.9	26.3	19	62.5	55	25.5

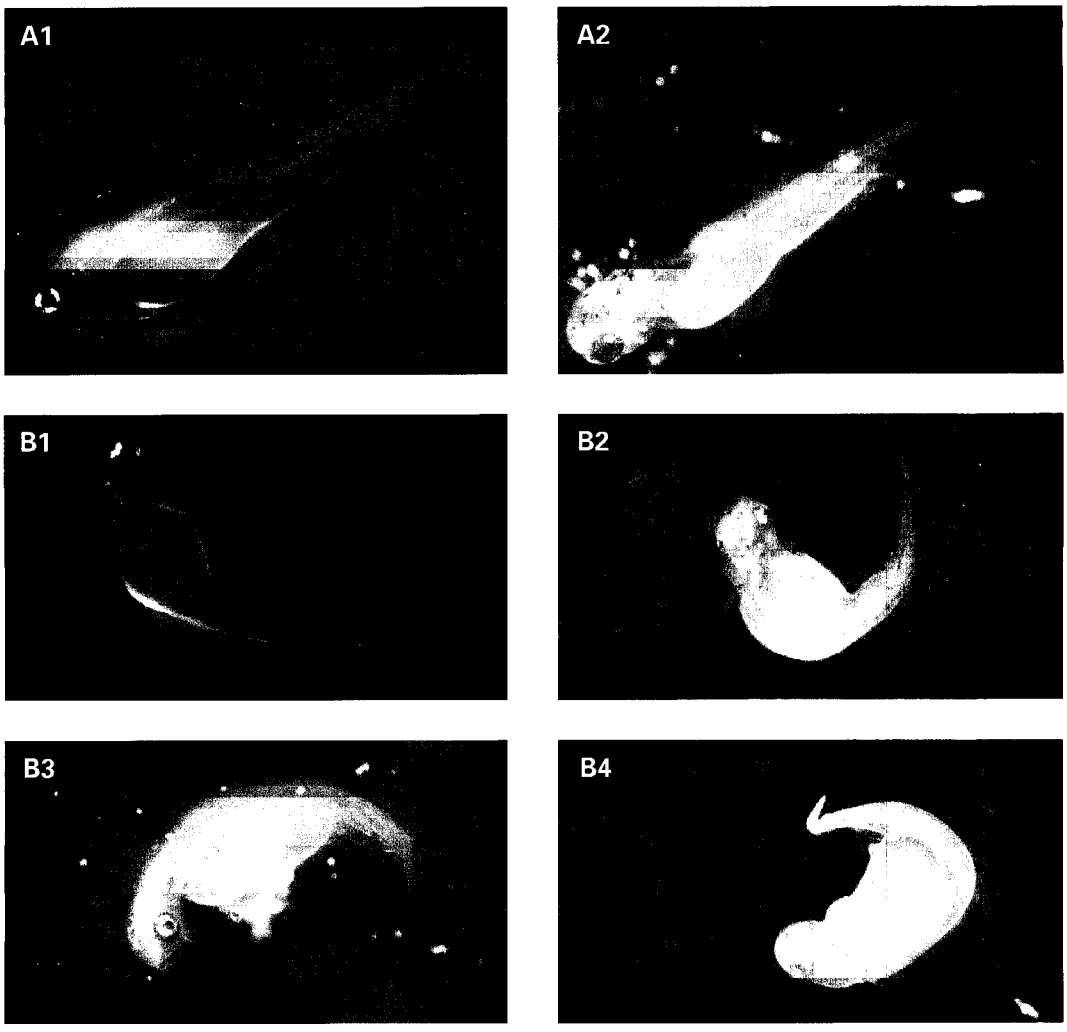

Fig. 1 Examples of post-hatching skeletal deformities observed in carp larvae. A1, A2 – Normal larvae. B1, B2, B3, B4 – Abnormal larvae.

4. DISCUSSION AND FINAL REMARKS

This study describes the anatoxin-a effects upon the early development stages of a freshwater fish. Therefore it is of interest to aquatic toxicology in particular to the assessment of ecological impacts caused by this neurotoxin so widely distributed.

The tested concentrations as well as the period of exposure used in both types of treatments were chosen in a way to be ecologically relevant. (10^6 –10^5 cell.ml^{-1} during 4 days). Higher concentrations and longer exposures could also happen in natural blooms but with this assay it is not possible to determine what would be the effects.

Results demonstrated that pure toxin was less toxic than cell extracts with the same concentration of anatoxin-a. This higher toxicity of the extracts may be caused by other substances that were also toxic. Other hypothesis is that those substances could have a synergistic effect over anatoxin-a or could have increased the uptake rate of the toxin.

Synergy of anatoxin-a and other cyanotoxin, microcystins-LR, has been described previously in mice after intranasal (i.n.) administration of both toxins: the pre-administration of a sub lethal dose of microcystin-LR (30 µg/kg bw, *i.n.)* to mice, before dosing with anatoxin-a, reduced the intranasal LD_{50} of the latter from 2000 µg/kg to 500 µg/kg bw (Fitzgeorge *et al.,* 1994).

Cyanobacteria lipopolyssacarides (LPS) could also be responsible for the toxicity found in the ANA 37 extracts. *Cyprinus carpio* has important innate immune factors that are present since embryonic phase and can elicit immune response to LPS. It was stated that those factors could play an important role in the carp development itself (Huttenhuis *et al.,* 2006). Therefore this could be the reason of the developmental toxicity caused by the cyanobacterial extracts.

Toxic effects of the cellular extracts could be also associated with toxic effects of mueggelone or lupenyl acetatate, two metabolites found in another cyanobacterial extracts of *Aphanizomenon* (Papendorf *et al.,* 1997). Mueggelone and lupenyl acetate were the likely causative agents for the mortality and developmental toxicity observed in zebra fish (*Danio rerio*) after incubation of eggs in media containing mueggelone and lupenyl acetate (Papendorf *et al.,* 1997). Nuclear magnetic resonance (NMR) experiments with ANA 37 would be an adequate approach in order to learn about the production of those two metabolites by ANA 37.

Comparing to the control, results clearly showed that pure anatoxin-a cause no mortality in early stages of carp development but at the highest concentration reduced the standard length of the newly hatched larvae. Whether that sub lethal effect could be compensated later in the development of the fish is beyond the scope of this assay but should be object of further investigations.

The ANA 37 cell extracts had a more evident effect than the pure anatoxin-a treatments. A similar situation was described by Oberemm and co-authors (1997 and 1999) after comparing pure microcystins–LR treatments with crude microcystins–LR producing cyanobacterial extracts.

This study demonstrating a remarkably higher toxic effect of the cellular extracts comparing to the pure toxin treatments, emphasizes the need for this kind of bioassays instead of the ones using only pure cyanotoxins. The fractionation of these cyanobacterial extracts added with specific chemical techniques, would be essential in determining what fraction is being responsible for the observed lethal effects on early stages of development in carp. This should be the approach for future works since it would contribute to understand the ecotoxicology of cyanobacteria in aquatic ecosystems. This idea is in agreement with Berry and co-authors (2007) who stated that zebrafish development should be used as a model to "lead" to toxins that could be later characterized using bioassay-guided fractionation and eventual chemical and biological identification.

The toxic effects of pure anatoxin-a and of cellular extracts on the eggs and larvae of carp that were described in this work clearly indicate that the occurrence of anatoxin-a producing cyanobacteria may alter aquatic communities.

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CONCLUSÃO GERAL E CONSIDERAÇÕES FINAIS

Até ao momento não existiam trabalhos publicados sobre ocorrência de anatoxina-a em Portugal. Esta lacuna é ao menos parcialmente preenchida por esta dissertação, que apresenta fortes evidências sobre a ocorrência da anatoxina-a em Portugal. Inclui os primeiros estudos sobre os efeitos da anatoxina-a em organismos aquáticos e também uma vasta revisão bibliográfica sobre esta importante neurotoxina de origem cianobacteriana. Assim, este trabalho constitui uma contribuição que julgamos importante para a compreensão da ecotoxicologia aquática, em particular sobre o impacto das cianobactérias em espécies aquáticas.

A falta de competências técnicas para a detecção da anatoxina-a em Portugal obrigava a recorrer a laboratórios estrangeiros ou então a não efectuar de todo as análises. Isto talvez tenha levado à omissão de ocorrências de anatoxina-a em Portugal. O trabalho técnico desenvolvido durante esta investigação, em colaboração com a Universidade de Vigo, permitiu assim a implementação de um protocolo experimental para a detecção e quantificação da anatoxina-a em Portugal.

Após considerar as várias técnicas disponíveis para a detecção e quantificação da anatoxina-a, a HPLC-FLD após SPE com WCX, foi eleita e implementada no nosso laboratório. Revelou-se uma técnica suficientemente sensível (LOD= 0.24 ng, 24 $ng.l^{-1}$) para a monitorização ambiental e para estudos de toxicidade em organismos aquáticos. Esta técnica foi testada em matrizes diferentes, nomeadamente água, células cianobacterianas e em tecidos de organismos aquáticos, tornando-se a ferramenta essencial nos trabalhos subsequentes de quantificação da anatoxina-a a em amostras de água e em bioensaios.

Embora não se tenha detectado anatoxina-a em amostras naturais de água, demonstrou-se que esta toxina é produzida por estirpes portuguesas, quando cultivadas em laboratório. Portanto, a potencial ocorrência de anatoxina-a em águas doces portuguesas deverá ser considerada. Tal como acontece com as outras cianotoxinas, os fenómenos tóxicos causados pela anatoxina-a não se podem prever mas podem-se identificar num determinada momento, num determinado local. É necessária, para isso, a implementação de técnicas de detecção como a apresentada nesta dissertação (HPLC-FLD) que permite determinar o grau de toxicidade de uma massa de água num certo momento. As estirpes portuguesas produtoras de anatoxina-a pertencem aos géneros *Anabaena, Aphanizomenon, Microcystis* e *Oscillatoria* produziram anatoxina-a numa gama de 0.06 $\mu g.g^{-1}$ a 24.62

$\mu g.g^{-1}$ de peso seco de cianobactérias.

Uma situação que pode gerar um risco importante é o consumo de organismos aquáticos contaminados com anatoxina-a. A falta de estudos sobre a acumulação de anatoxina-a em organismos aquáticos ou sobre a possível transferência de toxicidade para o organismo consumidor/predador, foram motivo impulsionador para esta dissertação. Ficou demonstrado que a carpa comum (*Cyprinus carpio*) submetida a uma suspensão de células produtoras de anatoxina-a, é capaz de acumular 0.073 µg de anatoxina-a por grama de peso fresco. Sabendo que o NOAEL oral para a anatoxina-a pura, em ratinho é 98 $\mu g.kg^{-1}$ por dia (Fawell *et al.*, 1999), aplicando o factor de incerteza 1000 (Humpage e Falconer, 2003) pode-se estimar que o TDI para a anatoxina-a em humanos seja 0.098 $\mu g.kg^{-1}$. Para uma criança de 10 kg, o TDI será 0,98 µg de antoxina-a. Se considerarmos que uma criança de 10 kg pode consumir 30 g de peixe contaminado numa refeição, ela ingeriria 2,19 µg de anatoxina-a, ou seja, um valor 2,23 vezes o TDI calculado anteriormente. Este cenário, embora muito cauteloso, é o único cientificamente comprovado e deverá por isso ser considerado até surgirem dados mais concretos.

O aumento da eutrofização das massas de água devido à pressão antropogénica é uma forte razão para o aumento das florescências cianobacterianas (Falconer, 2005). O facto de se recorrer cada vez mais à captação de água nas camadas superficiais e não no subsolo aumenta também o risco de contaminação da água de consumo por cianotoxinas. Métodos de vigilância e tratamento deverão ser definidos e implementados. Especial atenção deverá ser dada às regiões de maior pressão antropogénica. De salientar que a produção de anatoxina-a pode ocorrer em géneros cianobacterianos bentónicos e por isso dever-se-á vigiar também margens, fundos e todas as estruturas que possam constituir substratos para aqueles géneros.

A elevada proporção de estirpes produtoras de anatoxina-a em laboratório verificada nesta dissertação levantou a questão sobre a frequência deste metabolito nas cianobactérias. Possivelmente trata-se de um metabolito comum neste grupo de organismos, no entanto a sua detecção tem sido limitada pela sua baixa concentração de ocorrência e pela falta de programas de monitorização.

Relativamente ao estudo do efeito da anatoxina-a em filtradores aquáticos, este trabalho inclui os primeiros ensaios experimentais nesse sentido. O estudo da dinâmica de acumulação/depuração em mexilhões (*Mytilus galloprovincialis*) demonstrou que a anatoxina-a é provavelmente eliminada pelos mexilhões de uma forma activa e após 1 d da suspensão da exposição à toxina não foi possível detectar anatoxina-a nas partes comestíveis dos animais. A capacidade de acumulação da anatoxina-a pelos mexilhões foi baixa (*maximum accumulation efficiency*=10.92%) quando comparada com outras cianotoxinas. Afastando assim a existência de riscos para a saúde pública devido á ingestão de mexilhões provenientes de locais contaminados. Pois a depuração desta neurotoxina por *Mytilus galloprovincialis* é rápida e eficiente.

Nos três bioensaios efectuados com concentrações de anatoxina-a ou de extractos celulares ecologicamente relevantes, verificou-se que o grau de toxicidade desta neuroto-

xina dependeu do organismo em questão. Para os mexilhões, a concentração de 10^5 cell.ml^{-1} durante 15 d não causou a morte de nenhum exemplar. Submetendo juvenis de carpa a 10^7 cel.ml^{-1} verificamos uma letalidade de 100% às 29 h. Para os ovos de carpa, a concentração de $5{,}46 \times 10^6$ cel.ml^{-1} provocou a morte de toda a população testada em 4 d. Estes dados demonstram que os vários organismos aquáticos apresentam respostas diferentes a esta mesma neurotoxina. Reforça-se assim a necessidade de efectuar estudos de toxicidade em organismos e fases de desenvolvimento distintos se quisermos efectuar uma avaliação de risco real.

Os eventuais riscos para a saúde pública terão que ser avaliados através de uma amostragem contínua e presistente, abrangendo o maior número possível de locais, especialmente os locais utilizados para águas de recreio e de consumo. Segundo Falconer (2005), a identificação das cianobactérias até ao género, é uma medida suficientemente cautelosa para monitorização do risco das cianotoxinas para a saúde pública. Assim, a ocorrência dos géneros potencialmente produtores de anatoxina-a, (bentónicos e planctónicos), deverá constituir um factor de alerta. Ou seja, *Anabaena, Aphanizomenon, Cylindrospermum, Oscillatoria, Microcystis, Raphidiopsis, Planktothrix, Arthrospira, Nostoc* e *Phormidium* são géneros que deverão ser monitorizados.

Em situações de suspeita de ocorrência de anatoxina-a, as autoridades competentes deverão aplicar o princípio da precaucionariedade até possuírem dados concretos sobre a toxicidade da massa de água em questão. Para evitar episódios de intoxicação em locais não vigiados dever-se-ia implementar programas de sensibilização, explicando às populações os sinais de alerta e os cuidados a tomar.

OVERALL CONCLUSION AND FINAL REMARKS

Until now there was no published work about anatoxin-a in Portugal. This dissertation represents the first scientific evidence that anatoxin-a may in fact occur in Portugal. It is also includes the first studies about anatoxin-a effects in aquatic animals and a vast bibliographic review of this important neurotoxin of cyanobacterial origin. It is hoped that the present work may contribute to improve the knowledge about freshwater ecotoxicology, in particular about the impacts of cyanobacteria in aquatic organisms.

The lack of technical know-how on anatoxin-a detection methods in Portugal was imposing outsourcing of this technique to other countries. Other solution was not doing the analyses at all. This situation might have caused the omission about anatoxin-a occurrence in Portuguese fresh waters. Our collaboration with the University of Vigo made it possible to learn and implement an experimental protocol for the detection and quantification of anatoxin-a in Portugal.

After considering the various available chemical techniques to detect and quantify anatoxin-a, HPLC-FLD after SPE with WCX was chosen and implemented in our laboratory. A good degree of sensitivity for the following studies was achieved (LOD=0.25 ng, 24 $ng.l^{-1}$). This chemical technique was assayed in different matrices, namely, water, cyanobacterial cells and tissues of aquatic organisms. HPLC-FLD turned out to be an essential tool for the following experiments in this dissertation that involved anatoxin-a detection and quantification.

Although anatoxin-a was not found in nature, it was demonstrated that it can be produced by cultivated Portuguese cyanobacterial strains. This strongly indicates that anatoxin-a may occur in Portugal. As it happens with other cyanotoxins, anatoxin-a phenomena can not be predicted, they can only be detected at a certain moment in a certain water body. Therefore chemical techniques as the one shown in this dissertation (HPLC-FLD) should be implemented in order to measure anatoxin-a in Portugal. Portuguese cyanobacterial strains that produced anatoxin-a in laboratory belong to the genera *Anabaena, Aphanizomenon, Microcystis* and *Oscillatoria* and produced anatoxin-a within a range of 0.06 $\mu g.g^{-1}$ to 24.62 $\mu g.g^{-1}$ of cyanobacterial dry weight.

The consumption of aquatic organisms contaminated with anatoxin-a is an important issue in terms of risk. The lack of scientific evidence about accumulation of anatoxin-a by aquatic organisms and its transference along the aquatic and terrestrial food chains rep-

resented a further incentive for this dissertation. In one of the experiments presented, juvenile carp *(Cyprinus carpio)* accumulated 0.073 µg of anatoxin-a per gram of fresh weight after being exposed to ANA 37 (anatoxin-a producing strain) at 10^7 cell.ml^{-1}. The no observed adverse effect level (NOAEL) of pure anatoxin-a orally administered to mice was found to be 98.0 µg.kg^{-1} of bodyweight per day (Fawell et al., 1999). To make a human risk evaluation we can apply the overall uncertainty factor of 1000, used by Humpage and Falconer (2003) for cylindrospermopsin, and estimate that the maximum total daily intake (TDI) of anatoxin-a for humans is 0.098 µg.kg^{-1} of bodyweight. Considering a 10 kg child the TDI would be 0.98 µg of anatoxin-a. If the child was to eat 30 g of contaminated carp, he or she would ingest 2.19 µg of anatoxin-a that would correspond to 2.23 times the TDI estimated above. Although this is a very conservative scenario (based on this one experiment), it is the only one scientifically supported, therefore it should be considered before obtaining more data.

The anthropogenic pressure on aquatic systems has been causing the increase of cyanobacterial blooms (Falconer, 2005). Moreover, the fact that water captions for human consumption are being more frequently taken from the surface, increases the risk of cyanotoxin contamination by humans. Surveillance of water bodies and water treatment methods should be implemented. Special attention should be given to locals with high anthropogenic pressure. Another important issue in this subject of health risk evaluation is the fact that benthic cyanobacteria may also be potential anatoxin-a producers. This should direct the surveillance also for the water edges and benthos. Every structure in contact with water as deposits and pipes that can function as substrates for cyanobacteria should also be attentively observed.

The high frequency of anatoxin-a producing strains found in this dissertation opens up the question about the production of this metabolite by cyanobacteria. As happens with other alkaloids in plants, maybe anatoxin-a is a common metabolite of cyanobacteria but because usually it is produced in low concentration and there is a lack of surveillance programs, it has not been detected.

In order to determine the effects of anatoxin-a in aquatic organisms and subsequently in aquatic ecosystems, three bioassays were performed. This work includes the first experiment with aquatic filter feeders *(Mytilus galloprovincialis)* and anatoxin-a. The study of accumulation/depuration of anatoxin-a in this mussels indicated that anatoxin-a is probably eliminated actively by these organisms. One day after end of exposure period there was no detectable anatoxin-a in the edible parts of the mussels. The maximum accumulation efficiency (10.92%) was low when compared to other cyanotoxins. Therefore the health risk due to the ingestion of contaminated mussels by humans is low. Depuration of anatoxin-a by *Mytilus galloprovincialis* was fast and efficient. Submitting different organisms at different development stages to ecologically relevant concentrations of an anatoxin-a producing strain, gave distinct results. All mussels survived to 10^5 cell.ml^{-1} during 15 days. The exposure of juvenile carp to 10^7 cell.ml^{-1} caused 100% mortality at 29 h. For the carp eggs 10^5 cell.ml^{-1} were enough to kill all the tested population in 4 d. These data demon-

strate that organisms react differently to the toxin depending on their taxa and developmental stage. It reinforces the need for these types of studies in order to make correct evaluation of the risks caused by anatoxin-a producing cyanobacteria.

Possible public health risk of anatoxin-a should be evaluated based on continuous and persistent sampling including as many places as possible. More efforts should be directed to water systems for human usage as recreation and consumption. According to Falconer (2005), the identification of cyanobacteria to the Genera level is sufficient for appropriate public health surveillance. The potentially anatoxin-a producing Genera are *Anabaena, Aphanizomenon, Cylindrospermum, Oscillatoria, Microcystis, Raphidiopsis, Planktothrix, Arthrospira, Nostoc* and *Phormidium,* which should be monitored for by responsible authorities.

Whenever there is a suspicion about anatoxin-a occurrence, responsible health authorities should follow the precautionary principles until having the definite data about the toxicity of the water body in question. In order to avoid intoxication episodes in unmonitored locals, programs of public education on this mater should be implemented. That way local population could recognize the alert signs and thus could contribute to a more efficient measure of remediation.

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