The Cyanotoxin Anatoxin-a: Factors Leading to its Production and Fate in Freshwaters

by
Alexis Gagnon

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in the
Ottawa-Carleton Institute of Biology
Specialization in Chemical and Environmental Toxicology

Thèse soumise à la
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention d'une maîtrise

L'Institut de biologie d'Ottawa-Carleton

© Alexis Gagnon, Ottawa, Canada, 2012

Abstract

Anatoxin-a (ANTX) is a neurotoxin produced by several freshwater cyanobacteria and has been implicated in the death of livestock and domestic animals from consumption of tainted surface waters. ANTX is unstable under normal conditions and is somewhat problematic to extract and study. Accelerated solvent extraction (ASE) combined with liquid chromatography-mass spectrometry (LC/MS) was used to develop an efficient extraction and analytical method for both ANTX and the more commonly encountered hepatotoxic microcystins produced by cyanobacteria. The effects of nitrogen supply on the cellular production and release of ANTX was investigated in *Aphanizomenon issatschenkoi* (Ussaczew) Proschkina-Lavrenko (Nostocales). In contrast to the predictions of the carbon-nutrient balance hypothesis, the maximum production was observed under moderate N stress. In addition, steady state fugacity-based models were employed to investigate ANTX's distribution and fate in freshwater ecosytems. ANTX was not found to be very persistent in aquatic ecosystems and did not appear to bioaccumulate in fish, at least not from the dissolved phase.

Résumé

L'anatoxin-a (ANTX) est une cyanotoxine produite par plusieurs genres de cyanobactérie d'eau douce et a été responsable de la mort d'animaux domestiques et de bétail. L'ANTX est extrêment instable sous des conditions normales, ce qui est problématique quant à son extraction et à son étude. L'extraction accélérée au solvant (ASE), combinée à la chromatographie liquide et à la spectrométrie de masse (LC/MS), a été developpée en tant que méthode commune d'extraction efficace pour l'ANTX et l'hépatotoxine, microcystine (MC). L'effet de l'azote sur la production de l'ANTX chez *Aphanizomenon issatschenkoi* (Nostocales) a été déterminé dans cette étude. À l'encontre des théories courantes sur la production des substances secondaires, la concentration et le contenu cellulaire en ANTX était maximale sous des conditions de stress modéré. De plus, des modèles basés sur la fugacité, à état stationnaire, ont été utilisés pour définir la distribution et le sort de l'ANTX dans des écosystèmes d'eau douce. Lors des modélisations, aucune bioaccumulation chez les poissons n'a été déterminée et l'ANTX ne semble pas être persitente dans les écosystèmes aquatiques, du moins en ce qui a trait à la phase dissoute.

Remerciements

Tout d'abord à mon superviseur de thèse, Dr Frances Pick. Pour sa générosité, son partage de conaissances, sa spontanéité, sa franchise et son support précieux tout au long de cette expérience académique.

Aux membres de mon comité : Dr J.T. Arnason et Dr P. White de l'Université d'Ottawa pour leurs conseils indiqués. J'aimerais également remercier le Dr J. Blais pour son intérêt contagieux pour la limnologie et pour son aide en modélisation basée sur la fugacité. Au Dr Rocio Aranda-Rodriguez, pour m'avoir guidé dans mon projet de recherche et pour m'avoir si généreusement ouvert les portes de son laboratoire de Santé-Canada. À Zhiyun Jin pour avoir pris le temps d'analyser des échantllons pour moi. Au Ministère de l'environnement de l'Ontario, pour le support financier (Best in Science).

Je me dois également de remercier chaleureusement Ammar Saleem qui m'a appris beaucoup de la chimie analytique, de la patience, de la persévérence et de ce merveilleux petit bijou qu'est le Moni Mahal. À Linda Kimpe, pour sa générosité et son aide grandement appréciée la veille de nuits blanches passées au laboratoire. Au Dr Shabana Bhatti pour le partage de son savoir en ce qui a trait à la culture algale.

Je remercie toute l'équipe de laboratoire : Rebecca Dalton, Jacinthe Contant, Muriel Rolon Dos Santos Mérette, Justin Lajoie et plus particulièrement Arthur Zastepa ; pour son aide incommensurable, sa patience, son partage de connaissances, sa tendance à boucher la colonne analytique une journée avant l'injection de mes échantillons et pour avoir souffert de m'écouter parler, malgré mon accent français, sans jamais grimacer.

À Noel Alfonso, qui a été bien plus qu'un simple superviseur et, sans qui, je n'aurai peut-être pas entrepris cette maîtrise. À Martin Pelchat, pour avoir tendu une oreille, si ce n'est les deux, durant nos pratiques hebdomadaires entre deux « ripoffs » de Mercyful Fate. À Wened de Poméranie. À Ramin Deison, pour sa personnalité sans pareille et pour avoir partagé ces nombreux cafés et recettes perses. À la musique, en particulier celle de Geirr Tveitt et de Janis Kalifatidis, qui m'a accompagnée lors de l'écriture de cette thèse. Je remercie ma famille mais surtout mes parents, qui ont su apporter un support moral constant pendant ces années de recherche. Je remercie également Alfred B.N., pour son apport réconfortant à travers son indifférence.

Pour finir, j'aimerais remercier, avec toute mon affection, ma douce moitié, Camille Béland aka Colonel T.C., pour son support, son écoute, et sa compréhension. Nous voici enfin arrivé à la croisée des chemins; et c'est à trois que nous traverserons.

Table of Contents

Abstract ii
Résumé iii
Remerciements iv
Table of Contents vi
List of Tables vii
List of Figures viii
Chapter I: General Introduction 1
1.1 Cyanobacteria and their toxins 2
1.2 Anatoxin-a, homoanatoxin-a and anatoxin-a(s) : chemistry and toxicity 3
1.3 Anatoxin-a extraction and analyses 5
1.4 Factors influencing anatoxin-a production 6
1.5 Anatoxin-a: environmental fate 9
1.6 Thesis Objectives 10
Chapter 2: Development of a common extraction and analytical method for anatoxin-a and microcystins 15
2.1 Introduction 16
2.2 Material and Methods 20
2.3 Results and Discussion 24
Chapter 3: Effect of nitrogen on cellular production and release of the neurotoxin anatoxin-a in a nitrogen-fixing cyanobacterium 39
3.1 Introduction 40
3.2 Material and Methods 41
3.3 Results 44
3.4 Discussion 47
Chapter 4: Use of steady-state fugacity based-models to determine the fate of cyanobacterial freshwater neurotoxin anatoxin-a 57
4.1 Introduction 58
4.2 Material and Methods 61
4.3 Results and Discussion 64
Chapter 5: General Conclusion 73
References 77
Appendices 86

List of Tables

Table 1.1 : Types of cyanotoxins based on their mode of action and estimates of LD50. 12

Table 2. 1 : Anatoxin-a producing cultures. 28

Table 2. 2 : Comparison of ASE parameters for ANTX and MCs pressurized liquid extraction. 29

Table 2. 3 : MRM transitions of each analyte obtained after infusion into truboV electrospray source of 3200 QTRAP. 30

Table 2. 4 : Linearity and instrument limits of detection (LOD) and limits of quantification (LOQ) for each analyte. 31

Table 3. 1 : Average growth rate based on optical density changes, carbon to nitrogen (C:N) molar ratios and yield of biomass (dry weight mg/ml) for *Aphanizomenon issatschenkoi* grown at different nitrogen concentrations (as % of full strength BG11). 51

Table 4. 1 : Environmental properties of the EQC (standard environment) used in the fugacity-based model simulations. 68

Table 4. 2 : Half-life (hours) estimated and measured for ANTX. 69

Table A.1 : Composition of BG11 medium used for cyanobacterial cultures. 86

List of Figures

Figure 1.1 : Chemical structures of anatoxin-a, homoanatoxin-a and anatoxin-a (s) 13

Figure 1.2 : The ana gene cluster responsible for biosynthesis of anatoxin-a and homoanatoxin-a in cyanobacteria and postulated biosynthetic route leading to these neurotoxins 14

Figure 2.1 : Structures of anatoxin-a, phenylalanine and microcystin..................................32

Figure 2.2 : Extraction ion (149.2 amu) of two freeze-dried filtrate samples (10 ml) of *Aphanizomenon issatschenkoi* analyzed on the Sciex QTRAP 3200 LC-MS/MS. 33

Figure 2.3 : Chromatogram of an injection (25 µl) of ANTX-fumarate standard (5 µg /ml) using an HPLC-DAD at 229 nm detection. 34

Figure 2.4 : Chromatograms of an injection (25 µl) of 10 ml of ANTX producing cultures of *Aphanizomenon issatschenkoi*, *Anabaena flos-aqua* and *Oscillatoria sp.*..............................35

Figure 2.5 : Extraction ions chromatogram of 1 µl injection on column of standard mix at 5ppm 36

Figure 2.6 : Enhanced mass scan in positive polarity showing two major fragments of anatoxin-a obtained from a direct injection of ANTX standard. 37

Figure 2.7 : Enhanced mass scan in positive polarity showing two major fragments of L-phenylalanine obtained from a direct injection of L-phenylalanine standard.........................38

Figure 3.1 : Trichome of the cyanobacterium *Aphanizomenon issatschenkoi* (Live material under phase contrast 630X)..52

Figure 3.2 : Batch culture growth of *Aphanizomenon issatschenkoi* in 100%, 5% and 1% nitrogen-rich media. 53

Figure 3.3 : Chlorophyll-a levels (µg/L) and phycocyanin to chl-a ratios (627 nm:438 nm) of *Aphanizomenon issatschenkoi* grown in 100%, 5% and 1% nitrogen-rich media. 54

Figure 3.4 : ANTX culture concentrations (µg/L) of total toxin and intracellular toxin in *Aphanizomenon issatschenkoi* grown in 100%, 5% and 1% nitrogen-rich media. 55

Figure 3.5 : Anatoxin-a cellular content (µg/g) dry weight of *Aphanizomenon issatschenkoi* grown in 100%, 5% and 1% nitrogen-rich media. 56

Figure 4.1 : Non-toxic degradation products of ANTX A) A-dihydroanatoxin-a and B) B-epoxyanatoxin-a. 70

Figure 4.2: Fugacity-based model (level I) simulation of an emission of 200,000 kg of ANTX in an EQC environment...71

Figure 4.3 : Fugacity-based model (level II) simulation of an emission of 200,000 kg of ANTX in an EQC environment using a half-life in water of 120 hours.. 72

Chapter I: General Introduction

1.1 Cyanobacteria and their toxins

Cyanobacteria are one of the oldest and most successful life forms. The phylum includes gram-negative phototrophic prokaryotes found in virtually every habitat from marine and fresh waters to soil, ice, rocks and within other organisms as endosymbionts. They include important nitrogen fixing species and all have the capacity to use light at wavelengths that most other photosynthetic organisms cannot utilize efficiently (Whitton and Potts 2000). Cyanobacteria, or blue-green algae as they were previously named, are often labelled by the popular press as undesirable and toxic. However, they are not pathogens since they cannot cause diseases by colonizing, invading or growing in an animal host (Codd *et al.* 2005). What makes them potentially dangerous is the cyanotoxins some taxa produce. The adverse health effects of cyanotoxins on humans and other animals have been reported throughout the world and range from mild to fatal (Chorus and Bartram 1999).

Cyanotoxins can be divided into four main groups according to their mode of action: neurotoxins, hepatotoxins, cytotoxins and irritants including gastrointestinal toxins (Table 1.1, Chorus and Bartram 1999, Codd *et al.* 2005). Hepatotoxins, especially the cyclic heptapeptide microcystins (MCs), are among the most frequently detected cyanotoxins in freshwaters. MCs were first reported and described by Bishop *et al.* (1959) in *Microcystis aeruginosa*. Since that report, microcystins have been described and detected in a wide range of cyanobacterial genera including *Anabaena* and *Planktothrix*. Over 80 variants of the molecule have been reported thus far but the most common appear to be microcystin-LR, microcystin-LA, microcystin-RR and microcystin-YR (WHO 2008). Human exposure to MCs can cause a wide range of symptoms including: stomach cramps, fever, headache, muscle pain, liver damage and, in cases of acute exposure, mortality. MCs cause protein

phosphatase inhibition in cells resulting in oxidative stress (Rajaneesh and Jaswant 2010). Another cyanobacterial hepatotoxin that is also considered a cytotoxin is cylindrospermopsin (CYN), which was first isolated in the early 1990s (Ohtani *et al.* 1992). This toxin is produced by several species including *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Umezakia natans* and the benthic species *Lyngbya wollei* (Wood and Stirling 2003, Seifert *et al.* 2007). Three natural variants exist: cylindrospermopsin, 7-epicylindrospermopsin and deoxycylindrospermopsin (Bownik 2010). CYN may induce gastroenteritis, kidney malfunction, and hemorrhaging in rodents, and genotoxicity in human cell lines. Other cyanotoxins include irritants and gastrointestinal toxins (e.g. aplysiatoxin, debromoaplysiatoxin, lyngbyatoxin) that may also be tumour promoters. These are largely produced by marine cyanobacteria and have not been well studied in freshwater (Codd *et al.* 2005).

However, the most acutely toxic cyanobacterial metabolites in terms of lethal dose (LD_{50}) are the neurotoxins (Table 1.1). Neurotoxic saxitoxins have long been associated with blooms of dinoflagellates (red tides) in the marine environnement and can cause widespread fatalities from fish to birds, beluga whales and humans (Lair *et al.* 2002). Anatoxin-a (Fig. 1.1) is a chemically similar neurotoxin and is found in freshwaters worldwide (Osswald *et al.* 2007). Anatoxin-a is the subject of this thesis.

1.2 Anatoxin-a, homoanatoxin-a and anatoxin-a(s) : phytochemistry and toxicity

Prior to its chemical identification, anatoxin-a (ANTX) was labeled the Very Fast Death Factor (VFDF) due to its ability to induce death within four minutes after

intraperitonial injection (i.p.) in mice (Osswald *et al.* 2007). The toxin was identified in the literature based on its toxicological symptoms including tremors and gasps preceeding death (Gorham *et al.* 1964). In the mid 1960s, Gorham and Stavric (1966) isolated a toxic strain of *Anabaena* (NRC-44) from Canadian waters and reported that the toxic agent was a low molecular weight amine. In fact, ANTX is a secondary amine (2-acetyl-9-azabicyclo(4.2.1) non-2-ene)(Fig. 1.1) with a molecular weight of 165.

ANTX is considered to be the smallest toxic alkaloid yet characterized and has been synthesised from cocaine out of pharmacological interest due to the similarities in chemical structure (Devlin *et al.* 1976, Campbell 1977, Osswald *et al.* 2007). The biosynthesis of anatoxin-a was first investigated by Gallon *et al.* (1990) (cited in Osswald *et al.* 2007). Gallon *et al*. (1994) and Hemscheidt *et al.* (1995) hypothesized that the amino acid putresceine was involved in the biosynthesis of ANTX via the synthesis of pyrolline. Recently, Méjean *et al.* (2010) identified the gene cluster responsible for the synthesis of ANTX (Fig. 1.2) suggesting a biosynthetic route for anatoxin-a starting from the amino acid L-proline.

In 1992, a more stable homologue to ANTX was synthesized for research purposes related to its neurological effects and mode of action (Wonnacott *et al.* 1992). Homoanatoxin-a (2-(propan-1-oxo-1-yl)-9-azabicyclo[4.2.1]non-2-ene) has the same chemical structure as ANTX except for an additional methylene (CH_2) unit (MW=179) on the side chain (Fig. 1.1). Its toxicity is similar to ANTX (Cronberg and Annadotter 2006).

Anatoxin-a(s) is chemically different from ANTX (Fig. 1.1) and is a naturally occurring phosphate ester of a cyclic N-hydroxyguanine (MW = 252) (Cronberg and Annadotter 2006). It is produced by *Anabaena flos-aquae* (NRC 525-17) and *Anabaena*

lemmermannii from which it was isolated during a bloom (Matsunaga *et al.* 1989, Onodera *et al.* 1997). Anatoxin-a(s) is about ten times more toxic than ANTX (Carmichael *et al.* 1990). Both ANTX and anatoxin-a(s) are potent nicotinic cholinergic agonists and act as a post-synaptic depolarizing neuromuscular blocking agent (Carmichael *et al.* 1975, Devlin *et al.* 1977, Matsunaga *et al.* 1989). For ANTX, the LD_{50} estimated by Carmichael *et al.* (1975) is 200-250 µg/kg of mouse body weight injected intraperitonially. Under field conditions, death by respiratory arrest of livestock, pets and wildlife has been observed as a consequence of ingestion of ANTX producing cyanobacteria (Stevens 1991).

1.3 Anatoxin-a extraction and analyses

At present, New Zealand and Australia are countries with specific ANTX guidelines (Ministry of Health of New Zealand 2005, Hoehn and Lang 2002). As opposed to other cyanotoxins like microcystins, there are no guidelines yet for anatoxin-a in drinking or recreational waters in Canada (Health Canada) or the U.S.A. This is partly due to an absence of standardized extraction and analytical protocols.

Prior to analysis of anatoxin-a, sample pre-treatment is necessary. Cellular lysis is required to extract the intracellular toxin. However, cyanobacteria with their thick cell walls and, in some taxa, mucilaginous sheaths, are quite resistant to lysis. Common methods are to freeze-dry or freeze-thaw the cells prior to ultrasonication or shaking (e.g. Stevens and Kreiger 1988; Ojanperä *et al.* 1991; Van de Waal *et al.* 2009). Another effective extraction method is pressurized liquid extraction (PLE). This method exposes the cells to a high temperature and pressure and is an alternative to ultrasonication. It has proven efficient with microcystins (Aranda-Rodriguez *et al.* 2005). The solvents most commonly used for ANTX extraction are water, acidified water, acidified methanol and a mixture of water and methanol

(Osswald *et al.* 2007). After comparing water, 0.05M acetic acid-water, methanol and 0.05M acetic acid-methanol, Harada *et al.* (1989) concluded that aqueous acetic acid was the most effective solvent. In contrast, Rapala *et al.* (1993) found that water was the most efficient solvent in terms of ANTX extraction. Following the extraction of the intracellular fraction, a purification step is often required to isolate the cyanotoxin from the extracts in order to avoid analytical interferences. Solid phase extraction (SPE) is the most commonly used clean-up method (Osswald *et al.* 2007, James *et al.* 2005).

Chromatography is the main analytical method used for ANTX detection. So far, ANTX levels have been successfully quantified in environmental samples using High Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS), ultra-violet (UV) or fluorescence (FL) detection (Osswald *et al.* 2007). Gas chromatography (GC) coupled with (MS) has also been used (Aráoz *et al.* 2005). In addition, a non-radioactive ligand-binding assay quantifying ANTX based on the affinity of ANTX for nicotinic acetylcholine receptors (nAChRs) was recently developped (Aráoz *et al.* 2008). As with other toxins, aside from chromatography, bioassays have been used, notably the brine shrimp (*Artemia salina*) larvae test (Osswald *et al.* 2007)

1.4 Factors influencing anatoxin-a production

ANTX production appears to be strain specific but arises in several cyanobacterial genera including to date *Anabaena, Aphanizomenon, Microcystis, Planktothrix (Oscillatoria), Raphidiopsis, Cylindrospermum, Phormidium* and *Nostoc* (Osswald *et al.* 2007). These genera include members of the *Chlorococcales* as well as the *Nostocales* and include both nitrogen fixing and non-fixing species (Whitton and Potts 2000). These are

widespread genera and as a result, ANTX has been reported throughout the globe (Osswald *et al.* 2007). However, few studies have examined the environmental factors that lead to anatoxin production compared to the number of studies that have examined microcystins in freshwater. A Web of Science data base search of “anatoxin” yielded, 562 papers published since 1990 compared to 2150 for “microcystin” (March 10, 2011). Why anatoxin is found in some freshwater systems as opposed to others is not known.

In general, cyanotoxins are considered secondary metabolites and not part of the cell’s primary metabolism (Carmichael 1992, Gademann and Portmann 2008). Consequently, any factor limiting cyanobacterial growth should therefore inhibit or decrease the production of cyanotoxins. Few studies have examined the conditions that give rise to maximum ANTX production and the few results presented in the literature are not always in agreement. This may be the result of differences in cyanobacterial strains and/or growth conditions. Rapala *et al.* (1993) observed the highest ANTX concentrations in *Anabaena* and *Aphanizomenon* within the first 2 weeks of batch growth as did Peary and Gorham (1966). Other studies found the highest ANTX concentrations after 3 weeks and even after 6 to 7 weeks (Kiviranta *et al.* 1991; Bumke-Vogt *et al.* 1996). Regardless of the time frame, all these studies seem to indicate that the highest ANTX levels occurred somewhere in the exponential phase as opposed to the stationary phase. Bumke-Vogt *et al.* (1996) demonstrated that ANTX concentration (cellular and dissolved phases combined) in *Anabaena flos-aquae* followed the growth curve up to 6 to 7 weeks then reached a plateau and then declined before the stationary phase was reached. This suggests that the ANTX production rate might not be directly correlated to the growth rate. With respect to the effect of temperature, three studies reported that values from 19.8 to 22° C yielded the highest concentrations of ANTX in *Anabaena* sp. and *Aphanizomenon flos-aquae*, which generally

corresponded to the maximum growth rates (Rapala *et al.* 1993, Peary and Gorham 1966, Rapala and Sivonen 1998).

The availability of nutrients may influence the cellular investment in cyanotoxin production independently of the growth rate as suggested by the carbon-nutrient hypothesis (e.g. Hamilton *et al.* 2001). This hypothesis links the amount of secondary metabolites produced by plants to the relative abundance of nutrients and makes three assumptions: 1) growth takes priority over secondary metabolite production (Tuomi *et al.* 1991), 2) nutrient limitations constrain growth more than rates of photosynthesis and light limitation constrains photosynthesis more than growth (Bryant *et al.* 1983) and 3) the concentration of precursor molecules is the most important determinant of the rate of secondary metabolite production (Reichardt *et al.* 1991).

With respect to phosphate (PO_4^-), Rapala *et al*. (1993) reported that higher concentrations of PO_4^- led to higher cyanobacterial dry weight in batch-cultures of one strain of both *Aphanizomenon flos-aquae* and *Anabaena mendotae* and two strains of *Anabaena flos-aquae*. However, no significant difference in anatoxin-a concentration was observed, which contradicts the first assumption of the carbon-nutrient hypothesis. In a subsequent study, Rapala and Sivonen (1998) observed a relationship between P and ANTX in lake samples where higher levels of ANTX were associated with higher levels of PO_4-.

In the case of soluble nitrogen, its availability would allow cyanobacterial growth and production of nitrogen-rich secondary metabolites such as alkaloids and proteins if there was no other limiting-factor (Van de Waal *et al.* 2009). However, Rapala *et al.* (1993) observed that an increase in nitrate (NO_3^-) led to a decrease in ANTX concentration. Additionnaly, ANTX levels were higher in the presence of NH_4 or only N_2, which is suprising given the high energy requirement for atmospheric nitrogen (N_2) fixation. The carbon-nutrient balance

hypothesis does seem applicable to these results. However, it is important to emphasize that anatoxin-a ($C_{10}H_{15}NO$) □ although being an alkaloid □ has one nitrogen atom for 10 atoms of carbon. Thus, the availability or allocation of carbon might have a greater role to play in its production than nitrogen itself.

1.5 Anatoxin-a: environmental fate

Due to its small molecular weight (165.26 g/mol), anatoxin-a can pass through the cell membrane of its producer and consequently be found both extra- and intracellularly. It is not known, as yet, if anatoxin-a accumulates in cells, and if so, under what conditions, or if the cyanotoxin simply passes through the cell membrane as it is being produced (Osswald *et al.* 2007). A few studies have separately analysed the levels of intracellular and extracellular ANTX but the results are contradictory. Rapala *et al.* (1993) did not observe any extracellular ANTX in *Anabaena flos-aquae* and *Anabaena mendotae.* However, 4 to 18% of total anatoxin-a analysed in batch cultures was found extracellularly in *Aphanizomenon flos-aquae*. It was not clear if this difference was due to taxonomic/strain differences or to bacterial degradation in the media since the strains used were not axenic. Some bacteria (*Pseudomonas*) are able to degrade dissolved anatoxin-a (Kiviranta *et al.*1991). More recently, Bumke-Vogt *et al.* (1999) found that the extracellular portion represented 40 and 50% of the total anatoxin-a analysed in 80 water bodies in Germany.

In terms of its behaviour in the aquatic environment, unlike the microcystins, ANTX is quite soluble in water, which is a concern for drinking water supplies when toxic cyanobacterial blooms occur (Takino *et al.* 1999, Osswald *et al.* 2007). ANTX possesses a low octanol-water partition coefficient (K_{OW}) as estimated by the Environmental Protection

Agency (EPA) and available on Chemspider (www.chemspider.com). Under normal environmental conditions, the half-life in water of ANTX was estimated as 14 days at pH 8-10 by Smith and Sutton (1993). However, ANTX appears quite labile in sunlight and Stevens and Krieger (1991) estimated its half-life for photochemical breakdown as 1-2 hours at the pH measured within cyanobacterial blooms (pH 8-9).

Several studies have reported anatoxin-a in lakes (Bumke-Vogt *et al.* 1999, Takino *et al.* 1999, Bogiali *et al.* 2006) but none have examined its fate following a toxic cyanobacterial bloom. It is not known if it can travel through air as can other algal toxins and how long it persists. In the meantime, anatoxin-a partitioning and fate in freshwater ecosytems can be studied using theoretical models (e.g. Mackay *et al.* 2001).

1.6 Thesis Objectives

This thesis has three objectives. The first objective is to develop a common extraction and analytical method for both freshwater cyanotoxins, anatoxin-a and microcystins (Chapter 2). At present there are separate methods for these two commonly encountered cyanotoxins requiring separate extractions and analyses. Clearly, a common method would facilitate more rapid detection of potentially toxic blooms. Starting with a pressurized liquid extraction method, first developped for microcystin (Aranda-Rodriguez *et al.* 2005), a protocol was developped for analysing both toxins using liquid chromatography-mass spectrometry (LC-MS).

The second objective (Chapter 2) is to compare ANTX production in different cyanobacterial genera and test the carbon-nutrient hypothesis with the most productive strain

(Chapter 3). If the carbon-nutrient balance hypothesis is correct, one would predict higher total (both intra and extracellular) toxin levels under higher nutrient conditions.

The last objective of this thesis is to use fugacity-based models (Mackay *et al.* 2001) to evaluate the fate of anatoxin-a in freshwaters under specific environmental conditions (Chapter 4).

Table 1.1 : Types of cyanotoxins based on their mode of action and estimates of LD50.

Modified from Codd *et al.* 2005, WHO 2008 and Bownik *et al.* 2010.

Mode of action	Cyanotoxins	LD_{50} (i.p. mouse, µg/kg body wt)
Neurotoxins	Anatoxin-a	250
	Anatoxin-a(s)	20-40
	Saxitoxins	10–30
Hepatotoxins:	Microcystins	25-1000
	Nodularins	30-50
	Cylindrospermopsin	200-2100
Cytotoxins	Cylindrospermopsin	200-2100
Irritants and Gastrointestinal Toxins:	Aplysiatoxin,	n/a
	Debromoaplysiatoxin,	n/a
	Lyngbyatoxin	n/a
	Lipopolysaccharide endotoxins (LPS)	n/a

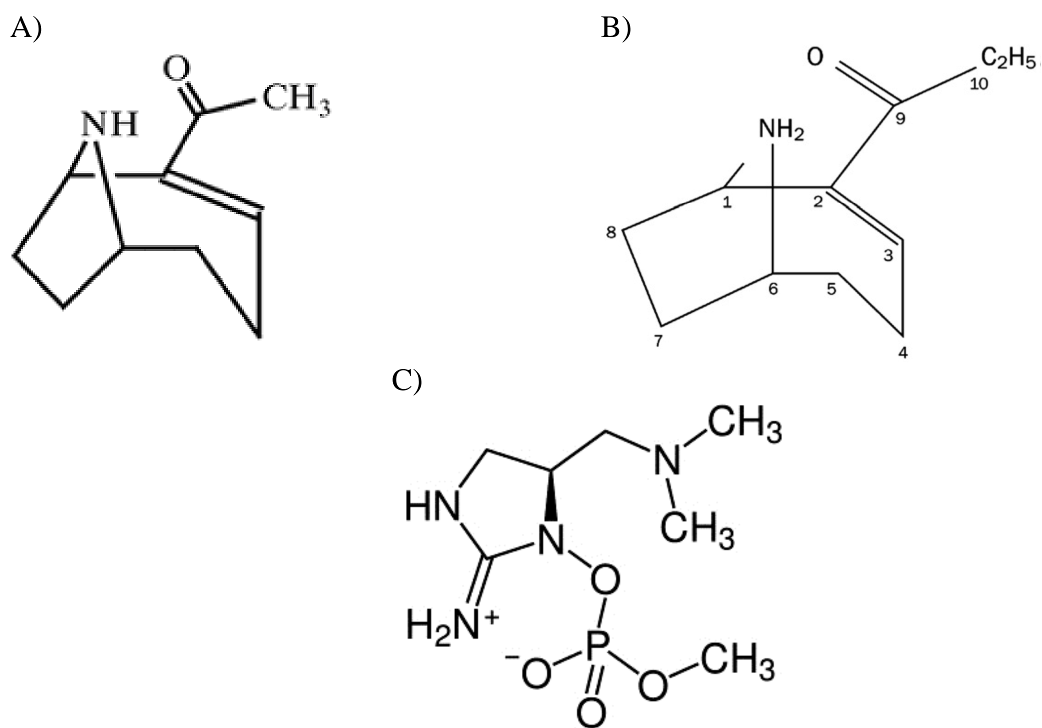


Figure 1. 1 : Chemical structures of A) anatoxin-a, B) homoanatoxin-a and C) anatoxin-a (s).

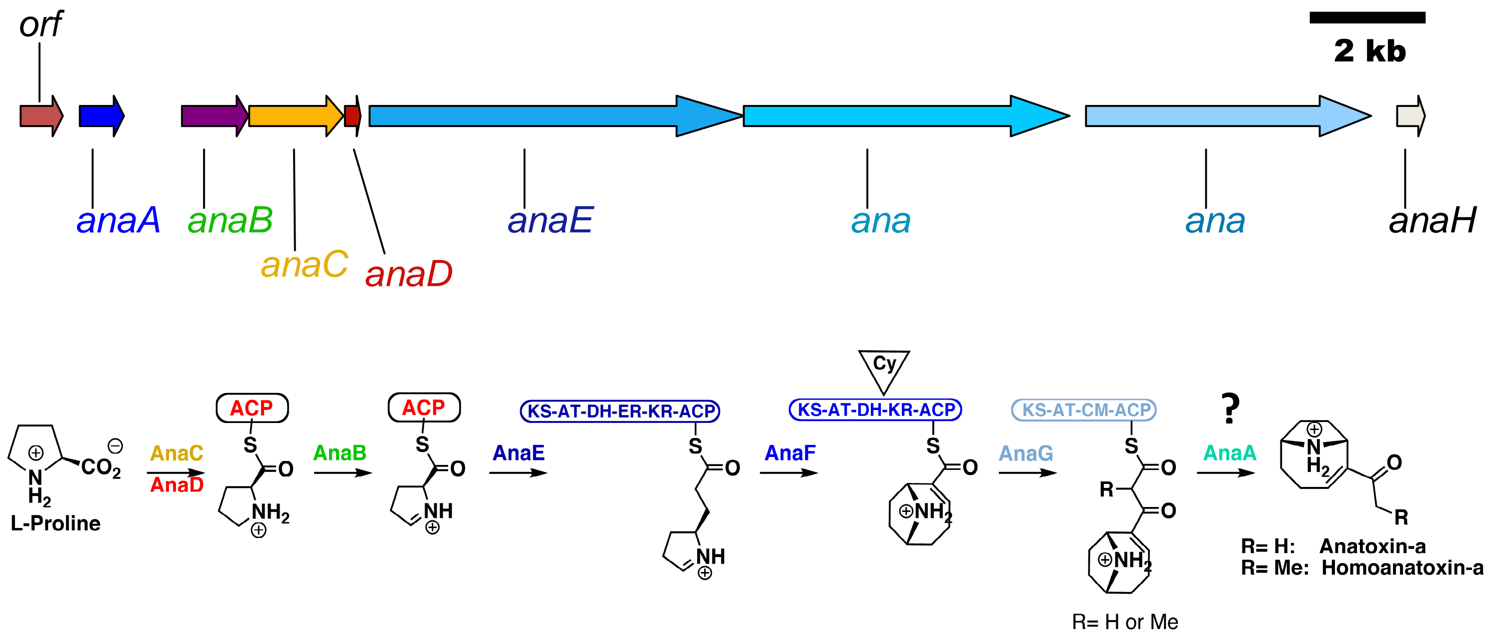


Figure 1. 2 : The ana gene cluster responsible for biosynthesis of anatoxin-a and homoanatoxin-a in cyanobacteria and postulated biosynthetic route leading to these neurotoxins. Used with permission from Dr Olivier Ploux (CNRS, France). The following abbreviations stand for: KS: ketosynthase, AT: acyltransferase, KR: ketoreductase, DH: dehydratase, ER: enoylreductase, ACP: acyl carrier protein, Cy: cyclase and CM: methyltransferase.

Chapter 2: Development of a common extraction and analytical method for anatoxin-a and microcystins

2.1 Introduction

Cyanobacterial toxins (CBTs) are a growing environmental and health concern worldwide (Chorus and Batram 1999, Sivonen and Jones 1999, Zurawell *et al.* 2005). The hepatotoxic microcystins (MCs) and the neurotoxic anatoxins are among the most commonly encountered in freshwater (WHO 2003, Codd *et al.* 2005). Different sample preparation (i.e. extraction) and analytical methods are being applied to detect them due to their different chemical properties. No universal extraction and analytical protocols that can be routinely applied in a high throughput manner to detect CBTs in aquatic samples have been developed and standardized.

Prior to the analysis of CBTs, special sample pre-treatment is normally a mandatory step in order to obtain acceptable extraction yields and minimize the matrix effects during the analysis. Cyanobacteria have thick gram-negative cell walls that require effective extraction techniques to ensure complete cell lysis and acceptable yields (Campinas and Rosa 2010). The most common extraction methods applied to MCs and anatoxins (ANTX) include the following steps: incubating the algal biomass with an extraction solvent (acetic acid, methanol or water), freeze-drying or freeze-thawing the cells, mixing (vortexing and shaking), sonicating and/or using solid-phase extraction (SPE) (Sangolkar *et al.* 2006, Osswald *et al.* 2007). Another method, used for microcystins, is accelerated solvent extraction (ASE) (Aranda-Rodriguez *et al.* 2005). ASE subjects the cells to a simultaneous high temperature, pressure and a fixed volume of solvent.

A few studies have compared the extraction efficiency of solvents for both toxins. Methanol (75%) has been reported as the most effective solvent to extract microcystins and produce better reproducibility (Fastner *et al.* 1998). However, the sample matrix plays an important role in the extraction process and it was reported that EDTA-sodium pyrophosphate was a suitable solvent for the extraction of MCs from sediments and soils (Chen *et al.* 2006). In the case of ANTX, water, acetic acid-water, methanol and acetic acid-methanol are the most commonly used extraction solvents (Sangolkar *et al.* 2006, Osswald *et al.* 2007). Harada *et al.* (1989) concluded that aqueous acetic acid (0.05M) was the most effective solvent. In contrast, Rapala *et al.* (1993) found that water was the most efficient solvent for the extraction of ANTX.

In order to minimize analytical interferences, a solid phase extraction (SPE) clean up followed by a concentration step is the most commonly used method to preconcentrate, purify and extract both microcystins and anatoxins from cyanobacterial cells and from the dissolved phase (Sangolkar *et al.* 2006, Osswald *et al.* 2007). For microcystins, normal-phase Cyano cartridges (CN), C_{18} reversed-phase octadecyl siloxane (ODS) silica gel cartridges and immunoaffinity cartridges are commonly used (Pyo and Shin 2002, Rapala and Lahti 2002) to improve detection and identification (Aranda-Rodriguez *et al.* 2003). The immunoaffinity cartridges, consisting of monoclonal or polyclonal antibodies, are not common due to their high cost and time required for use (Sangolkar *et al.* 2006). Metcalf and Codd (2000) used microwave extraction from boiling water for MCs to avoid organic solvents in order to reduce the matrix effects during analysis. For anatoxin-a, an ODS silica gel or a weak cation-exchange (COOH) are the most commonly used SPE cartridges (Harada *et al.* 1989, James *et al.* 1998, Namikoshi *et al.* 2003). Solid phase micro-extraction (SPME) has been used to simultaneoulsy extract, concentrate and clean-up samples, but the recovery

results appear less satisfactory (Namera *et al.* 2002, Rallén *et al.* 2007). SPME is still not as widely used as SPE.

Once extracts are ready for analysis, several analytical techniques have been used from mouse and invertebrate bioassays to gas or liquid chromatography coupled with different analytical techniques (Lahti K *et al.* 1995, Pérez and Aga 2005, Osswald *et al.* 2007). High performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection and mass spectrometry (MS) are common analytical methods for microcystins (Robillot *et al.* 2000, Sangolkar *et al.* 2006). Separation by liquid chromatography generally involves a C_{18} silica column. In the case of anatoxin-a, UV detection coupled to HPLC is not very sensitive, although it has been used with some success on environmental samples (Carrasco *et al.* 2007, Osswald *et al.* 2007). The sensitivity can be considerably increased with fluorimetric detection (FL) when ANTX is derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (James *et al.* 1998). Mass spectrometry (MS) is more widely used for anatoxin-a detection. For microcystins and anatoxin-a, different ionization techniques have been used for MS analysis including electrospray ionization (ESI), atmospheric pressure ionization (API), thermospray (TSP), matrix-assisted laser desorption/ionization (MALDI), fast atom bombardment (FAB), time of flight coupled to MALDI (Msagati *et al.* 2006, Sangolkar *et al.* 2006). Mass spectrometry (MS) allows mass confirmation, hence improving the accuracy of identification (Spoof *et al.* 2003).

As an alternative to LC, gas chromatography coupled to MS or electron capture detection (ECD) has been used for anatoxin-a (Sivonen *et al.* 1989, Aráoz *et al.* 2005). ANTX must be derivatized in order to get an enhanced sensitivity with the gas chromatographic analysis (Osswald *et al.* 2007). GC/MS methods were developed as well for

microcystins but only for screening as it is not possible to distinguish MC variants with such a method and it is time-consuming in comparaison to LC (Sangolkar *et al.* 2006).

One challenge in the analysis of anatoxin is that ANTX can be misidentified with the isobaric naturally occurring amino acid phenylalanine (PHE). Additionally, both compounds have similar molecular structures and fragmentation patterns (Fig. 2.1) (Furey *et al.* 2005). PHE has been misidentified as ANTX in forensic investigations (Dimitrakopoulos *et al.* 2010). Few studies have successfully distinguished both compounds in environmental samples. Furey *et al.* (2005) evaluated different strategies to avoid misidentification of ANTX by using tandem mass spectrometry. They distinguished these molecules by conducting MS^3 experiments on a time-of-flight (QqTOF) mass spectrometer. Dimitrakopoulos *et al.* (2010) developed an analytical method for the detection of anatoxin-a using LC-MS/MS with phenylalanine-d_5 as an internal standard which clearly distinguished PHE from ANTX.

The purpose of this study was to develop an extraction and analytical method for anatoxin-a and microcystin (using nodularin, a similar cyanotoxins but absent from freshwater, as an internal standard) to improve their recovery and detection in environmental samples. In 1995, Aranda-Rodriguez *et al.* developped a pressurized liquid extraction method for MCs. Two variants of microcystin (MC-LR and MC-RR) were successfully extracted using an ASE method with recovery values ranging from 79% to 105%. However, ASE provided lower (ca. 50%) extraction recovery for anatoxin-a even at lower temperature values such as 40 °C. In this study, this method was modified to allow extraction of both MCs and ANTX. A spike recovery monitoring approach was employed to maximize the extraction yield and achieve better SPE conditions. To monitor the recovery, a selective LC-MS/MS method on a 3200 QTRAP system with a sub-2 micron particle size column was

used. Selective MRM transitions (2 transitions for each molecule) were obtained for each analyte. To achieve optimal separations within a very short run time, suitable mobile phase composition and linear gradient conditions, which enabled the identification of the target molecules in a single run in a high throughput manner, were applied.

2.2 Material and Methods

Cultures, chemicals and standards

Several major algal culture collections were surveyed in order to obtain different genera of potential ANTX producers. Unfortunately, few strains are available from culture collections and further isolations are needed. In order to compare ANTX production between different genera, two species of *Nostocales* and one species of *Oscillatoriales* were obtained (Table 2.1). *Anabaena flos-aquae* (Lyngb.) de Breb. (Nostocaceae) (UTEX LB 2383) was isolated from Burton Lake, Saskatchewan, Canada and obtained from the University of Texas culture collection, U.S.A. *Oscillatoria sp.* (PCC 6407) was isolated in California (exact location is unknown), U.S.A. and obtained from the Pasteur Institute, France. *Aphanizomenon issatschenkoi* (Ussaczew) Proschkina-Lavrenko (Nostocales) (CAWBG02) was obtained from Cawthron Institute, New Zealand and isolated from Lake Hakanoa in New Zealand (Fig. 3.1) (Wood *et al.* 2007). Cultures were grown at a light intensity of 85 µE $\cdot m^{-2} \cdot s^{-1}$ and a 12:12 light: dark cycle at 20° Celsius ± 1°C in a Conviron growth chamber (E-15). BG11 growth media was used and purchased as a concentrate from Sigma Aldrich, Canada (Andersen 2005).

Microcystin variants (MC-LR, MC-RR, MC-YR, 7dmLR and MC-LA) and nodularin were purchased from Cedarlane, Toronto, Canada. Anatoxin-a fumarate was purchased from

Tocris Bioscience, U.S.A and L-Phenylalanine from Sigma-Aldrich, Oakville, Canada. Hydromatrix (flux-calcined diatomaceous earth) was obtained from Varian, Montreal, Canada. Water, methanol, acetonitrile, hexane, acetone, formic acid and ammonium formate were obtained from Sigma-Aldrich (Oakville, Canada). CHROMASOLV® HPLC grade solvents were used for extraction, and CHROMASOLV® LC-MS grade were used for the solubilisation and the analysis of the extracts and standards. Stock solutions of microcystin, nodularin, anatoxin-a fumarate and L-Phenylalaline standards were dissolved in 50% aqueous MeOH to a final concentration of 10 ppm for developing MRM transitions. A standard mix was prepared to develop optimal separation conditions at a final concentration of 10 ppm.

Sample preparation

Cultures (10 ml) were filtered through Whatman GF-C filters that were preignited at 500 °C for ~2 h and weighed prior to use. Filtrates were collected in solvent rinsed (hexane and acetone) amber scintillation vials. Filters with wet biomass were oven-dried at ~50 °C for about 24 h, weighed and stored with filtrates at –20 °C prior to extraction.

Extraction of anatoxin-a from solid and dissolved phases

In preliminary tests, a sonicating bath proved inefficient for cell lysis to release the toxins as compared to the Accelerated Solvent Extraction (ASE) (data not presented). The latter technique was chosen due to its speed, automation and low solvent consumption. The extraction of ANTX from cyanobacterial cells was performed using an ASE 200 device Dionex Corporation (Bannockburn, IL, USA). GF-C filters with frozen biomass were thawed and inserted in stainless-steel ASE cells (11 ml), which were packed with pre-washed

hydromatrix. ASE parameters were initially set according to the protocol of Aranda-Rodriguez *et al.* (2005). A series of spike recovery extractions were undertaken at concentrations of ANTX ranging from 100 to 2000 ppb. The following parameters were optimized to achieve the best recoveries of target compounds: ASE temperature, ASE pressure, extraction without Hydromatrix, Turbovap (Zymark) water bath temperature and rinsing solvents and their volumes. All extracts were collected in solvent rinsed (acetone and hexane) amber vials to avoid photodegradation of ANTX. In order to adjust the pH so that ANTX remained stable during the extraction process (Aranda-Rodriguez, personnal communication), all extracts obtained from cyanobacterial cells and filtrates were spiked with formic acid prior to evaporation. Stevens and Krieger (1991) observed that anatoxin-a was more stable in acidic conditions due to the reactivity of its free base at lower pH values. Volumes of 0 µl, 50 µl and 100 µl formic acid were tested to evaluate those that led to better analyte recovery. Extracts were then transfered to solvent rinsed Turbovap tubes (Zymark) and evaporated to dryness using a Turbovap II (Zymark) device with water at 59 °C and under a gentle nitrogen flow. No difference in recovery was observed between water bath temperature values of 50 °C and 59 °C, although the latter temperature provided faster evaporation. Tube walls were rinsed and evaporated to dryness. Five ml of 50% MeOH and then five ml of 100% MeOH were used to rinse the walls of the Turbovap tubes. Extracts were then re-suspended in 1 mL of 50% MeOH in water. Filtrates were freeze-dried under vacuum at –40 °C using a Super Moduylo freeze-dryer (Thermo Fisher Scientific Ottawa, Canada). The walls of the scintillation vials were rinsed with 1ml of MS grade 50% MeOH/Water for resupension. To effectively remove cellular debris from the extracts and filtrates without losing the compounds of interest, a comparison between centrifugation

(4000 rpm at room temperature) and filtration with syringe filter (Acrodisc, 0.2 µm, PTFE) was performed prior to analysis.

LC-MS/MS Analysis

The method was optimized on a QTRAP 3200 LC-MS/MS (ABSciex, Toronto, Canada). The system consisted of a 1200 series Agilent liquid chromatograph with a high performance autosampler (model G1376B), a binary pump (model G1312A), a column thermostat (model G1316A) and a triple quadrupole linear ion trap mass spectrometer equipped with a turbospray ion source. The acquisition of data was performed using electrospray ionization (ESI) in positive mode and Analyst software (version 1.4.1). Chromatographic separations were achieved with a Zobrax SB-C18 Rapid Resolution column (50 × 2.1 mm I.D., 1.8 µm particle size column) and a guard column (12.5 × 2.1 mm I.D, 5 µm) (Agilent Technologies, Canada) at 40 °C. The optimal mobile phase conditions were: MeOH in water with 0.1% formic acid and ammonium formate 40 mM with a constant flow rate of 0.3 ml/min. Injection volume for samples was set to 1 µl. The needle was washed with 50% methanol, 50% acetonitrile and 0.01% formic acid at the flush port (3×) after each injection to minimize carry over.

MCs, ANTX and PHE standards of 1 to 20 ppm were dissolved in 50% methanol and individually infused in the MS with a 4.6 mm I.D Harvard syringe at a flow rate of 10 µL/min**.** Multiple reaction monitoring (MRM) scans were performed for each standard by selecting the protonated molecular ion $[M + H]^{+1}$ with the first mass filter quadrupole Q1. Two product ions yielded by collision-induced dissociation using the second mass filter quadrupole Q2 were scanned by the third quadrupole (Q3). The source parameters were

optimized using flow injection analysis (FIA) using the mobile phase and appropriate flow rate suitable for the column.

Method Validation

Standard mix solutions containing all eight standards were prepared at the following concentrations: 1 ppb, 10 ppb, 100 ppb, 500 ppb and 1000 ppb in 50% methanol (MeOH) and water and stored in amber vials at –20 °C. After the optimization of chromatographic and mass spectrometric parameters, the calibration curve and linearity were determined by performing a linear regression analysis of the plotted standard peak area against the respective standard concentration average value of three injections. The instrument limit of detection (LOD) for each analyte was defined as three times the signal to noise ratio (3 x S:R) for a standard mix sample directly injected on column. The limit of quantification (LOQ) for each analyte was defined as 9 times S:R for a spike recovery sample performed on the ASE. Intra-day precision was evaluated by triplicate analysis of samples injected within the same analytical run. Method recovery was defined as the ratio of the intensity of spike recovery samples and standard injection on column for the same concentration.

2.3 Results and Discussion

Optimization of ASE

In this study, spiking an extract with 50 µL of formic acid led to an increase of about 6 fold in ANTX recovery between two 10 ml filtrate samples of *Aphanizomenon issatschenkoi* (Fig. 2.2). This can probably be explained by the fact that protonation provided higher stability for the toxin in the extraction process, thus preventing degradation. Samples

spiked with 100 µl of formic acid provided similar recovery to those obtained with 50 µl spikes. An insignificant variation in spike recovery (1-3%) was observed between the two volumes; hence, all further samples were spiked with a volume of 50 µl of formic acid.

Additionally, ASE parameters (Table 2.2) were modified to improve ANTX recovery. Pressure was increased to 2000 psi and offered the best method recovery values for both MCs and ANTX (results not shown). High speed centrifugation proved to be more efficient than filtration with Acrodisc filters (Pall LC 25mm 0.2um) and led to no apparent loss of the analyte. Acrodisk filters retained about 25% of ANTX present in a 1 ml sample of standard. In contrast, microcystin was filtered with no apparent loss using the same method and filters. Method recovery values for intracellular and extracellular ANTX were 53% and 64% respectively. The recovery for MCs variants and nodularin ranged from 60 to 90%.

LC-MS/MS Analysis of ANTX

A 25 µL injection of ANTX standard at a minimum concentration of 5 ppm was needed to clearly identify the toxin on the HPLC chromatogram (Fig. 2.3). No detectable ANTX was found in 10 mL of cultures of *Aphanizomenon issatschentkoi*, *Anabaena flos-aqua* and *Oscillatoria* sp. due to the low sensitivity of the HPLC-DAD (Fig. 2.4). Hence, further analyses were carried out on the LC-MS for better detection. The optimized LC parameters yielded the separation of all the analytes. ANTX and PHE eluted first at 0.6 min and 0.65 min respectively followed by MC variants (7.51 to 8.30 min) and nodularin (7.74 min) (Fig. 2.5). The analytical column was thermostated to 40 °C and the mobile phase gradient started at 90% water. Unambiguous Q1/Q3 pair transitions were obtained by manually optimizing the declustering potential to get characteristic product ions of isobaric ANTX and PHE (Table 2.3). This approach yielded unique product ions of ANTX at (*m/z*)

149.20 and PHE *m/z* 120. Thus, both compounds were clearly distinguished and misidentification was avoided. Their MRM scan, fragmentation pattern and characteristic ions are featured in Figures 2.6 and 2.7 respectively.

Method validation

The calibration data (Table 2.4) for ANTX, PHE, nodularin, MC-RR and MC-LA standards were linear; r^2 = (0.992-0.999) within the range of 30 and 2000 µg/L and gave a relative standard deviation (% RSV) of ≤ 13.6 (N=3). In the case of MC-7dmLR, MC-YR and MC-LR, linear regressions provided r^2 results of 0.939-0.971, with a range between 30 and 1000 µg/L and with a higher % RSV (23.9, N=3) for MC-LR. The calibration curves for each analyte were obtained using a weighted (1/x) linear regression of peak-area ratios plotted against the respective analytes' concentrations in the standard mix. Instrument limits of detection were calculated for each analyte contained in the standard mix solutions using the software Analyst Data. Detection values varied from 1.07 to 58.82 pg (S/N=3) on column (Table 2.4).

ANTX production in three cyanobacterial genera

All three cultures were grown under identical conditions and subsampled during their exponential phase (between 20 and 30 days of growth). The mean intracellular ANTX concentrations of triplicate samples of *Oscillatoria* sp., *Anabaena flos-aqua* and *Aphanizomenon issatschenkoi* were 189, 156 and 426.1 $\mu g \cdot g^{-1}$ dry weight respectively. Based on these results, *A. issatschenkoi* produced the highest intracellular concentrations of ANTX under current conditions and genotype.

With respect to the extracellular fraction, no detectable ANTX was found in the media of *Anabaena flos-aqua* (n=2) and about 1.9 $\mu g \cdot L^{-1}$ (n=3) was measured for *Aphanizomenon issatschenkoi*. *Oscillatoria sp.* yielded the highest mean concentration of extracellular ANTX with a value of 5 $\mu g \cdot L^{-1}$ (n=2). However, since ANTX degradation could not be assessed, it is hard to properly measure how much ANTX was released in the media. Cultures of the *Oscillatoria* strain showed a lot of clumping of colonies compared to the other two. Consequently, ANTX released in the media of *Oscillatoria* sp. might have been exposed to higher irradiance than in cultures of *Anabaena* and *Aphanizomenon* leading to a faster rate of degradation since ANTX is photodegradable.

Recovery values obtained with the method presented in this chapter are not as good for ANTX as they are for MCs. The sample preparation and extraction processes could hence be improved in order to increase the recovery of ANTX. Using a different matrix, or none at all, might avoid any kind of potential adsorption of ANTX to the matrix in the extraction process. Additionally, ANTX is very unstable under normal conditions and, despite the acidification of the extracts, might degrade in the evaporation process. Using solid phase extraction (SPE) to isolate ANTX might be an appropriate alternative to evaporation. However, since MCs and ANTX are chemically different, both cannot be extracted with the same SPE cartridge. Using either two different SPE cartridges, or a combination of SPE and evaporation, might lead to better recovery. In the meantime, the method presented in this study provides a way to simultaneously extract and analyse MCs and ANTX in the same environmental sample.

Table 2. 1 : Anatoxin-a producing cultures.

Genus	Species	Catalogue number	Obtained from	Reference
Anabaena	*flos-aquae*	LB 2383 (NRC-44h)	UTEX	Gorham *et al.* 1964
Oscillatoria	sp.	PCC 6407	Pasteur Institute	Araoz *et al.* 2005
Aphanizomenon	*issatchenkoi*	CAWBG02	Cawthron Institute	Wood SA *et al.* 2007

Table 2. 2 : Comparison of ASE parameters for ANTX and MCs pressurized liquid extraction.

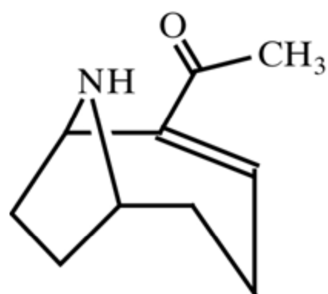
Parameters	Experimental conditions	Values from Aranda-Rodriguez *et al.* (2005)
Pressure (psi)	2000	1015
Temperature (°C)	80	60, 80
Preheat (min)	1	1
Heat(min)	5	5
Static (min)	5	5
Flush (%)	100	100
Purge (s)	120	80
Number of cycles	2	2

Table 2. 3 : MRM transitions of each analyte obtained after infusion into truboV electrospray source of 3200 QTRAP.

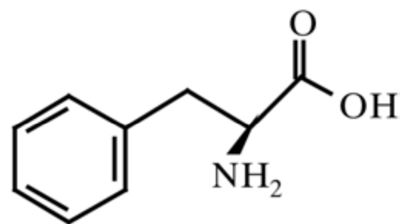
Analytes	Q1 transition	Q3 transition
Nodularin	825.5	135.1
MC-RR	520.1	135.1
MC-LR	995.6	135.1
MC-7dmLR	981.4	135.1
MC-YR	1045.4	135.1
MC-LA	910.6	135.1
Anatoxin-a	166.1	149.2
Phenylalanine	166.1	120.0

Table 2. 4 : Linearity, instrument limits of detection (LOD) and limits of quantification (LOQ) for each analyte.

Analytes	Instrument LOD (pg)	Instrument LOQ (pg)	Retention time (min)	r^2	Range (µg/L)
Anatoxin-a	1.07	3.56	0.6	0.9993	30-1000
Phenylalanine	3.14	10.46	0.65	0.9982	30-2000
MC-RR	4.33	14.43	7.51	0.9973	30-2000
MC-YR	30.00	100	7.73	0.9713	30-1000
Nodularin	2.77	9.23	7.74	0.9958	30-2000
MC-LR	58.82	196.06	7.92	0.9555	30-1000
MC-7dmLR	23.08	76.93	8.00	0.9385	30-1000
MC-LA	20.98	69.93	8.30	0.9922	30-2000



(A)



(B)

(C)

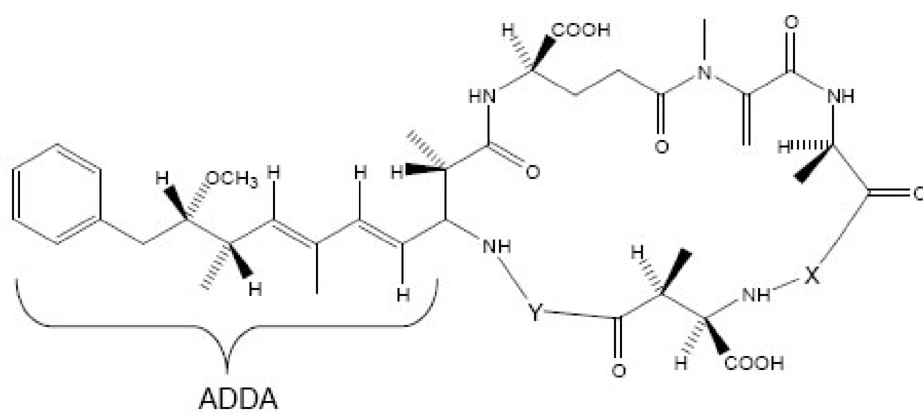
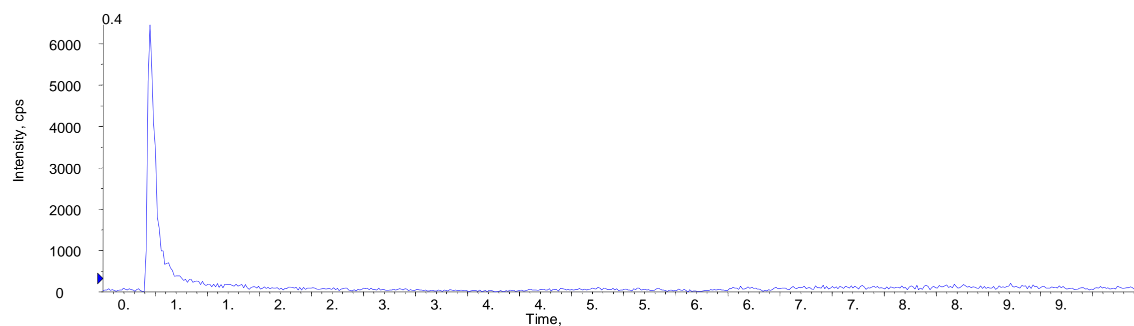


Figure 2.1 : Structures of A) anatoxin-a, B) phenylalanine and C) Microcystin.

A



B

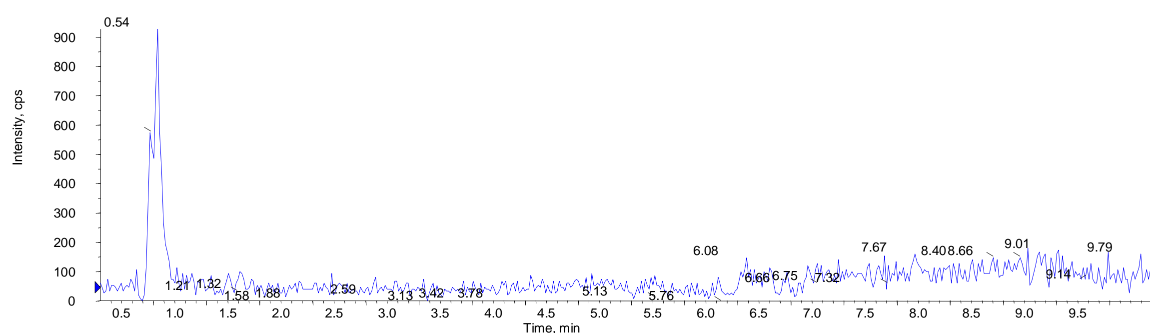


Figure 2.2 : Extraction ion (149.2 amu), corresponding to ANTX (RT: 0.6 min), of two freeze-dried filtrate samples (10 ml) of *Aphanizomenon issatschenkoi* analyzed on the Sciex QTRAP 3200 LC-MS/MS.

Sample A has been spiked with 50 µl of formic acid prior to freeze-drying. No formic acid was added to sample B.

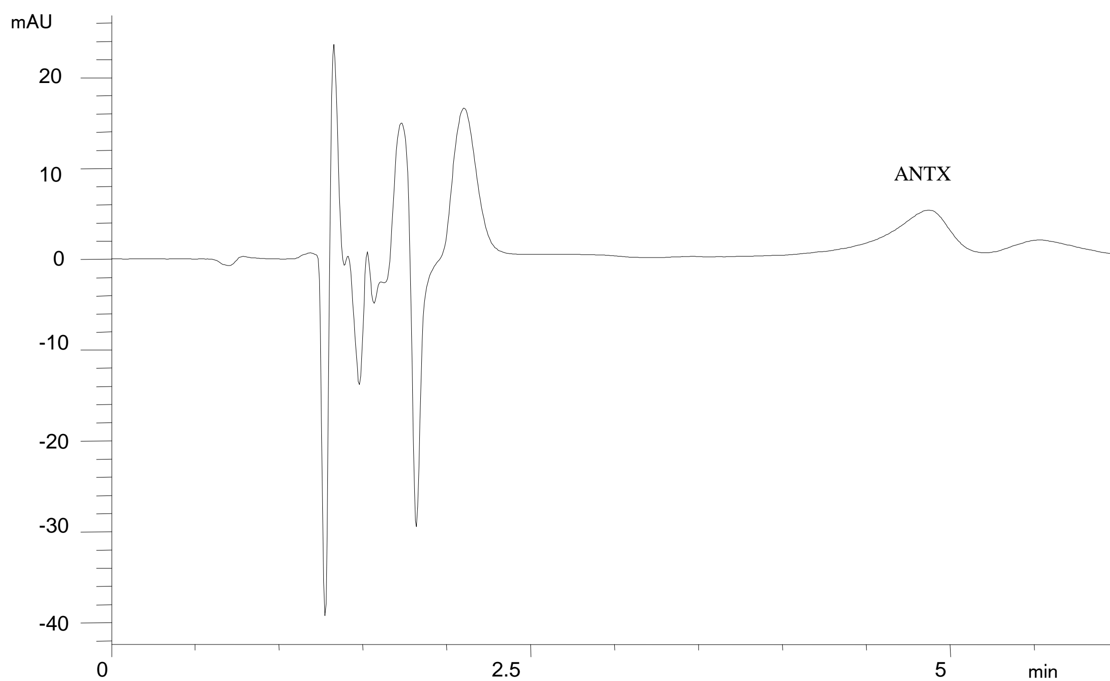


Figure 2.3 : Chromatogram of an injection (25 µl) of ANTX-fumarate standard (5 µg /ml) using an HPLC-DAD at 229 nm detection.

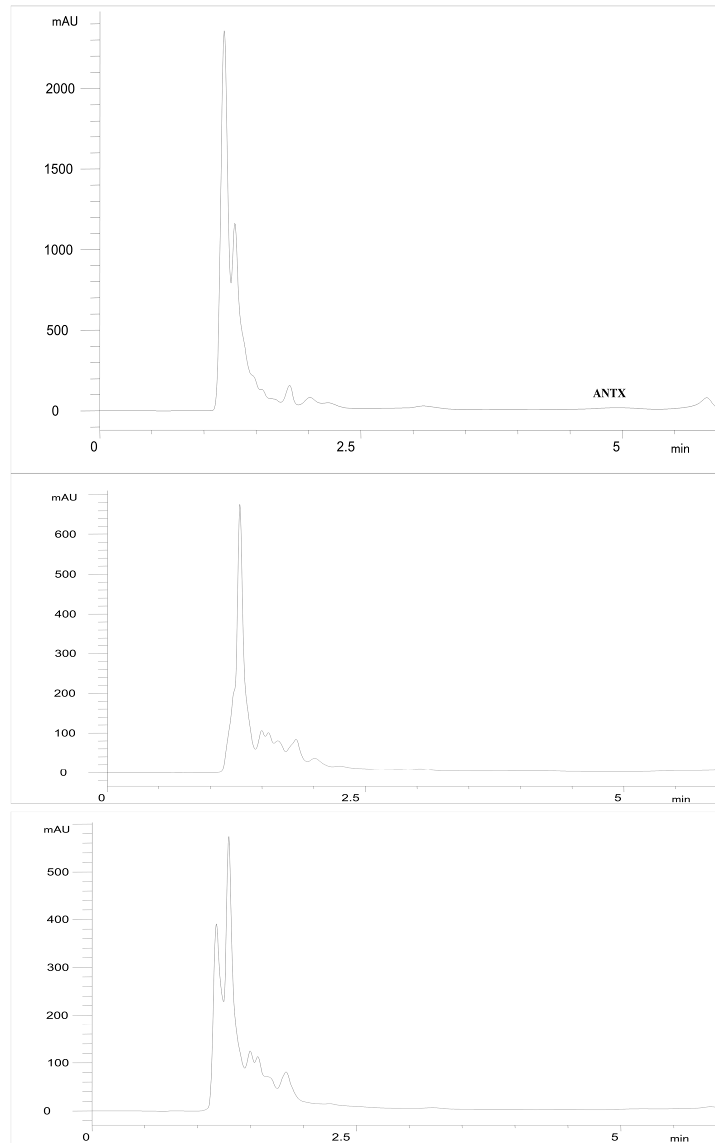


Figure 2.4 : Chromatograms of an injection (25 µl) of 10 ml of ANTX producing cultures of *Aphanizomenon issatschenkoi* (upper panel), *Anabaena flos-aqua* (middle panel) and *Oscillatoria* sp. (lower panel).

Extraction was performed on an ASE 200 (Dionex) and analysis was done using an HPLC-DAD at 229 nm detection.

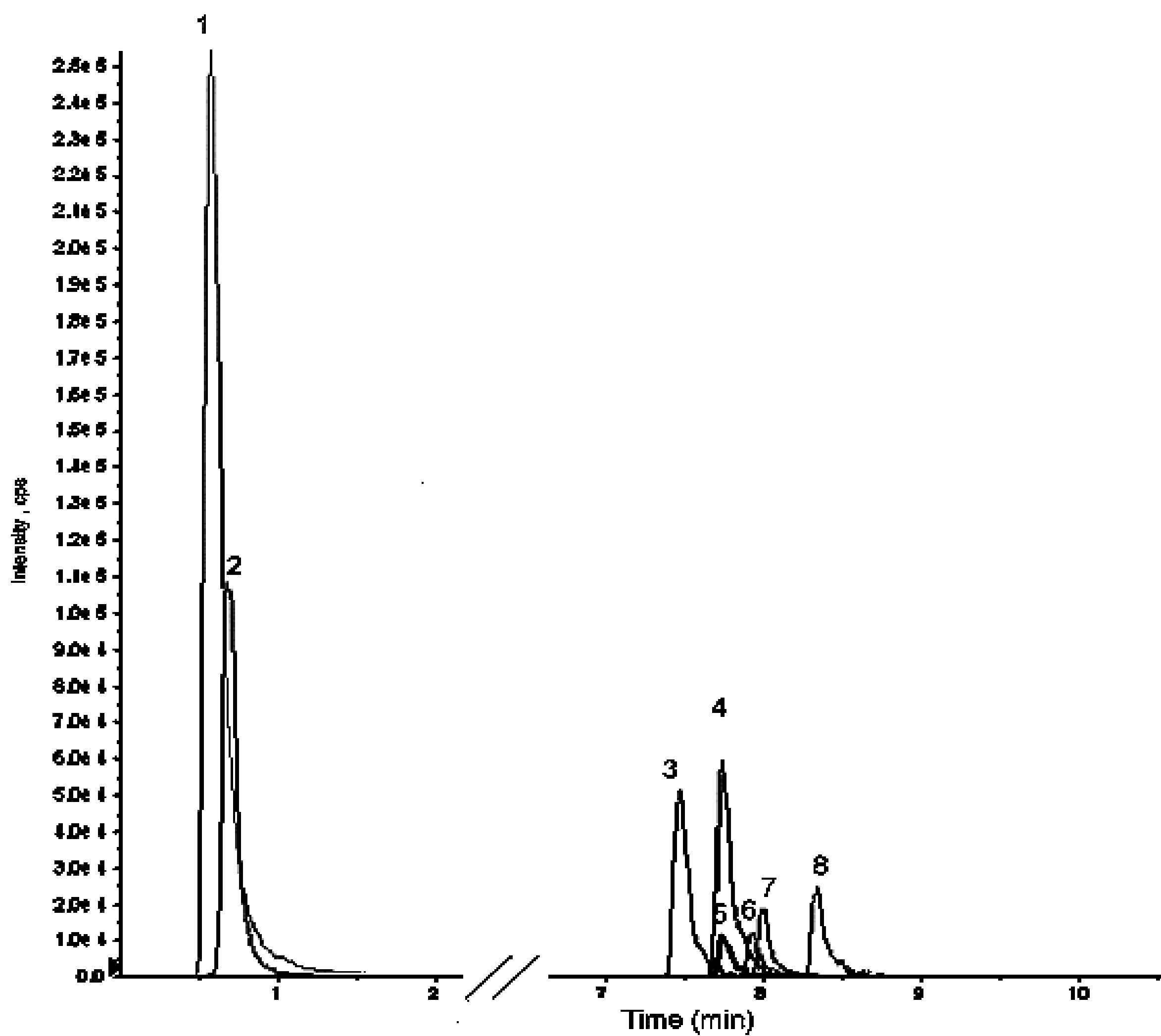


Figure 2.5 : Extraction ions chromatogram of 1 µl injection on column of standard mix at 5ppm

containing 1)ANTX (RT: 0.6 min), 2)PHE (RT: 0.65 min), 3)MC-RR (RT: 7.51 min), 4)Nodularin (RT: 7.74 min), 5)MC-YR (RT: 7.73 min), 6)MC-LR (RT: 7.92 min), 7)MC-7dmLR (8.00 min) and 8)MC-LA (RT: 8.30 min). The time (x) axis was modified by cutting off <5 minutes for a better representation of analyte peaks.

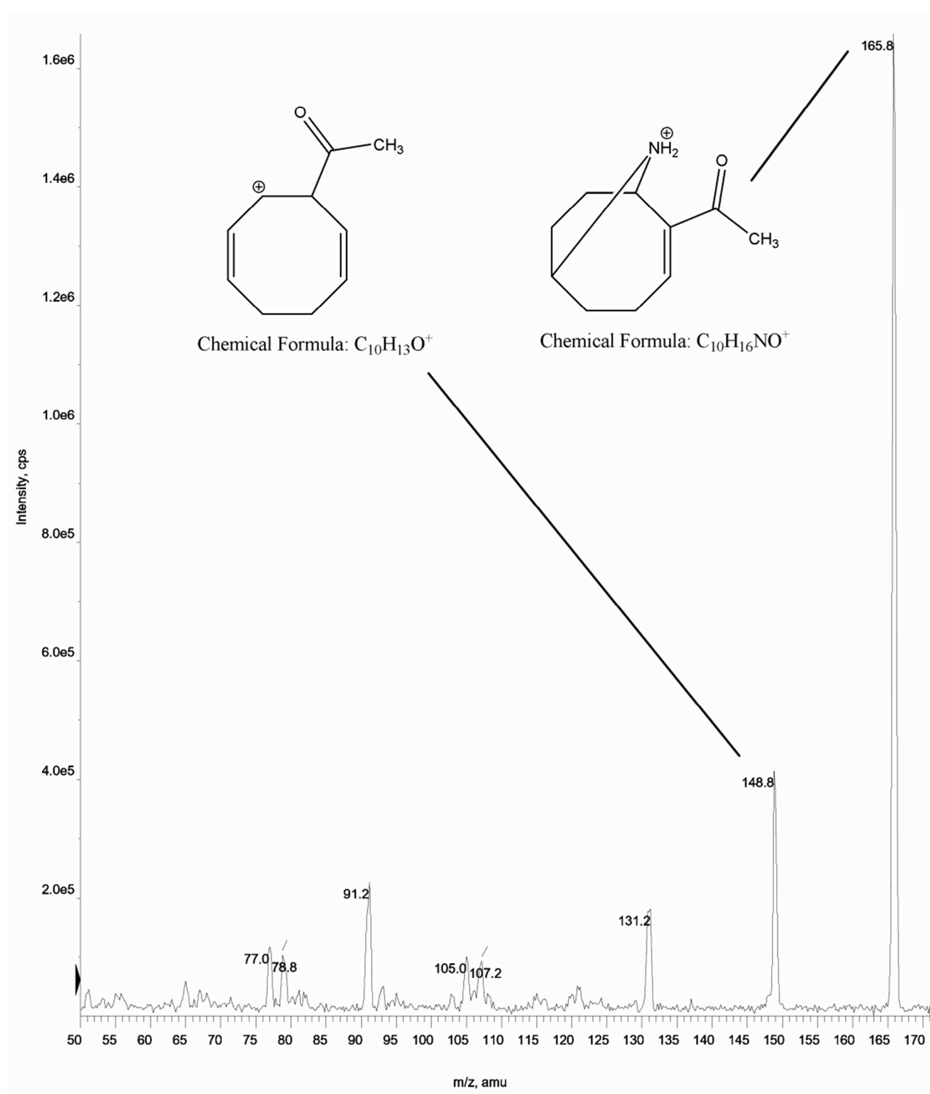


Figure 2.6 : Enhanced mass scan in positive polarity showing two major fragments of anatoxin-a obtained from a direct injection of ANTX standard.

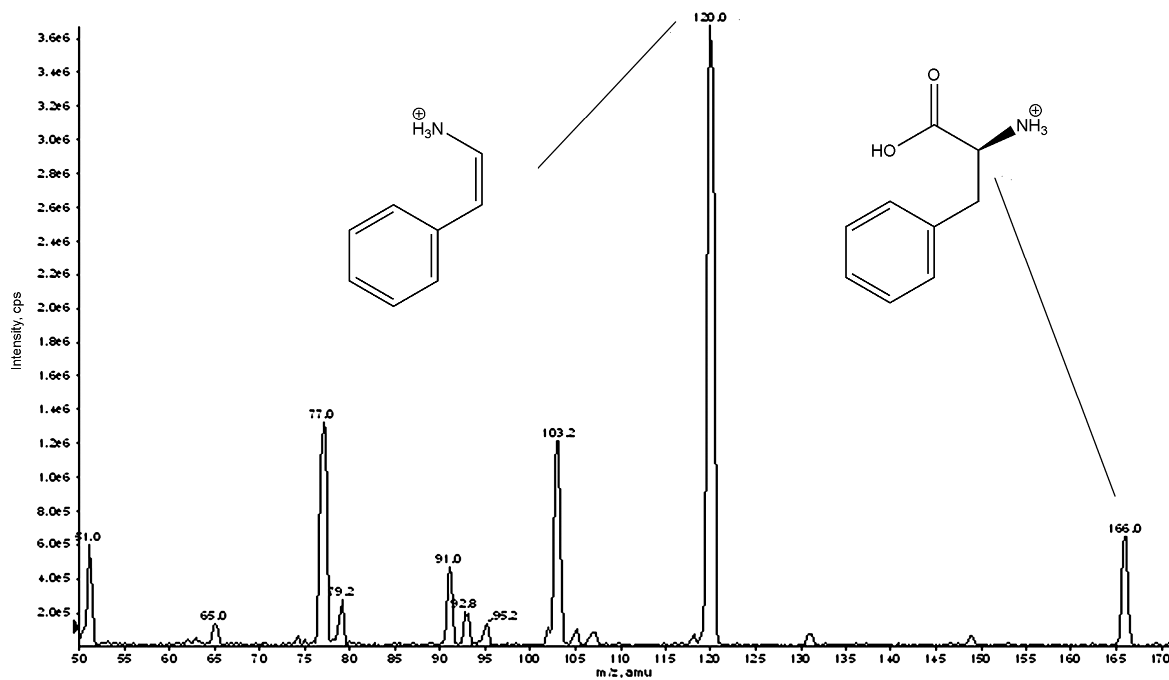


Figure 2.7 : Enhanced mass scan in positive polarity showing two major fragments of L-phenylalanine obtained from a direct injection of L-phenylalanine standard.

Chapter 3: Effect of nitrogen on cellular production and release of the neurotoxin anatoxin-a in a nitrogen-fixing cyanobacterium

3.1 Introduction

Many cyanobacteria are capable of producing compounds that are toxic to other organisms. The cyanotoxins have been classified into four main groups according to their mode of action. In addition to the hepatoxins, cytotoxins and gastrointestinal toxins, there are several types of neurotoxins (Codd *et al.* 2005). An important neurotoxin synthesised by freshwater cyanobacteria is anatoxin-a (ANTX). ANTX can be found in lakes and rivers throughout the world and has been reported in Europe, Oceania, Asia and North America (Wood *et al.* 2003; Codd *et al.* 2005, Cadel-Six *et al.* 2007). Several different cyanobacterial genera are able to produce ANTX at varying concentrations (Araoz *et al.* 2008) with the maximum concentration reported to date produced by *Phormidium favosum* (8000 $\mu g \cdot g^{-1}$ per dry weight, Gugger *et al.* 2005).

Several hypotheses have been proposed as to why these cyanotoxins are produced. Anatoxin-a might be produced as a defence mechanism against grazers or other organisms in order to gain ecological advantage. It might also act as a cell-mediated signal to communicate with other organisms (Wiegand *et al.* 2005). ANTX and other cyanotoxins have been labelled as secondary metabolites as they do not appear to be involved in primary metabolism (Carmichael 1992). In this sense, an increase in demand for resources at the cellular level would in theory lead to a decrease in ANTX production.

Anatoxin-a production seems to be strain specific although many factors, such as temperature and light intensity, affect its production (Osswald *et al.* 2007). According to the carbon-nutrient balance hypothesis (Hamilton *et al.* 2001), the availability of carbon and nitrogen may also play a role in the production of secondary metabolites, including nitrogen containing alkaloids such as ANTX. Recently, the effect of nitrogen to carbon supply ratios

(N:C) was investigated in the freshwater cyanobacterium *Microcystis aeruginosa* in relation to the production and composition of microcystins (MCs) (Van de Waal *et al.* 2009). Consistent with the carbon-nutrient balance hypothesis, Van de Waal *et al.* (2009) reported that a high N: C ratio in the external media and within the cells was more likely to lead to higher levels of the nitrogen-rich MC variant MC-RR in comparison to to lower N: C ratios. The strain of *M. aeruginosa* studied produced up to 50 times more MC-RR per cell when cultured in chemostats containing three to six times more nitrogen. Despite the fact that less than 1% of the total cellular nitrogen is invested in microcystins, the availability of inorganic nitrogen and carbon seemed to have an impact on secondary metabolite concentrations and composition. Nitrogen availability may also play an important role in the production of other nitrogen containing secondary metabolites such as ANTX.

In the following study, the relationship between nitrogen availability and ANTX production was investigated in *Aphanizomenon issatschenkoi* (Ussaczew) Proschkina-Lavrenko (CAWBG02, Woods *et al.* 2007). *A. issatschenkoi* is a colonial planktonic member of the *Nostocales*, which is able to produce heterocysts for atmospheric nitrogen (N_2) fixation when exposed to low soluble inorganic nitrogen concentrations (Moustaka-Gouni *et al.* 2010). The process of nitrogen fixation being energy demanding, this study hypothesized that nitrogen fixation might lead to further reductions in the production of secondary metabolites containing nitrogen.

3.2 Material and Methods

Culture conditions and sampling

Cultures of *A. issatschenkoi* were obtained from the Cawthron Institute in New Zealand. Batch cultures of *A. issatschentkoi* were grown at a light intensity of 85 $mE \cdot m^{-2} \cdot s^{-1}$ on a 12:12 light: dark cycle at 20° C ± 1°C in a Conviron growth chamber (E-15) using 500 ml Erlenmeyer flasks. Three different growth media were used with sodium nitrate ($NaNO_3$) as 100%, 5% and 1% of full strength BG11 (Andersen 2005) corresponding to 1500 $mg \cdot L^{-1}$, 75 $mg \cdot L^{-1}$ and 15 $mg \cdot L^{-1}$ of $NaNO_3$ respectively. Total culture volumes were 250 ml and inocula for all the experimental cultures came from the same parent culture in exponential phase. Under these conditions, *A. issatschenkoi* grew as single trichomes and minimal clumping occurred that facilitated subsampling.

Two ml from each flask were sub-sampled every 3 to 4 days to measure optical density using a Pye-Unicam SP-100 UV-spectrophotometer at 750 nm. The optical density at 750 nm provides a measure of turbidity of the culture and is a function of both cell density and cell size. Growth rates were obtained by plotting the natural logarithm of absorbance readings at 750 nm during the exponential growth and using linear analysis (Guillard 1973). Optical densities at 627 nm and 438 nm were also measured as an estimate of phycocyanin to chlorophyll a changes (Van de Waal *et al.* 2009). Phycocyanin is a protein and accessory pigment found in cyanobacteria and involved in light harvesting (Whitton and Potts 2000).

For toxin extraction from cells, 10 ml from each flask was filtered on days 10, 20, 30 and 40 of growth using pre-ashed and pre-weighed Whatman GF/C filters. A subsequent 10 ml was filtered from each flask using the same filter type for the analysis of particulate organic carbon (C) and nitrogen (N). Filters were oven dried for 24 hours, weighed and stored frozen at -20° C. The dry biomass for each batch culture ($mg \cdot ml^{-1}$) was also monitored. During filtrations, one 20 ml of filtrate was collected from each culture in solvent rinsed (acetone and hexane) scintillation vials wrapped in foil. Filtrates were spiked with 50

µL formic acid and frozen immediately at -20° C. For chlorophyll a analysis, 5 ml were sub-sampled and filtered using 934-AH glass microfiber filters with approximately the same pore size as GF/C. Filters were inserted in clean 15 ml plastic test tubes and stored at -20° C for subsequent analysis. The experiment was terminated when the 1% and 5% N cultures had clearly reached stationary phase.

Extraction and analysis of chlorophyll a and anatoxin

Chlorophyll *a* was extracted by incubating the frozen 934-AH filters with biomass in 13 ml of 95% ethanol at 4° Celsius for about 24 hours (Jespersen and Christoffersen 1987). Anatoxin-a extraction from the particulate phase was performed using an ASE200 Accelerated Solvent Extractor (Dionex, Canada) as described in Chapter 2. Extracts were collected in solvent (acetone and hexane) rinsed amber vials and then spiked with 50 µL formic acid to adjust pH to a value of about 3. Extracts were then evaporated down to dryness at 59° Celsius under a gentle nitrogen flow using a Zymark Turbovap II and then resuspended in 1 ml of 50% methanol and water. Filtrates were freeze-dried and then resuspended in 1 ml of 50% methanol and water. All extracts, both particulate and liquid phase, were filtered with pre-conditionned 0.2 µm Acrodisc filters and stored at -20° C prior to analysis. Particulate organic C and N were measured using a Costech elemental analyzer (ECS 4010; Costech, Valencia, California, USA) following Frost *et al.* (2009).

Chlorophyll a concentration was calculated based on absorbance readings of the extracts at the following wavelengths 750 nm, 665 nm and 649 nm (Bergmann and Peters 1980). Toxin analyses were performed on a Sciex QTRAP 3200 LC-MS/MS as described in Chapter 2. All samples were analysed on the same run to minimize any day to day instrument

variability.

Statistical analyses

In order to compare the effects of N treatments, one-way analyses of variance (ANOVA) were performed on the following end-points: total and cellular toxin concentrations, growth rates, chlorophyll a, phycocyanin to chlorophyll a ratios and particulate organic carbon to nitrogen (C:N) ratios for each day of sampling. In the case of significant differences ($p \leq 0.05$), Tukey's pairwise comparison test was subsequently used to determine differences between treatments. All statistical analyses were done using SigmaStat (version 3.1) software.

3.3 Results

Growth and nitrogen stress

All cultures entered the exponential phase after about 18 days and reached the stationary phase after approximately 34 days for the 1% N treatment and 40 days for both 5% and 100% N treatments. The growth curves based on the optical density between the three nitrogen treatments were clearly different (Fig. 3.2). By days 30 ($P = 0.006$), 34 ($P = <0.001$) and 40 ($P = 0.001$), the mean optical density of the 100% N treatment was significantly higher than the other two treatments. On days 34 and 40, the treatments were all significantly different from one another, with the lowest optical densities corresponding to the 1% N treatment. The growth rates (Table 3.1) ranged from 0.03 to 0.07 day^{-1} and showed significant differences (Tukey's test) between treatments 100% and 1% N. The average biomass as dry weight (Table 3.1) of both the 1% and 5% N treatments was significantly

lower than that of 100% N treatment on all four days of exponential and early stationary growth (day 10, 20, 30 and 40). Chlorophyll a concentrations (µg·L^{-1}) (Fig. 3.3a) showed a similar trend: the 1% N treatment was significantly different on day 18 and all three treatments were significantly different on subsequent days (30 and 40). The highest chl-a levels were measured in the 100% N treatment and the lowest in the 1% N treatment.

With respect to cell composition, the particulate organic carbon to nitrogen ratios (Table 3.1) started to differ on day 20 and onward. Batch cultures grown in 100% N medium were significantly different from the other two treatments with a lower mean ratio on day 20 (P = <0.001) and 30 (P = <0.001). At day 40 however, no difference between the C:N mean ratios of batch cultures grown in 100% N and 1% N was detected using Tukey's all pairwise test. This was due to the high standard error (SE=1) between triplicates of the 1% N treatment. However, a Duncan all pairwise test showed a significant difference (P = 0.045) between the 100% N treatment and the other two treatments. As for the ratios measured for the phycocyanin and chl-a pigments, the 100% N batch cultures had clearly the highest mean ratio and the 1% N the lowest (Fig. 3.3b). Significant differences between, either all treatments or the 1% and 100% N treatments were observed at days 7 (P = 0.028), 14 (P = <0.001), 18 (P = <0.001), 30 (P = 0.016) and 34 (P = 0.032). On day 40, the mean ratio for the 1% N was significantly different (P = <0.001) from the other two treatments.

Anatoxin-a

Total ANTX concentrations analysed ranged from 4 to 146 µg·L^{-1}.The total anatoxin-a production (Fig. 3) in *A. issatschenkoi* was not significantly different between all three treatments except on day 10. On this day, the highest total ANTX mean concentration corresponded to the 5% N treatment and the lowest to the 1% N. The batch cultures grown at

5% N had the highest total ANTX concentrations with a mean concentration maximum of 111 ±18 $\mu g \cdot L^{-1}$ on day 30. The cultures that produced the lowest amount of total ANTX were the ones grown in 100% N medium with maximum total ANTX concentrations ranging from 51 to 74 $\mu g \cdot L^{-1}$.

The extracellular ANTX concentrations ranged from <1 $\mu g \cdot L^{-1}$ to 13 $\mu g \cdot L^{-1}$. Cultures grown in 100% N medium had significantly ($P < 0.001$) lower ANTX extracellular concentrations than the other two treatments on days 20 and 30. The highest mean extracellular concentrations were obtained in the 1% N treatment on days 30 and 40, reaching 9 ± 0.12 $\mu g \cdot L^{-1}$. The mean extracellular concentrations of ANTX were higher in the 5% cultures than in the other two treatments on every day of sampling, although the difference was statistically significant only on days 10 and 30. The mean percentages of total toxin represented by the extracellular fraction ranged from 2.98% to 46.8%. Two cultures had higher concentrations of ANTX in the extracellular phase compared to the intracellular phase: one on days 10 and 20 and the other on day 20. Both of these cultures were grown in 1% N medium.

ANTX cellular content ranged from 6 to 1683 $\mu g \cdot g^{-1}$ dry weight with the highest values corresponding to 30 days of culturing or the late exponential phase. Ballot *et al.* (2010) reported a maximum ANTX content in CAWBG02 of 2354 ±273 $\mu g \cdot g^{-1}$ fresh weight. In this study, ANTX content (Fig. 4) was significantly higher for the 5% N treatment compared to the other 2 treatments on days 20 ($P = 0.018$) and 30 ($P = 0.003$). The 5% and 1% treatments were significantly different on day 10 and every treatment was significantly different from one another by day 40. The highest ANTX cellular content was measured in the 5% N treatment on day 30 with a mean of 1408 ±181 $\mu g \cdot g^{-1}$. As with the total culture concentrations, the 5% N and 1% N treatment had higher ANTX cell content than the batch

cultures grown in 100% N with the 5% N treatment resulting in the highest amounts except at day 40, which corresponded to the onset of stationary phase.

3.4 Discussion

Nitrogen clearly limited the biomass yield of *Aphanizomenon issatschenkoi* in the cultures as evidenced by lower dry weight concentrations under the 1% and 5% relative to 100% N (Table 3.1). The growth rate was also reduced in the 1% cultures and, to a lesser extent, in 5% cultures. Nitrogen deficiency was also reflected in several cellular constituents: in particular lower chlorophyll a concentrations and overall lower ratios of phycocyanin to chlorophyll a. The phycobiliproteins, being nitrogen rich accessory pigments, are typically reduced relative to chlorophyll a under nitrogen stress in cyanobacteria (Turpin 1991). The cellular carbon to nitrogen ratio was expected to rise under nitrogen deficiency and this was observed through time in each culture with the highest ratios found in the 1% cultures.

When soluble inorganic nitrogen becomes limiting in the environment, many cyanobacteria belonging to the *Nostocales*, such as *Aphanizomenon*, are capable of fixing atmospheric nitrogen to compensate for their needs for that specific nutrient. Hence, the particulate organic nitrogen cell content should have been more or less constant throughout the 40 days in all three treatments if N_2 fixation had occurred to compensate for nitrogen limitation. On days 20 and 30, the C:N ratios were significantly higher for cultures grown in 1% and 5% N compared to 100% N treatment. This suggests that nitrogen was still limiting and that N_2 fixation did not compensate for the N deficiency, particularly in the 1% N treatment. In fact, heterocysts were not evident in the cultures and were either non-existent or too small. Regardless of the treatment, the particulate organic nitrogen content of cells observed in this study (Table 3.1) seemed relatively high when compared to the Redfield

ratio (Redfield 1934). According to Redfield (1934), the elemental composition of organic matter in the oceans, including phytoplankton, is constant when nutrients are not limited. Redfield molar element ratio of carbon and nitrogen (C: N) is 6.63 for all organic matter present in nutrient abundant conditions. For example, Van De Waal *et al.* (2009), for cultures of *Microcystis aeruginosa* grown in chemostats where nitrogen and carbon were not a limiting factor, reported molar C: N ratios ranging from 6.25 to 7.14. Cultures of *Aphanizomenon issatschenkoi* in the present study, had overall lower C: N molar ratios. Nevertheless, higher ratios of C: N were clearly found in the cultures grown in the lower nitrogen media.

According to the carbon-nutrient balance hypothesis, nutrients should be allocated to the production of secondary metabolites once growth is assured (Hamilton *et al.* 2001). Since cyanotoxins are considered secondary metabolites (Carmichael, 1992), their production should therefore increase whenever the availability of essential nutrients increases. A higher production of anatoxin-a, an alkaloid that contains a nitrogen atom, was expected under higher nitrogen availability. In this study, higher nitrogen concentrations corresponded to higher growth rates (Table 3.1) of CAWBG02 but not to higher total ANTX concentrations (Fig. 3.4). In fact, the highest concentrations of total ANTX were produced by cultures grown in 5% N, while the lowest mean concentrations of total ANTX were found in the 100% N treatment; which contradicts the carbon-nutrient balance hypothesis. The same trend was observed for ANTX cellular contents (Fig. 3.5) since the cultures grown in 5% N produced the highest content and the 100% N treatment corresponded to the lowest measured. Cultures grown in 100% N had the highest growth rate, the highest biomass yield, the highest levels of particulate organic nitrogen and the highest phy:chl-a ratios (Table 3.1) yet, they produced the lowest concentrations – both for total toxin ($\mu g \cdot L^{-1}$) and cellular

content ($\mu g \cdot g^{-1}$). If ANTX is a type of defense compound then, its production might be a mechanism that confers ecological advantage under low nutrient conditions which are common in aquatic systems.

The mean extracellular to total toxin concentration ratios were always higher in 1% N treatment than in the other two treatments. Stress conditions in cyanobacteria can lead to transient permeability changes of the cell membrane which result in leakage (Potts and Whitton 2000). However, the extracellular ANTX concentrations presented in this study might have been underestimated as ANTX is quite labile under light and thus may degrade rapidly once released into the medium (Stevens and Krieger 1988). Hence, this must be taken into consideration in regards to the extracellular ANTX concentrations presented.

This study suggests that toxin production may be higher when cyanobacterial cells are under moderate nutrient stress. Recently, Kurmayer (2011) reported that *Nostoc* produced higher levels of microcystin cell contents under stress conditions - particularly under low phosphorous (P-PO_4) and low light irradiance – even though growth rates were reduced up to >100-fold compared to the control. As a result, microcystin contents per cell were negatively correlated to P-PO_4 and irradiance. However, the relationship between the net production rates of the toxin and the daily cell division was still highly significant. Even if *Nostoc* produced higher levels of microcystin per cell when grown under stress conditions, the total toxin production was still lower than when grown under optimal conditions. In contrast, in this study, both cellular and total ANTX concentrations in *A. issatschenkoi* were higher in cultures grown at intermediate N concentrations. Similarly, Rapala *et al.* (1993) had observed that ANTX cellular contents were higher in *Aphanizomenon flos-aquae* and *Anabaena flos-aquae* grown in nitrogen-free media as opposed to nitrogen-rich control media. However, ANTX cellular mean concentrations for CAWBG02 grown in 100% and

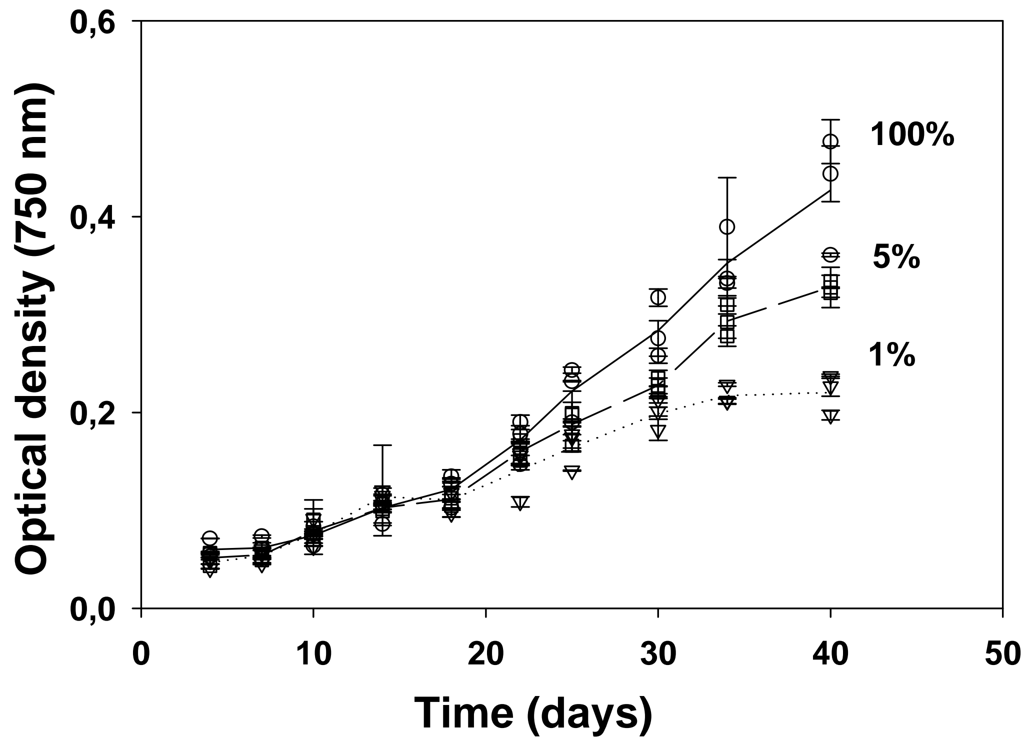
1% N media were both significantly lower than that of the 5% N treatment in this study. Stress conditions may increase ANTX cellular production to a certain point where cyanobacterial cells are still capable of producing the toxin without compromising their survival. Above this threshold however, overstressed cells cannot respond to what is needed in order to produce more toxins. ANTX cellular mean concentrations for 1% N were higher than for cultures grown in 100% N, but the difference was not significant. Based on the cellular consituents, cultures grown in 1% N were clearly more stressed compared to the other two treatments and the 1% may have been just too limiting for toxin production.

The results presented in this study suggest that ANTX production may increase, to a certain extent, in *A. issatschenkoi* grown under nitrogen limited conditions. According to the carbon-nutrient balance hypothesis, the opposite would be expected. However, growth rates were different for all three treatments, and growth rate variation might have had an effect on the final ANTX concentrations observed in *A. issatschenkoi*. Hence, it is not known if nitrogen limitation is the only factor that contributed to the increase in ANTX concentrations. Using a different culturing technique (i.e., chemostats) to simultaneously control nitrogen availability and growth rates might help further explain the influence of nitrogen on ANTX production.

Table 3.1: Average growth rate based on optical density changes (± standard error), carbon to nitrogen (C: N) molar ratios and yield as weight biomass (mg/ml) for *Aphanizomenon issatschenkoi* grown in different nitrogen concentrations (shown as % of full strength BG11 media). Within the same column (parameter) values with different letters are significantly different from each other as determined by a one way ANOVA and Tukey's post-hoc pairwise comparisons (n=3), $p < 0.05$.

Treatment Nitrogen	Growth rate (d^{-1})	Time (days) 10		20		30		40	
		C: N	Biomass	C: N	Biomass	C: N	Biomass	C: N	Biomass
100% N	0.07 (0.009)a	0.96 (0.22)a	0.098 (0.002)a	1.56 (0.07)a	0.134 (0.005)a	2.59 (0.08)a	0.157 (0.005)a	2.92 (0.3)a	0.220 (0.015)a
5%	0.05 (0.002)ab	4.04 (1.49)a	0.041 (0.005)b	4.63 (0.1)b	0.060 (0.005)b	5.16 (0.1)b	0.080 (0.004)b	5.17 (0.05)b	0.135 (0.003)b
1%	0.03 (0.042)b	2.79 (0.75)a	0.049 (0.008)b	5.00 (0.36)b	0.061 (0.002)b	6.01 (0.49)b	0.092 (0.001)b	5.49 (1.00)b	0.093 (0.007)b

Figure 3.1 : Trichome of the cyanobacterium *Aphanizomenon issatschenkoi (*Live material under phase contrast 630X).



.

Figure 3.2 : Batch culture growth of *Aphanizomenon issatschenkoi* in 100% (circles, n=3), 5% (squares, n=3) and 1% (triangles, n=3) nitrogen-rich media. The error bars are standard errors of mean.

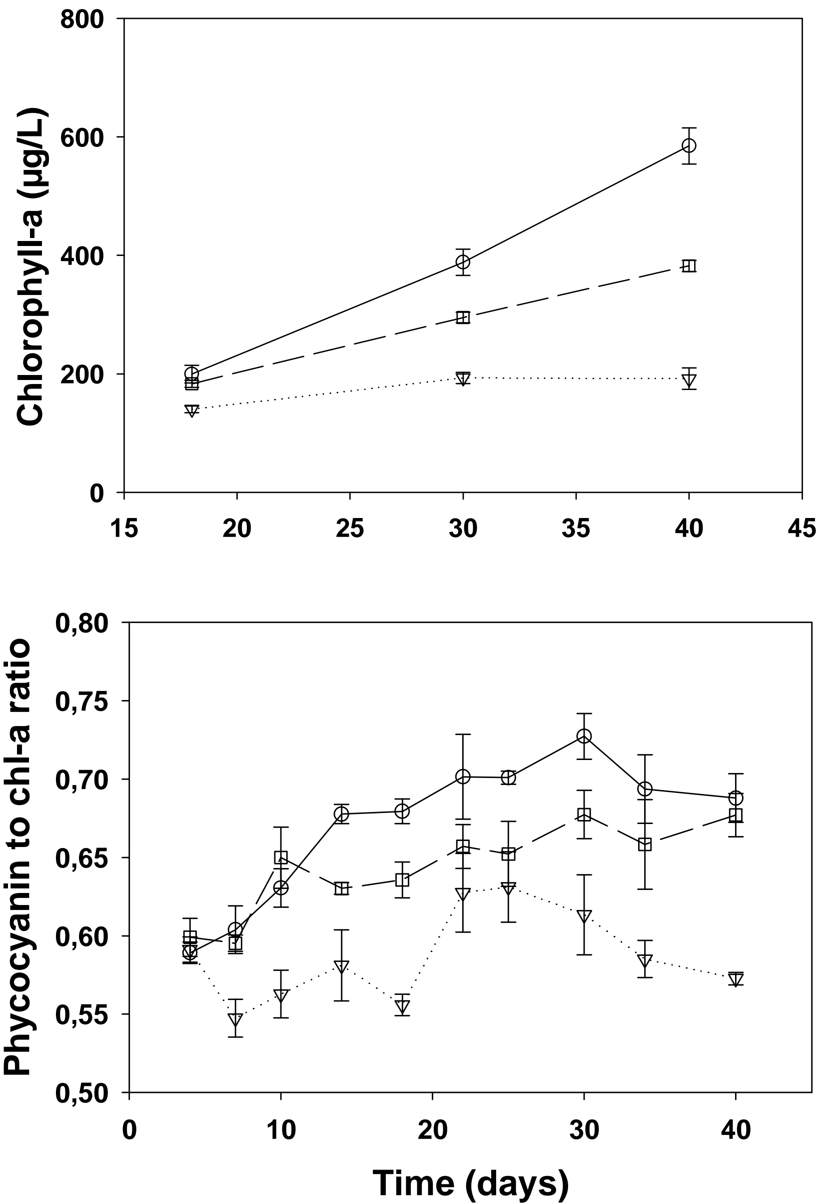


Figure 3.3 : Chlorophyll-a concentrations (µg/L) (upper panel) and phycocyanin to chl-a ratios (627 nm: 438 nm) (lower pannel) of *Aphanizomenon issatschenkoi* grown in 100% (circles, n=3), 5% (squares, n=3) and 1% (triangles, n=3) nitrogen-rich media. The error bars are standard errors of mean.

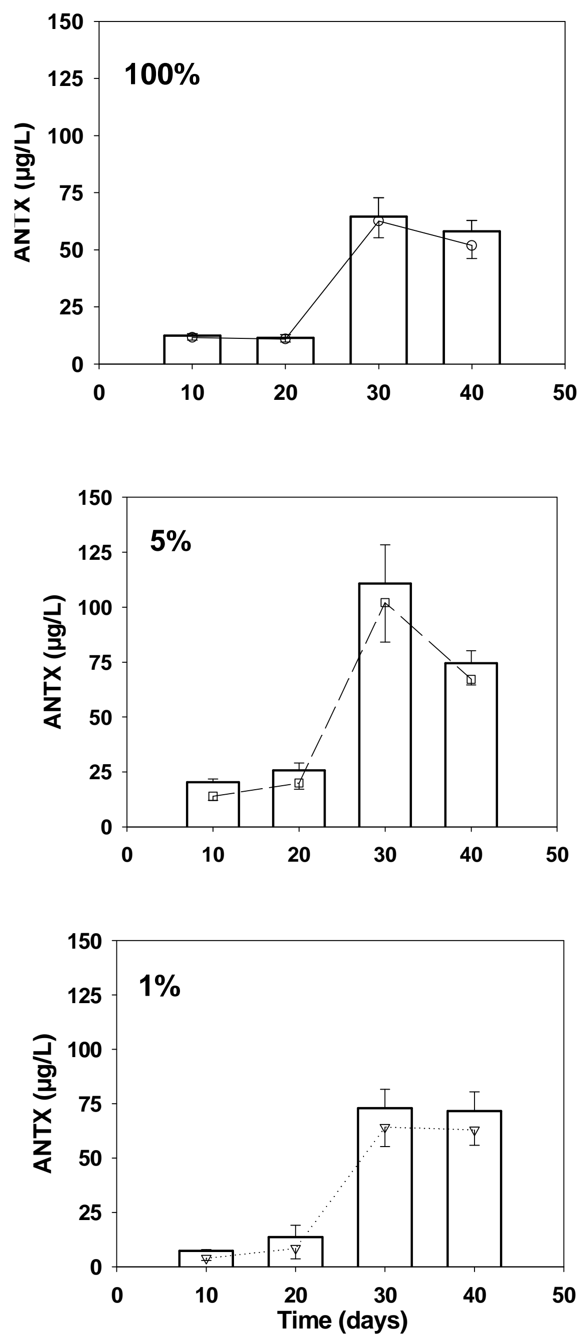


Figure 3.4 : ANTX culture concentrations (μg/L) of total toxin (vertical bars) and intracellular toxin (points) in *Aphanizomenon issatschenkoi* grown in 100% (n=3), 5% (n=3) and 1% (n=3) nitrogen-rich media. The error bars are standard errors of mean.

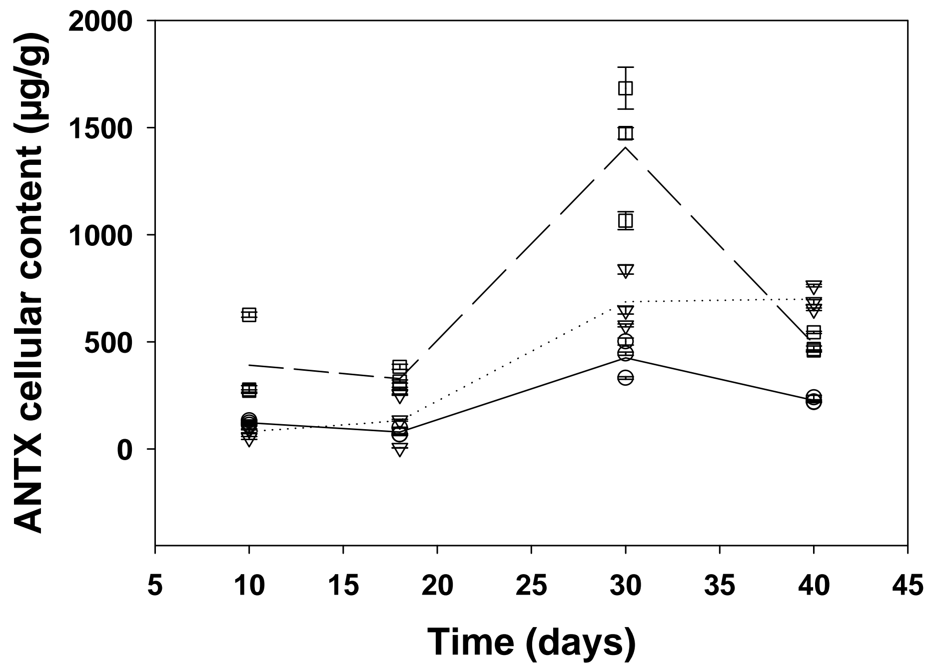


Figure 3.5 : Anatoxin-a cellular content (µg/g dry weight) of *Aphanizomenon issatschenkoi* grown in 100% (circles), 5% (squares) and 1% (triangles) nitrogen-rich media. The error bars are standard errors of mean (n=3).

Chapter 4: Use of steady-state fugacity based-models to determine the fate of cyanobacterial freshwater neurotoxin anatoxin-a

4.1 Introduction

In general, the fate of algal toxins in aquatic environments is not well understood, despite the ecosystem and human health risk toxins pose. The cyanotoxin anatoxin-a (ANTX) is a potent neurotoxin produced by several genera, such as *Anabaena, Aphanizomenon, Microcystis and Planktothrix,* and found in freshwaters worldwide (Osswald *et al.* 2007). However, at least in North America, ANTX seems less prevalent than the more commonly encountered microcystins (Hedman *et al.* 2008). ANTX is an alkaloid, produced intracellularly, that can pass through the cell wall due to its small molecular weight (165.26 g/mole, Lewis 2000). Guidelines for ANTX only exist in Australia and New Zealand and as of yet have not been developed elsewhere due in part to the lack of a standardized extraction and analytical methods. As a consequence, there has been little ANTX monitoring and even less fate determination in freshwater ecosytems.

Under natural conditions, ANTX breaks down into two non-toxic products (Fig. 4.1): the alkaloid dihydroanatoxin-a and anatoxin-a epoxide (Smith and Lewis 1987, Harada *et al.* 1993). The four main routes of degradation of the compound in water are: dilution, adsorption, photolysis and non-photochemical biodegradation (i.e., microbial) (Stevens and Krieger 1991). Along with volatilization, dispersion, and adsportion, dilution is an important natural attenuation mechanism labeled as non-destructive (http://toxics.usgs.gov). ANTX being highly soluble in water, dilution should be considered as a significant degradation route.

ANTX adsorption to sediments taken from four different lakes was reported to range from 50 to 82 µg of ANTX per ml of sediment by Rapala *et al.* (1994). It was also recently suggested that the sorption mechanism of ANTX to sediment is mainly controlled by clay

and, to a lesser extent, organic carbon through cation exchange mechanism. Klitzke *et al.* (2001) investigated the sorption of ANTX to four different sediment textures: clayey, moderately organic, sandy and silty. Values ranged from 47 to 656 µg of ANTX per kg of dry sediment with the highest sorption strength corresponding to clay-rich and the lowest to sandy sediments.

Photolysis of ANTX in sunlight appears to be dependant on both pH and light intensity and is independant of oxygen (Stevens and Krieger 1991). The ANTX half-life can be several days in the absence of light as opposed to a few hours in the presence of sunlight. Furthermore, the lower the pH the greater the stability of ANTX. Photolysis does not occur at pH 2, even under high light intensity (Stevens and Krieger 1991). High pH values however, cause ANTX to degrade readily (James *et al*. 1998).

With respect to the fourth pathway of degradation, micro-organisms can also degrade ANTX, including bacteria naturally occurring in lakes (Kiviranta *et al*. 1991, Rapala *et al.* 1994). Kiviranta *et al*. (1991) reported that a *Pseudomonas* sp. broke down ANTX at a rate of 2 to 10 µg/ml per day.

These above results are based on laboratory experiments and thus cannot fully represent or predict the fate of ANTX once it is released in aquatic ecosystems. From a theoretical stand point, mass balance models may estimate the behaviour and fate of compounds in a specific ecosystem (i.e., closed environment, lake or a waste water treatment plant). Along with environmental monitoring, the use of mass balance models could lead to a better understanding of the fate of ANTX. Fugacity-based models are mass balance models that, instead of being conventionnaly based on the chemical's concentration in every environmental compartment, are based on the principle of fugacity (Mackay *et al.* 2001). The concept of fugacity was first introduced by G.N. Lewis in 1901 as a criterion of equilibrium.

It is similar to chemical potential but is not proportional to concentration (Webster *et al.* 2005) Fugacity-based models include four different levels.

The first model (Level I), simulates the behaviour of a chemical compound in a closed environment (Mackay *et al.* 2001). The compound will partition from one phase to another, depending on its physicochemical properties, until the equilibrium is reached. In such a model, each phase possesses the same fugacity, meaning that the fate of the compound is based on its physicochemical properties only and not the capacity of a certain phase to “accept” (or not) the chemical in question. A level I fugacity-based model is meant to provide a general idea of the phase a chemical has a preference for.

The second model (Level II) differs from the first in the way that the chemical is introduced into the theoretical environment (Mackay *et al.* 2001). Instead of a fixed quantity introduced once at the same time, the model simulates a constant inflow of chemical into a closed environment. Additionnally, the processes of degradation and advection are taken into account. A chemical reaction is calculated for the degradation rate of the compound based on its half-life relative to each phase. With respect to advection, the model calculates how much of the chemical exits the modelled environment due to mechanical forces (i.e., wind transport and/or water currents) set by the user. Equilibrium is attained as soon as the rate of chemical entering the environment equals the quantity of the chemical exiting it. Such model enables a better understanding of the fate of the chemical based on its physicochemical properties.

As opposed to the first two models, the Level III model takes into account the fugacity capacity of each compartment. Since there is no equilibrium in between phases, a new concept is introduced: the interphase transportation. Although this model incorporates the degradation concept and interphase transportation, it is not considered to be dynamic (Webster *et al.* 2005). A dynamic model would allow the simulation of a non steady-state

flow of chemical in the environment. Such a model (i.e. Level IV) does exist but is not covered in this chapter. All three models described above are considered to be steady-state models where every variable remains constant through time (Webster *et al.* 2005).

This study included three objectives: 1) to define what would be the partitioning behaviour of ANTX in a closed aquatic environment, 2) to determine the impact of photolyis and microbial biodegradation on the persitence of ANTX in an aquatic environment, 3) to predict interphase transportation when a constant inflow of ANTX is limited to water exclusively.

4.2 Material and Methods

Anatoxin-a parameters and half-life values

A scientific literature research was performed in order to compile different values, either measured or estimated, for the physicochemical characteristics of ANTX. The Environmental Protecton Agency's (EPA) Office of Pollution Prevention Toxics and Syracuse Research Corporation (SRC), developed the Estimation Program Interface Suite™ (EPI Suite™). The EPI Suite™ is a Windows®-based suite of physicochemical parameters and environmental fate estimation programs that includes several individual models responsible for the estimation of different parameters (www.epa.gov). The logarithm of the octanol water partition coefficient ($LogK_{OW}$) was estimated using the model KOWWIN v1.67. The vapour pressure and melting point parameters were both estimated with the MPBPWIN v1.42 model. Water solubility was estimated with WSKOW v1.41 using the $LogK_{OW}$ estimation value provided by KOWWIN v1.67.

The ACD/PhysChem suite is software developed by ACD/Labs for the prediction of physicochemical properties of chemicals (www.acdlabs.com). The $LogK_{OW}$, vapour pressure and water solubility were estimated based on the ANTX molecular weight. The water solubility for ANTX was predicted at different pH values ranging from <6 to 9 using the model ACD/Labs Software v8.14.

Half-life values for ANTX in water found in the literature were compiled. First, considering biodegradation and adsorption as the main degradation routes, Smith and Sutton (1993) measured the half-life of ANTX in reservoir water containing bed sediment. Reservoir water samples were spiked with ANTX at different pH values. Solid phase extraction followed by HPLC-UV was used for analysis. ANTX's half-life in the presence of sediments was also measured by Rapala *et al.* 1994. ANTX was incubated in vials with non-sterilized sediments from four different lakes over a 22-day period in the dark. The concentration of ANTX was determined with an HPLC-UV. Both half-life values were used to predict ANTX's persistence without any photodegradation.

The impact of photolysis on ANTX stability was investigated by Stevens and Krieger (1991) who measured the half-life of ANTX in distilled water at different pH values ranging from 2 to 12. ANTX was quantified using NMR analysis.

With respect to atmospheric degradation, the half-life of ANTX was estimated based on the reaction with photochemically-produced hydroxyl radicals using AopWin v1.92 (Toxnet). Half-life values for ANTX in the soil and sediment were estimated by the Syracuse Research Corporation (SRC) and available on the Chemspider website.

Simulations

Fugacity-based models were obtained though the Centre for Environment Modelling and Chemistry (CEMC), on Trent University's website (http://www.trentu.ca/academic/aminss/envmodel/welcome.html). The EPI Suite™ predicted vapour pressure; melting point and $LogK_{OW}$ parameters for ANTX remained the same and were used for every simulation, considering a temperature of 25° C. ANTX's fate in a closed environment was simulated using a Level I model considering the following pH values: 6, 7, 8 and 9. The predicted ACD/PhysChem suite water solubility values for ANTX respective to the simulated pH values were used. An EQC standard environment (Table 4.1) was used for all simulations. The emission source of ANTX was arbitrarily set to 20,000 Kg, which corresponds to a maximum concentration in water of 1 ppb. Accumulation in water, air, soil, sediment and biota (fish) were measured. A lipid factor in fish of 0.05 $g \cdot g^{-1}$ was employed.

A Level II simulation was performed using the three different half-life values measured for ANTX in water at pH 9 (Stevens and Krieger 1991, Smith and Sutton 1993, Rapala *et al.* 1994). Residence times were predicted and compared for all values considering the following toxin elimination routes: adsorption, biodegradation and photolysis. To reflect toxin dilution, advective flow residence times were set to the following values: 100, 1000 and 50,000 hours for air, water and sediment (burial), respectively. The emission flow rate was set to 200,000 kg per hour. The same simulations were undertaken using a Level III model to consider interphase transportation. As opposed to the other simulations, the chemical emission rate inflow of ANTX was limited to water exclusively as it is the natural source of production.

4.3 Results and Discussion

ANTX Parameters and half-life values

Based on the EPI Suite^{TM} prediction of physicochemical parameters, ANTX has a $LogK_{OW}$ of 1.12, which is in the range of hydrophilic chemicals. The estimated vapour pressure at a temperature of 25° C, and the melting point for ANTX were 0.00581 mmHg and 61.26° C, respectively. Different values for the estimation of the water solubility of ANTX were obtained. The EPI Suite^{TM} (WSKOW v1.41) predicted a value of 72.6 $g \cdot L^{-1}$. According to the ACD/PhysChem, estimated water solubility values for ANTX fluctuated depending on the pH. For pH values of 6 and below, water solubility was estimated at 1000 $g \cdot L^{-1}$ whereas 960 $g \cdot L^{-1}$, was estimated for pH 7. At pH 8 and 9, water solubility decreased to 120 $g \cdot L^{-1}$ and 16 $g \cdot L^{-1}$ respectively.

With respect to ANTX half-life values, Smith and Sutton (1993) measured a half-life of 120 hours (5 days) for ANTX incubated in reservoir water with bed-sediment at a pH of 9. Rapala *et al.* (1994) reported that up to 48 % of ANTX was degraded when incubated in the dark with non-sterilized lake sediment for 22 days. Degradation in both studies was attributed to biodegration (i.e., microorganisms) and sediment adsorption.

Photolysis was investigated by Stevens and Krieger (1991) who measured a half-life of 1 to 2 hours for ANTX in the presence of sunlight although irradiance levels were not specifically measured. The half-life was measured at pH 8-9, which is expected in cyanobacterial blooms in most northern temperate climate The EPI Suite^{TM} estimations for ANTX's half-life in the air, water, soil and sediment were 1.1, 360, 720 and 3240, hours respectively.

Simulations

In the closed environment simulation (Level I), 98.8 % of the total mass of ANTX migrated to the water compartment (Fig 4.2). Regardless of the mass value simulated, the final total mass percentages of ANTX migrating in each environmental compartment remained identical. Anatoxin-a is extremely water soluble due to its low $LogK_{OW}$. The partitioning behaviour of ANTX was not impacted by a change in water solubility estimated at pH values ranging from 6 to 9. A decrease in water solubility, from 960 to 16 $g \cdot L^{-1}$, lead to a negligeable transfer of only 0.1 % of the total mass of ANTX from water to the air compartment. This suggests that even at lower water solubility corresponding to a pH of 9, ANTX's affinity for the water phase is higher than that for any other compartment.

With respect to ANTX accumulation in fish lipids, a concentration of 0.651 $ng \cdot g^{-1}$ of ANTX was calculated in fish in contrast to a concentration in water of 988 $ng \cdot g^{-1}$. This suggests a low bioconcentration factor with a difference in ANTX concentration of more than 1500 fold between water and fish. The low octanol to water partition coefficient ($LogK_{OW}$) is responsible for such results. ANTX being hydrophilic, it is less likely to accumulate in lipids. However, the bioaccumulation in fish presented above may be significantly underestimated due to the fact that a dietary route was not considered.

When taking into consideration degradation (Level II), 99.3 % of the total ANTX was found in the water phase at equilibrium for all three half-life values simulated (Fig. 4.3). The percentage of total ANTX in water is slightly higher than that predicted in a closed environment simulation (level I). This is due to the ANTX half-life in the air being lower than that predicted in any other compartment, including water. Since ANTX degrades more rapidly in the air, more accumulation may occur in water before the equilibrium between all

environmental compartments is reached. No difference was observed between all three ANTX water solubility values corresponding to pH 7, 8 and 9.

The chemical residence times predicted in an EQC environment ranged from 2.9 to 430 hours. The lowest value was obtained using ANTX's half-life in water measured by Stevens and Krieger (1991) during photolysis. ANTX is labile under sunlight and degrades rapidly thus, it is not expected to be very persistent in natural environments. However, light irradiance is negatively correlated with depth in a lake; hence, ANTX may reside for a longer period of time in deeper and darker waters. Based on both Sutton and Smith (1993) and Rapala *et al.* 1994 measured half-life values, the predicted chemical residence times of ANTX exposed to sediment micro-organisms varied from 148 hours to 430 hours, respectively. Rapala *et al.* (1994) observed that ANTX's degradation rate was different when incubated with sediment from different lakes. This suggests that certain populations of sediment micro-organisms can degrade ANTX more rapidly than others and might explain the difference in ANTX half-life values in water reported by Smith and Sutton (1993) and Rapala *et al.* (1994).

When considering an emission rate of ANTX directly into water only (Level III), 100 % of the total ANTX remained in the water phase. No interphase transportation occurred, even at the lower water solubility value of 16 $g \cdot L^{-1}$. The combination of ANTX's high water solubility and low vapour pressure is responsible for the absence of volatilization. No sedimentation was predicted either, due to the high water solubility of ANTX.

Steady-state mass balance models, like the ones used in this chapter, may help to assess the partitioning behaviour of ANTX and its persistence, but only with state variables that are constant through time. ANTX's fate is dependant on parameters that are naturally changing through time and space (e.g. . light irradiance). Using a dynamic model (Level IV)

would thus be more appropriate for predicting the neurotoxin's fate but would also present a greater challenge. The rate at which ANTX is released from the cyanobacteria remains unknown but could be measured experimentally at least under laboratory conditions. Additionally, measuring ANTX's half-life instead of using estimations would allow a better representation of the toxin's stability in the environment. More experimental data, combined with the use of mass-balance models, would therefore lead to a better understanding of ANTX's environmental fate.

Table 4. 1 : Environmental properties of the EQC (standard environment) used in the fugacity-based models simulations.

Environmental Properties

Volumes (m^3)		**Densities ($kg \cdot m^3$)**	
Air	1e+14	Air	1.185
Aerosol	2000	Aerosol	2000
Water	2e+11	Water	1000
Suspended particles	1e+6	Suspended particles	1500
Fish	2e+5	Fish	1000
Soil	9e+9	Soil	1500
Sediment	1e+8	Sediment	1280
Organic Carbon ($g \cdot g^{-1}$)			
Lipid Fraction (fish)	0.05	Suspended Particles	0.2
Sediment	0.04	Soil	0.02

Table 4. 2 : Half-life values (hours) estimated and measured for ANTX. Values measured by Smith and Sutton (1993) and Rapala *et al.* (1994) correspond to ANTX incubated with non-sterilized sediments in the dark at pH 9. Value measured by Stevens and Krieger (1991) correspond to ANTX exposed to sunlight at pH 8-9.

	Phases			
	Water	**Air**	**Sediments**	**Soil**
EPI Suite™	360	1.1	3240	720
Smith and Sutton (1993)	120	-	-	-
Rapala *et al.* (1994)	528	-	-	-
Stevens and Krieger (1991)	2	-	-	-

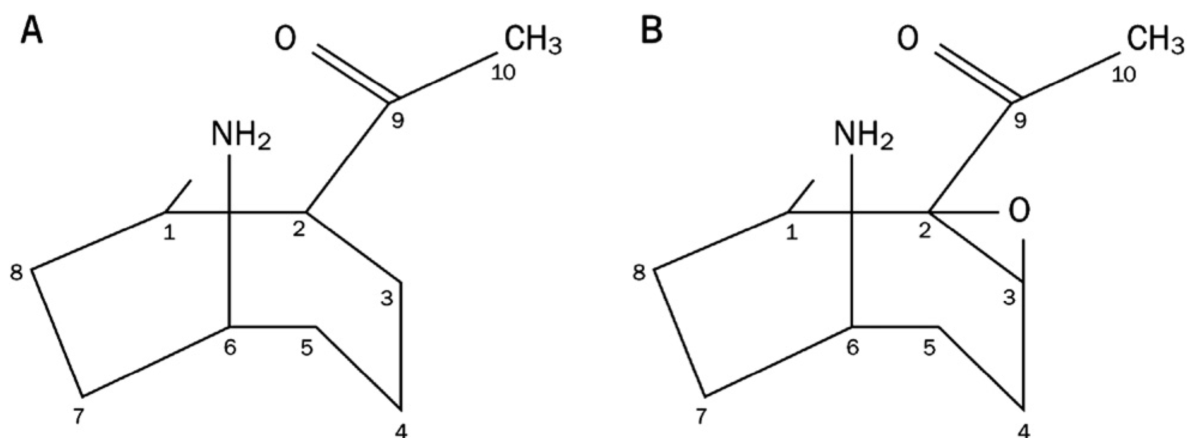


Figure 4. 1 : Non-toxic degradation products of ANTX A) A-dihydroanatoxin-a and B) B-epoxyanatoxin-a taken (Osswald et al. 2007).

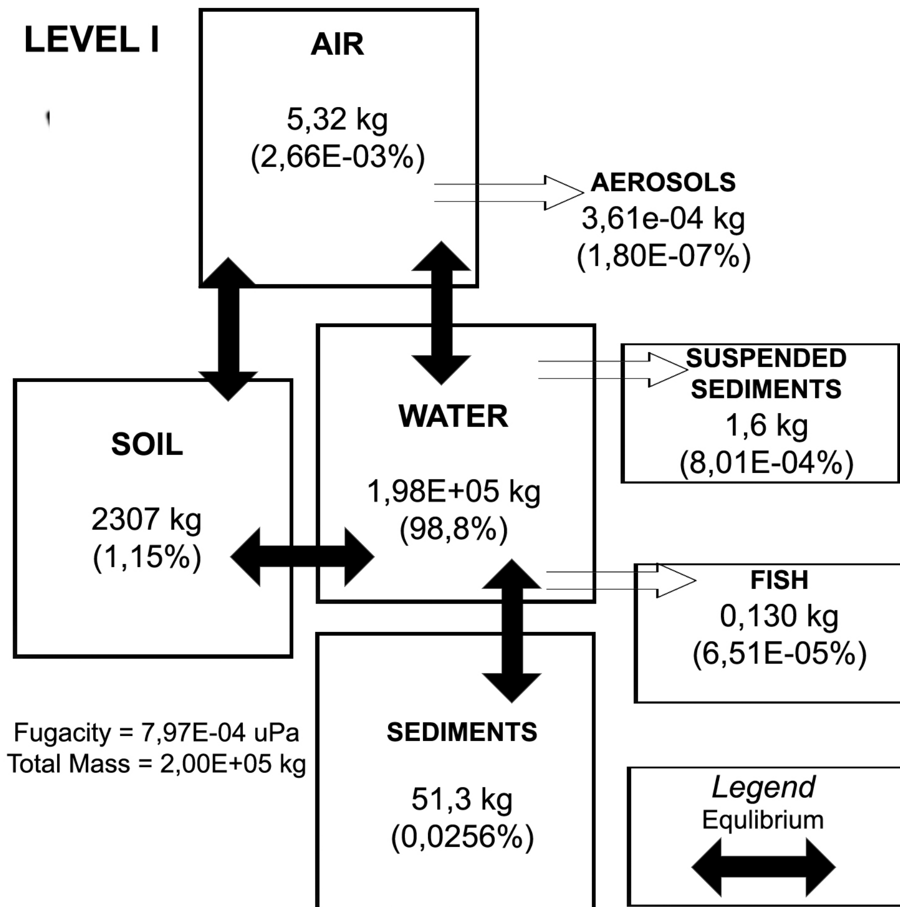


Figure 4. 2: Fugacity-based model (level I) simulation of an emission of 200,000 kg of ANTX in an EQC environment. The model used (Version 3.0) was obtained from the CEMC, Trent University, Peterborough.

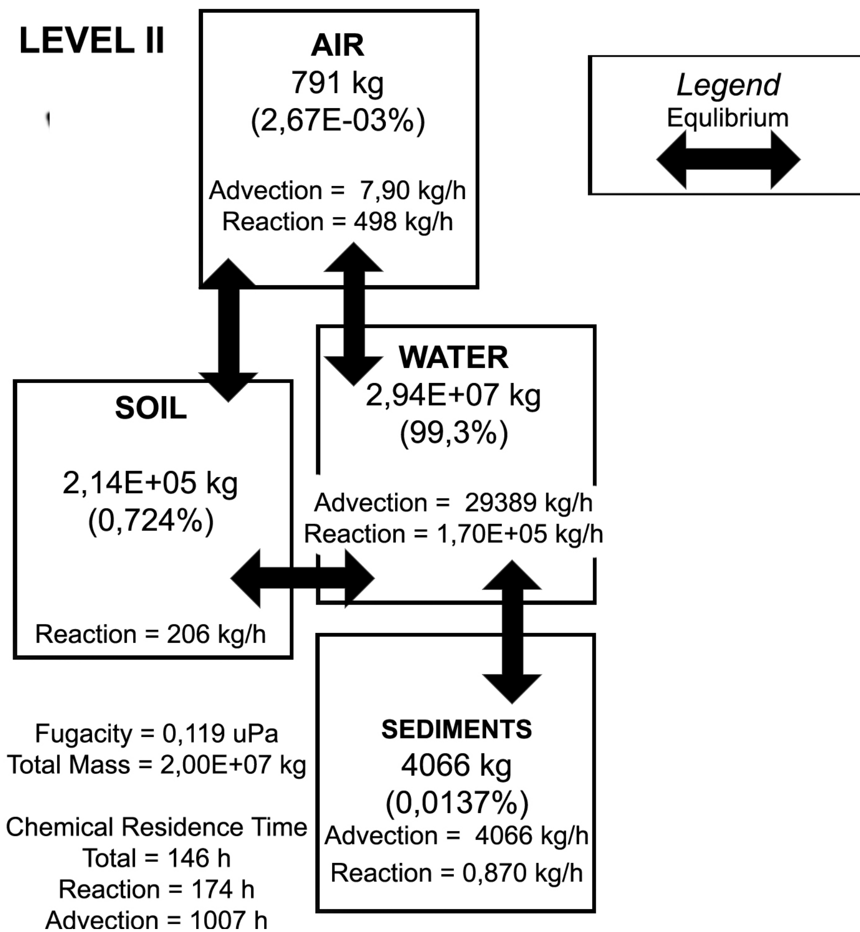


Figure 4. 3 : Fugacity-based model (level II) simulation of an emission of 200,000 kg of ANTX in an EQC environment using a half-life in water of 120 hours. The model used (Version 3.0) was obtained from the CEMC, Trent University, Peterborough.

Chapter 5: General Conclusion

Claims to originality

In this research, a common extraction and analytical method for anatoxin-a (ANTX) and microcystins (MCs) was successfully developed to facilitate a more rapid detection of potentially toxic blooms. The extraction method was modified from the one proposed by Aranda-Rodriguez *et al.* (2005). Different parameter values were tested to improve the extraction efficiency and formic acid was used to lower the pH and stabilize ANTX in the extraction process. In order to obtain an original analytical method, an enhanced scan was performed for each analyte and selective MRM transitions (2 transitions for each molecule) were acquired for proper identification. Different mobile phases, with different additives, were tested to obtain the best chromatographic separation possible. The extraction efficiency obtained for MCs in this new method was comparable to that reported for previous protocols (Aranda-Rodriguez *et al.* 2005). For ANTX, the extraction efficiency was comparable to that reported by Aranda-Rodriguez *et al.* (2005). However, the analytical method presented in this study is unique in that it can simulteanously analyse five variants of microcystin, nodularin, ANTX and phenylalanine using a quadrupole LC-MS/MS.

The factors regulating the production of ANTX by cyanobacteria are not well understood. Since ANTX, like other cyanotoxins, has no known function in primary metabolism, the assumption has been that it is a secondary metabolite. As a result anatoxin production should be a function of resource availability, with maximum production arising under high resource conditions. However, in batch culture experiments where the dissolved inorganic nitrogen concentrations were varied, the production of ANTX in *Aphanizomenon issatchenkoi* (a nitrogen fixing species of Nostocales) was in fact highest under intermediate N supply.

These results are in contrast to those of Rapala *et al.* (1993) who investigated ANTX concentrations in other cyanobacteria (*Aphanizomenon flos-aquae* and *Anabaena flos-aquae*) in relation to the availability of soluble nitrogen. However, a recent study by Kurmayer (2011) similarly found that the production of microcystins in *Nostoc* sp. was highest at intermediate levels of phosphorus stress. Both the Kurmayer study and the present results for ANTX are not consistent with current theories of resource allocation and secondary metabolite production.

The fate of ANTX in the environment has not been investigated to date in the scientific literature. By using fugacity-based models (Mackay 2001), the partitionning and the fate of dissolved ANTX was investigated based on available (albeit limited) physicochemical characteristics of ANTX and reported for the first time in this thesis. Various simulations showed that, at equilibrium, more than 98% of ANTX partitions into water as opposed to other phases. A similar partition behaviour, as well as minimal interphase transportation, was observed when the fugacity capacity for each phase was considered. ANTX is not likely to persist or bioaccumulate in food chains, at least in comparison to some other cyanotoxins such as microcystins.

Future research directions

With respect to the analytical method described in this thesis, the recovery values obtained were less satisfying for ANTX than for the MC variants. Adsorption to Hydromatrix might have been responsible for some loss of analyte in the extraction process and should be examined more closely. Furthermore, ANTX is extremely unstable under normal conditions and degradation might have occurred during sample preparation despite the acidification of the extracts. More work should be undertaken on the extraction process

and sample preparation in order to attain higher recovery values for ANTX. In addition, to further validate the method, a wider range of sample types needs to be tested including, most importantly, environmental samples from cyanobacterial blooms.

Growing cyanobacteria in batch cultures was a simple way to simulate an algal bloom. However, it was observed that the growth rates were different between nitrogen treatments. Hence, differences obtained in ANTX production among treatments might not be attributable to nitrogen supply exclusively, as other factors could be contributing to physiological changes under batch growth (e.g., changing light levels, accumulation of other cell products). It would be worthwhile to repeat these experiments with chemostats that would enable control of the growth rate while varying the nitrogen supply.

The partitioning and fate of dissolved ANTX in freshwater ecosystems was investigated using steady-state fugacity-based models. However, since most of the physicochemical parameters were estimated, more experimental data are needed to better represent the actual behaviour of ANTX in the environment. In particular, the rate at which ANTX is released from cyanobacterial cells is still unknown and is required to determine the toxin's environmental fate. In addition, bioaccumulation in fish through the dietary route should also be considered in future work. This would allow for a more complete understanding of the fate of both dissolved and particulate ANTX in aquatic environments.

References

Allen M.M. 1984. Cyanobacterial cell inclusions. Annu. Rev. Microbiol. 38: 1–25.

An J., Carmichael W.W. 1994. Use of a colorimetric protein phosphatase assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. Toxicon. 12: 1495–1507.

Andersen R., Algal Culturing Techniques, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME USA.

Aranda-Rodriguez R., Tillmanns A., Benoit F.M., Pick F.R., Harvie J., Solenaia L. 2005. Pressurized liquid extraction of toxins from cyanobacterial cells. Environ. Toxicol. 20(3): 390–396.

Aranda-Rodriguez R, Kubwabo C., Benoit F.M. 2003, Extraction of 15 microcystins and nodularin using immunoaffinity columns. Toxicon. 42(6): 587–599.

Aráoz R., Nghiêm H.O., Rippka R., Palibroda N., Tandeau de Marsac N., Herdman M. 2005. Neurotoxins in axenic oscillatorian cyanobacteria: coexistence of anatoxin-a and homoanatoxin-a determined by ligand-binding assay and GC/MS. Microbiology. 151: 1263–1273.

Aráoz R., Herman M., Rippka R., Ledreux A., Molgo J., Changeux J, Tandeau de Massac N., Nghiêm H.O. 2008. A non-radioactive ligand-binding assay for detection of cyanobacterial anatoxins using Torpedo electrocyte membranes. Toxicon. 52(1): 163-174.

Aráoz R, Molgó J, Tandeau de Marsac N. 2010, Neurotoxic cyanobacterial toxins, Toxicon, 56: 813-828.

Ballot A., Krienitz L., Kotut K., Wiegand C., Metcalf J., Codd G.A. 2004., Cyanobacteria and cyanobacterial toxins in three alkaline Rift Valley lakes of Kenya-Lakes Bogoria, Nakuru and Elmenteita. Journal of Plankton Research. 26(8): 925–935.

Ballot A., Fastner J., Lentz M., Wiedner C. 2010. First report of anatoxin-a producing cyanobacterium *Aphanizomenon issatschenkoi* in northeastern Germany. Toxicon. 56(6): 964-971.

Bazin E., Mourot A., Humpage A.R., Fessard V. 2010. Genotoxicity of a Freshwater Cyanotoxin, Cylindrospermopsin, in Two Human Cell Lines: Caco-2 and HepaRG. Environmental and Molecular Mutagenesis. 51: 251-259.

Bishop C.T., Gorham P.R. 1959. Isolation and identification of the fast-death factor in *Microcystis aeruginosa* NRC-1. Can. J. Biochem. Physiol. 37: 453–471.

Bergmann M., Peters R.H. 1980. A Simple Reflectance Method for the Measurement of Particulate Pigment in Lake Water and its Application to Phosphorus-Chlorophyll-Seston Relationships. Can. J. Fish. Aquat. Sci.. 37: 111-114.

Bogiali S., Bruno M., Curini R., Di Corcia A., Lagana A. 2006. Simple and rapid determination of anatoxin-a in lake water and fish muscle tissue by liquid-chromatography-tandem mass spectrometry. Journal of Chromatography A. 1122(1-2) : 180-185.

Bownik A. 2010. Harmful algae: effects of alkaloid cyanotoxins on animal and human health. Toxin Reviews. 29(3–4): 99–114.

Bumke-Vogt C., Mailahn W., Rotard W., Chorus I. 1996. A highly sensitive analytical method for the neurotoxin anatoxin-a, using GC-ECD, and first application to laboratory cultures. Phycologia. 35(6 supplement): 51–56.

Bumke-Vogt C., Mailahn W., Chorus I. 1999. Anatoxin-a and neurotoxic cyanobacteria in German lakes and reservoirs. Environ. Toxicol. 14: 117–125.

Cadel-Six S., Peyraud-Thomas C., Brient L., Marsac N.T., Rippka R., Méjean A. 2007. Different genotypes of anatoxin-producing cyanobacteria coexist in the Tarn River, France. Appl. Environ. Microbiol. 73: 7605–7614.

Campbell H.F., Edwards O.E., Kolt R. 1977. Synthesis of nor-anatoxin-a and anatoxin-a. Can. J. Chem. 55: 1372–1379.

Campinas M., Rosa M.J. 2010. Evaluation of cyanobacterial cells removal and lysis by ultrafiltration. Separation and Purification Technology. 70(3): 345-353.

Carmichael W.W., Biggs F., Gorham P.R. 1975. Toxicology and pharmacological action of *anabaena flos-aquae* toxin. Science. 187: 542-544.

Carmichael W.W. 1992. Cyanobacteria secondary metabolites - the cyanotoxins. Journal of Applied Bacteriology. 72: 445-459.

Carrasco D., Moreno E., Paniagua T. 2007. Anatoxin-a occurrence and potential cyanobacterial anatoxin-a producers in Spanish reservoirs. Journal of Phycol. 43: 1120-1125.

Chen W., Li L., Gan N., Song L. 2006. Optimization of an effective extraction procedure for the analysis of microcystins in soils and lake sediments. Environ. Pollut. 143(2): 241–246.

Chorus I., Bartram J. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. WHO and UNEP by E&FN Spon

Codd G.A., Morrison L.F., Metcalf S.J. 2005. Cyanobacterial toxins: risk, management for health protection. Toxicol. App. Pharmacol. 203(3): 264–272.

Cronberg G., Annadotter H. 2006. Manual on aquatic cyanobacteria, A photo guide and a synopsis of their toxicology. Intergovernmental Oceanographic Commission of UNESCO, International Society for the Study of Hramful Algae.

Devlin J.P., Edwards O.E., Gorham P.R., Hunter N.R., Pike R.K., Stavric B. 1977. Anatoxin-a, a toxic alkaloid from *Anabaena flos-aquae* NRC-44H. Canadian Journal of Chemestry. 55(8): 1367-1371.

Dimitrakopoulos I.K., Kaloudis T.S., Hiskia A.E., Thomaidis N.S., Koupparis M.A. 2010. Development of a fast and selective method for the sensitive determination of anatoxin-a in lake waters using liquid chromatography–tandem mass spectrometry and phenylalanine-d_5 as internal standard. Anal. Bioanal. Chem. 397: 2245–2252

Fastner J., Flieger I., Neumann U. 1998. Optimized extraction of microcystins from field samples a comparison of different solvents and procedures. Water Res. 32 (10): 3177–3181.

Furey A., Crowley J., Hamilton B., Lehane M., James K. J. 2005. Strategies to avoid the mis-identification of anatoxin-a using mass spectrometry in the forensic investigation of acute neurotoxic poisoning. Journal of Chromatography A. 1082: 91–97.

Frost P.C., Kinsman L.E., Johnston C.A., Larson J.H. 2009. Watershed discharge modulates relationships between landscape components and nutrient ratios in stream seston. Ecology. 90(6): 1631-1640.

Funari E., Testai E. 2008. Human Health Risk Assessment Related to Cyanotoxins Exposure. Critical Reviews in Toxicology. 38: 97–125.

Gademann K., Portmann C. 2008. Secondary Metabolites from Cyanobacteria: Complex Structures and Powerful Bioactivities. Current Organic Chemistry. 12: 326-341.

Gallon J.R., Chit K.N., Brown E.G. 1990. Biosynthesis of the tropane-related cyanobacterial toxin anatoxin-a: role of the ornithine decarboxilase. Phytochemistry. 29(4): 1107–1111.

Gallon J.R., Kittakoop P., Brown E.G. 1994. Biosynthesis of Anatoxin-a by *Anabaena flos-aquae*: examination of primary enzymic steps. Phytochemistry. 35(5): 1195–1203.

Ghassempour A., Najafi N.M., Mehdinia A., Davarani S.S.H., Fallahi M., Nakhshab M. 2005. Analysis of anatoxin-a using polyaniline as a sorbent in solid-phase microextraction coupled to gas chromatography-mass spectrometry. J. Chromatogr. 1078: 120–127.

Gorham P.R., McLachlan J., Hammer U.T., Kim W.K. 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Bréb. Verh. Internat. Verein. Limnol. 15: 796–804.

Gugger M., Lenoir S., Berger C., Ledreux A., Druart J., Humbert J-F. 2005. First report in a river in France of the benthic cyanobacterium Phormidium favosum producing anatoxin-a associated with dog neurotoxicosis. Toxicon. 45: 919–928.

Guillard R.R.L. 1973. Division rates. In Handbook of phycological methods, ed. by J. R. Stein, 289-312. Cambridge, Cambridge University Press.

Hamilton J.G., Zangerl A.R., De Lucia E.H., Berenbaum M.R. 2001. The carbon-nutrient balance hypothesis: its rise and fall. Ecology Letters. 4: 86-95.

Harada K.I., Kimura Y., Ogawa K., Suzuki M., Dahlem A.M., Beasley V.R. 1989. A newprocedure for the analysis and purification of naturally occurring anatoxin-a from the blue-green alga *Anabaena flos-aquae*. Toxicon. 7(12): 1289–1296.

Harada K.I., Nagai H., Kimura Y., Suzuki M., Park H.,Watanabe M.F. 1993. Liquid chromatography/mass spectrometric detection of anatoxin-a, a neurotoxin from cyanobacteria. Tetrahedron. 49(41): 9251–9260.

Hedman C.J., Krick W.R., Karner Perkins D.A., Harrahy E.A., SonzogniW.C. 2008. New measurements of cyanobacterial toxins in natural waters using high performance liquid chromatography coupled to tandem mass spectrometry. Journal of Environmental Quality. 37 (5): 1817-1824.

Heresztyn T., Nicholson B.C. 2001. Determination of cyanobacterial hepatotoxins directly in water using a protein phosphatase inhibition assays. Water Res. 35(13) : 3049–3056.

James K.J., Crowley J., Hamilton B., Lehane M., Skulberg O., Furey A. 2005. Anatoxins and degradation products, determined using hybrid quadrupole time-offlight and quadrupole ion-trap mass spectrometry: Forensic investigations of cyanobacterial neurotoxin poisoning. Rapid Commun Mass Spectrom. 19(9): 1167–1175.

James K.J., Furey A., Sherlock I.R., Stack M.A., Twohig M., Caudwell F.B. 1998. Sensitive determination of anatoxin-a, homoanatoxin-a and their degradation products by liquid chromatography with fluorimetric detection. J. Chromatogr. A. 798(1–2): 147–157.

Jespersen A., Christoffersen K. 1987. Measurements of chlorophyll-a from phytoplankton using ethanol as extraction solvent. Archiv. Fur. Hydrobiologie. Stuttgart. 109(3): 445-454.

Kaarina S., Gary J. 1999. Toxic cyanobacteria in water : A guide to their public health consequences, monitoring and management. WHO and UNEP by E&FN Spon.

Kiviranta J., Sivonen K., Lahti K., Luukkainen R., Niemel S.I. 1991. Production and biodegradation of cyanobacterial toxins - a laboratory study. Arch. Hydrobiol. 121: 281-294.

Kurmayer R. 2011. The Toxic Cyanobacterium *Nostoc* sp. strain 152 produces highest amounts of microcystin and nostophycin under stress conditions, Journal of phycology. 47: 200-207.

Lahti K., Ahtiainen J., Rapala J., Sivonen K., Niemela S.I. 1995. Assessment of rapid bioassays for detecting cyanobacterial toxicity. Lett. App. Microbiol. 21: 109–114.

Lair S., Starr M., Measures L., Scarratt M., Michaud S., Michaud R., Béland P., Robert M., Quilliam M. 2002. Multispecies Mortalities Associated with Saxitoxin Intoxication Resulting from a Harmful Algal Bloom of *Alexandrium tamarense* in the St. Lawrence Estuary, Quebec, Canada, WSAVA 2002 Congress.

Lewis R.J. 2000. Sax's Dangerous Properties of Industrial Materials. Vol. 1-3, 10th ed., John Wiley & Sons Inc., New York, NY. p. 257.

Lopez C.B., Jewett E.B., Dortch Q., Walton B.T., Hudnell H.K. 2008. Scientific Assessment of Freshwater Harmful Algal Blooms. Interagency Working Group on Harmful Algal Blooms, Hypoxia, and Human Health of the Joint Subcommittee on Ocean Science and Technology. Washington, DC.

Mackay D. 2001. Multimedia Environmental Models: The Fugacity Approach - Second Edition. Lewis Publishers. Boca Raton, pp.1-261.

Matsunaga S., Moore R.E., Niemczura W.P., Carmichale W.W. 1989. Anatoxin-a(s), a potent anticholinesterase from *Anabaena flos-aquae*. Journal of the American Chemical Society. 111(20): 8021-8023.

Méjean A., Mann S., Vassiliadis G., Lombard B., Loew D., Ploux O. 2010. *In Vitro* Reconstitution of the First Steps of Anatoxin-a Biosynthesis in Oscillatoria PCC 6506: From Free L-Proline to Acyl Carrier Protein Bound Dehydroproline. Biochemistry. 49: 103-113.

Meriluoto J.A., Spoof L.E. 2008. Cyanotoxins: sampling, sample processing and toxin uptake. Adv. Exp. Med. Biol.. 619: 483-499.

Metcalf J.S., Codd G.A. 2000. Microwave oven and boiling waterbath extraction of hepatotoxins from cyanobacterial cells. FEMS Microbiol. Lett. 184 (2): 241–246.

Mikhailov A., Härmälä-Braskén A.S., Meriluoto J., Sorokina Y., Dietrich D., Eriksson J.E.E. 2001. Production and specificity of mono and polyclonal antibodies against microcystins conjugated through N-methyldehydroalanine. Toxicon. 39: 477–483.

Moustaka-Gouni M., Kormas K.A., Polykarpou P., Gkelis S., Bobori D., Vardaka E. 2010. Polyphasic evaluation of *Aphanizomenon issatschenkoi* and *Raphidiopsis mediterranea* in a Mediterranean lake. Journal of Plankton Research. 32(6): 927-936.

Msagati T.A.M., Siame B.A., Shushu D.D. 2006. Evaluation of methods for the isolation, d etection and quantification of cyanobacterial hepatotoxins. Aquat. Toxicol. 78 (4): 382–397.

Namera A., So A., Pawliszyn J. 2002. Analysis of anatoxin-a in aqueous samples by solid-phase microextraction coupled to high-performance liquid chromatography with fluorescence detection and on-fiber derivatization. J Chromatogr A. 963(1–2): 295–302.

Namikoshi M., Murakami T., Watanabe M.F., Oda T., Yamada J., Tsujimura S. 2003. Simultaneous production of homoanatoxin-a, anatoxin-a, and a new non-toxic 4-hydroxyhomoanatoxin-a by the cyanobacterium *Raphidiopsis mediterranea* Skuja. Toxicon. 42(5): 533-538.

Oehrle S.A., Southwell B., Westrick J. 2010. Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectrometry. Toxicon. 55: 965–972.

Ohtani I., Moore R.E., Runnegar M.T.C. 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. J. Am. Chem. Soc. 114: 7941–7942.

Ojanperä I., Vuori E., Himberg K., Waris M., Niinivaara K. 1991. Facile detection of anatoxin-a in algal material by thin-layer chromatography with Fast Black-K salt. The Analyst. 116: 265–267.

Onodera H., Oshima Y., Henriksen P., Yasumoto T. 1997. Confirmation of anatoxin-a(s), in the cyanobacterium *Anabaena lemmermannii*, as the cause of bird kills in Danish Lakes. Toxicon. 35(11): 1645-1648.

Osswald J., Rellan S., Gago A., Vasconcelos V. 2007. Toxicology and detection methods of the alkaloid neurotoxin produced by cyanobacteria, anatoxin-a. Environment International. 33(8): 1070–1089.

Park H.D., Kim B., Kim E., Okino T. 1998. Hepatotoxic Microcystins and Neurotoxic Anatoxin-a in Cyanobacterial Blooms from Korean Lakes. Environmental Toxicology And Water Quality. 13(3): 225-234.

Peary J.A., Gorham P.R. 1966. Influence of light and temperature on growth and toxin production by *Anabaena flos-aquae*. J. Phycol. 2(Suppl 2): 3.

Pérez S., Aga D.S. 2005. Recent advances in the sample preparation, liquid chromatography tandem mass spectrometric analysis and environmental fate of microcystins in water. Trends in Analytical Chemistry. 24(7): 658-670.

Poon G.K., Griggs L.J., Edwards C., Beattie K.A., Codd G.A. 1993. Liquid chromatography-electrospray ionization-mass spectrometry of cyanobacterial toxins. J. of Chromatogr. 628(2): 215–233.

Powell, M.W. 1997. Analysis of Anatoxin-a in Aqueous Samples. Chromatographia. 45: 25-28.

Pyo D., Shin H. 2002. Extraction and purification of microcystins RR and LR cyanobacteria using a cyano cartridage. J. Biochem. Biophys. Methods. 51(2): 103–109.

Rajaneesh P., Jaswant S. 2010. The toxicology of microcystins and its implication on human health, Journal of Ecophysiology & Occupational Health. 10(1-2)

Rapala J., Sivonen K., Luukainen R., Niemalä S.I. 1993. Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and non-toxic *Anabaena* strains - a laboratory study. J. Appl. Phycol. 5: 581–591.

Rapala J., Lahti K., Sivonen K., Niemelä S.I. 1994. Biodegradability and adsorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. Lett. App. Microbiol. 19: 423–428.

Rapala J., Sivonen K. 1998. Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. Microb. Ecol. 36: 181–192.

Rapala J., Lahti K. 2002. Methods for detection of cyanobacterial toxins. In: Franca, Palumbo, GiulianoZiglip, Andre'Van der Beken (Eds.), Detection Methods for Algae, Protozoa and Helminthes in Fresh and Drinking Water. Water Quality Measurement Series.Wiley. New York., pp. 107–128

Redfield A.C. 1934. On the proportions of organic derivations in sea water and their relation to the composition of plankton. In *James Johnstone Memorial Volume*. (ed. R.J. Daniel). University Press of Liverpool. pp. 177-192.

Rellán S., Osswald J., Vasconcelos V., Gago-Martinez 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. 1156: 134–140.

Robillot C., Vinh J., Puiseux-Dao S., Hennion M.C. 2000. Hepatotoxin production kinetics of the cyanobacterium *Microcystis aeruginosa* PCC 7820, as determined by HPLC-

mass spectrometry and protein phosphatase bioassay. Environ. Sci. Technol. 34: 3372–3378.

Sangolkar L.N., Maske S.S., Chakrabarti T. 2006. Methods for determining microcystins (peptide hepatotoxins) and microcystin-producing cyanobacteria. Water Research, 40(19): 3485-3496.

Seifert M., McGregor G., Eaglesham G., Wickramasinghe W., Shaw G. 2007. First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck. Harmful Algae. 6 : 73–80.

Selwood A.I., Holland P.T., Wood S.A., Smith K.F., Mcnabb P.S. 2007. Production of Anatoxin-a and a Novel Biosynthetic Precursor by the Cyanobacterium *Aphanizomenon issatschenkoi.* Environ. Sci. Techno. 41(2): 506-510.

Sivonen K., Himberg K., Luukkainen R., Niemelä S.I., Poon G.K., Codd G.A. 1989. Preliminary characterization of neurotoxic cyanobacteria blooms and strains from Finland. Toxic Assess. 4: 339–352.

Smith R.A., Lewis D. 1987. A rapid analysis of water for anatoxin-a, the unstable toxic alkaloid from *Anabaena flos-aquae*, the stable non-toxic alkaloids left after bioreduction and a related amine which may be nature's precursor to anatoxin-a. Vet. Human Toxico.l: 29(2):153–154.

Smith C., Sutton, A. 1993. The persistence of anatoxin-a in reservoir water. Foundation for Water Research, UK Report No. FR0427.

Spoof L., Vesterkvist P., Lindholm T., Meriluoto J. 2003. Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatographyelectrospray ionization mass spectrometry, J. Chromatogr. A. 1020 : 105–119.

Stavric B., Gorham P.R. 1966. Toxic factors from *Anabaena flos-aquae* (Lyngb.) de Breb clone NRC-44 h. Proceedings of the Canadian Society of Plant Physiology. Annual meeting University of British Columbia.

Stevens D.K., Krieger R.I. 1991. Stability studies on the cyanobacterial nicotinic alkaloid Anatoxin-a. Toxicon. 29(2): 167–179.

Stevens D.K., Krieger R.I. 1988. Analysis of Anatoxin-a by GC/ECD. J. Anal. Toxicol. 12(3): 126–131.

Takino M., Daishima S., Yamaguchi K. 1999. Analysis of Anatoxin-a in freshwaters by automated on-line derivatization–liquid chromatography–electrospray mass spectrometry. Journal of Chromatography A. 862(2): 191-197.

Tillmanns A.R., Pick F.R., Aranda-Rodriguez R. 2007. Sampling and analysis of microcystins: Implications for the development of standardized methods. Environmental Toxicology. 22(2): 132-143.

Turpin D.H. 1991. Effect of Inorganic N Availability on Algal Photosynthesis and Carbon Metabolism. Journal of Phycology. 27: 14-20.

Van de Waal D.B., Verspagen M.H., Lürling M., Van Donk E., Visser P.M., Huisman J. 2009. The ecological stoichiometry of toxins produced by harmful cyanobacteria: an experimental test of the carbon-nutrient balance hypothesis. Ecological Letters. 12: 1326-1335.

Watanabe M.F., Tsujimura S., Oishi S., Niki T., Namikoshi M. 2003. Isolation and identification of homoanatoxin-a from a toxic strain of the cyanobacterium *Raphidiopsis mediterranea* Skuja isolated from Lake Biwa, Japan. Phycologia. 42 : 364–369.

Webster E., Mackay D., Wania F., Arnot J., Gobas F., Gouin T., Hubbarde J., Bonnell M. 2005. Development and Application of Models of Chemical Fate in Canada : Modelling Guidance Document, CEMN, Report to Environment Canada, 129 p.

Whitton B.A., Potts M. 2000. Introduction to the Cyanobacteria, The ecology of cyanobacteria: their diversity in time and space, Kluwer Academic Publishers, The Netherlands: 1-11.

WHO. 2008. Guidelines for Drinking Water Quality, Second Addendum to Third Edition, World Health Organization: Geneva, Switzerland.

Wiegand C., Pflugmacher S. 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol. App. Pharmacol. 203: 201–218.

Wood S.A., Stirling D.J. 2003. First identification of the cylindrospermopsin-producing cyanobacterium *Cylindrospermopsis raciborskii* in New Zealand. New Zealand Journal of Marine and Freshwater Research. 37(4): 821-828.

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

Zurawell R.W., Chen H., Burke J.M., Prepas E.E. 2005. Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. J. Toxicol. Environ. Health B. 8: 1-37.

Appendices

Table A.1 : Composition of BG11 medium obtained from Andersen 2005.

Chemicals	Concentration ($mg \cdot L^{-1}$)
calcium chloride dihydrate	36.7
citric acid	5.6
dipotassium hydrogen phosphate	31.4
disodium magnesium EDTA	1
ferric ammonium citrate	6
magnesium sulfate	36
sodium carbonate	20
sodium nitrate	1500