CONTRIBUTIONS TO RISK ASSESSMENT OF MICROCYSTINS, CYANOBACTERIAL TOXINS - DEVELOPMENT OF IMMUNOANALYSIS AND CHARACTERIZATION OF MICROCYSTIN VARIANTS

Pia Vesterkvist

Department of Biosciences
Åbo Akademi University
Turku, Finland
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Pia Vesterkvist

Department of Biosciences
Åbo Akademi University
Turku, Finland
2012

Supervised by

Docent Jussi Meriluoto
Biochemistry, Department of Biosciences
Åbo Akademi University
Turku, Finland

Reviewed by

Ph.D. Risto Tanner
Laboratory of Chemical Physics
National Institute of Chemical Physics and Biophysics
Tallinn, Estonia

and

Docent David Fewer
Food and Environmental Sciences
Division of Microbiology
University of Helsinki
Helsinki, Finland

Opponent

Professor Geoffrey A. Codd
Professor emeritus in Microbiology
College of Life Sciences,
University of Dundee
Dundee, UK

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Till mina föräldrar
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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers:

I. Pia Mehto, Matti Ankelo, Ari Hinkkanen, Andrey Mikhailov, John E. Eriksson, Lisa Spoof and Jussi Meriluoto. **A time-resolved fluoroimmunometric assay for the detection of microcystins, cyanobacterial peptide hepatotoxins.** *Toxicon 39, 831 – 836, 2001*

II. Tore Lindholm, Pia Vesterkvist, Lisa Spoof, Catharina Lundberg-Niinistö and Jussi Meriluoto. **Microcystin occurrence in lakes in Åland, SW Finland.** *Hydrobiologia 505, 129 – 138, 2003*

III. Lisa Spoof, Pia Vesterkvist, Tore Lindholm and Jussi Meriluoto. **Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography-electrospray ionisation mass spectrometry.** *Journal of Chromatography A, 1020, 105 – 119, 2003*

IV. Pia Vesterkvist and Jussi Meriluoto. **Interaction between microcystins of different hydrophobicities and lipid monolayers.** *Toxicon 41, 349 – 355, 2003.*

V. Pia Vesterkvist, Julia Misiorek, Lisa Spoof, Diana Toivola and Jussi Meriluoto. **Comparative cellular toxicity of hydrophilic and hydrophobic microcystins on Caco-2 cells.** Manuscript.

CONTRIBUTION OF THE AUTHOR

The author has contributed to the papers in the thesis as follows:

In paper I, the main work was designed, conducted and manuscript prepared by the author together with the supervisor. Antibody production was performed by Dr. Andrey Mikhailov.

In paper II, the TR-FIA was performed and interpreted by the author.

In paper III, the ELISA was performed and interpreted by the author.

In paper IV, the work was designed, conducted and manuscript prepared by the author together with the supervisor and Professor J. Peter Slotte.

In paper V, the biophysical work was designed and conducted by the author together with the supervisor and Professor J. Peter Slotte. The cell biology work was designed by the author together with the supervisor and Docent Diana Toivola, and conducted with the help of MSc Julia Misiorek who also maintained the cells and performed the morphology experiments. The protein phosphatase inhibition assay was performed by Dr. Lisa Spoof who also provided purified toxins.

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Pia Vesterkvist

ABBREVIATIONS

ACN	Acetonitrile
Adda	3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid
AET	Aminoethanethiol
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
BSA	Bovine serum albumine
CPS	Counts per second
CTL	Cholesta-5,7,9(11)-trien-3-beta-ol
Cys	Cysteine
DAD	Diode-array detection
Dhb	Dehydrobutyrine
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
Dm (dm)	Demethyl
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DW	Dry weight
EDA	Ethylenediamine
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionization
Glu	Glutamic acid
GSH	Glutathione
GST	Glutathione S-transerase
HPLC	High-performance liquid chromatography
IC_{50}	Concentration giving half-maximal inhibition
i.p.	Intraperitoneal
LDH	Lactate dehydrogenase
LC-MS	Liquid chromatography – mass spectrometry
LC-MS-MS	Liquid chromatography – tandem mass spectrometry
LC-MS-SIR	Liquid chromatography – mass spectrometry - single ion recording
LD_{50}	Lethal dose that kills 50% of the test organisms
Leu	Leucine
LOAEL	Lowest observed adverse effect level
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption ionization – time-of-flight
MC	Microcystin
Mdha	N-methyldehydroalanine
MeAsp	Erythro-β-methyl-D-aspartate
MeOH	Methanol
MS	Mass spectrometry
m/z	Mass-to-charge ratio
MW	Molecular weight

NOAEL	No observed adverse effect level
OATP	Organic anion-transporting polypeptide
p-NPP	p-nitrophenyl phosphate
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PP	Protein phosphatase
PPIA	Protein phosphatase inhibition assay
TFA	Trifluoroacetic acid
TMA-DPH	1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene
TRF	Time-resolved fluorescence
TR-FIA	Time-resolved fluoroimmunoassay
Tyr	Tyrosine
WHO	World Health Organization
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

1 REVIEW OF THE LITERATURE

1.1 CYANOBACTERIA IN THE ENVIRONMENT

Cyanobacteria are ancient organisms dating back as far as 3.5 billion years (Schopf, 1993). The phylum Cyanobacteria is a diverse group of photosynthetic gram-negative bacteria responsible for creating the oxygenic atmosphere on earth (Blank and Sánchez-Baracaldo, 2010). Morphology, physiology and habitat differ between species, and enable the cyanobacteria to adapt and colonize a great variety of different environments. The characterization and classification of cyanobacteria is challenging and molecular, morphological, physiological and biochemical data are combined (Komárek et al., 2003). The taxonomic category "species" is problematic among cyanobacteria in the light of modern classification criteria but traditionally about 2000 species belonging to 150 genera have been estimated to exist (van den Hoek et al., 1995; Wilmotte and Herdman, 2001).

1.1.1 Cyanobacterial blooms

Aquatic cyanobacteria occur in fresh water, brackish water and marine environments. Cyanobacteria vary in shape and appearance, single cells being about 1 to 10 µm in diameter or length and appearing unicellularly or as colonies of many shapes, or formations of filaments (Garcia-Pichel, 2009). One colony may contain hundreds or thousands of cells and grow to millimeters in diameter. In favorable environmental conditions cyanobacterial blooms may develop. A dense cyanobacterial bloom seriously deteriorates the water quality, also by decreasing the oxygen level when decaying. Some cyanobacteria produce slime and odorous compounds that can cause problem in the water treatment (Srinivasan and Sorial, 2011). The most serious ecological problem and health risk to humans and animals is the production of cyanobacterial toxins (Codd et al., 2005).

A bloom arises when cyanobacteria dominate the freshwater environment. The out-competition of other phytoplankton members originates from several distinct properties (Carey et al., 2012). Many cyanobacteria can differentiate into particular cell types, like heterocysts that have thick cell walls and are able to fix nitrogen. Special resting cells, akinetes, enable cyanobacteria to survive in unfavourable conditions and regenerate when conditions improve. Gas vesicles regulate the buoyancy of cyanobacteria, allowing them to adopt an optimum depth in the water column. Cyanobacteria tolerate low light conditions and for example *Planktothrix*, is low light adapted and can form dense populations in the epilimnion of a lake (Reynolds et al., 1987; Lindholm and Meriluoto, 1991).

1.2 SECONDARY METABOLITES IN CYANOBACTERIA

Cyanobacteria produce a variety of unique secondary metabolites (Fujii et al., 2000; Welker et al., 2006; Van Wagoner et al., 2007). Several of these are small but complex alkaloids or peptides whose true function has not yet been elucidated (Harada, 2004). Some cyanobacteria produce toxins as secondary metabolites and they deserve attention due to their impact on human society and the environment (Codd et al., 2005; Wiegand and Pflugmacher, 2005). The cyanotoxins are both chemically and toxicologically a diverse group of natural toxins (Sivonen and Jones, 1999).

Cyanotoxins seem to be more toxic to mammals than to aquatic organisms (Ibelings and Havens, 2008). According to traditional bacterial systematics at least 46 cyanobacterial species have been reported to produce toxins (Sivonen and Jones, 1999).

1.2.1 Hepatotoxins

The compounds most often implicated in cyanobacterial toxicosis are hepatotoxins called microcystins and nodularins. The large and diverse heptapeptide family of microcystins has been the most frequently reported cyanobacterial toxin (more detailed information regarding microcystins is found in the section Microcystins and in Risk Assessment for Human Health). Microcystins are typically found in species of the freshwater genera *Microcystis, Planktothrix, Anabaena, Nostoc, Phormidium, Hapalosiphon, Fischerella* and *Anabaenopsis* amongst others (Krishnamurthy et al., 1986; Meriluoto et al., 1989; Sivonen et al., 1990; Prinsep et al., 1992; Moore, 1996; Izaguirre et al., 2007; Fiore et al., 2009). The smaller and less diverse pentapeptide family of nodularins comprises about 10 different variants, produced predominantly by *Nodularia* (Rinehart et al., 1988; Sivonen and Jones, 1999). These closely related peptides are potent protein phosphatase inhibitors with a primary effect on hepatocytes (Falconer and Yeung, 1992; Runnegar et al., 1993).

1.2.2 Neurotoxins

Cyanobacteria produce a variety of heterocyclic alkaloid neurotoxins. The main neurotoxins are divided into three groups: a) anatoxin-a and analogues, b) anatoxin-a(S), and c) saxitoxin and analogues.

Anatoxin-a and homoanatoxin-a are bicyclic secondary amines with potent neuromuscular blocking effects (Devlin et al., 1977; Carmichael, 1992). Anatoxin-a binds to neuronal nicotinic acetylcholine receptors at neuromuscular junctions, acting as a nicotinic (cholinergic) agonist (Swanson et al., 1986). LD_{50} values for anatoxin-a and homoanatoxin-a are 200 – 250 µg/kg body weight (Devlin et al., 1977; Carmichael et al., 1990; Skulberg et al., 1992). Cyanobacterial species of *Anabaena, Aphanizomenon, Cylindrospermum, Microcystis, Oscillatoria* and *Planktothrix* can produce anatoxin-a and/or homoanatoxin-a (Sivonen and Jones, 1999).

Anatoxin-a(S) is an organophosphate that inhibits acetylcholinesterase at the neuromuscular junction (Mahmood and Carmichael, 1987). Despite its name it is not chemically related to anatoxin-a, but it was isolated from the same species (*A. flos-aquae*). The LD_{50} value of anatoxin-a(S) is approximately 50 µg/kg (mouse, i.p.) (Mahmood and Carmichael, 1986). Some strains of the genus *Anabaena* produce anatoxin-a(S) (Onodera et al., 1997a).

Saxitoxins, also known as paralytic shellfish poisons (PSP), are a large group of sodium channel antagonists. The toxicities of the variants differ significantly with saxitoxin being the most toxic one with an LD_{50} of 10 µg/kg (mouse, i.p.) (Carmichael et al., 1990; Funari and Testai, 2008; Humpage, 2008). These toxins are produced by e.g. *Anabaena circinalis, Lyngbya wollei* and *Cylindrospermopsis raciborskii*, although they were originally isolated from shellfish and marine dinoflagellates (Humpage et al., 1994; Onodera et al., 1997b; Lagos et al., 1999).

1.2.3 Cytotoxins

Cylindrospermopsin is a cyclic guanidine alkaloid the main toxic effect of which is to irreversibly inhibit protein synthesis (Terao et al., 1994; Runnegar et al., 2002). The acute effect seems to be mediated by CYP450 generated metabolites which inhibit the synthesis of glutathione (Runnegar et al., 1995b; Norris et al., 2002; Froscio et al., 2003; Humpage et al., 2005). Cylindrospermopsin has two further structural variants, the toxic 7-epicylindrospermopsin (Banker et al., 2000) and the 7-deoxycylindrospermopsin, whose level of toxicity is still under debate (Runnegar et al., 2002; Neumann et al., 2007). Cylindrospermopsin and its variants are produced by e.g. *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum, Raphidiopsis curvata, Anabaena lapponica* and *Lyngbya wollei* (Ohtani et al., 1992; Norris et al., 1999; Banker et al., 2000; Li et al., 2001; Spoof et al., 2006; Seifert et al., 2007).

1.2.4 Lipopolysaccharides

Lipopolysaccharides (LPS) are endotoxins found in the outer membrane of several gram-negative bacteria including cyanobacteria. The biological activity of LPS is attributed to the detailed structure of lipid A (Stewart et al., 2006). No cyanobacterial lipid A structure has so far been described or published (Stewart et al., 2006). However, cyanobacterial lipopolysaccharides are considered to be less toxic than the LPS of other bacteria like e.g. *Salmonella* (Raziuddin et al., 1983; Rapala et al., 2002; Best et al., 2003) but can give rise to pyrogenic, irritant and allergenic responses in humans and animals (Ressom et al., 1994). Some studies claim that the endotoxicity of cyanobacterial LPS is comparable to heterotrophic bacteria (Bernardova et al., 2008). Co-exposure to cyanobacterial lipopolysaccharides and microcystins has been shown to reduce microcystin detoxification capabilities in zebra fish (Best et al., 2002). The life history of *Daphnia galeata* was shown to be affected in a context dependent manner by the microcystin-producing strain and concentration together with the LPS source (Dionisio Pires et al., 2011).

1.3 MICROCYSTINS

Microcystins are not essential for cyanobacterial primary metabolism (Carmichael, 1992; Tillett et al., 2000; Christiansen et al., 2003). In healthy growing cyanobacteria most of the microcystins reside within the cell (Lam et al., 1995). When cell lysis occurs the cell-bound toxins are released into the water (Lam et al., 1995). Cyanobacterial species can simultaneously produce many different variants of microcystins (Rantala et al., 2006). Both changes in the environment and genetic factors lead to variations in the production and composition of microcystins (Sivonen, 2009; Jähnichen et al., 2011).

1.3.1 Structure and nomenclature

Microcystins are cyclic heptapeptides with the following structure: cyclo(-D-Ala^{1}-X^{2}-D-MeAsp^{3}(iso-linkage)-Z^{4}-Adda^{5}-D-Glu^{6}(iso-linkage)-Mdha^{7}), where MeAsp is *erythro*-β-methylaspartate and Mdha is *N*-methyldehydroalanine (Carmichael et al., 1988; Rinehart et al., 1994). The structure was elucidated by Botes et al. (Botes et al., 1984; Botes et al., 1985) and Rinehart et al. (Rinehart et al., 1988). The β-amino acid Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid, is an uncommon and unique amino acid found in microcystins and nodularins

(Botes et al., 1984; Botes et al., 1985; Rinehart et al., 1988). Microcystin variants are named after their structure by using the single letter code of amino acids. The most frequently found microcystin is microcystin-LR, abbreviated to MC-LR (Fig 1). Substitution of the L-amino acids at position 2 (X) and 4 (Z) and the demethylation of the amino acids at position 3 (MeAsp) and/or 7 (Mdha) are the most common variations in the structure. However, all of the amino acids can vary (Sivonen and Jones 1999). At the moment over 100 different microcystin variants are described in the literature (partially reported in Sivonen and Jones, 1999; Brittain et al., 2000; Matthiensen et al., 2000; Sano et al., 2004; Spoof, 2004). New structural variants within the microcystin family are reported continuously.

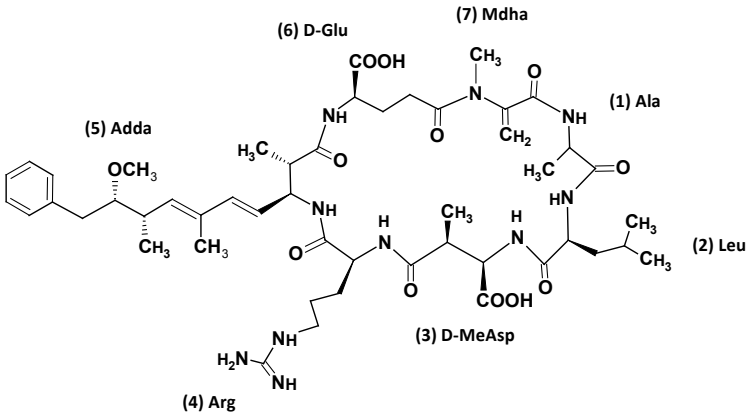

Figure 1. Structure of microcystin-LR, the most frequently encountered microcystin.

1.3.2 Physicochemical properties

Microcystins are chemically stable molecules, not particularly sensitive to hydrolysis, oxidation or heating (Harada, 1996). Microcystins are not destroyed by boiling (Metcalf and Codd, 2000). Chlorination, ozonation and reverse osmosis treatments are effective procedures in water works to remove microcystins (Tsuji et al., 1997; Hitzfeld et al., 2000; Liu et al., 2010). In natural environments, microbial decomposition of microcystins is usually slow, often requiring an extended lag time (Lahti et al., 1997; Holst et al., 2003). Dissolved microcystins can be degraded by photodegradation, and the rate is accelerated when cyanobacterial pigments are present (Tsuji et al., 1994; Wörmer et al., 2010). The distribution ratio of microcystin-LR in octanol/water (log D_{ow}) was shown to vary between 2.18 and -1.76, depending on the pH (de Maagd et al., 1999). The low log D_{ow} at higher pH values suggested a low tendency of MC-LR to bioconcentrate from water to biota (de Maagd et al., 1999). The 1-octanol/water partition coefficients (log P) of MC-LW and -LF have been estimated to be 3.46 and 3.56, respectively (Ward and Codd, 1999).

The arrangement of amino acid residues in small peptides is crucial to the overall hydrophobicity of the molecule (Liu et al., 2005). Microcystins are amphipathic molecules with hydrophobic groups in combination with polar groups of hydrophilic character. The amino acid Adda gives a certain degree of hydrophobicity to the rather hydrophilic molecule. All seven amino acids that compose the microcystin molecule contribute to the properties, physicochemical as well as toxicity

and protein phosphatase inhibition capacity, of the different variants (Stotts et al., 1993). In Table 1 a few common variants of microcystins are listed along with their molecular weight and toxicity data. Certain parts of the molecular structure are crucial for MC-LR to retain toxicity, e.g. the cyclic structure (Choi et al., 1993), the trans-isomeric structure at C-6 in the Adda double bond (Harada et al., 1990) and the non-esterified carboxy group in the glutamate position 6 (Rinehart et al., 1994) since modification at these positions resulted in non-toxic microcystins.

Table 1. Molecular weights and toxicity data (LD_{50}, µg/kg, mouse, i.p.) for some common microcystin variants. Table modified from Sivonen & Jones 1999.

Microcystin	MW	Toxicity LD_{50} (µg/kg, mouse, i.p.)
MC-LR	994	50
MC-LA	909	50
MC-YR	1044	70
MC-LY	1001	90
[D-Asp^3]-MCLR	980	160-300
[Dha^7]-MC-RR	1023	180
[Dha^7]-MCLR	980	250
[D-Asp^3]-MCRR	1023	250
MC-RR	1037	320-600

1.3.3 Interaction with membranes

There are numerous bioactive peptides, among them microcystins, that need to be taken up by transport proteins or penetrate the cell membrane in order to elicit their action. The ability of a compound to interact with biological membranes might influence the compound's biological activity, as well as pharmacological parameters like absorption, biotransformation, half-life and excretion of the compound (de Matos Alves Pinto et al., 2006). Small molecules that interact with lipids, or proteins, in the membrane or at the membrane interface, affect the properties of the membrane (MacCallum et al., 2008). Although the effect of a compound is initially subtle and local, the consequence of the interaction might be amplified and spread throughout the whole membrane surface and volume (Bagatolli and Gratton, 2000; Garcia and Perillo, 2002). Compounds can have different effects on a membrane. Some peptides, or aggregates of peptides, are able to form e.g. inclusions within the membrane and influence the bilateral pressure profile of lipids (Cantor, 2002). E.g. magainin-2 can form small, transient openings in the membrane and ceratotoxins a transmembrane channel (Bessin et al., 2004; Burkhard, 2005; Bechinger and Lohner, 2006). Some molecules, e.g. filipin, have been shown to specifically interact with certain sterols or proteins in, or associated to, cell membranes (Loura et al., 2001). Amphiphilicity is an important feature of peptides able to absorb to or disrupt membranes (Strömstedt et al., 2010). E.g. melittin, a thoroughly studied peptide from bee venom, is efficient in lysing biological membranes (Hristova et al., 2001). Membrane effects of microcystins have not been studied thoroughly although MC-LR has been shown to induce membrane instability (Hermansky and Stohs, 1991; Sicinska et al.,

2006). Theoretically, the hydrophobic properties of microcystins could enhance their penetration capacity across cell membranes.

Interactions of small molecules with biological membranes are difficult to study due to the complexity and heterogeneity of the membrane (Lee, 2011). Biological membranes are composed of a mixture of lipids organized as a bilayer matrix and associated with different kinds of proteins. The lipid molecules in bilayer membranes are orientated so that their polar head groups are faced towards the bulk water and the non-polar acyl chains are directed towards each other (Fig.2). Conclusions on how small molecules possibly interact with intact biological membranes can be drawn by using different kinds of membrane models with defined lipid compositions (Papo and Shai, 2003). The study of properties and interactions with monolayers is of direct significance to bilayers and consequently to biological membranes (Brezesinski and Möhwald, 2003). Monomolecular layers are simple model membranes that can be established at an air-water interface (Jones and Chapman, 1995). By combining a relatively large hydrophobic group with a polar or charged headgroup a layer, one molecule thick, can be formed at the aqueous-air interface. The hydrophilic nature of the polar headgroup anchors the molecule to the aqueous interface and allows the hydrophobic group to protrude towards the air phase (Fig. 2).

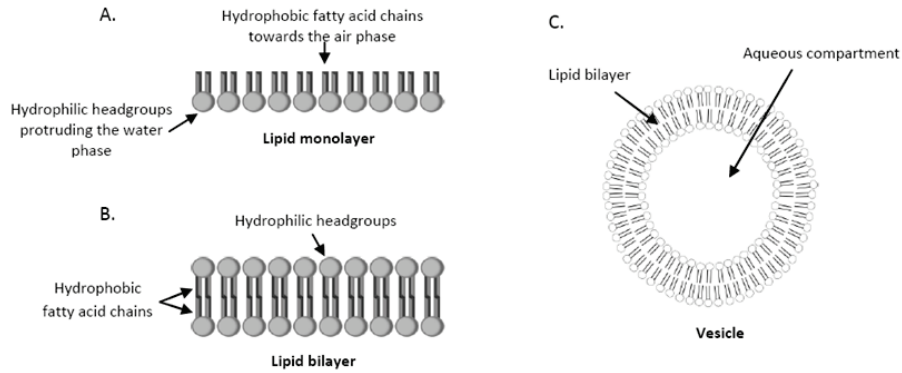

Figure 2. A lipid monolayer (A), a lipid bilayer with the hydrophobic fatty acid chains protruding towards each other (B), and a unilamellar vesicle enclosing an aqueous compartment (C).

Monolayers as well as bilayers exist in different phase states, depending on temperature and pressure (Brockman, 1999). The interfacial surface density of molecules in the monolayer is controlled by the amount of amphiphile placed on the interface and the area available to it (Jones and Chapman, 1995). When compressing a monolayer, the area per molecule at the interface is reduced, and a number of phases can be clearly identified. The gaseous phase (G), liquid expanded phase (LE), liquid-condensed phase (LC) and finally the solid (S) phase are recognized (Fig. 3). In between, mixtures of two different phases can be seen. From the gas phase to the solid phase the headgroup separation is decreasing, the order is increasing and the conformational freedom of the alkyl chains is lost (Jones and Chapman, 1995).

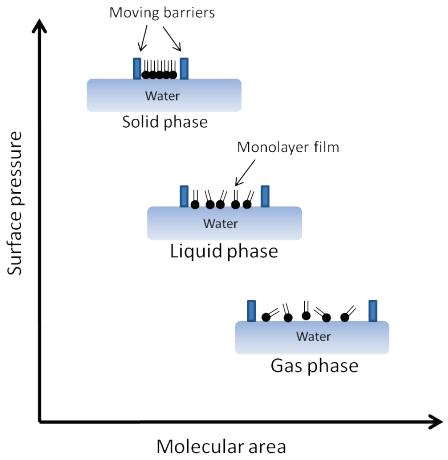

Figure 3. Different phases (solid, liquid and gas) that can be identified as a monolayer is compressed. Picture drawn by the author inspired by KSV Nima, Espoo, Finland.

1.4 ANALYTICAL METHODS FOR MICROCYSTINS

The presence of microcystins in drinking water and in food is a matter of concern for human health and has led to the development of sensitive analytical methods for detection of microcystins and microcystin-producing cyanobacteria (Sivonen, 2008). The World Health Organization has set a provisional guideline value of 1 µg microcystin-LR per litre drinking water since microcystins pose a threat to human health (WHO, 2011). The occurrence of microcystins is often reported as microcystin-LR equivalents, despite which variants are present in the sample. When choosing which analytical method to use e.g. cost, time, sensitivity and specificity need to be considered.

From an analytical point of view the structural diversity among microcystins constitutes a demanding task. Several microcystin variants usually exist in a sample and different microcystins have different toxicity (Vasconcelos et al., 1996; Sivonen and Jones, 1999). A natural bloom contains several cyanobacterial species (Stal et al., 2003). Within a species, like *Microcystis aeruginosa*, there are non-toxic, toxic and highly toxic strains. A highly toxic strain can produce several microcystins, e.g. eleven different variants (Krüger et al., 2010).

1.4.1 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a reliable and widely accepted chromatographic technique used in detection of microcystins (Lawton et al., 1994; Meriluoto, 1997). Microcystins in a sample can be separated in order to be identified, quantified or purified (Meriluoto, 1997; Lawton and Edwards, 2001). Depending on the composition of the stationary phase and the composition and gradient of the mobile phase, analytes with e.g. varying polarity can be separated in a single run (Mant and Hodges, 2006; Neffling et al., 2009). When separating peptides, the mobile phase is often composed of water-acetonitrile gradients in the presence of trifluoroacetic acid (Garcia, 2005).

A large number of detectors have been developed for liquid chromatography. In common use in microcystin analysis is UV absorbance detection with either a variable-wavelength or a diode-array detector (Meriluoto, 1997). Several compounds absorb UV light in the range of 200 to 350 nm and the intensity of UV light absorbed in the detector cell can be related to analyte concentration using Lambert-Beers' law. A diode-array detector (DAD) detects eluted analytes over a range of wavelengths simultaneously and peak spectra can be used for verification of analyte identity. UV detection is not specific since almost all microcystins have a UV absorbance maximum at 238 nm, together with many other compounds (Nageswara and Nagaraju, 2003). When analysing a chromatogram standards are needed since the microcystin variants are identified based on their retention times (Meriluoto, 1997). Commercially available standards can be found for some of the microcystin variants, such as MC-LR, -RR, -YR, -LA, -LW and -LF. A UV absorbance detector have limitations in complex analyses as it lacks specificity and the matrix, coeluting compounds, solvents and additives can influence the analyte spectra (Meriluoto, 1997; Williams, 2004). The sample needs to be pretreated, often by solid-phase extraction, concentration and filtering of the sample.

Developments in HPLC aim at improved resolution and selectivity, sharper peak shape and faster run times. With HPLC-DAD and liquid chromatography-mass spectrometry (LC-MS) microcystin variants can be separated and quantitated (Harada et al., 1996; Meriluoto et al., 2000; Spoof et al., 2009). In LC-MS for microcystins the sample is separated by liquid chromatography usually on narrower columns and online loaded onto the MS instrument (Neffling et al., 2009). In the ion source of the mass spectrometer the solvent flow undergoes vaporisation to remove solvent and generate gas-phase ions. Electrospray ionisation (ESI) is a technique used for a broad range of analytes. A fine aerosol of charged droplets is produced when the sample exits a metal capillary kept at a specific potential and is nebulised by nitrogen. The droplets are decreased further until repulsion causes the sample ions to be ejected from the droplet surface (ion evaporation). The ions are then separated by their mass-to-charge ratio in the mass analyser. The commonly used quadrupole mass analyser consists of four parallel metal rods with opposing potentials. When applying a radio frequency field, the ions pass according to their mass-to-charge ratio through the quadrupole to the detector. In a triple quadrupole MS instrument (LC-MS-MS) there are three quadrupoles in a linear series and this allows the study of fragments, or daughter ions (for microcystins, see e.g. Neffling et al., 2010). The first quadrupole usually acts as a filter that only allows passage of an ion of a specific m/z. The second quadrupole (collision cell) fragments that ion further. The third quadrupole scans the fragments produced. Detection of cyanobacterial toxins by mass spectrometry makes liquid chromatography (LC-MS) a powerful analytical technique with high sensitivity and specificity (Kaya et al., 2001; Barco et al., 2002; Neffling et al., 2009). LC-MS can deal with complex samples, trace levels and small sample volumes (Barco et al., 2004; Shan et al., 2011).

1.4.2 Protein phosphatase inhibition assay

Protein phosphatase inhibition assays (PPIA) for microcystins are based on inhibition of the activity of the catalytic subunits of serine/threonine protein phosphatases (PP) type 1 and type 2A (MacKintosh et al., 1990; An and Carmichael, 1994). Protein phosphatases dephosphorylate proteins, removes a phosphate group (PO_3) from a protein (MacKintosh et al., 1990). Many

enzymes and receptors are regulated by reversible phosphorylation caused by kinases and phosphatases. By inhibiting protein phosphatases the phosphorylation state of several proteins within the cell is increased. Microcystins inhibit the activity of PP1 and PP2A in a dose-dependent manner (Solter et al., 1998). Different microcystin variants have different affinities for a specific protein phosphatase (Mountfort et al., 2005). Protein phosphatase inhibition is however not directly correlated to acute toxicity (Blom and Juttner, 2005). Protein phosphatase inhibition assays are sensitive methods, with detection limits like 0.17 µg/l (Sassolas et al., 2011). Lower detection limits has also been reached (Ikehara et al., 2008). Although enzyme assays usually are highly specific, the protein phosphatase inhibition assay is not exclusively specific for microcystins (Honkanen et al., 1994). For example anabaenopeptins, produced by several microcystin-producing genera, are also protein phosphatase inhibitors (Harada et al., 1995).

Enzyme assays are used to measure the activity of an enzyme. In assays developed for microcystins either PP1 or PP2A are used, and a substrate, e.g. p-nitrophenyl phosphate is added. The consumption of the substrate or the development of the product is measured after a time-point or continuously during the experiment. Performing the assay in microtiter plates makes it possible to analyse several samples at the same time (Ward et al., 1997). Several detection methods are available, spectrophotometric, fluorometric, luminometric, and radiometric, among others. Enzymes are however prone to easily be inactivated, and attention must be paid to e.g. salt concentration, temperature and pH (Silvestre et al., 2011). Moreover, cofactors are sometimes necessary, the lifetime of enzymes is limited and interfering factors might exist in the sample (Silvestre et al., 2011).

1.4.3 Immunoassays

In immunoassays specific polyclonal or monoclonal antibodies (or antibody-like molecules) are used for identification and quantification of an analyte, an antigen. Antibodies aimed against microcystins are versatile tools for monitoring, detecting and quantifying microcystin-contaminated water (Campas and Marty, 2007; Long et al., 2009; Xia et al., 2011). Using immunoassays, such as ELISA (enzyme-linked immunosorbent assay) or TR-FIA (time-resolved fluoroimmunoassay), the concentration of microcystins in natural waters can be determined without any need to concentrate the sample (Lei et al., 2004; McElhiney and Lawton, 2005). Immunoassays have potential for microcystin analysis since they are easy to use, sensitive and specific (Metcalf and Codd, 2003). The functionality of the assays relies on the binding reaction between the antibody and the antigen, and the detection method.

In immunoassays, the interaction between antibody and the antigen is connected to a reaction that gives an observable signal. This interaction is dependent on non-covalent bonds, such as hydrogen bonding, ion bonding, hydrophobic interaction and van der Waals forces (Deshpande, 1996). The intensity of the interaction depends on the number of these weak non-covalent bonds.

In quantitative assays the calibrators consist of a negative sample with no analyte present and positive samples having different concentrations of the analyte. The specificity of an immunoassay is determined by the cross-reactivity with structurally similar molecules to the analyte (Saito et al., 1994). Antibodies for microcystins developed so far cross-react with different microcystin variants

(Metcalf et al., 2000b; Young et al., 2006). Since the purpose of immunoassays developed for microcystins usually is to detect all the microcystins present cross-reactivity is a useful property. However, in some assays developed, some variants, e.g. [ADMAdda5]-microcystins, show low cross-reactivity (Laub et al., 2002). When the purpose is to determine the presence of specific microcystin variants, the demands on the antibody specificity are increased and monoclonal antibodies with extraordinary sensitivity and selectivity for e.g. [4-arginine]microcystins have been developed (Zeck et al., 2001a). Cross-reacting substances not related to the studied analyte affect the immunoassay, depending on their concentration compared to the analyte, their affinity against the antibody and the setup of the analysis (Price and Newman, 1997). Antibodies developed for microcystins usually also bind to microcystin conjugates of glutathione, cysteine-glycine and cysteine produced in detoxification processes of microcystin (Metcalf et al., 2000a; Metcalf and Codd, 2003).

The quantitative determination by immunoassays requires the results to be compared to a standard curve based on known concentrations. If MC-LR or an appropriate surrogate is used as a calibrating agent for determining the microcystin concentration, the amount of toxin can be reported as microcystin-LR equivalents (Harada et al., 1999). The result from the immunoassay can thus not directly be correlated to the toxicity of the sample when other variants than MC-LR are present.

Although immunoassays can be classified according to many different criteria, a common way is to divide them according to the construction of the method. In a direct competitive immunoassay, the antibodies are immobilized on the microtiter plate wells. The sample is added to the well together with the labelled antigen. If there are antigens in the sample they compete with the added labelled antigens for the binding to the antibodies. Antigens not bound to antibodies are washed away, reagents that react with the label are added and the product is detectable as a result. The amount of labelled antigen that is bound to the antibody can be measured and the result is inversely proportional to the concentration of the antigen in the sample. A high concentration of antigen in the sample gives a low signal. Many immunoassays developed for microcystins are based on this method (Chu et al., 1989; Chu et al., 1990; An and Carmichael, 1994; Liu et al., 1996; Carmichael and An, 1999; Mikhailov et al., 2001; Zeck et al., 2001a; Zeck et al., 2001b).

A secondary antibody is quantitatively determined in an indirect competitive immunoanalysis. A sample with a primary antibody is added together with the sample to the microtiter well immobilized with antigen and is allowed to react. Unbound analytes are washed away and a labelled secondary antibody that binds to the primary antibody is added. Free secondary antibodies are washed away and a compound that reacts with the label gives a product that can be detected. The indirect competitive immunoanalysis is the most commonly used immunoassay that have been developed for microcystins (Chu et al., 1989; Saito et al., 1994; Nagata et al., 1995; Ueno et al., 1996b; Nagata et al., 1997; Tsutsumi et al., 1998; Metcalf et al., 2000b; Mikhailov et al., 2001; Lindner et al., 2004). It is important both in the direct and indirect competitive immunoassays that the relative amount of the labelled antigen and the antibody are kept constant because the amount of free antigen is compared to a series of standards for a quantitative result.

Nagata and co-workers developed a sensitive sandwich ELISA with a detection limit of 2 ng microcystin per liter water (Nagata et al., 1999). Sandwich assays are non-competitive two-site immunoassays in which antibodies are immobilized in the microtiter well, the sample is added and the antibodies bind an epitope on the sample antigen. The well is washed, whereafter a labelled secondary antibody is allowed to react with another epitope on the antigen. Small molecules like microcystins might not bind two or more antibodies at the same time due to steric hindrance. However, anti-idiotype antibodies have been developed that recognize the antibody-microcystin complex (Tsutsumi et al., 1998). The free antibodies are washed away, the developing solution is added and the product can be measured. The result is directly proportional to the concentration of the antigen because the labelled antibody does not bind if the antigen is not present.

1.4.3.1 Microcystin as antigen

Antigens with low molecular weights (~300-2000 Da) such as microcystins are called haptens and show antigenicity, as they can be recognized by antibodies, but have to be conjugated to a carrier to be immunogenic and give rise to a proper immune response (Baier et al., 2000). Many different proteins, such as bovine serum albumin (Chu et al., 1989), keyhole limpet hemocyanin (Zeck et al., 2001a), ovalbumin (Nagata et al., 1995; Zeck et al., 2001b) and soybean trypsin inhibitor (Mikhailov et al., 2001), have been used as carriers. However, some proteins, such as BSA, require modification for more efficient binding to microcystin (Chu et al., 1982). In many publications the carboxy group in glutamate, at position 6 in microcystins, are used for the conjugation to the carrier protein (Chu et al., 1989; Nagata et al., 1995; Ueno et al., 1996a). The spacer arm between the microcystin and the carrier has usually been carbodiimide (Chu et al., 1982; Chu et al., 1989). By varying the spacer arm and the linking location, e.g. aminoethylation of the *N*-methyldehydroalanine in microcystins (Moorhead et al., 1994; Mikhailov et al., 2001), antibodies with specificity for different epitopes are obtained (Luo et al., 2011). MC-LA conjugated to a carrier, polylysine, was an effective immunogen in mice when used in combination with an adjuvant, muramyl dipeptide (Botes et al., 1982; Kfir et al., 1986). Microcystin conjugated to bovine serum albumin (BSA) through ethylendiamine (EDA) was shown to be an even better immunogen in mice (Chu et al., 1989). For antibody generation, immunization with protein conjugated microcystin has to be combined with adjuvants like lipopeptides (Baier et al., 2000).

1.4.3.2 Monoclonal and polyclonal antibodies against microcystins

The production of monoclonal antibodies demands a considerably heavier work load than the production of polyclonal antibodies. When an animal is immunized with an antigen the immune response gives rise to polyclonal antibodies, which are heterogenic mixtures of different antibodies that each and everyone are specific for an epitope. It is not possible to directly purify monoclonal antibodies from a mixture of polyclonal antibodies. Therefore the normal active antibody producing B cell and a myeloma cell (a cancer cell) are fused to a hybrid cell, a hybridoma (Kohler and Milstein, 1975). Hybridomas divide unrestrictedly as myeloma cells do and possess the antibody producing properties of the B cells, and high amounts of monoclonal antibodies can be produced. Monoclonal antibodies that originate from a separate hybridoma clone are often preferred in both research and diagnostics and in therapeutic purposes.

In the production of antibodies against microcystins mice have often been used (Kfir et al., 1986; Saito et al., 1994; Nagata et al., 1995; Liu et al., 1996; Baier et al., 2000; Zeck et al., 2001a; Zeck et al., 2001b), sometimes also rabbits (Chu et al., 1989; Baier et al., 2000; Metcalf et al., 2000b; Mikhailov et al., 2001), and occasionally even sheep (Fischer et al., 2001) and chicken (McDermott et al., 1995).

The first antibodies against microcystins were monoclonal and developed by Kfir et al. (1986). They were not developed to an immunoassay but the affinity was determined and it was shown that they bound to six different microcystin variants with equal affinity (Kfir et al., 1986).

The first polyclonal antibodies reported against microcystins were produced by Brooks and Codd (Brooks and Codd, 1988). Chu et al. (1989) developed the first enzyme-linked immunoassay that was based on polyclonal antibodies by which many natural algal blooms were analyzed. The lowest detection limit for microcystins was 0.2 μg/l. The affinity for MC-RR, -LR and -YR was shown to be good, but the antibodies cross-reacted poorly with -LY and -LA (Chu et al., 1989). The cross-reactivity of the antibodies was later tested for 18 different microcystins and nodularins. The stereochemistry of the double bond system in Adda was shown to be important for the specificity (An and Carmichael, 1994) as well as the arginine in position 4 (Chu et al., 1989). Other polyclonal antibodies that cross-reacted with many of the microcystin variants and nodularins were produced and developed to an ELISA with a detection sensitivity of 1 μg/l (Metcalf et al., 2000b). The cross-reactivity showed that MC-LA had the highest affinity, followed by -LR, -LF, -LW, -D-Asp^3-RR, -LY and nodularin. A good correlation (r^2=0.96) was found between results from HPLC-UV (DAD) and ELISA (Metcalf et al., 2000b). An even more sensitive ELISA with polyclonal antibodies was developed with a detection limit of 0.12 μg microcystin-LR per litre (Sheng et al., 2006). Broad cross-reactivity between several microcystins was reported (Sheng et al. 2006).

To increase specificity monoclonal antibodies that specifically recognize a region on the microcystin molecule have been developed. Nagata et al. (1995) produced 6 different monoclonal antibodies against MC-LR, with epitopes recognising arginine in position 4 and Adda in position 5. The best antibody was chosen for developing an ELISA with a detection limit of 25 ng/l and more than 1 100 samples collected from Japan, Thailand, Germany and Portugal were analysed (Ueno et al., 1996b). The sensitivity of an ELISA developed by Fischer et al. (2001) was 20 ng/l and the cross-reactivity of the antibodies against 7 other microcystins was very good. A sensitivity of 70 ng/l was obtained by antibodies from Zeck et al. (2001b) and the cross-reactivity was tested for 12 microcystin variants and 3 Adda-derivatives and nodularin. Monoclonal antibodies especially for microcystins with arginine at position 4 (4-R-microcystins) were produced and developed to a sensitive ELISA with a detection limit of 6 ng/l (Zeck et al. 2001a). The cross-reactivity between 4-R-microcystins was good, whereas the antibodies did not recognize -LY, -LW, -LF and Adda derivatives. In another ELISA based on the same commercially available antibodies the detection limit was 4 ng MC-LR per liter (Lindner et al. 2004). Khreich et al. recently developed an immunoassay able to detect microcystins at 10 ng MC-LR per liter water (Khreich et al., 2009).

Parts of antibodies can be used as such in immunoassays. Genetically modified antibody fragments consisting of one single chain was developed by McElhiney et al. (2000) against microcystins. The

antibody fragments were isolated from an assembly of bacteriophages that express different antibody fragments on their surfaces (McCafferty et al., 1990). The fragments that were chosen were expressed in considerable quantities in bacteria. By choosing an appropriate antibody library, sensitive antibodies were found (Strachan et al., 2002). Production of antibody fragments in bacteria is fast and cheap and the ethical questions of using animals are avoided (McElhiney et al., 2000).

1.4.3.3 Enzyme-linked immunosorbent assay (ELISA)

The detection of the antibody or the antigen can be made by many different methods. Among non-isotopic labels, enzymes compose a versatile group with possibilities of measuring low concentrations. Several conjugation possibilities, sensitivity and size have made the horseradish peroxidase, HRP, to a popular labelling enzyme (Azevedo et al., 2003). HRP is a commonly used enzyme in ELISA and has almost exclusively been used in microcystin labelling, although in one publication alkaline phosphatase was used (McDermott et al. 1995). A spectrophotometer is used for detection of the signal from HRP. HRP is considered to be a small enzyme though its molecular weight is 44000 Da. The high molecular weight of most enzymes is a clear disadvantage. Labelling of small molecules with large enzymes causes a large structural change in the antigen, even though they still bind to the antibody (Selby, 1999). Labelling of antibodies with enzymes is usually less problematic since antibodies themselves are large molecules (Selby, 1999)

1.4.3.4 Time-resolved fluoroimmunoassay

Time-Resolved FluoroImmunoAssay (TR-FIA) is an immunoassay where the antibodies or the antigen has been labelled with a fluorescent lanthanide, e.g. europium (Soini and Hemmilä, 1979; Soini, 1990). Time resolved fluorescence in immunoassays has been used since the 1980s, mostly in human diagnostics, but also many other applications have been reported (Hemmilä and Laitila, 2005). In this thesis, a TR-FIA for the analysis and detection of microcystins was developed.

Many compounds are in practice weakly fluorescent and some molecules have more intense fluorescent properties, like aromatic compounds. Atoms on the other hand are usually non-fluorescent, with lanthanides being an important exception. Lanthanide ions themselves have weak fluorescence due to their poor molar absorptivities and low quantum yields (Bunzli and Eliseeva, 2011). The fluorescence of lanthanides can be sensitized and enhanced by forming a complex between the lanthanide and a suitable ligand (Soini and Hemmilä, 1979; Bunzli and Eliseeva, 2011).

Fluorescence requires that light has been absorbed, or that energy from outside the system has been provided so that an electronic excited state can be accomplished. A molecule that has reached the excited state rapidly returns to the ground state. At the same time a photon is emitted. The process between absorption and emission of light can be illustrated with a Jabłoński diagram (Fig. 4).

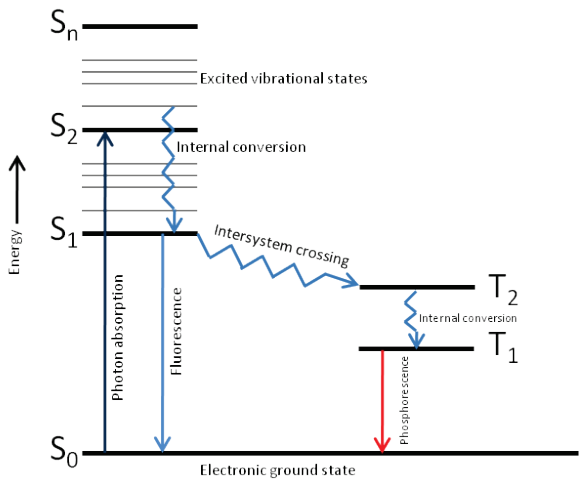

Figure 4. A Jabłoński diagram, modified from Lacowicz, 1999. S_0, S_1 and S_2 are singlet ground, first and second electronic states, respectively. Transition to S_0 is called fluorescence. Some fluorophores can undergo a spin conversion (intersystem crossing) to a triplet state, denoted T. Transition from T_1 to S_0 is called phosphorescence.

The lifetime of a fluorescent compound is the time from the excitation to the return to the ground state. The chelating ligand absorbs excitation energy and transfers it to the central atom which is excited from the ground state (S_0) to a higher state (usually S_1 or S_2). Energy is then transferred through intersystem crossing to the triplet (T) state (Fig. 4) and thereafter, for e.g. europium, to the central atom (Soini and Hemmilä, 1979). When the metal returns to the ground state from the excited state, a photon is emitted.

Due to the short lifetime of conventional fluorophores the excitation is usually done with a continuous light source and the emission is observed continuously. When using time resolved fluorescence the sample is exposed to a pulsed source with a very short light flash of 1 µs from a xenon light source (Soini and Hemmilä, 1979). Fluorescence is usually a short-lived phenomenon with most of the fluorophores having a lifetime around 10 ns. Lanthanide ions have several ground states and the f-f transition is forbidden. Lanthanides show long-lived fluorescence since the higher orbitals are protected, with lifetimes up to a few milliseconds for chelates of europium and terbium (Hemmilä and Mikola, 1990; Hemmilä and Mukkala, 2001). The emission continues long after the instant background fluorescence has disappeared. For example, the fluorescence of europium is measured in 400 µs after a delay time of 400 µs (Fig. 5). After 1 ms the excitation is repeated and the whole measurement cycle is repeated 1000 times per second. All the photons that are emitted during the measurement cycle are expressed as cps (counts per second). Hereby the intensity is integrated for a specific time period.

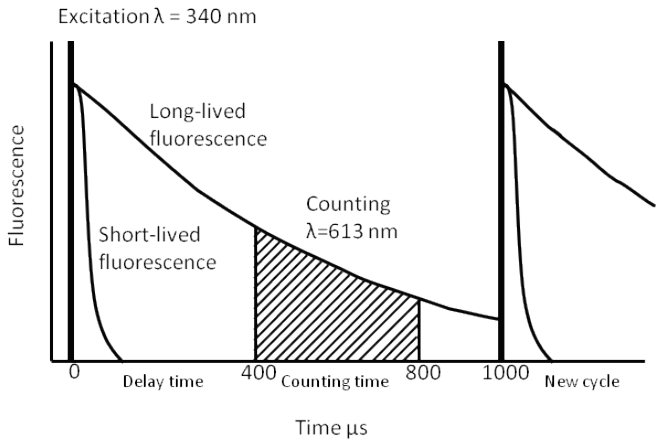

Figure 5. Time-resolved fluorescence measurements of europium chelates. Excitation occurs at 340 nm. Fluorescence is measured in 400 µs after a delay time of 400 µs, at 613 nm. Picture drawn by the author according to Soini and Hemmilä, 1979.

The different lanthanides are distinguished according to their quenching time, which is nanoseconds for the chelates of ytterbium, neodymium and erbium, and milliseconds for dysprosium chelate (Hemmilä and Mikola, 1990; Hemmilä and Mukkala, 2001). This property of the lanthanides results in a simple and efficient way to eliminate interference from the background (Soini and Lövgren, 1987). When using time-resolved fluorometry the emission is measured after a delay time (10-400 µs) has elapsed from the excitation. This makes it possible to avoid the short lived fluorescence of 10 ns that the non-specific background gives rise to. Usually TR-FIA has better sensitivity than ELISA because the sensitivity in other immunoassays is restricted by non-specific background noise (Hemmilä and Mikola, 1990). Interfering noise can be avoided by measuring the specific signal after a time delay, which is possible with time-resolved fluorescence (Soini and Lövgren, 1987).

Energy of the emission is usually less than that of the absorption, and fluorescence usually occurs at a lower energy and at a longer wavelength. This phenomenon is called Stokes shift and was named after Sir G.G. Stokes who made the observation for the first time in 1852 (Stokes, 1852). Energy is lost for instance at the rapid decay to the lowest vibrational levels. The europium chelate has a Stokes shift of 270 nm, which means that the distance between the wavelength of the excitation and the emission is very wide (Hemmilä, 1988). Moreover, with narrow emission profiles (10 nm) the specific signal can easily be separated from the non-specific light scattering and the background (Soini and Hemmilä, 1979).

A stable europium carrier allowing simple and reliable labelling of antibodies or analytes is e.g. EDTA derivatives (Soini and Hemmilä, 1979). At excitation, energy is transferred from the chelate to the metal ion. Due to weak fluorescent properties of the conjugate, a new complex needs to be formed. By adding an enhancer solution containing e.g. a β-diketone like 2-naphtoyl trifluoroacetate a new highly fluorescent chelate is produced at low pH conditions (Hemmilä et al.,

1984). The chelate that is formed has the chromophore needed for the light absorption that can be transferred to the lanthanide through resonance energy transfer. To keep the fluorescence efficient, water needs to be expelled since it acts as an efficient quencher (Hemmilä et al., 1984). This can be accomplished by adding a fatty acid derivative, e.g. trioctylphosphine, dissolved in a detergent, e.g. Triton X-100, since they form an insulating sheath around the lanthanide complex by formation of a micelle (Hemmilä et al., 1984).

1.5 RISK ASSESSMENT FOR HUMAN HEALTH

Risk assessment is the scientific discipline of the risk management process and includes the process of evaluating the impact of contaminants on the well-being of individuals and populations.

Cyanobacteria produce a variety of bioactive compounds, some of which cause adverse health effects in mammals and/or aquatic organisms due to toxic properties (Codd et al., 2005). Health effects related to cyanobacteria are derived from consumption of or contact with contaminated water (Falconer and Humpage, 2005). For the estimation of hazard to human health, the total risk of a specific toxin should take into consideration the toxic potential of the substance, dose response relationship, possible routes for human exposure and, prevalence and concentration of the toxin.

The relationship between water and health is complex. Several diseases, e.g. cholera, have a direct connection to water supplies and spread easily with water (Reidl and Klose, 2002). Cyanobacteria however are unable to colonize, invade and grow in human and animal hosts and are therefore not regarded as waterborne pathogens in the safety assessment of drinking water (Codd et al., 2005).

1.5.1 Hazard identification

Human and animal poisoning episodes as well as toxicological studies show that cyanobacterial toxins can cause adverse human health effects (Codd et al., 2005; Wiegand and Pflugmacher, 2005; Golubic et al., 2010). Toxicosis in humans is diverse, ranging from mild to fatal, and includes symptoms of gastroenteritis, abdominal pain, kidney and liver damage, nausea, vomiting, sore throat, blistered mouth, flu-like symptoms, ear and eye irritation, rashes etc (Codd, 2000; Codd et al., 2005).

Epidemiological evidence from the Caruaru dialysis center in Brazil 1996 shows that 76 people died following intoxication by microcystin (Jochimsen et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002). In Armidale, Australia 1981, human liver damage was observed due to microcystins in the water released from cells after an algicide treatment of a heavy cyanobacterial bloom (Falconer et al., 1983). Complete epidemiological data are lacking in several cases but cyanobacterial toxins were involved in human injuries for example in Ohio (Tisdale, 1931), Harare, Zimbabwe (Zilberg, 1966) and Palm Island, Australia (Byth, 1980). Several cases of microcystin causing adverse effect in wildlife population have been reported (Stewart et al., 2008). Linking the observed adverse effect with exposure to microcystins can however be difficult, since the exposure has often been to a mixture of substances.

Toxicological experiments, conducted under controlled laboratory conditions, provide important information on the toxic potential of microcystins. Direct conclusions from animal or cell experiments can seldom be directly extrapolated to human health effects due to e.g. physiological differences between species (Dybing et al., 2002). Moreover, results from extrapolations are subject to great uncertainty and translations cause problems due to multifactorial etiology, e.g. in cancer (Dybing et al., 2002). The way microcystins enter the organism, organs and cells has a crucial effect on the distribution of microcystins and their toxicity *in vivo* (Gaudin et al., 2008). Especially data regarding other variants than MC-LR is limited.

Microcystins exhibit their toxic action by inhibiting protein phosphatases 1 and 2A (Eriksson et al., 1990; MacKintosh et al., 1990; Runnegar et al., 1995a; Craig et al., 1996). Also other members of serine/threonine phosphatases (PP3, PP4, PP5) have been suggested to be inhibited (Prinsep et al., 1992; Hastie et al., 2005). The conformation of MC-LR allows the molecule to bind with high affinity to PP1 (Goldberg et al., 1995). The long hydrophobic Adda side chain fits smoothly into a hydrophobic groove in PP1, and the leucine side chain packs closely to Tyr272 near the active site (Goldberg et al., 1995). Structural and conformational changes in the molecule have been shown to affect the protein phosphatase inhibition capacity (Mountfort et al., 2005). However, structure-activity relationships are scarcely available for microcystins, especially for other microcystin variants than MC-LR. After an initial, rapid, non-covalent binding, mediated principally by the Adda moiety (Goldberg et al., 1995), *N*-methyldehydroalanine undergoes a covalent linkage to Cys 273 in PP1 or Cys266 in PP2A (MacKintosh et al., 1995; Runnegar et al., 1995a). This makes the inhibition irreversible. Hyperphosphorylation of many cellular proteins is the primary acute effect of microcystins. Especially hepatocytes are susceptible as they have several types of organic anion transport proteins, also responsible for e.g. bile acid uptake. Some of these proteins actively take up microcystins with different selectivity (Fischer et al., 2005; Monks et al., 2007; Fischer et al., 2010). Other transporters might also be involved, for example ABC transporters have been suggested as a route of entry for microcystins (Pearson et al., 2004; Fischer et al., 2010). Due to the active uptake the main target of microcystins is the liver. The cytoskeleton of cells is disrupted which causes loss of cell-cell contacts and intra-hepatic haemorrhage and death occurs due to hypovolemic shock (Runnegar and Falconer, 1986; Falconer and Yeung, 1992; Runnegar et al., 1993). Microcystins also affect liver aldehyde dehydrogenase 2 and inhibits ATP synthase and these changes have been associated with apoptosis (Mikhailov et al., 2003; Chen et al., 2006).

Uptake of microcystins occur through specific organic anion transporters, and as mentioned earlier, different microcystins variants have different affinities for the OATP:s (Runnegar et al., 1991; Runnegar et al., 1995a; Fischer et al., 2005). Microcystins exhibit a predominantly hepatic organotropism, although several other cell types and organs are affected as well (Falconer, 2008). In addition to whole-animal experiments cell experiments with cells derived from humans, mice, monkeys, fish etc have been done. The kidneys show *in vitro* and *in vivo* toxic effects of microcystins (Nobre et al., 1999; Milutinovic et al., 2002; Milutinovic et al., 2003; Gaudin et al., 2008; Alverca et al., 2009). The intestine as well as the heart, brain, lungs, immune system and the reproductive system have been shown to be affected by microcystins (Humpage et al., 2000; Lankoff et al., 2004; Picanco et al., 2004; Dietrich and Hoeger, 2005; Fischer et al., 2005; Ding et al., 2006; Zegura et al., 2008; Herfindal et al., 2009; Qiu et al., 2009).

Microcystins cause an excessive formation of reactive oxygen compounds and consequently to oxidative damage in cells (Ding et al., 1998; Ding and Nam Ong, 2003; Amado and Monserrat, 2010). The increased levels of reactive oxygen species have been shown to be associated with genotoxic and carcinogenic effects (Zegura et al., 2003; Zegura et al., 2004; Dittmann and Wiegand, 2006). Repeated consumption of low levels of microcystin have been shown to cause increased liver weight, hepato-histological damage (Heinze et al., 1999), liver tumours (Nishiwaki-Matsushima et al., 1992; Bouaicha et al., 2005; Herfindal and Selheim, 2006; Li et al., 2009; Martinez Hernandez et al., 2009) and kidney damage (Milutinovic et al., 2003). Epidemiological studies have shown that microcystins are linked to the high prevalence of primary liver cancer in risk areas, and to cancer in the large intestine and the rectum (Yu, 1995; Ueno et al., 1996a; Zhou et al., 2002; Herfindal and Selheim, 2006).

1.5.2 Dose-Response Assessment

Comprehensive dose-response studies in pigs, mice and rats have resulted in LOAEL (lowest observed adverse effect level) and NOAEL (no observed adverse effect level) values (Falconer et al., 1994; Fawell et al., 1994; Heinze, 1999). The Tolerable Daily Intake (TDI) for microcystin-LR, established by WHO, is 0.04 μg/kg body weight per day (WHO, 2011). This dose level is considered not to cause any kind of adverse effect over a life time. The value is based on the NOAEL value that is 40 μg/kg/day in mice (Fawell et al., 1994) and a security factor of 1000. The security factor takes into account inter-species extrapolation, intra-species variation and less-than-lifetime-exposure. The assumptions were: adult body weight of 60 kg, drinking water consumption of 2 l daily, and an allocation factor for the exposure from drinking water 0.8 (WHO, 2011). The provisional guideline value adopted by WHO for MC-LR have been criticized because new toxicity data are available for many of the variants and a tightened guideline value has been discussed (Dietrich and Hoeger, 2005). The effect of long-term exposure is also quite unclear since the study on pigs lasted for only 44 days. Experience from China where the increased rate of primary liver cancer, and other related diseases, have been linked to exposure of microcystins, implies a stricter guideline value (Ueno et al., 1996a). Toxin exposure resulting from contaminated food (e.g. seafood) has not been considered in the NOAEL studies (Ibelings and Chorus, 2007).

The potency of a toxin is expressed as the amount required to produce an effect of given intensity. Most microcystins showed an acute LD_{50} value of 50-600 μg/kg (mouse, i.p.). Pure MC-LR, injected intraperitoneally, has an LD_{50} value (mouse) of 50 μg/kg, whereas orally administrated MC-LR had an LD_{50} value of 10.9 mg/kg (Yoshida et al., 1997). Another study showed that the LD_{50} value in mouse varies from 250 μg/kg, i.p. or intranasal administration, to 3000 μg/kg, gastric intubation (Fitzgeorge et al., 1994). Microcystins that have hydrophobic amino acids such as, tyrosine (MC-LY), tryptophan (MC-LW) and phenylalanine (MC-LF) may be more toxic than MC-LR in some organisms (Ward & Codd 1999). For example the cells of *Tetrahymena pyriformis* (a freshwater protozoa commonly used in bioassays) were more sensitive to the tyrosine-containing MC-YR compared to the hydrophilic MC-LR but most sensitive to MC-LW and MC-LF (Ward and Codd, 1999). However, no LD_{50} value has been determined for MC-LW and MC-LF.

Detoxification of microcystin-LR in the liver occurs at least in part by conjugation to glutathione (GSH) catalysed by glutathione S-transferase (GST) (Kondo et al., 1996; Pflugmacher et al., 1998; Takenaka, 2001). GSH is thought to be one of the most important non-enzymatic antioxidants and is the first protection against reactive oxygen compounds. Hepatocytes exposed to MC-LR show an initial increase, followed by a decrease of cellular glutathione (Ding et al., 2000). Microcystins induce the glutathione synthesis, and the increase of the GST activity is regulated at the transcription level (Wiegand et al., 1999; Gehringer et al., 2004). Also conjugation of microcystins with cysteine is possible (Kondo et al. 1996). The complex formed after the conjugation is transported to the kidneys and the intestine for secretion (Ito et al., 2002).

1.5.3 Exposure assessment

In exposure assessment there is a need to estimate the extent, frequency and duration of contact between cyanobacterial toxins and an individual or a population. The dose that is received by the individual or the population varies for example with location (distance to source), lifestyle and physiology. Particular care needs to be taken to determine the exposure of susceptible populations. Contact with microcystins occurs via the environment as well as through consumer products, like consumption of contaminated drinking water, food and dietary supplements. Oral, inhalation and cutaneous exposures to microcystins are the most likely routes of contact. Two episodes of human microcystin exposure by the intravenous route have been documented (Jochimsen et al., 1998; Soares et al., 2006).

A cyanobacterial bloom is a mass occurrence of cyanobacteria at the water surface. The blooms are dynamic and vary both with weather and time. The increased prevalence of the cyanobacterial blooms can often be associated with eutrophication, often caused by humans (Anderson et al., 2002). Usually cyanobacterial blooms occur in summertime (in the northern latitudes) because most cyanobacteria have their maximal growth at temperatures over 25°C (Sivonen and Jones, 1999). Global warming might promote the growth of cyanobacteria (Hallock, 2005). The intensity and the extent of cyanobacterial blooms are difficult to predict. Calm weather promotes cyanobacterial growth, whereas wind and currents can transport the cyanobacterial masses long distances, disperse them or flush them on shore (Dziallas and Grossart, 2011). Microcystin concentrations detected in natural waters are usually between 0.1 – 10 µg/l, but higher concentrations have been reported, with an extreme case of 36000 µg/l (Ueno et al., 1996b; Sivonen and Jones, 1999). Sometimes a bloom can occur temporarily, other times for a longer period of time. Some cyanobacteria problems can be connected to the increased ship traffic (Lindholm et al., 2001; Doblin et al., 2007). Cyanobacteria are transported in ballast water and contribute to spreading of toxin-producing species around the world (Lindholm et al., 2001; Doblin et al., 2007). Nutrients are also available from the bottom sediments, both due to natural processes and anthropogenic loadings (Lindholm et al., 2001; Sellner et al., 2003; Doblin et al., 2007). Increase in temperature and phosphorous concentrations seemed to promote growth of toxic *Microcystis* cells (Davis et al., 2009). On the other hand, different cyanobacterial species respond to nutrient concentrations in different ways and there is no simple relationship (Kaebernick and Neilan, 2001; Vezie et al., 2002; Vuorio et al., 2005; Håkanson et al., 2007).

Microcystins have different bioavailability and toxicity *in vivo* (Sivonen and Jones, 1999; Gupta et al., 2003; Chen et al., 2007). 80 % of the microcystin exposure is estimated to occur through intake of drinking water (Kuiper-Goodman et al., 1994). The risk for the exposure through drinking water can be estimated to be high in areas where toxic cyanobacteria occur, drinking water resources are restricted and the water purification is limited. Especially in these areas monitoring of the drinking water is of importance. Use of algicides (e.g. copper sulphate and hydrogen peroxide) in drinking water reservoirs can rapidly release large amounts of toxins into the water and some of the short-term human exposure incidences can directly be linked to bloom clearance actions (Jones et al., 1994; Kuiper–Goodman et al., 1999; Ross et al., 2006; Qian et al., 2010; Jancula and Marsalek, 2011).

Microcystins are able to bioaccumulate in various aquatic species, including fish, bivalves, molluscs, zooplankton, crabs and crayfish (Eriksson et al., 1989; Vasconcelos, 1995; Carbis et al., 1997; Wiegand and Pflugmacher, 2005; Malbrouck and Kestemont, 2006). The main accumulation site is the digestive tract in bivalves, the hepatopancreas in crayfish, and the liver in fish (Vasconcelos, 1995; Carbis et al., 1997; Vasconcelos et al., 2001). The accumulation in e.g. fresh water molluscs is most pronounced in highly contaminated waters and in adult animal species (Gérard et al., 2009). Differences in the uptake and detoxification mechanisms affect microcystin bioaccumulation (Zurawell et al., 2005). Microcystins can also be present in plants, e.g. lettuce and mustard (Kurki-Helasmo and Meriluoto, 1998; Codd et al., 1999; McElhiney et al., 2001; Wang et al., 2011). Microcystins have been shown to covalently bind to target proteins (Craig et al., 1996). The bioavailability of the covalently bound toxins for the next trophic level is not known (Dietrich and Hoeger, 2005). The proportion of the covalently bound toxins from the total contamination has been reported to vary in different organisms and exposure conditions.

Chronic exposure to microcystins have revealed an average value of 0.39 ng/ml in human serum samples taken from people exposed to microcystins below the range of WHO guidelines (Chen et al., 2009). A positive correlation was found between microcystin concentrations and the concentration of the enzymes alanine and aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase (Chen et al., 2009). The WHO guideline value of microcystin-LR has been suggested to be reassessed since liver damage might occur at low exposure levels (Chen et al., 2009).

Cyanobacterial blooms are seldom monotypic, rather they contain different species of cyanobacteria (Fewer et al., 2009). Blooms that produce microcystins have been reported from all over the world (Wood et al., 2008). The probability that a bloom produce toxins was shown to be between 25 and 95% (Sivonen and Jones, 1999). The cyanobacterial blooms in Finland had toxins in 45% of the cases (Sivonen et al., 1995). The presence of toxins cannot be predicted by determining the dominating species, since only half of the blooms by *Microcystis* are hepatotoxic (Namikoshi and Rinehart, 1996). There are morphologically identical strains that can be either toxic or non-toxic (Ohtake et al., 1989). Studies made in Denmark, Germany, Czech Republic and South Korea showed that 80-90% of the blooms dominated by *Microcystis* spp. were hepatotoxic (Chorus, 2001). If the bloom is dominated by *Planktothrix* the risk for hepatotoxins seemed to be

even greater (Chorus, 2001). At growth-limited conditions microcystin producing *Planktothrix* strains seemed to outcompete non-microcystin producing *Planktothrix* strains (Briand et al., 2008).

Cyanobacteria are able to produce several microcystins simultaneously although only a few usually dominate in any single strain (Mikalsen et al., 2003; Fewer et al., 2009; Kurmayer and Christiansen, 2009). Variations in the production and the composition of microcystins are determined by genetic factors as well as environmental factors (Sivonen and Jones, 1999; Tooming-Klunderud et al., 2008; Kurmayer and Christiansen, 2009). In Australia, 23 different microcystins were characterized in a bloom of *Microcystis aeruginosa*, none of which was MC-LR (Jones et al., 1995). *Microcystis* spp. often produce the microcystin variants -LR, -YR and -RR (Watanabe et al., 1988). In Finland the variants MC-LR and MC-RR, and especially their demethylated variants (amino acid 3 and/or 7) are most common (Sivonen et al., 1995). *Anabaena* species produce mainly 2-4 of these microcystins whereas *Planktothrix* species often produce only one microcystin, which usually is a demethylated variant of MC-LR or MC-RR (Sivonen et al., 1995). Exposure to different microcystins raises needs to evaluate also other variants than MC-LR for their chemical, physical and biological properties. Especially those variants that have amino acids of more hydrophobic character as they have been implied to be more toxic (Ward and Codd, 1999; Fischer et al., 2010).

1.5.4 Risk characterization

Risk characterization is the final phase in the health risk assessment process and sums up the three former phases: hazard identification, dose-response assessment and exposure assessment. The quantity and quality of the data, the biological relevance and the consistency of the study results are considered carefully and conclusions are drawn regarding the health risk implicated in exposure to microcystins. The more comprehensive data that is available, the lower uncertainty in risk assessment and the more precise guideline values can be estimated. There are insufficient data to determine a health-based guideline value for most cyanobacterial toxins (Donohue and Orme-Zavaleta, 2008). Several countries have regulations or guidelines for microcystins in drinking water, and in some cases also in water used for recreation and agriculture (Chorus, 2005; Burch, 2008). Regarding microcystins there is a wide variation in the toxicity due to the several variants (Feurstein et al., 2010). There is a need to increase the toxicology database for microcystin variants other than MC-LR. Also the congener specific uptake of different microcystins needs to be elucidated (Feurstein et al., 2010). Risk characterization should be performed for a variety of microcystin analogues.

Additive, synergistic, potentiating or antagonistic effects caused by other compounds in a cyanobacterial extract complicate further the estimation of toxicity (Donohue and Orme-Zavaleta, 2008; Jokela et al., 2010). Cyanobacteria produce a whole variety of bioactive compounds and several studies indicate that there are many non-identified components along with chemically characterized cyanotoxins. These new components can evoke toxic effects that can be even more pronounced (Pietsch et al., 2001; Šuput et al., 2002; Buryskova et al., 2006). Some components seem to mediate the release of other compounds which can amplify the toxic effect of known cyanotoxins (Sedmak and Suput, 2002), and may induce systemic genotoxicity in mammals (Sedmak et al., 2008).

Identification and classification of different microcystin variants has proven challenging. When analysing microcystins in water the method chosen should be 1) sensitive enough to meet the health risk assessment requirements and 2) able to characterise the concentration of specific microcystin variants. Moreover, a rapid speed of response is desirable. Efforts should be put on developing detection methods for analysing microcystin variants other than MC-LR.

2 AIMS

The overall aim of this PhD thesis was to provide tools and insight for risk assessment. The work consisted of the following steps:

I. Development of a sensitive time-resolved immunoassay TR-FIA for microcystins.
II. Screening of waterbodies on Åland Islands, SW Finland, for microcystins, with special interest to compare TR-FIA and ELISA to other detection methods.
III. Elucidation of interactions of different microcystin variants with artificial lipid membranes in order to study membrane effects of more hydrophobic microcystins.
IV. Comparison of the Caco-2 cellular toxicity of MC-LR, MC-LW and MC-LF.

3 MATERIALS AND METHODS

3.1 REAGENTS AND EQUIPMENT

L-α-phosphatidylcholine (egg) was purchased from Avanti Polar-Lipids, Inc (Alabaster, AL), cholesterol from Sigma Chemicals (St Louis, MO), and TMA-DPH from Molecular Probes (Eugene, OR). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. 6-dodecanoyl-2-dimethylaminonaphtalene (Laurdan) was obtained from Molecular Probes (Leiden, The Netherlands). Cholesta-5,7,9(11)-trien-3-beta-ol (CTL) was synthesized according to the method described by Fischer et al. (Fischer et al., 1984) and purified using reversed-phase HPLC (Björkbom et al., 2008). Culture media and supplements used for the maintenance of Caco-2 cells were purchased from Sigma. The water used was purified on a Milli-Q instrument (Millipore, Molsheim, France) to 18.2 MΩcm. All other reagents and buffers were of analytical or chromatographic grade.

Polystyrene microtiter plates were from FluoroNunc (Nunc, Roskilde, Denmark). The plates were washed with a 1296-026 Delfia Platewash (PerkinElmer, Turku, Finland) and a 1296-003 Delfia Plateshake (PerkinElmer, Turku, Finland) was used. Excitation at 340 nm and emission at 613 nm was measured with a 1232 Delfia Fluorometer (PerkinElmer, Turku, Finland) or a 1420 Victor multilabel fluorometer (PerkinElmer, Turku, Finland).

3.2 PURIFICATION OF MICROCYSTINS

MC-LR, MC-LW and MC-LF were purified from *Microcystis* PCC 7820, cultured in the laboratory in Z8 medium according to Staub (Staub, 1961) under constant illumination (PowerGlo T8 1800K 15 W) (R.C. Hagen Inc, West Yorkshire, UK) and aeration (Spoof and Meriluoto, 2005). Briefly, the cyanobacteria were grown in room temperature and diluted (1:20) into fresh growth medium every 3-4 weeks. For harvesting, the cyanobacteria were filtered on GF/A filters. Purification of microcystins started with freeze-thawing of harvested cyanobacteria and extraction with methanol in a bath sonicator, dilution with water, filtration, and binding to BondElut C18 columns (Varian, Walnut Creek, CA) (Spoof and Meriluoto, 2005). Fractions were collected after preparative C18 HPLC and concentrated by BondElut columns (Spoof and Meriluoto, 2005). Reversed-phase HPLC was used to quantify MC-LR, MC-LF and MC-LW (Meriluoto et al., 2000). *Anabaena* sp. 90 (culture collection of prof. Kaarina Sivonen, Helsinki University, Finland), cultured in Z8 medium without nitrogen addition and under constant illumination and aeration, was extracted with methanol and microcystins were analysed by HPLC.

3.3 PRODUCTION OF ANTIBODIES

Monoclonal antibodies were produced by conventional methods as described in Mikhailov et al., 2001.

3.4 DERIVATISATION OF MICROCYSTIN

Labelling of MC-LR included preparation of the toxin, the actual labelling and purification of the labelled product. Aminoethanethiol-MC-LR was made by adding a 3000-fold molar excess of 2-aminoethanethiol to MC-LR in 0.1 M sodium bicarbonate buffer, pH 9 (Moorhead et al., 1994). The mixture was incubated for 1 h at 50°C, cooled to room temperature and acetic acid was added to stop the reaction according to the manufacturer's description (PerkinElmer, Turku, Finland). The product, aminoethanethiol-MC-LR (Fig. 6) was purified on a BondElut column.

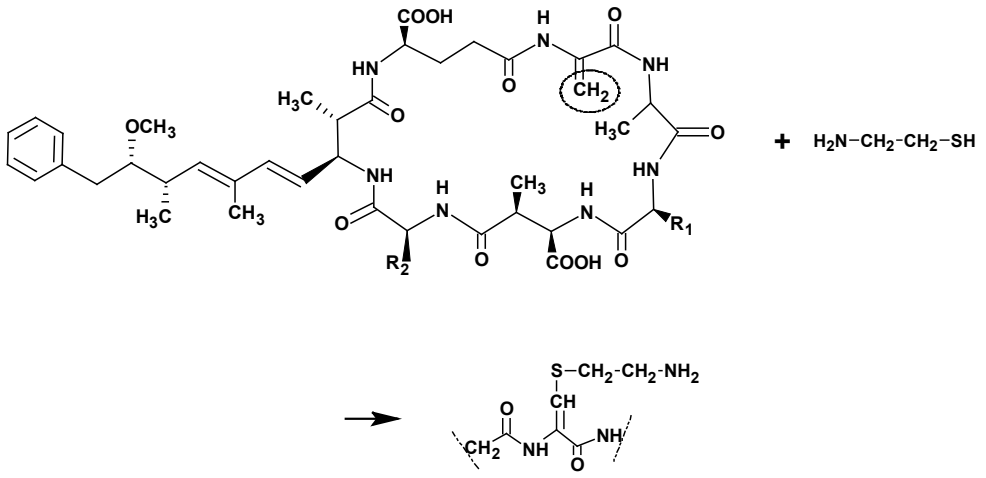

Figure 6. Conjugation of MC-LR with aminoethanethiol.

Aminoethanethiol-MC-LR was dissolved in labelling buffer (50 mM $NaHCO_3$, 0.15 M NaCl, pH 8.5) and mixed with the Eu-Labelling reagent, DTTA (europiumchelate of N1-(p-isotiocyanatobenzyl)-dietylentriamine-N1,N2,N3,N3-tetraacetic acid; Fig. 7; PerkinElmer, Turku, Finland). DTTA forms a stable complex with Eu^{3+} and the aromatic isothiocyanate group reacts with the free amino group and a stable, covalent thiourea bond is formed. After incubation overnight at room temperature, fractions were collected in semipreparative C18 HPLC and the specific activity of the europium chelate was measured. Mass of the final product, MCLR-Eu, was verified by MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization – Time-of-Flight Mass Spectrometry; Finnigan MAT Ltd, Thermo BioAnalysis, UK), and performed by the Centre of Biotechnology (Turku, Finland). Since europium is released from the chelate at the low pH used in MALDI-TOF MS analysis the final product was detected only without europium.

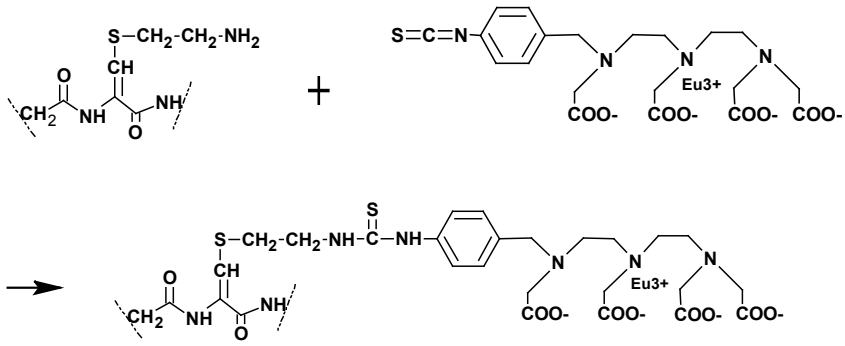

Figure 7. Conjugation of aminoethanethiol-MC-LR to the europium chelate.

3.5 EXTRACTION OF FIELD SAMPLES

In year 1999 field samples were collected from 55 lakes on the Åland Islands and analysed by HPLC. The samples, 25 - 200 ml, were filtered through Whatman GF/C filters (diameter 25 mm). The filters were air-dried, frozen in glass tubes, thawed and 100 µl of water was added to each filter. The filters were freeze-thawed once more and extracted with 2 ml methanol in a bath sonicator (Branson B2210, Danbury, CT) for 15 min. The extraction was repeated twice. Argon at 50°C was used to evaporate the combined extracts, about 6 ml, to dryness. After redissolving in 200 µl methanol, the residue was centrifuged at 10000 x g in 10 min and the supernatant was analysed with HPLC.

In year 2000 field samples were collected from another 79 lakes on the Åland Islands, making it a total of 134 lakes. Pretreatment of the samples were simplified: filters were freeze-thawed twice and 1 ml 75% methanol was added for the extraction in the bath sonicator for 15 min. Use of 75% methanol improves the extraction of MC-RR in comparison with 100 % methanol (Fastner et al., 1998). The extracts were centrifuged as described earlier and analysed by HPLC, TR-FIA and PPIA.

In 2001 field samples were filtered on GF/C filters (diameter 25 mm), air dried and freeze-thawed twice and extracted with 1.2 ml 75% methanol in a bath sonicator (Branson 2519E-MT, Danbury, CT) for 15 min. GF/C filters with a diameter of 47 mm were extracted with 2 ml 75% methanol. The samples were further sonicated with a probe sonicator (Branson Sonifier II W-250, Danbury, CT) for 1 min. The extracts were centrifuged at 10000 x g for 10 min and divided into aliquots and analysed by HPLC-UV, LC-MS and ELISA.

3.6 ANALYSIS BY TR-FIA

The monoclonal antibodies used in paper I were incubated overnight at +4°C in 0.2 M phosphate buffer, pH 7.0, in polystyrene microtiter plates. The next day the wells were washed with 5 mM Tris–HCl pH 7.8, 0.15 M NaCl, 0.05% Tween 20. Field samples that potentially contained microcystins were added to wells in triplicate together with MCLR-Eu dissolved in Assay Buffer

(PerkinElmer). Standards of different MC-LR concentrations and negative controls were dissolved in 4.5% methanol. After 1 h shaking the washing procedure was repeated and 100 µl Enhancement Solution (PerkinElmer) was added. Measurement of time-resolved fluororescence (exc. 340 nm, em. 613 nm) was performed after 15 min incubation.

3.7 HPLC

Microcystins in *Anabaena* sp. 90 extract and field samples from 1999, 2000 and 2001 were analysed by HPLC. For information on columns, mobile phases, gradient and flow rate used see Table 2. Semipreparative HPLC was used to purify MCLR-Eu. The HPLC system used consisted of a Shimadzu LC-10AT (Kyoto, Japan) pump coupled to a UV diode array detector Merck–Hitachi L-7450A (Darmstadt, Germany). The quantitative calculations were made on chromatograms at 238 nm. Samples were injected with a Shimadzu SIL-9A autoinjector. The column temperature was 40°C.

3.7.1 Semipreparative HPLC-UV

For purification of MCLR-aminoethanethiol a Nucleosil 100 5 C_{18} column (Macherey-Nagel, Düren, Germany), 250 mm×4 mm i.d. was used. Mobile phase consisted of solvent A (27% acetonitrile and 73% 13.5 mM ammonium acetate) and B (80% acetonitrile and 20% 13.5 mM ammonium acetate). A gradient was used and the percentage of solvent B changed in a linear manner according to following: at 0 min concentration of B was 0%, at 12 min 0%, at 30 min 25%, at 32 min 25% and at 34 min 0%. Flow rate 1 ml/min and the total run time was 35 min.

For purification of MCLR-Eu a Nucleosil 100 7 C_{18} column (Macherey-Nagel, Düren, Germany), 250 mm×10 mm i.d. was used. Mobile phase consisted of solvent A (2 volumes of water and 1 volume of solvent B) and B (27% acetonitrile and 73% 13.5 mM ammonium acetate). A gradient was used and concentration of solvent B changed in a linear manner according to following: at 0 min concentration of B was 0%, at 20 min 100%, at 45 min 100% and at 50 min 0%. Flow rate used was 2 ml/min and the total run time was 51 min.

3.7.2 Analytical HPLC-UV

To elucidate the microcystin profile in cultured cyanobacteria as well as in environmental samples the following columns, mobile phases, gradients and flow rates were used (Table 2).

Table 2. Stationary phase and mobile phases used in analytical HPLC-UV work

	Column	Mobile phase	Gradient		Flow rate
			Time (min)	Solution B (%)	ml/min
Anabaena extract	Nucleosil 100 3 C_{18}, 150 mm×2 mm i.d. (Phenomenex, Torrance, CA)	Solution A: 20% ACN + 80% 13.5 mM ammonium acetate. Solution B: 100% ACN	0 30 32 34	0 20 20 0	0.2
Field samples 1999	Nucleosil 100 3 C_{18}, 150 mm×2 mm i.d.	Solution A: 0.05% TFA Solution B: 100% ACN	0 35 36 38 60	25 70 70 25 25	0.25
Field samples 2000	Discovery RP-Amide C_{16}, 150 mm×2.1 mm i.d. (Supelco, Bellefonte, PA)	Solution A: 0.05% TFA Solution B: 100% ACN	0 25 27 28 45	20 65 65 20 20	0.3
Field samples 2001	Discovery RP-Amide C_{16}, 150 mm×2.1 mm i.d.	Solution A: 0.05% TFA Solution B: 100% ACN	0 25 27 28 45	20 65 65 20 20	0.3

3.7.3 HPLC-ESI-MS

The LC-MS instrument consisted of a HPLC Agilent 1100 (Agilent Technologies, Waldbronn, Germany) coupled to a Micromass (Manchester, United Kingdom) Quattro Micro triple quadrupole mass spectrometer with an electrospray interface. The toxins were separated on Merck Purospher STAR RP-18e column, 30 mm×4 mm i.d. Mobile phase consisted of solution A (0.5% formic acid) and B (acetonitrile). Concentration of solution B was changed in a linear manner according to following: concentration of B at 0 min was 25%, at 10 min 70%, at 11 min 70% and at 11.1 min 25%. Flow rate was 0.5 ml/min. Capillary voltage was 3.8 kV and the cone voltage 80 V. Temperature of the desolvation gas (nitrogen) was 350°C and flow rate 615 l/h. Temperature of the ion source was 120°C.

3.8 IMMUNOASSAY

For ELISA 50 µl from the field sample extract was evaporated to dryness at 50°C. The samples were redissolved in 300 µl 75 % methanol. Further dilutions were made in water and the final methanol concentration varied from 4 to 9.5 %. The commercial ELISA was EnviroGard Microcystin Plate Kit (Strategic Diagnostics, Newark, DE). Manufacturer's instructions were followed. 100 µl were added to the wells and the plate was incubated at room temperature for 30 min. The plate was washed four times with 5 mM Tris–HCl pH 7.8, 0.15 M NaCl, 0.05% Tween 20 and incubated for 30 min

after addition of 100 µl substrate. Stop solution was added and the plate was measured after 15 min with a spectrophotometer at 450 nm.

TR-FIA in paper II was conducted in the same manner as the commercial ELISA with the following exceptions. No stop solution was added and the microcystin-enzyme conjugate was replaced by MCLR-Eu (2 µg/l, 100 µl) and substrate by Enhancement Solution, 100 µl. The Enhancement Solution is an acidic proprietary detergent solution that dissociates Eu^{3+} from the bound Eu-labelled microcystin and chelates it to form a highly fluorescent micelle (PerkinElmer, Turku, Finland). Measurement was conducted within 15 min after addition of Enhancement Solution with a time-resolved fluorometer.

3.9 PROTEIN PHOSPHATASE INHIBITION

In paper II a fluorimetric protein phosphatase inhibition method was modified from Fontal et al. and Gee et al. (Fontal et al., 1999; Gee et al., 1999). Heating the samples to 80°C for 15 min inactivated endogenic phosphatases. Protein phosphatase 1 (PP1) was diluted with TRIS buffer (50 mM TRIS, 0.1 M EDTA, 0.2 mg/ml BSA, 5 mM ditiotreitol, pH 7) and 0.025 units of PP1 was incubated in each well for 10 min. Standards and samples were added and incubated with enzyme for 10 min. Substrate, DiFMUP, was added to a final concentration of 100 µM. After 1 h incubation, fluorescence was measured (exc. 355 nm, em. 460 nm) with a fluorometer.

In paper V a colorimetric protein phosphatase inhibition assay protocol similar to that described by An and Carmichael (1994) was used. Samples and calibrators, dissolved in water, were added to microtiter plate wells together with 10 µl PP1 (activity of 1.67 U/ml, dissolved in buffer). The negative control contained only PP1. Substrate (15 mM p-NPP) was added and after two hours of incubation at 37°C the absorbance was measured at 405 nm on 1420 Victor multilabel counter (PerkinElmer, Turku, Finland). The assay was repeated three times with samples, calibrators and negative controls performed in duplicates. The inhibition percentages were calculated as: average (OD of calibrator 0 – average OD of standard or sample) × 100 / average OD of standard. IC_{50} was calculated as the concentration of toxin that inhibited half of the protein phosphatase activity compared with the uninhibited control reaction.

3.10 MODEL MEMBRANES

3.10.1 Surface barostat technique

The capacity of MC-LR, MC-LF and MC-LW to penetrate membranes was studied by the surface barostat technique in monolayers consisting of egg phophatidylcholine and cholesterol (7:3, molar ratio). Lipids dissolved in hexane/isopropanol (3:2, vol/vol) were spread on a sub-phase (Verger and Pattus, 1976) consisting of 20 mM Tris–HCl and 145 mM NaCl, pH 7 (Fig. 8). The toxins were injected in the subphase (final concentration of 1 mM) and the change in the surface pressure ($\Delta\pi$) was registered with a platinum Wilhelmy plate (Fig. 8; (Bougis et al., 1981). Penetration of toxins into the lipid monolayer was determined at room temperature with a KSV 3000 surface barostat (KSV Instruments Ltd, Helsinki, Finland).

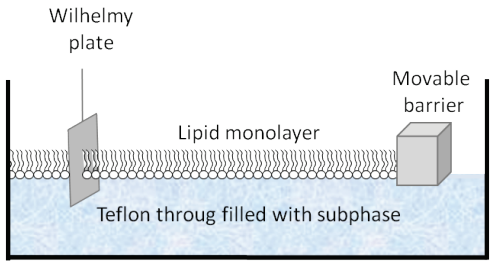

Figure 8. Schematic picture of a throug used in the surface barostat technique (KSV Nima, Espoo, Finland). Lipids are carefully spread on a sub-phase. The Wilhelmy plate registers the surface pressure and the monolayer can be compressed or expanded with the movable barrier. Picture drawn by the author, inspired by Verger and de Haas (1973)

3.10.2 Steady-state fluorescence anisotropy

Vesicles of egg phosphatidylcholine (100 mM) were made in a buffer containing 20 mM Tris–HCl and 145 mM NaCl. Unilamellar vesicles were prepared after brief sonication by repeated extrusion through 100 nm polycarbonate filters (Costar Corp., Cambridge, MA) using a Lipextruder (Lipex Biomembranes, Vancouver, BC) according to Hope et al. (1985). TMA-DPH, dissolved in ethanol, was added to the vesicles (final concentration 1 mole %). Anisotropy was measured in the presence and absence of microcystins (final concentration 1 mM). A PTI Quantamaster 1 spectrofluorometer was used for the steady-state fluorescence anisotropy measurements with the software program Felix (Photon Technology International, Canada). After excitation at 360 nm, the emission was registered with two channels at 430 nm. The excitation polarizer was first placed in the vertical orientation, and the ratio of the parallel and perpendicular signals (RV) was measured. Then the ratio was measured using horizontally polarized excitation, both emission channels observing the emission (RH). These values, which include the system specific G factor, were used to calculate the anisotropy, r.

3.10.3 Fluorescence emission maximum

The fluorescence emission maximum of tryptophan in MC-LW (final concentration 7.5 mM) was measured after excitation at 280 nm in the presence and absence of egg phosphatidylcholine vesicles (final concentration 750 mM). The toxin was dissolved in buffer (20 mM Tris–HCl and 145 mM NaCl, pH 7).

3.10.4 Generalized polarization

Lipid vesicles of DPPC were dissolved in a buffer of 20 mM Tris-HCl and 145 mM NaCl, pH 7. After sonication (duty cycle 40 %, output control 5) for 2 minutes with a Branson probe sonifier W-250 (Branson Ultrasonics, Danbury, CT), unilamellar vesicles were prepared by repeated extrusion using a Lipextruder (Lipex Biomembranes, Vancouver, BC) through 100 nm polycarbonate filters (Costar Corp., Cambridge, MA) according to Hope et al. (1985). Laurdan (final concentration of 1 mole%) was dissolved in ethanol and incorporated in the vesicles by shaking for 30 min. After excitation at 365 nm, laurdan emission spectra were recorded between 400 and 580 nm with a PTI

QuantaMaster 1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ). A temperature probe was submerged in the sample and the temperature (40°C) was controlled by a Peltier element. The generalized polarization (GP) was calculated according to

$$GP_{ex} = \frac{I_{440} - I_{475}}{I_{440} + I_{475}}$$

where I_{440} is the fluorescence intensity at 440 nm, the characteristic wavelength of the liquid disordered phase, and, I_{475} is the emission intensity at 475 nm, the characteristic wavelength of the liquid ordered phase (Parasassi et al., 1990). The emission spectra of Laurdan was measured and compared in the absence and presence of toxins (1 and 10 µM final concentration). Tamoxifen was used as a successful positive control (Engelke et al., 2001).

3.10.5 Fluorescence resonance energy transfer

Lipid vesicles were made of POPC and CTL (molar ratio 99:1) by probe sonication. The phospholipid was dried under argon at 40°C with excess solvent removed by vacuum drying for 20 min, and resuspended in argon-purged Tris-HCl buffer. CTL was added and the lipids were briefly vortexed and sonicated (duty cycle 20 %, output control 5) for 2 min using a Branson W-250 probe sonifier (Branson Ultrasonics, MA, USA). After excitation at 290 nm the fluorescence emission intensity of CTL was measured at 374 nm using PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ). The intensity of the fluorescence emission was compared in the absence and presence of MC-LW (5 µM final concentration).

3.11 CACO-2 CELL LINE CULTURE

Caco-2 cells (human epithelial colorectal adenocarcinoma cells, DSMZ, Braunschweig, Germany) were grown at 37°C in a humidified atmosphere (5 % CO_2, 95 % O_2) in plastic dishes in Dulbecco's modified Eagle medium supplemented with 10 % Fetal Calf Serum, 1 % L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. After trypsinization, staining with trypan blue and counting using a cell haemocytometer (Neubauer improved, Marienfeld, Germany) the cells were seeded the day before the start of the cell proliferation and cytotoxicity assays in 96-well microtiter plates (BD Falcon, Helsinki, Finland) at densities of 80000 cells/ml. Fresh media was added to all wells at the start of the experiments. Control cells were treated with 5 µl pure PBS and the experiment cells with 5 µl of each toxin dissolved in PBS (final toxin concentration 1, 10 or 50 µM), every concentration was run at least in triplicates.

3.11.1 Cell viability and morphology

To study the effects of microcystins on Caco-2 cell morphology, cells were exposed to MC-LR, MC-LW and MC-LF at 50 µM for 22 h and 44 h, and photographed under phase contrast in a Leica DMIL light microscope using a Leica EC3 digital camera.

3.11.2 Cell proliferation

The effects of microcystins on Caco-2 cell proliferation were studied with the Cell Proliferation Reagent WST-1 (Roche Applied Sciences, Mannheim, Germany). Mitochondrial dehydrogenases

catalyse the cleavage of tetrazolium salt WST-1 to dark red formazan (Berridge et al., 2005). An increase of viable and metabolically active cells leads to increased mitochondrial dehydrogenases and increased cleavage of formazan. Cells treated in 96-well microtiter plates with MC-LR, MC-LW and MC-LF were compared to non-treated cells and controls, according to the WST-1 kit description. The amount of formazan dye formed was measured on a multiwell spectrophotometer (Varioskan Flash, Thermo Fisher Scientific Inc, Vantaa, Finland) at 450 nm after the addition of 10 µl of Cell Proliferation Reagent WST-1.

3.11.3 Cytotoxicity assay

Damage on the cell membrane was estimated by lactate dehydrogenase (LDH) leakage using a Cytotoxicity Detection Kit (Roche Applied Sciences, Mannheim, Germany). The test was performed according to the LDH assay protocol. The reaction mixture provided with the kit was added to all wells to monitor the leakage of LDH into the extracellular fluid. A lysis solution was added to the high control wells for the maximum cellular amount of LDH. Cells that were not treated with toxins represent the spontaneous baseline leakage of LDH. The % LDH leakage from cells treated with MC-LR, MC-LW and MC-LF was calculated from the maximum cellular LDH leakage.

4 RESULTS AND DISCUSSION

4.1 DEVELOPMENT OF TR-FIA (I)

4.1.1 Labelling of microcystin with europium

The use of lanthanides in immunoassays requires a chelating group that binds the lanthanide ion and a functional group for the attachment to biomolecules (Soini and Hemmilä, 1979; Hagan and Zuchner, 2011). There is a wide variety of different chelators for constructing lanthanide chelates to be used in time-resolved fluoroimmunoassays (Hagan and Zuchner, 2011). The DELFIA system developed by PerkinElmer uses the major class of non-luminiscent polyaminocarboxylates that carry the lanthanide as a tag (Soini and Hemmilä, 1979; Soini and Kojola, 1983; Lövgren et al., 1984). The labelling reagent chosen here was an isothiocyanate derivative of the stable europium chelate that reacts readily with e.g. amino groups (Mathis and Bazin, 2011). A free amino group was therefore first introduced to MC-LR. After derivatisation with 2-aminoethanethiol, the product, MCLR-aminoethanethiol (MCLR-AET), was purified from MC-LR according to the retention time, 9.37 min for MCLR-AET versus 17.4 min for MC-LR, on HPLC (Fig. 9).

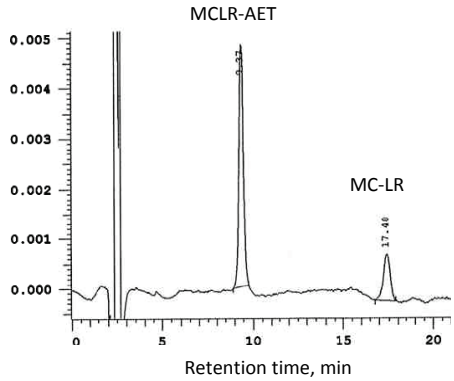

Figure 9. Separation of MC-LR and MCLR-AET on a Nucleosil 100 5 C18 column, 250 mm×4 mm i.d.. Mobile phase: solvent A (27% acetonitrile and 73% 13.5 mM ammoniumacetate) and B (80% acetonitrile and 20% 13.5 mM ammoniumacetate) with the following linear gradient programme: 0 min concentration of B was 0%, 12 min 0% B, 30 min 25% B, 32 min 25% B and 34 min 0% B. Detection wavelength 238 nm. Flow rate 1 ml/min, total run time 35 min.

MCLR-AET with the free amino group was then linked to the europium chelate. Efficient labelling (about 70%) was reached and MC-LR conjugated to the Eu chelate (retention time 27.93 min) was easily separated from non conjugated MC-LR (36.97 min) with HPLC (Fig. 10). The Eu chelate increased the molecular weight of the final product from 994 Da to 1700 Da. The product also became more hydrophilic. As the principal chromophore, the conjugated diene in Adda, was not modified, the same molar absorptivity factor that was used for MC-LR (ε=39810) was used for the conjugated product.

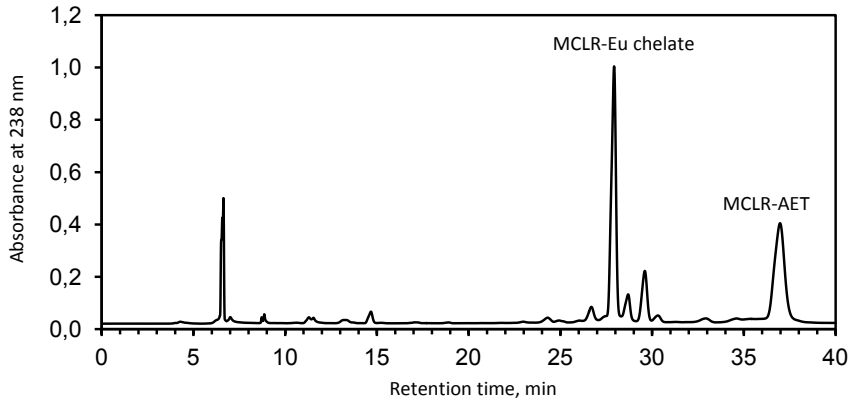

Figure 10. Separation of MCLR-AET and MCLR-Eu chelate on a Nucleosil 100 7 C18 column, 250 mm×10 mm i.d. Mobile phase: solvent A (2 volumes of water and 1 volume of solvent B) and B (27% acetonitrile and 73% 13.5 mM ammonium acetate) with the following linear gradient programme: 0 min 0% B, 20 min 100% B, 45 min 100% B and 50 min 0% B. Detection wavelength 238 nm. Flow rate was 2 ml/min and total run time was 51 min.

4.1.2 TR-FIA in detection of microcystins

The efficacy of TR-FIA as an analytical method for microcystins was evaluated. Pure MC-LR and a cyanobacterial extract were analyzed in parallel by using TR-FIA and HPLC. The concentration of purified MC-LR was determined by HPLC and dilutions were made for TR-FIA. A typical sigmoidal standard curve was obtained with TR-FIA for MC-LR concentrations of 0.01-50 µg/l (Fig. 11, black squares). Standard deviation was low (±2.7%, max 6%), calculated as average of three replicates for each concentration.

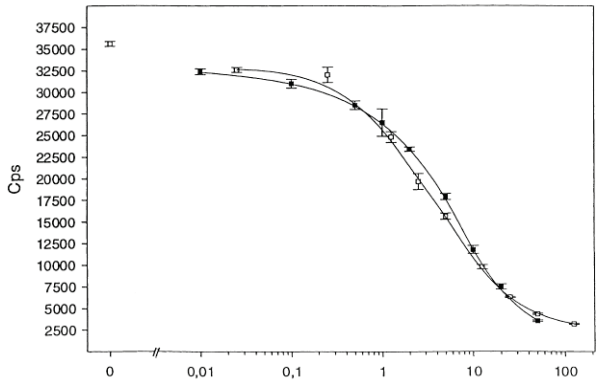

Figure 11. TR-FIA standard curve for MC-LR concentrations 0.01-50 µg/l (black squares). TR-FIA response curve for Anabaena sp. 90 extract (white squares). All values are average of three replicates.

Environmental samples usually contain several different microcystins. The performance of TR-FIA was tested by analyzing an extract of *Anabaena* sp. 90, that produced four different microcystins. Toxin variants in *Anabaena* sp. 90 had been determined by others (Rapala et al. 1997) and were verified by us with mass spectrometry and amino acid analysis (results not shown). The methanolic extract of *Anabaena* sp. 90 was analyzed with HPLC and contained 16 µg/ml MC-LR (retention time 19.05 min), 11 µg/ml MC-RR (21.73 min), 8 µg/ml [D-Asp^3]-MC-LR (22.32 min) and 5 µg/ml [D-Asp^3]-MC-RR (21.11 min), i.e. the total content of microcystins was 40 µg per ml (Fig. 12). MC-LR constituted 40% of the total microcystin amount. The extract was diluted into water and used as a representative of an environmental sample. Since the WHO guideline value for microcystin-LR is given in µg per litre water the same unit was chosen here.

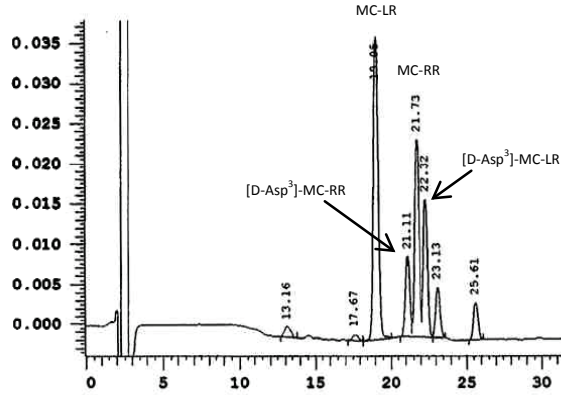

Figure 12. Separation of microcystins in Anabaena sp. 90 on a Nucleosil 100 3 C18 column, 150 mm×2 mm i.d. Mobile phase: solution A (20% acetonitrile and 80% 13.5 mM ammonium acetate) and B (100% acetonitrile), with the following linear gradient programme: 0 min 0% B, 30 min 20% B, 32 min 20% B and 34 min 0% B. Flow rate 0.2 ml/min and total run time 51 min.

The *Anabaena* extract was diluted to fit to the concentration range of the TR-FIA standard curve. The response curve of the *Anabaena* extract matched the standard curve of pure MC-LR (Fig. 11). Therefore it was concluded that an estimation of microcystins in environmental samples can be done by comparing the result to a standard curve of pure MC-LR.

Depending on which analytical method was chosen for the detection of microcystins different aspects needed to be considered. TR-FIA was suitable for screening purposes, while HPLC was conducted for the identification and quantification of microcystin variants. Although the WHO guideline specifically refers to MC-LR, it is of importance to analyze the total amount of microcystins. That includes all microcystin variants in the sample. Antibodies used in TR-FIA cross-reacted with structurally similar molecules, i.e. other microcystin variants. Analyzing total amount of microcystins with HPLC required standards. The standards available were MC-LR, MC-RR, MC-YR, MC-LY, MC-LF, MC-LW, dmMC-LR, dmMC-RR, didmMC-LR. Moreover, HPLC usually required a

time-consuming pretreatment of the sample due to e.g. higher detection limit and cleanup needs, while untreated water samples could be analyzed with TR-FIA.

4.1.3 TR-FIA versus ELISA

TR-FIA was compared, with respect to detection limits, to a commercially available ELISA kit (SDI EnviroGard ELISA, Strategic Diagnostics Inc, Newark, DE). A higher level of sensitivity was reached with TR-FIA compared to ELISA when using the antibodies and assay conditions optimized for the commercial ELISA. The lowest concentration used (100 pg/ml) showed a decrease in the fluorescence of TR-FIA that was statistically different from the negative control (Fig. 13). TR-FIA was shown to be a useful technique for screening microcystins in water. The response curve in TR-FIA was more even and more smooth at low concentrations (Fig. 13). The sensitivity of TR-FIA using Eu chelates has been found to be higher compared to ELISA, e.g. Siitari showed a 30-60-fold increase in sensitivity (Siitari, 1990). Several other studies have shown increased sensitivities with time-resolved fluorescence assays compared to conventional ELISA (Ogata et al., 1992 ; Allicotti et al., 2003; Butcher et al., 2003). Detection limits for different non-radioactive labelling systems were summarized in Diamandis, 1990. For conventional enzyme markers the detection limit was 10^{-10}–10^{-11} mol/l, and the lowest detection limit was seen with europium chelates in TR-FIA, 10^{-12}–10^{-13} mol/l (Diamandis, 1990). In TR-FIA a higher sensitivity has been reached also when using smaller sample volumes, allowing assay minituarization development (Daijo and Sportsman, 1999; Lavery et al., 2001 ; Zuck et al., 2005). Smaller sample volumes allow larger dilutions with e.g. buffers used in the assay and matrix effects can thereby be eliminated more efficiently (Peruski et al., 2002).

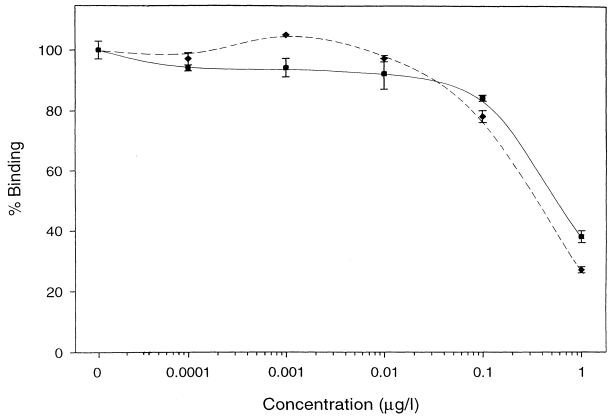

Figure 13. Concentration curve of TR-FIA (squares) and ELISA (dashed line). % Binding was calculated as the mean fluorescence/absorbance of each concentration (B) divided with the mean zero standard value (B_0). All values are average of three replicates.

The total analysis time was decreased from 1½ h to 1 h using TR-FIA, since the last incubation step in ELISA (addition of a stop solution) is not required in TR-FIA. In screening purposes savings in time are of interest (Munoz-Olivas, 2004). Several ELISA kits that are commercially available for microcystin analysis use colorimetric detection of the signal produced by HRP together with its

substrate, e.g. EnviroLogix QuantiPlate Kit for Microcystins, Abraxis Microcystins-ADDA ELISA and Envirogard Microcystins QuantiTube Test Kit. Change of color intensity is easy to detect and often precise enough for most analysis. However, the detection method is restricted by several characteristics of HRP and colorimetry (Gosling, 1990). HRP is easily inactivated and sensitive to several inhibitors, even though well preserved enzyme conjugates usually stay intact for several years. Europium chelates on the other hand have been shown to have a long-term stability. The lanthanide chelates are easy to handle and not sensitive to temperature or light (Sevéus et al., 1994).

HRP has complicated kinetics and it can be inhibited by high substrate concentrations. In colorimetry the intensity of the monochromatic light that has not been absorbed by the sample is measured, which means that at low concentrations small differences in intensity must be measured and this demands a high light intensity which in turn limits the detection level. In the colorimetric detection of ELISA, the optical absorbance measurements allow a pretty narrow dynamic scale. A wider dynamic range has been reached when using TR-FIA (Peruski et al., 2002). A considerably broader range was shown in one publication where a scale of 2 – 160000 ng/l was reached (Madersbacher et al., 1993). That would reduce multiple dilution steps and remeasurement of samples when concentrations fall outside the range of the standard curve. The unique fluorescent properties of lanthanide chelates allow sensitive and specific detection of an analyte. Europium, the most used lanthanide, can form highly fluorescent chelates which have a long fluorescence lifetime and a large Stokes' shift.

4.2 PREVALENCE OF MICROCYSTINS ON THE ÅLAND ISLAND (II, III)

During the years 1999-2001 samples from practically every fresh water lake on the Åland Islands were analysed for microcystins and nodularins (Table 3 and 4). Microcystins were analysed by HPLC, PPIA and TR-FIA. Surveys covering such a high local area are quite rare. A large variety of lake types exist on the Åland mainland and on the larger islands of the Åland archipelago (Lindholm, 1996). Several lakes and drinking water reservoirs have a history of toxic cyanobacteria occurrence (Lindholm et al., 1989; Lindholm and Eriksson, 1990; Lindholm et al., 1999). The summer 1999 was sunny and visible cyanobacteria blooms were observed in approximately half of the 55 examined lakes, of which 4 contained microcystins, determined by HPLC. In summer 2000 several lakes in forest areas were included and cyanobacterial blooms were found in 40 lakes of 134 lakes analysed. Microcystins were found in 14 lakes. Especially the eutrophic lakes contained microcystins but also in some dystrophic lakes and even in one oligotrophic lake, microcystins were found. One of the lakes was a drinking water reservoir, Tjudö träsk, and several other lakes were used for crop irrigation.

Satisfactory correlations were achieved in 2000 with the three analytical methods, TR-FIA, PPIA and HPLC (Table 3). In one lake, Träsket, microcystins was detected only with HPLC, although HPLC had the highest detection limit of 0.25 μg microcystin per litre water. For six other lakes microcystins were detected only with TR-FIA and/or PPIA. The reason why microcystins were not detected with HPLC could be the limited number of available standards or that several minor microcystin variants were present but below the detection limit. In three cases there were

disagreements between TR-FIA and PPIA. The positive response obtained with TR-FIA (Glosholm lake, 11.9.2000) but not with HPLC or PPIA may be related to the dominating presence of the cyanobacterium *Chroococcus*. The cyanobacterium *Chroococcus* is not a known microcystin producer but could have produced other compounds that cross-reacted with the antibodies in TR-FIA. The positive responses obtained only with PPIA (Gesterby tjärnan 25.8.2000 and Bokhällamossen 6.9.2000) could have been due to the presence of other protein phosphatase inhibitors than microcystins. E.g. oscillamides and anabaenopeptins are protein phosphatase inhibitors as well, and produced by cyanobacteria (Harada et al., 1995; Sano et al., 2001). Toxins of non-cyanobacterial origin, like okadaic acid, tautomycin and calyculin A, have also been shown to have protein phosphatase inhibition capacity (Bagu et al., 1997). In several lakes the cyanobacterial bloom lasted until late autumn, as for example in Nåtö Hemviken where microcystins were found in every collected sample. The highest microcystin level, 12 μg/l, was detected with PPIA in Nåtö Hemviken in October 2000. Several different species of *Microcystis* were present at that time in the lake. However, microcystins were not detected with HPLC, at least not with the available standards. TR-FIA showed a microcystin concentration of 7.2 μg/l. The high concentration detected with PPIA suggested that compounds that inhibit protein phosphatase 1, both microcystins and others, might have been present in the lake.

Based on microscopic examination of the samples collected 1999-2000, the most likely microcystin producers in the lakes were *Planktothrix agardhii, Microcystis aeruginosa* and *Anabaena lemmermannii.* Annual variations seemed to be common as none of the four positive lakes in 1999 had any microcystins detected the next year, although samples were taken at nine different occasions from e.g. Östra Kyrksundet (Lindholm et al., 2002). Different cyanobacteria showed a tendency to dominate on an annual basis. Mass occurrence of *Planktothrix agardhii* was detected in lake water samples from Vargsundet in the year 1997 and *Microcystis aeruginosa* in the year 2000 (Lindholm et al., 1999).

Table 3. Microcystins (μg/l) detected on the Åland Islands in 1999 and 2000. nd stands for "not detected".

Date	Location	HPLC	TR-FIA	PPIA
7.9.1999	Östra Kyrksundet	6.4	Not examined	Not examined
19.9.1999	Gloskärs träsk	2.2		
22.9.1999	Långsjön	1		
30.9.1999	Syllöda träsk	5.8		
28.6.2000	Brantsböle träsk	nd	1.6	0.5
29.6.2000	Nåtö Hemviken	nd	7.2	12
5.7.2000	Tjudö träsk	4.7	5	8
6.7.2000	Olofsnäs	1.3	1	0.3
3.8.2000	Prästträsket	5.7	4	4.5
18.8.2000	Lemböte Byträsk	5.1	3	6
25.8.2000	Gesterby tjärnan	nd	nd	0.5
3.9.2000	Godby träsk	nd	0.4	0.3
6.9.2000	Bokhällamossen	nd	nd	1.4
11.9.2000	Glosholms träsk	nd	0.6	nd
18.9.2000	Träsket	1.4	nd	nd
18.9.2000	Vargata träsk	0.7	0.8	2
22.9.2000	Vargsundet	0.4	0.3	0.2
6.11.2000	Strömma träsk	1.3	1.1	2.8

In 2001, 93 lakes on the Åland Islands were analysed (113 water samples) for microcystins and nodularins. Three different methods, ELISA, HPLC-UV and LC-MS, were compared and the results were shown to be comparable (Table 4). Microcystin concentrations exceeding 0.2 μg/l were detected with at least one technique in 31 water samples (16 different lakes) and with all three techniques in 21 water samples (9 lakes). The sum of the total microcystin concentrations in the positive samples was 179 μg/l for HPLC-UV, 200 μg/l for LC-MS and 135 μg/l for ELISA (Table 4). The lowest concentrations were often seen with ELISA. The antibody cross-reactivity towards different microcystin variants is known to vary. The cross-reactivity in the commercial ELISA used for the analysis was relatively equal for the few microcystin variants that had been tested, with EC_{50} values for MC-LR 0.31 μg/l, MC-RR 0.32 μg/l and MC-YR 0.38 μg/l. The cross-reactivity for other variants, such as demethylated microcystins, had not been reported.

The microcystins most often found were MC-RR, -LR and -YR with different degrees of demethylation. The more hydrophobic variants, MC-LF and MC-LY, occurred in only one case, Katthavet. The highest microcystin concentration according to ELISA was 30 μg/l (Vargata lake, August). The same sample also showed the highest concentration detected with HPLC-UV 42 μg/l and with LC-MS 39 μg/l. The lake Prästträsket in June showed according to LC-MS the highest microcystin concentration 42 μg/l whereas HPLC-UV detected 34 μg/l and ELISA 16 μg/l. When the

sample was collected, *Planktothrix agardhii* dominated. A month later the concentrations had decreased to 3.4, 3.1 and 3.0 μg/l respectively according to LC-MS, HPLC-UV and ELISA. The discrepancy at the higher concentrations could be related to the high presence of demethylated variants. Depending on the cross-reactivity ELISA can over- or underestimate the concentration of microcystins in a sample (Gago-Martinez, 2007). Usually antibodies used in ELISA have been raised against a specific microcystin variant and therefore cross-reactivity against other variants might be considerably lower (Fischer et al., 2001). When a sample contains microcystin variants not particularly well recognised by the antibodies, immunoassays tend to underestimate the microcystin concentration.

In paper II TR-FIA was compared to HPLC and PPIA (2000) and in paper III to ELISA, HPLC-UV and LC-MS (2001). All the methods used were found to be useful for real water samples. The best agreement between different detection methods was shown with samples that mainly contained MC-LR and/or MC-YR. The half maximum inhibition concentration (IC50) for those variants together with MC-RR has often been reported when antibodies have been developed for immunoassays (Chu, 2000). More seldom have IC50 values been determined for demethylated microcystin variants (Fischer et al., 2001).

Parallel methods to analyze microcystins have been used in several publications, either to directly compare and evaluate the methods or to combine fast screening with variant analysis (Rapala et al., 2002; Muñiz Ortea et al., 2004; Gkelis et al., 2005; Mountfort et al., 2005). ELISA and protein phosphatase inhibition assay (PPIA) were used in parallel to estimate the concentration of microcystins with an intention to evaluate the toxicity of the microcystins (Carmichael and An, 1999; Mountfort et al., 2005). Rapala et al. (2002) showed that protein phosphatase inhibition assay, ELISA and HPLC correlated well when analysing pure toxin, laboratory cultures, water samples and cyanobacterial samples for several microcystin variants. However, [D-Asp^3]-microcystins and hydrophobic microcystins constituted exceptions.

Table 4. Occurence of microcystins (µg/l) on Åland Islands 2001 (nd = not detected). The ELISA results are reported in MC-LR equivalents.

Location	Date	HPLC-UV	LC-MS-SIR	ELISA	Detected MC variants
Baststjärnan	June 18	0.13	0.34	nd	LR, RR, dmLR, dmRR
Brantsböle träsk	June 1	0.26	0.47	0.5	LR, RR, dmLR
Brantsböle träsk	June 27	0.86	1	0.4	LR, YR, dmLR
Brantsböle träsk	June 27	0.62	0.49	0.4	LR, YR, dmLR
Brantsböle träsk	June 27	0.67	0.74	0.4	LR, YR, dmLR
Brantsböle träsk	Aug. 9	3.1	1.4	1.5	LR, YR, dmLR
Brantsböle träsk	Oct. 7	0.21	0.37	0.1	LR, YR, dmLR
Gloskärs träsk	June 26	0.2	0.1	0.1	LR, YR, dmLR
Gloskärs träsk	June 26	0.25	0.11	0.1	LR, YR, dmLR
Godby träsk	June 7	1.4	0.48	0.2	LR, RR, dmRR
Godby träsk	July14	5.1	3.6	4.8	LR, RR, dmRR
Hägnträsk	Aug. 8	0.44	<0.10	nd	RR, YR, dmLR
Högbolstad	June 6	0.07	0.22	0.15	RR, dmRR
Kaldersfjärden	June 28	0.7	<0.10	0.1	RR, YR, dmRR, dmLR
Katthavet	July 30	1.7	1.8	0.9	LR, LF, LY
Lemböte Byträsk	July 6	2.3	3.5	3	LR, YR, dmLR
Lemböte Byträsk	July 21	6	8.1	4	LR, YR, dmLR
Lillfjärden	July 29	0.16	0.21	0.1	LR, dmLR
Norra Långsjön	July 23	0.1	0.12	0.2	LR, dmLR, nod
Nåtö Hemviken	June 25	6.4	7.4	7.6	LR, RR, YR
Prästträsket	June 7	12.1	25.7	8	LR, RR, dmLR, dmRR, didmRR
Prästträsket	June 29	33.9	42	16	LR, RR, YR, dmRR, didmRR
Prästträsket	July 30	3.1	3.4	3	LR, RR, YR
Prästträsket	Aug. 9	7.5	8.5	9.1	LR, RR, YR
Strömma träsk	June 28	1	1.5	0.8	RR, dmRR, didmRR
Strömma träsk	Oct. 7	nd	<0.10	0.3	dmLR, dmRR, didmRR
Vargata träsk	July 13	5.4	3.9	6.8	LR, RR, YR
Vargata träsk	July 17	4.6	4.7	4.8	LR, RR, YR
Vargata träsk, NE	Aug. 9	36.8	32.8	30	LR, RR, YR
Vargata träsk, SW	Aug. 9	42.3	38.6	30	LR, RR, YR
Överby insjö	July 24	0.94	0.97	0.5	LR, dmLR

4.3 INTERACTION OF MICROCYSTINS WITH MEMBRANES AND CELLS (IV, V)

4.3.1 Microcystin interaction with monolayers (IV)

The membrane penetrating capacity of MC-LR, MC-LW and MC-LF was studied by the surface barostat technique in monolayers at various initial pressures and at constant area. The toxins were injected beneath the monolayer film (in the subphase) and the surface pressure was observed. The penetration capacity, or the surface activity, of MC-LR was low using artificial monolayer membranes made of egg phosphatidylcholine:cholesterol (molar ratio 7:3). MC-LR did not cause an increase in the membrane surface pressure at initial pressures between 9 and 26 mN/m (Fig. 14). However, the more hydrophobic microcystins, MC-LW and MC-LF, showed clear surface pressure dependent membrane penetrating capacities, as indicated by their effect on the monolayer surface pressure. MC-LW and especially MC-LF, induced a rapid increase in the surface pressure. The packing density in the monolayer was increased by the presence of toxins as the surface area available for the monolayer was not allowed to expand, and this was reflected as an increased surface pressure (Phillips and Krebs, 1986). Extrapolating their linear relationship of the surface pressure change versus initial surface pressure to $\Delta\pi=0$ gave exclusion pressures of 21.3 mN/m for MC-LF and 22.9 mN/m for MC-LW (Fig. 14).

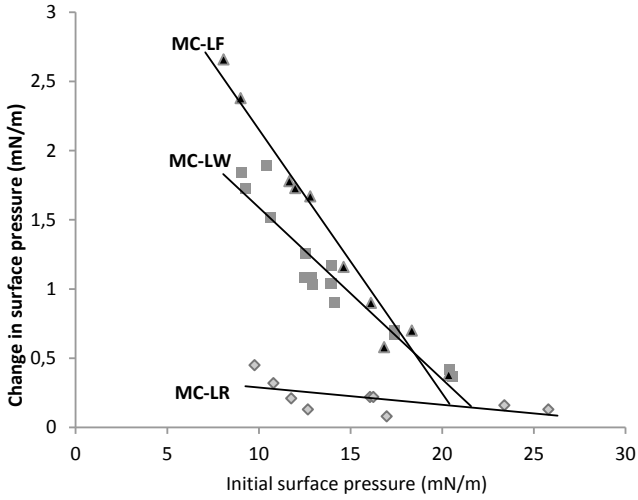

Figure 14. Interaction of microcystins with monolayers as studied by the surface barostat technique. Microcystins were injected in the subphase beneath the lipid monolayer and the change in surface pressure was monitored as the monolayer area available was not allowed to change.

The physiological pressure in biomembranes is close to 30 mN/m (Marsh, 1996) and considering the exclusion pressure of 21.3 mN/m for MC-LF and 22.9 mN/m for MC-LW no spontaneous penetration is likely to occur. However, the surface pressure of biomembranes can fluctuate ±15 mN/m due to compressibility properties of the membranes (Phillips et al., 1975). Thereby, it might be possible that membranes with packing defects could be more affected by the more

hydrophobic toxins. Evidence of membrane effects of MC-YR was found in a study by Lankoff and Kolataj (Lankoff and Kolataj, 2001). The authors showed that MC-YR caused a destabilisation of lysosomal membranes. MC-LF and MC-LW, being more hydrophobic, could perhaps give rise to an even more pronounced effect on the membranes. In paper IV it was shown that there are differences in how hydrophilic and hydrophobic microcystins interact with lipid membranes. These differences could have an effect on the organotropism, toxicokinetics and bioaccumulation of microcystins. Microcystins are considered to need the OATP system for entering the cell (Fischer et al., 2010). However, virtually all eukaryotic cells internalize pieces of their plasma membrane through pinocytosis. If some (hydrophobic) microcystins would be associated with the plasma membrane these toxins could more readily enter the cell. Bacteria are known to produce toxins that enter the cell through e.g. internalization (Montecucco et al., 1994; Montecucco, 1998). Possible non-specific uptake of different microcystin variants in the endocytotic pathway deserves some attention in the future.

4.3.2 Steady-state fluorescence anisotropy studied by TMA-DPH (IV)

The membrane fluidity was measured using TMA-DPH as a probe located in the membrane interface. An anisotropy value of 0.190 for pure phosphatidylcholine vesicles was achieved (Fig. 15). Anisotropy is a dimensionless quantity with a maximum value of about 0.4 due to unoriented samples and the angular dependence of photoselection. Addition of the different microcystins to a final concentration of 1 mM decreased the calculated anisotropy value to 0.170 for MC-LR and to 0.163 for MC-LW and MC-LF (Fig. 15). A decreased anisotropy correlates to increased rotational freedom of the probe. Since all three toxins decreased TMA-DPH anisotropy similarly and MC-LR is not likely to affect membrane packing, as shown in the monolayer experiments, it appeared that the toxins directly affected the anisotropy of TMA-DPH in the membrane, perhaps by altering the hydrogen bonding network at the water/membrane interface.

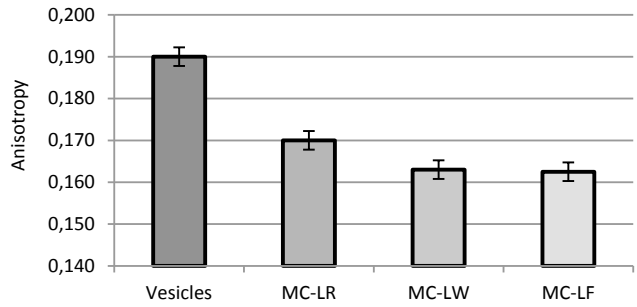

Figure 15. Steady-state fluorescence anisotropy of TMA-DPH. Microcystins were added to the vesicle solution and the fluorescence of TMA-DPH was measured in the absence and presence of microcystins, whereupon the anisotropy was calculated.

Steady-state fluorescence anisotropy was used to achieve information about the membrane environment around the fluorescent probe, TMA-DPH. When exciting the fluorophore with polarized light the emission is expected to be polarized. Rotational diffusion of the fluorophore can depolarize the emission. The extent of depolarization is described in terms of anisotropy. Membrane-bound fluorophores are able to reveal the internal viscosity of the membrane and whether the lipid composition is affected. Fluorescence anisotropy of the nonpolar fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has been used by Hermansky and Stohs to investigate fluidity in microsomal membranes in presence of MC-LR but no alterations in the membrane fluidity was observed (Hermansky and Stohs, 1991). By using TMA-DPH that partitions into the water/membrane interface, and egg phosphatidylcholine vesicles, a statistically significant decrease in fluorescence anisotropy could be observed when microcystins were present (Fig. 15).

4.3.3 Fluorescence emission maximum (IV)

MC-LW with the intrinsic fluorophore in the indole group of tryptophan was used in the steady-state fluorescence emission maximum experiments. The indole group in tryptophan has an emission spectrum that is dependent on the polarity of the local environment. The microenvironment around the tryptophan in the aqueous solvent changed when lipid vesicles were added. When comparing the fluorescence emission spectra recorded for MC-LW in the presence and absence of vesicles, an increased blue shift of the spectra in the presence of vesicle membranes was revealed (Fig. 16). This implied that the tryptophan in MC-LW translocated into a more hydrophobic environment as the toxin was allowed to interact with the bilayer membrane.

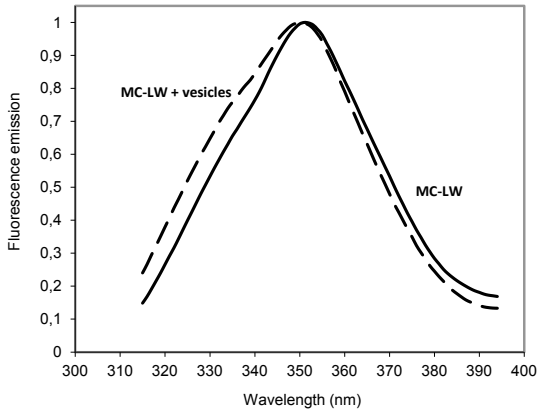

Figure 16. Fluorescence emission spectra of tryptophan in MC-LW in the presence and absence of vesicles. MC-LW was injected into the vesicle solution and the fluorescence emission spectrum was recorded after excitation at 280 nm.

4.3.4 Laurdan emission spectra (V)

Generalised polarisation (GP) values for Laurdan incorporated in DPPC bilayers in the presence and absence of toxins were calculated and the emission spectra were analyzed. The GP values were not influenced and the relative proportion of the two phases that DPPC show at the chosen temperature was not shifted in the presence of microcystins. DPPC have at 40°C two co-existing phases, liquid ordered and liquid disordered phases. If some of the phases had been stabilized by addition of a microcystin it could have been indicated by the fluorescence emission profile of the incorporated fluorophore Laurdan as the spectral properties of Laurdan are altered in response to lipid packing (Parasassi et al., 1990). The emission profile was not shifted and the emission intensity was not changed. None of the microcystin variants, added externally to the DPPC vesicles, had any observable effect studied by this technique on the phase behaviour of the bilayer lipids.

A single, well-studied phospholipid, DPPC, was preferred here due to its appropriate characteristics. The order of the hydrocarbon chains in a DPPC bilayer are decreased by high temperature due to the increased lateral and rotational motions of the lipid molecules (Purdon et al., 1976). To study whether the physical order of a vesicle bilayer is changed by microcystins, Laurdan was chosen as the fluorescent probe. Laurdan has an equal distribution in the liquid ordered and the liquid disordered phases that co-exist in DPPC vesicles at 40°C (Bagatolli, 2006). Laurdan, incorporated in vesicle membranes, can reflect the relaxation rate of water molecules present at the interfacial region of the membrane bilayer (Bagatolli, 2006).

4.3.5 Resonance energy transfer (V)

In an attempt to try to elucidate the location of tryptophan in MC-LW in relation to an incorporated sterol, CTL, in bilayers, resonance energy transfer measurements were performed. After excitation at 290 nm the emission intensity was measured at 374 nm. Although a good spectral overlap between the emission of tryptophan and the absorption of cholestatrienol was predicted (Holt et al., 2008), the emission intensity did not increase. It was concluded that energy transfer between the two fluorophores is not likely to occur.

Resonance energy transfer can occur if there is a spectral overlap of the emission spectrum of a donor with the absorption spectrum of an acceptor, provided that also other criteria are met, e.g. the distance between the two molecules and the orientation of the molecules (Clegg, 1995). The tryptophan in MC-LW was used as the intrinsic fluorescent donor since it is able to transfer energy in its excited state to another chromophore (Lakowicz, 1999). The fluorescent probe CTL located in the membrane interface acted as the acceptor (Holt et al., 2008). Cholestatrienol is a fluorescent analogue of cholesterol and partitions in the vesicle membrane much like cholesterol do (Schroeder et al., 1988).

4.3.6 Caco-2 cell viability and morphology (V)

Morphological changes in Caco-2 cells were investigated and compared for MC-LR, MC-LF and MC-LW. Morphological examination of Caco-2 cells exposed to microcystins revealed remarkable differences between different microcystin analogues. Cells exposed to relatively high

concentrations of MC-LR (50 µM) did not show changes in cell morphology after 22 h and 44 h of treatment, and resembled unaffected control cells (Fig. 17). On the other hand, cells treated with MC-LW and especially MC-LF, showed clear morphological alterations including apoptotic features with shrinkage, blebbing and loss of cell contact (Fig. 17). These changes appeared in a time and dose dependent manner (not shown). All three toxin variants changed the spreading of cells with minor effects by MC-LR, and a severe loss of cell number and cell-cell adhesion by MC-LF. Furthermore, blebbing was particularly evident for MC-LF.

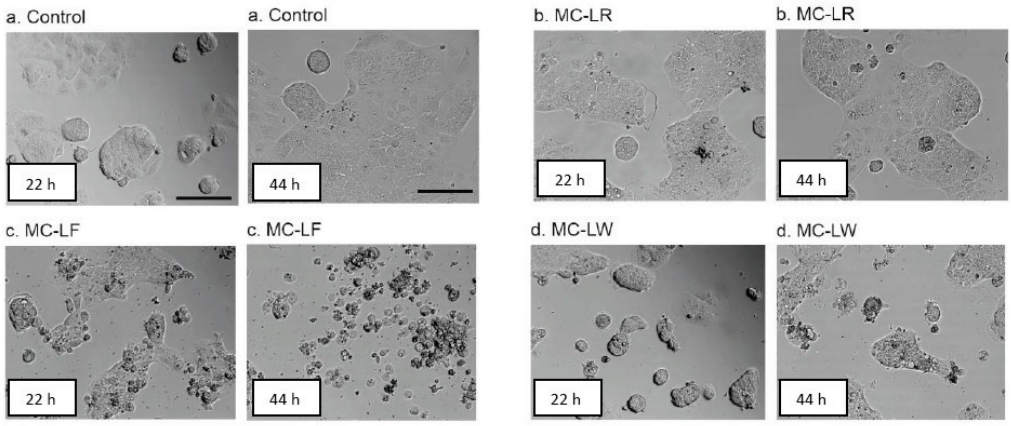

Figure 17. Morphological effects of microcystins in Caco-2 cells. Phase contrast microscopy images of Caco-2 cells after treatment with 50 µM microcystin for 22 h and 44 hours: MC-LR (b), MC-LW (c) and MC-LF (d). The control cells were treated with PBS (a). Scale bar is 100 µm.

4.3.7 Caco-2 cell proliferation and cytotoxicity (V)

To estimate plasma membrane damage, leakage of LDH was measured from Caco-2 cells treated with 50 µM toxins for 48 hours. Caco-2 cells not treated with toxins released spontaneously the same amount of LDH as MC-LR, about 22% of total cellular LDH (Fig. 18). Treatment with MC-LW and MC-LF caused an increased release of LDH, 45% and 52%, respectively, which was statistically significant ($p \leq 0.05$, t-test) from controls and MC-LR treated Caco-2 cells (Fig. 18). The % of LDH released from cells treated with toxins was calculated from the maximum cellular LDH. Lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells. When the plasma membrane is damaged, LDH is rapidly released into the cell culture supernatant (Korzeniewski and Callewaert, 1983). MC-LW and MC-LF showed an increase in the LDH activity indicating a loss of plasma membrane integrity of the exposed cells.

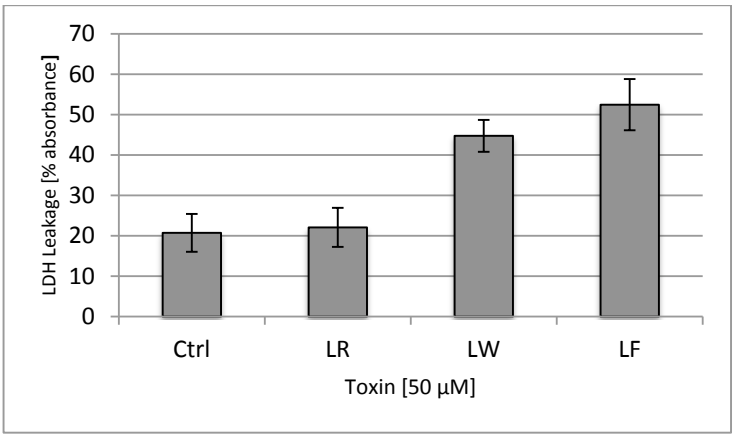

Figure 18. LDH leakage in Caco-2 cells after exposure to 50 µM of MC-LR, MC-LW and MC-LF. Control cells represent the spontaneous leakage. %LDH leakage was calculated from the maximum cellular LDH leakage. Values are mean ± standard deviation of four wells each. Data shown is a representative of 3 individually performed experiments.

A toxin concentration of 50 µM did not affect the activity of mitochondrial dehydrogenases for MC-LR, but reduced it to 45% for MC-LW and 19% for MC-LF after 48 hour toxin exposure compared to the control (Fig. 19). At a toxin concentration of 10 µM the activity was reduced to a lesser extent, 87% for MC-LW and 75% for MC-LF, compared to the control (Fig. 19). At 1 µM, cell proliferation was not affected by any of the microcystins tested.

In this experimental setup, the striking effects of MC-LR on Caco-2 cells that Botha et al. (2004) described were not observed (Botha et al., 2004). The culture conditions for Caco-2 cells have an effect on both morphology and the carrier-mediated transporters of the cells (Behrens and Kissel, 2003). Here a higher concentration of cells was used and the toxins were added at a time when cells had already formed a stable monolayer, i.e. one day after seeding. It was shown that the more hydrophobic microcystin variants MC-LF and MC-LW clearly inhibited Caco-2 cell proliferation to a larger extent than MC-LR. The proportion of metabolically active cells diminished considerably, measured by formazan cleavage after treatment of 10 µM of MC-LF and MC-LW. Metabolically active cells are able to cleave WST-1 and the amount of formazan measured can be directly correlated to healthy cells. The obvious effect caused by MC-LW and MC-LF is in agreement with previous results, where it was concluded that the more hydrophobic microcystin variants were clearly more toxic than MC-LR *in vitro* and thus potentially also *in vivo* (Monks et al., 2007; Fischer et al., 2010).

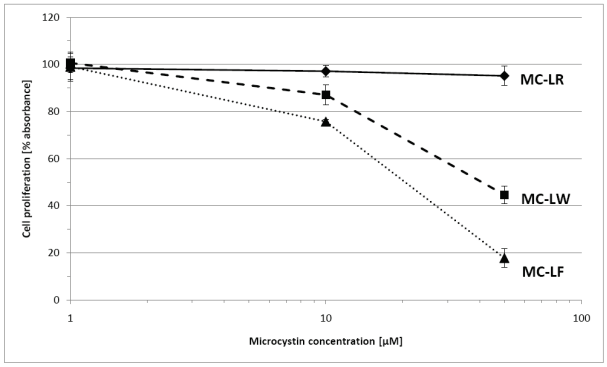

*Figure 19. Caco-2 cell proliferation, assayed by the WST-1 test. Cells were exposed to 1 µM, 10 µM and 50 µM of MC-LR (**—**), MC-LW (---) and MC-LF (···). On the y-axis is the % absorbance of the amount of formazan dye formed which correlates to metabolically active cells, compared to control cells. Values are mean ± standard deviation of four wells each. Data shown is representative of 3 individually performed experiments.*

4.4 PROTEIN PHOSPHATASE INHIBITION (V)

MC-LR, MC-LW and MC-LF were compared with respect to their capacity to inhibit PP1A. All three toxins inhibited protein phosphatase PP1A, MC-LF and MC-LW to a lesser extent than MC-LR. The normalized relative IC_{50} values calculated were 3.0 for MC-LF and 3.8 for MC-LW, compared to 1.0 for MC-LR. MC-LR appears to be the strongest inhibitor of the phosphatases among the studied microcystins (Eriksson et al., 1990; Monks et al., 2007; Fischer et al., 2010). In this study the protein phosphatase inhibition capacity and the acute toxicity did not correlate with each other. The more hydrophobic variants were weaker protein phosphatase inhibitors but showed more pronounced cytotoxic effects against Caco-2 cells (Fig. 18 and 19). Other mechanisms besides inhibition of protein phosphatases, as well as differences in uptake, transport, detoxification or targets, have been suggested to modulate the toxicity of different microcystin variants (Blom and Juttner, 2005).

5 CONCLUSIONS

Mass occurrences of toxic cyanobacteria limit the use of water for the purposes of drinking water production, recreation and fishing. Microcystins, common toxins produced by cyanobacteria, have documented adverse effects on human health, ranging from mild dermatitis to death. This PhD thesis contributed to risk assessment of microcystins.

a) Development of immunoanalytical methods and analyses of environmental samples

As the microcystin concentrations in environmental waters are highly unpredictable it is important that fast, convenient and sensitive analytical methods for the detection of microcystins are available.

In paper I, a time-resolved fluoroimmunoassay, TR-FIA, was developed. In an attempt to increase the sensitivity of immunoassays, time-resolved fluorescence was used as the detection method. To avoid steric problems in the recognition of antigens by their antibodies a relatively small label, europium, was coupled to MC-LR. Lanthanide chelates show a longlived fluorescence which made it possible to efficiently avoid interfering background fluorescence. Moreover, the large Stokes shift and the narrow emission enabled the fluorescent signal to be measured with high specificity. TR-FIA was shown to have a lower detection limit compared to ELISA as the detection limit was determined to 100 pg/l. That implies a broader dynamic range for TR-FIA.

In paper II and III, the developed immunoassay, TR-FIA, and a commercially available ELISA were used for screening of waterbodies on Åland Islands, SW Finland. The immunoassays were compared to chromatographic techniques and protein phosphatase inhibition assay in the detection of microcystin. Both immunoassays were shown to be sensitive, reliable and practical analytical methods for direct measurement of microcystins in field samples, without need to concentrate the samples. Microcystins were detected in roughly 10% of the studied lakes. The highest microcystin concentration was 30 µg/l (according to ELISA).

The development of immunoanalytical methods as well as the analyses of environmental samples generated tools, insight and data for exposure assessment which is an integral part of the risk assessment of microcystins.

b) Characterization of more hydrophobic microcystin variants

Several countries have adopted the WHO guideline value of 1 µg MC-LR per litre drinking water. The guideline value is based on data available from e.g. hazard and dose-response assessments. However, the data available is limited and many toxicity studies are based either on one common variant, MC-LR, or on cell extract preparations containing multiple toxins. The toxicity of microcystins varies greatly, and in this thesis focus was put on the more hydrophobic variants, MC-LF and MC-LW, in order to complement the numerous toxicological experiments conducted with MC-LR.

In paper IV and V interactions of MC-LR, MC-LW and MC-LF with artificial lipid membranes were studied to see whether the hydrophobicity of a microcystin has an effect on the lipid membrane. The relatively hydrophobic microcystin variant studied, MC-LF, showed the highest surface activity potential with the surface barostat technique used, closely followed by MC-LW. It was concluded that the more hydrophobic microcystins were clearly more surface active compared to MC-LR. Moreover, the fluorescence emission profile of MC-LW was shifted when interacting with vesicles. It was concluded that MC-LW and MC-LF locate closer to the membrane-water interface compared to MC-LR. Although an association between the more hydrophobic microcystins and lipids seemed to exist, any spontaneous penetration of microcystins across a lipid membrane was not interpreted to occur.

In paper V, the toxicities of three different microcystin variants were compared on Caco-2 cells. Apoptotic features deduced from morphological examination, with shrinking and blebbing of cells was most evident in MC-LF treated cells closely followed by MC-LW. Cytotoxicity, quantified by lactate dehydrogenase leakage, and cell proliferation and viability, assessed by mitochondrial dehydrogenases, showed obvious deteriorations when cells were exposed to MC-LF and MC-LW. Altogether, exposure to hydrophobic microcystins can result in different organotropism, toxicokinetics and bioaccumulation compared to that of e.g. microcystin-LR.

The characterization of (more hydrophobic) microcystins contributed to both hazard identification and dose-response assessment.

c) General statement concerning the overall risk caused by microcystins

Microcystins were frequently found in the study area (Åland Island's water environment) at concentrations which exceeded the WHO guideline level for microcystin-LR in drinking water. However, based on available research and knowledge it can be expected that modern drinking water treatment would in most cases reduce the detected microcystin concentrations to a safe level. None of the found microcystin concentrations were alarmingly high with regard to the recreational use of water. The prevalence of the potentially more harmful microcystins, i.e. the more hydrophobic variants, was very low according to the investigations reported in this PhD thesis. In the light of the presented results it can be concluded that the overall risk which microcystins constitute to the water users in the study area is fairly low as long as lake water is not directly used for drinking water, and mass occurrences of toxic cyanobacteria are avoided during recreational use of water.

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