



LUND UNIVERSITY

Biosensor-based Methods for Detection of Microcystins as Early Warning Systems

Lebogang, Lesedi

2014

[Link to publication](#)

Citation for published version (APA):

Lebogang, L. (2014). *Biosensor-based Methods for Detection of Microcystins as Early Warning Systems*. [Doctoral Thesis (compilation)].

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

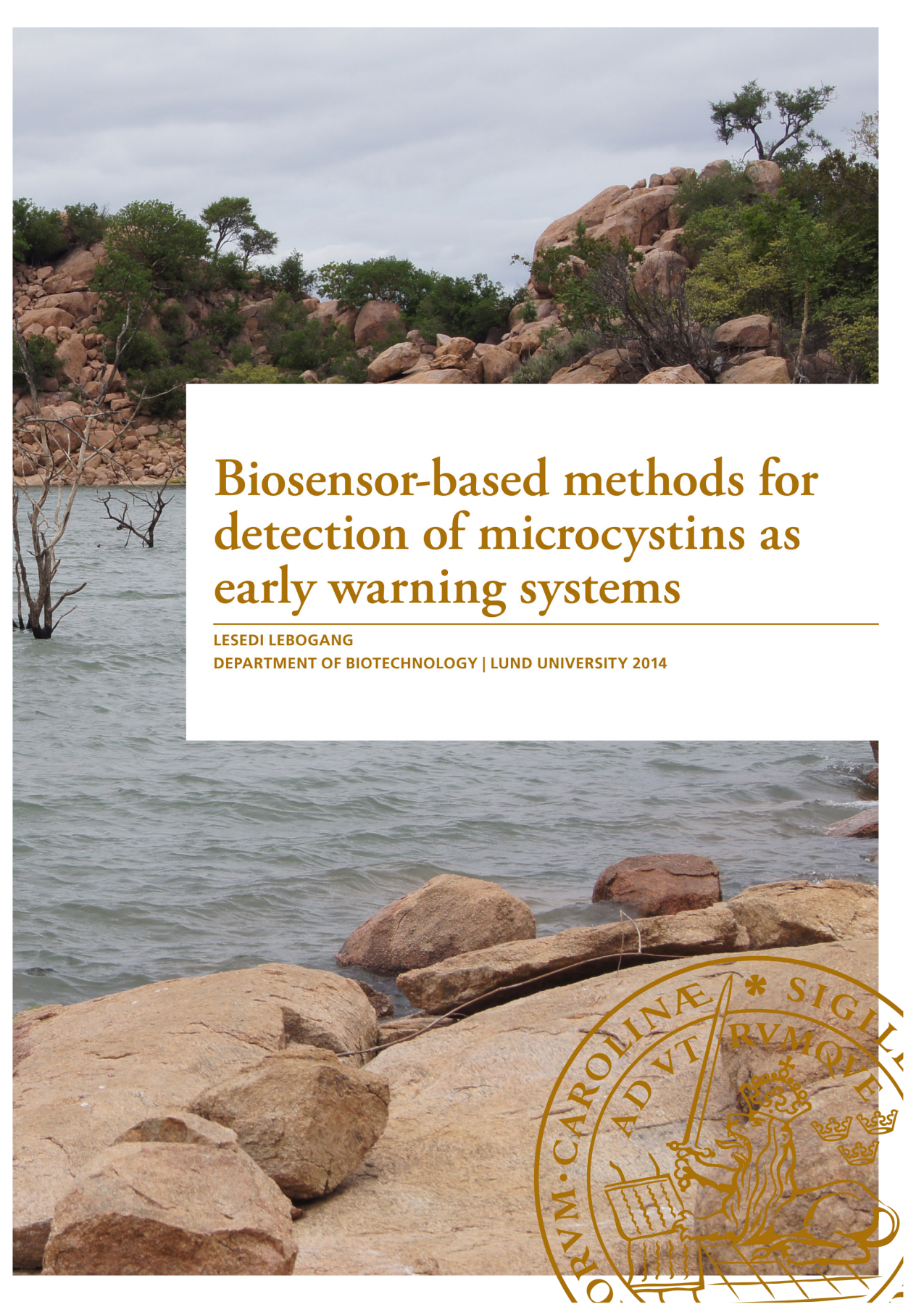
Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



Biosensor-based methods for detection of microcystins as early warning systems

LESEDI LEBOGANG

DEPARTMENT OF BIOTECHNOLOGY | LUND UNIVERSITY 2014



Biosensor-based methods for detection of microcystins as early warning systems



LUND
UNIVERSITY

Lesedi Lebogang

Department of Biotechnology
Doctoral Thesis
September 2014

Academic thesis, which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Wednesday, the 18th of September, 2014 at 10:30 a.m. in lecture hall B at the Center of Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Prof. Håkan Håkansson, Astrad AB, Astrakanvägen 6,
SE-22456 Lund, Sweden.

Doctoral Thesis 2014
Department of Biotechnology
Lund University
P.O. Box 124, 22100
Lund, Sweden

Cover photos by the author

Fig 1.1 Illustrated by Refilwe Lebogang

© Lesedi Lebogang

ISBN 978-91-7623-082-4

ISRN LUTKDH/TKBT--14/1155--SE

Printed in Sweden by Media-Tryck, Lund University
Lund 2014



Biosensor-based methods for detection of microcystins as early warning systems



LUND
UNIVERSITY

Lesedi Lebogang

Department of Biotechnology
Doctoral Thesis
September 2014

Organization LUND UNIVERSITY Department of Biotechnology P.O. Box 124, 22100 Lund, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue 2014-08-25	
Author (s) Lesedi Lebogang		Sponsoring Organization Botswana Int. University of Science and Technology	
Title and subtitle Biosensor-based Methods for Detection of Microcystins as Early Warning Systems			
Abstract <p>Cyanobacteria blooms are a water menace since they produce potent toxins that have been implicated in poisonings and deaths of humans and animals after consumption or contact with cyanotoxin-contaminated water. Of particular concern are cyanotoxins called microcystins, which are hepatotoxic cyclic peptides known to promote development of liver tumors in humans and animals. The high toxicity and increased occurrence of microcystins in drinking and surface waters have stimulated worldwide investigations and prompted the design of analytical techniques for the early stages of detection in order to protect human exposure to these fatal toxins. The major challenge for the accurate determination of microcystins is the large number of naturally occurring variants. To date, there are more than 80 different microcystin variants, and depending on the substituted amino acids in the structure, the hydrophobicity varies making it difficult for a single assay determination. Another problem is the low concentrations of individual toxins, which often are below the detection limits of existing assays. In order to achieve low concentrations detection, there is a need for a sensitive assay platform that gives a fast, quantitative determination of the microcystins below the stipulated limit.</p> <p>Regulations set for the monitoring of allowable microcystin levels in water are also becoming more stringent for water authorities to meet. Also, the predication of the algal blooming patterns becomes increasingly complicated. Of recent, algal blooms have shown to appear unexpectedly or come as late blooms due to changing weather patterns. Increased awareness of microcystin intoxications to the public that relies on surface waters has led to the World Health Organization (WHO) setting a guideline with a concentration limit for microcystins in drinking water of 1µg/L (10^{-9} M). Since these toxins are not efficiently removed during the conventional water treatment process, emphasis on alternative analytical methods are indeed required for the protection of drinking water supplies. In addition, the detection of cyanotoxins at very low concentrations is necessary for making a rapid intervention before the toxin concentrations reach harmful levels.</p> <p>In this thesis work, biosensor-based methods for ultra-sensitive detection of microcystins have been developed. Different biosensor configurations were investigated where the first study involved the development of a capacitive immunoassay using specific antibodies able to recognize a special part in microcystin structure called 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda). The study showed that microcystins could be detected at very low concentrations (down to 10^{-14} moles per liter) within 37 minutes. The developed biosensor was further applied for total analysis of microcystins produced in a batch culture of microcystin, aided by mass spectrometry to identify the different microcystin variants. A flow-ELISA-amperometric biosensor was also developed and investigated and showed an apparent rapid assay of 16 minutes (limit of detection 10^{-11} M). Finally, a micro-contact based molecularly imprinted polymer technique was explored as a cost-effective alternative for the expensive antibodies as ligands in the capacitive biosensor assay. In all cases, the developed biosensors showed both high selectivity and sensitivity and met the allowable detection limit set by WHO.</p> <p>In conclusion, the studies presented in this thesis demonstrated that low toxin determination with minimal sample preparation can be achieved using the investigated biosensor technology, and that miniaturization of the system can allow for portability and can be helpful for <i>in situ</i> monitoring of microcystins where sophisticated infrastructure is lacking. Also, the studies emphasize the need for more development of biorecognition molecules that will be able to monitor the group of microcystins at the same sensitivity while being able to discriminate against other non-related molecules. Automated and integrated system configurations used in all the experiments facilitated the analysis process by decreasing time and eliminating possible manual sampling handling errors.</p>			
Key words Microcystins, Drinking water, Adda-specific monoclonal antibodies, Biosensors, Capacitive, Amperometric, Flow-ELISA, Microcontact imprinting.			
Classification system and/or index terms (if any)			
Supplementary bibliographical information:		Language English	
ISSN and key title:		ISBN 978-91-7623-082-4	
Recipient's notes	Number of pages	Price	
	Security classification		

Distributed by (name and address)

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all the reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature: _____



Date: 2014-08-12

To my grandmother
(R.I.P)

Table of contents

Abstract	9
Popular summary	11
List of papers	12
My contribution to the papers	13
List of abbreviations	14
1. Introduction	17
1.1 Pollutants of emerging concern	17
1.2 Aims and Objectives of the thesis	19
1.3 Surface water pollution	20
1.3.1 Recreational water	20
1.3.2 Wastewater	21
1.3.3 Drinking water	22
2. Microcystin structure, biosynthesis and toxicity	25
2.1 General structure and description	25
2.2 Microcystins intoxication effects	27
2.3 Microcystin biosynthesis and toxicity	28
2.3.1 Genes involved in biosynthesis of microcystins	28
2.3.2 Mode of intoxication	29
2.4 Removal of microcystins from water	30
2.4.1 Adsorption and membrane filtration	30
2.4.2 Degradation	31

3. Conventional methods for detection of microcystins	35
3.1 Bioassays and immunoassays	35
3.1.1 Bioassays	35
3.1.2. Enzyme bioassay	36
3.1.3 Immunoassay	37
3.2 Chromatography	37
3.2.1 Liquid chromatography	38
3.2.2 Mass spectrometry	38
4. Biosensing	41
4.1 Principle of a biosensor	42
4.1.1 Biosensor categorization	42
4.2 The biorecognition platform	43
4.2.1 Antibodies	43
4.2.2 Molecularly imprinted polymers	44
4.3 Surface architecture and signal amplification	45
4.3.1 Surface functionalization	45
4.3.2 Nanomaterials and nanoenvironments as signal enhancement	47
4.4 Transductions methods	48
4.4.1 Amperometric biosensors	49
4.4.2 Capacitive biosensors	52
4.4.3 Sequential injection and automation of electrochemical biosensors	58
5. Challenges in microcystin analysis and biosensor development	61
5.1 Conclusions and future perspectives	61
Acknowledgements	63
References	65

Abstract

Cyanobacteria blooms are a water menace since they produce potent toxins that have been implicated in poisonings and deaths of humans and animals after consumption or contact with cyanotoxin-contaminated water. Of particular concern are cyanotoxins called microcystins, which are hepatotoxic cyclic peptides known to promote development of liver tumors in humans and animals. The high toxicity and increased occurrence of microcystins in drinking and surface waters have stimulated worldwide investigations and prompted the design of analytical techniques for the early stages of detection in order to protect human exposure to these fatal toxins.

The major challenge for the accurate determination of microcystins is the large number of naturally occurring variants. To date, there are more than 80 different microcystin variants, and depending on the substituted amino acids in the structure, the hydrophobicity varies making it difficult for a single assay determination. Another problem is the low concentrations of individual toxins, which often are below the detection limits of existing assays. In order to achieve low concentrations detection, there is a need for a sensitive assay platform that gives a fast, quantitative determination of the microcystins below the stipulated limit.

Regulations set for the monitoring of allowable microcystins levels in water are also becoming more stringent for water authorities to meet. Also, the predication of the algal blooming patterns becomes increasingly complicated. Of recent, algal blooms have shown to appear unexpectedly or come as late blooms due to changing weather patterns. Increased awareness of microcystin intoxications to the public that relies on surface waters has led to the World Health Organization (WHO) setting a guideline with a concentration limit for microcystins in drinking water of $1\mu\text{g/L}$ (10^{-9} M). Since these toxins are not efficiently removed during the conventional water treatment process, emphasis on alternative analytical methods are indeed required the protection of drinking water supplies. In addition, the detection of cyanotoxins at very low concentrations is necessary for making a rapid intervention before the toxin concentrations reach harmful levels.

In this thesis work, biosensor-based sensitive methods for determination of microcystins have been developed. Different biosensor configurations were investigated. The first study involved the development and application of a capacitive immunoassay using specific antibodies that recognize a special part in microcystin structure called 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

(Adda). The study showed that microcystins could be sufficiently detected at very low concentrations of 10^{-14} M within 37 minutes. The developed biosensor was further applied for total analysis of microcystins produced in a batch culture of microcystin, aided by mass spectrometry technique to identify the different microcystin variants. Furthermore, a flow-ELISA-amperometric biosensor was developed and investigated resulting in a rapid assay of 16 minutes (LOD 10^{-11} M). Lastly, a micro-contact based molecularly imprinted polymer technique was explored as a possible cheap alternative for expensive antibodies as sensor surface ligands. In all cases, the developed biosensors showed both high selectivity and sensitivity and met the allowable detection limit set by WHO.

In conclusion, the studies presented in this thesis demonstrated that low toxin determination with minimal sample preparation can be achieved using the biosensor technology, and that miniaturization of the system can allow for portability and be helpful for *in situ* monitoring of microcystins where sophisticated infrastructure is lacking. Also, this work emphasizes the need for further development of biorecognition molecules that will be able to monitor the group of microcystins at the same sensitivity while being able to discriminate against other non-related molecules. Automated and integrated system configurations used in all the experiments facilitated the analysis process by cutting down time and eliminating possible manual sampling handling errors.

Popular summary

Global invasion of our precious watercourses by cyanobacteria poses great threat to human- and animal health by deteriorating water systems. In fact, this problem is not likely to be solved in the near future as cyanobacteria are associated with pollution of waterways. Consequently, water sources are subject to contamination by natural toxins produced by abundant growth of cyanobacterial blooms all over the world. The produced toxins, which may be present at low levels without harm to humans or animals, can rapidly increase to become a dire health risk under certain environmental conditions. In addition, the rising global temperatures intensify the situation and excessive nutrient loading of watercourses through increased human activities by the expanding global populations, resulting in growth promotion of the potent toxin-producing blooms in surface waters.

In most cases, surface water supplies potable water to communities and hence any bloom expansion raises concern over human health safety. Communities with resource-limited settings often depend directly on water sources with limited treatment infrastructure available. As such, the presence of these potent cyanotoxins especially microcystins limit the use of the water. Although potent microcystins are known to occur in surface waters, there is still no known general method for their removal from contaminated water when the concentration increases and reaches lethal levels, thus reflecting a need for emergent mitigations. Several detection methods are available for detecting microcystins but are neither sensitive enough to measure low concentrations nor user-friendly. Therefore, the pressure to detect these toxins in trace amounts has led to increased research on development of analytical systems such as biosensors to overcome this problem.

In this thesis, sensitive biosensors have been developed for microcystin detection. Biosensor configurations combined with the advances in nanotechnology result in platforms with improved detection sensitivities compared to traditional analytical methods. It is expected that this new approach will dramatically reduce the analysis time, thereby reducing overall analysis costs without any loss of sensitivity and precision. These systems are made into small portable devices that can be taken to the site of contamination and quickly analyze the water. Mass production of such detection devices could help the communities out there who are in desperate need for clean and microcystin-free water.

List of papers

This thesis is based on the following papers, referred to by their Roman numerals in the text. The papers are attached as appendices at the end of the thesis. Reprints are published with permission of the respective journals.

- I. **Lesedi Lebogang**, Martin Hedström, Bo Mattiasson. Development of a real-time capacitive biosensor for cyclic cyanotoxic peptides based on Adda-specific antibodies. **Analytica Chimica Acta** 2014, 826:69-76.
- II. **Lesedi Lebogang**, Bo Mattiasson, Martin Hedström. Capacitive sensing of microcystin variants from *Microcystis aeruginosa* using a gold immunoelectrode modified with antibodies, gold nanoparticles and polytyramine. **Microchimica Acta** 2014, 181:1009-1017.
- III. **Lesedi Lebogang**, Jongjit Jantra, Martin Hedström, Bo Mattiasson. Amperometric-based Flow-ELISA for determination of microcystins using the versatile automated continuous flow system (VersAFlo). **Manuscript**.
- IV. **Lesedi Lebogang**, Martin Hedström, Bo Mattiasson. Micro-contact imprinting technique for capacitance-based detection of microcystin using a fully automated biosensor device. **Manuscript**.
- V. Bo Mattiasson, Kosin Teeparuksapun, **Lesedi Lebogang**, Martin Hedström. Nanoenvironmental effects influence the sensitivity of immunoassays dramatically. **Manuscript**.

My contribution to the papers

This thesis is based on the work contained in the following papers, referred to by roman numerals in the text. The papers are attached as appendices at the end of the thesis. All work presented in this thesis was performed under the supervision of Professor Bo Mattiasson and Assoc. Prof. Martin Hedström.

- Paper I.** I planned and performed all the experimental work and wrote the first draft of the paper. I edited and revised the paper together with other co-authors.
- Paper II.** I planned and performed all the experimental work, wrote the first draft of the paper. I was responsible for correcting the manuscript after comments from other co-authors.
- Paper III.** Jongjit and I planned and optimized the method. I performed the experimental work. I wrote the first draft of the paper. I finalized the manuscript together with other co-authors.
- Paper IV.** I performed all the experimental work and wrote the draft of the manuscript.
- Paper V.** I performed some parts of the experimental work. Bo wrote the first draft and I was responsible for finalizing the manuscript together with other co-authors.

List of abbreviations

ABTS	2,2'-azinobis (3-ethylbenzothiazolinesulfonic acid)
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
AuNPs	Gold nanoparticles
C18	Octadecylsilica
CCL	Candidate Contaminant List
CID	Collision induced dissociation
CIPPIA	Colorimetric immuno-protein phosphatase inhibition assay
CNBr	Cyanogen bromide
CV	Cyclic voltammetry
Da	Daltons
EDCs	Endocrine disrupting compounds
EDL	Electrical double layer
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
HPLC	High-performance liquid chromatography
HRP	Horse-radish peroxidase
IARC	International Agency for Research on Cancer
IUPAC	International Union of Pure and Applied Chemistry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
mAbs	Monoclonal antibodies
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MCLR	Microcystin-leucine (L) arginine (R)
MIPs	Molecularly imprinted polymers
MRM	Multiple-reaction monitoring
NRPS	Non-ribosomal protein synthases
ORFs	Open reading frames
pAbs	Polyclonal antibodies
PDA	Photodiode array
PKS	Polyketide synthase

POROS	Polystyrene-divinylbenzene
PP1	Protein phosphatase type 1
PP2A	Protein phosphatase type 2A
PPI	Protein phosphatase inhibition
SAM	Self-assembled monolayers
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization

1. Introduction

The great amount of chemicals and nutrients released into the environment in recent years has led to contamination and eventual eutrophication of surface waters. Contamination of the watercourses can result in loss of amenities that they provide such as drinking water, fishing and recreational activities. Drinking water is of most concern since eutrophic sources lead to excessive growth of algal blooms that produce toxins [1]. As such, environmental water monitoring is crucial for protection of human health and requires immediate actions in order to minimize potential risks associated to the polluted environments. Monitoring of hazardous contaminants and toxins in source water is thus imperative and of particular importance are the ‘pollutants of emerging concern’.

1.1 Pollutants of emerging concern

Pollutants of emerging concern are those contaminants that have not been given attention/scrutiny before and are now thought to be potential environmental and health hazards. Most of these pollutants are currently not covered by any environmental-quality regulation. While some have been recently discovered due to new improved analytical techniques, most are not necessarily new compounds since their presence in the environment have been known for years, though their environmental and health effects were not thoroughly evaluated [2, 3]. Hence, the phrase ‘pollutants of emerging concern’ is deemed more appropriate than ‘emerging pollutants’.

Micropollutants of emerging concern have recently received increased attention as alarming concerns have been raised against them. The most well-known and commonly discussed emerging contaminants are the endocrine disrupting compounds (EDCs), which include pharmaceutical residues [4]. Naturally occurring, are the cyanobacterial toxins. Cyanotoxins, particularly microcystins, have lately been a topic of concern after studies linked them to animal and human poisonings around the world [2]. Although most of the pollutants of emerging concern were unknown or not well studied before, microcystins studies goes way back in history by Francis [5], but it was not until late the 1980s when their health impact was realized on a larger scale, that they were characterized and intensively studied [6].

The perception regarding the potential risks posed by microcystins was not realized over a prolonged period of time by water authorities, regulatory bodies and the public in general, since a link between human/animal intoxications and microcystins exposure from water supplies was not identified. By the late 1900s, there was still limited concern due to insufficient knowledge about microcystins and their presence in water [6].

Among the first confirmed cases relating to microcystin exposure and acute poisoning included the death of cows and dogs in the Buffalo lake in 1960, the death of two army personnel who fell into and swallowed cyanotoxin-contaminated water in Staffordshire, England in 1989 [7]. These were followed by the most exposed incident involving haemodialysis patients in Caruaru, Brazil, in year 1996, which resulted in more than 50 deaths from the 130 patients treated with microcystin-contaminated water [8-10]. This is the tragedy that probably focused more attention towards the realization of the potential harm posed by microcystins. There were no regulations for microcystins in water until 1998 when USEPA listed cyanobacteria and their toxins on the Candidate Contaminant List (CCL) [1, 2, 6, 11, 12]. Increased awareness of microcystin intoxications to populations relying on surface waters led to the World Health Organization setting a guideline limit for drinking water at 1.0 µg/L microcystin-LR for daily consumption [13]. This guideline is still used in most countries such as Brazil, New Zealand and France. However, other countries have set their own microcystin limits for drinking water e.g. Australia and Canada have 1.3 µg/L and 1.5 µg/L respectively [1, 2, 9]. These demands led to increased efforts in the development of detection methods for microcystins and other closely related toxins.

The apparent massive proliferation of algal blooms that produce cyanotoxins and increased mammalian poisonings is a growing concern. Many reports have attributed toxic algal blooms to rising climate temperatures due to global warming and nutrient enrichment from agricultural run-offs and post-treatment sewage disposals [14, 15]. As high nutrient loading in watercourses occurs, so will the toxin-producing cyanobacteria. Of particular concern are microcystin-producing genera, such as *Microcystis*, which are ubiquitous and thrive in high-light, warm, nutrient-rich and stagnant water around the world [16, 17]. Moreover, in water-scarce countries such as Botswana, building of dams across the rivers will contribute further to this problem.

Microcystins are generally known for their acute poisoning effects on humans and animals, where exposure to these toxins elicits liver damages and fatal haemorrhage [16]. In addition to hepatotoxic effects, in 2006, the International Agency for Research on Cancer (IARC) declared microcystin a carcinogen [18, 19]. Moreover, microcystins have already been indicated to have EDCs effects since recent reports implicated them in male rats sperm decline [20, 21], thus making them high risk water contaminant that needs extensive monitoring programs.

Despite the widespread risk of microcystin exposure through drinking water, microcystins are currently not part of routine monitoring of water treatment facilities in many countries of the world (e.g. Botswana) due to lack of guidelines as well as

stringent regulations on cyanobacteria. Water and health authorities normally act on alerts, which could mean that the toxins have already spread and potentially have caused harm.

1.2 Aims and Objectives of the thesis

Cyanotoxins are one of the emerging environmental contaminants of concern that the drinking water industry is faced with throughout the world. Detailed information about microcystin group of cyanotoxins has revealed new and structurally different microcystins. The discovery of new variants then renders the existing microcystin detection methods inefficient because they targeted a few variants when they were developed. Considering water safety, the ideal situation will be to measure all the existing variants in the water for any detection to be effective against these potent cyanotoxins. Thus, development of detection methods with no discrimination against related toxins is highly desirable, but first and foremost researchers must be aware of the multivariation displayed by microcystins before deciding to develop any method.

A successful analytical method for microcystin detection requires high selectivity for microcystin group. In addition, the method must be sensitive and robust to work in real matrix situations. The capability to rapidly measure microcystin at real-time is also desirable. Employment of biosensors detection has great potential in environmental testing since they offer various advantages including portability for both laboratory and field analyses.

The research reported in this thesis involved developing sensitive biosensors for detection of microcystins at levels below the allowable limit set by WHO. The emphasis is put on the variations that exist among microcystin variants and should be carefully considered when developing analytical methods. Different types of biosensors were therefore evaluated on selectivity and sensitivity to determine trace amounts of microcystins. **Paper I** deals with the development and optimization of a sensitive capacitive biosensor, which is aimed at cumulatively detecting broad spectrum of microcystins. Cumulative detection is achieved by using monoclonal antibodies that are specifically designed for selective recognition of a common element in the microcystin structure. **Paper II** reports successful application of the capacitive immunosensor developed in **paper I** for the cumulative detection of microcystin variants. This demonstrated that direct monitoring of environmental contaminants in the field and real-time detection with minimum sample preparation and handling is possible.

The work reported in **paper III** was aimed at developing a simple and quick Flow-ELISA coupled to amperometric detection for determination of microcystins. The same antibodies used in **papers I and II** were immobilized in a micro column. The immunocolumn was used as a reaction chamber for a two-step competitive ELISA employing an automated sequential injection system.

The stability of the biosensing layer is desirable when pursuing sensitivity and reliable biosensing systems. In further development to achieve more stable biosensing layers, molecularly imprinted polymers (MIPs) were considered for an attractive antibody alternative. In a preliminary study MIPS for MCLR were designed using a microcontact imprinting technique, and investigated for use with the capacitive detection (**paper IV**).

Factors that influence sensitivity of capacitive immunosensors are discussed in **paper V**. Capacitive measurements directly measure binding kinetics at a very small space intimate with the transducer surface, insinuating that sensitivity will depend mostly on density of immobilized antibodies on the transducer surface and the ultimate surface thickness after binding event. The concentration of antibodies at the electrode surface dictates the amount of antigen binding and hence the capacitive response of the immunosensor. The transducer surface thickness determines the starting (baseline) capacitance, which have an influence on subsequent capacitance change upon antigen binding according to the electrical double layer theory discussed in sections of this thesis.

1.3 Surface water pollution

Water pollution occurs when contaminants that result from un- or partially treated harmful compounds enter the watercourses and cause dire consequences on aquatic life. Water pollution greatly affects drinking water sources such as rivers and lakes all over the world. In many water-strained countries, it is the major cause of water-related health issues when out of no choice people drink from these polluted sources. A large part of the world population relies on surface source water [22, 23]. Wastewater contributes immensely to pollution of water sources as large volumes of wastewater from households, industries and agriculture are produced every day and disposed of into rivers that communities depend on for various activities.

1.3.1 Recreational water

The presence of cyanotoxins, in particular microcystins in recreational water is now recognized as a potential public risk and is receiving increased attention the world over. Water bodies used for recreational activities (fishing, swimming, boating and wind

surfing) are not as highly regulated as drinking water sources. However, there are measures put on how to respond to blooming events; (1) by alerting the public of the presence of toxic blooms via warning signs or restricting public access by closing the affected areas, (2) if the area has persistent bloom occurrence the recreational facilities can be relocated to areas with low potential of bloom risk [9, 24, 25]. Individuals should avoid water-related activities in areas with dense blooms since toxins present therein can be absorbed from the water via ingestion or inhalation and can cause skin irritations [26].

1.3.2 Wastewater

There is a tendency for populated settlements to be near major waterways for easy access to water. Hence, the long-lasting upwards-going trend for people to move towards water-resourced areas continues. Unfortunately, these movements put enough stress on the aquatic environments as they are subjected to many types of pollution. Eventually, these vital resources that many people especially resource-limited communities directly depend on for daily life will be lost.

Historically, water in rivers, lakes and streams has been used as waste disposal by diluting and washing away pollutants. With growing populations, this practice has excessively increased and led to overloading of watercourses with nutrients [27]. The nutrient-rich wastewater becomes a big concern as it affects ecosystems well beyond the discharge area and poses human health risk. Inevitably, these discharges lead to eutrophication of receiving waters and stimulation of algal blooming. Algal blooms are harmful to livestock, humans and other organisms when they release some of the potent toxins (microcystins) into the water. As such, the degradation of the water sources will interfere with the use of the water for aquaculture, fisheries, recreation, drinking and livestock watering.

Pollution from unspecified points and runoffs is referred to as nonpoint sources. Eventhough nonpoint source pollution is mostly linked to seasonal agricultural activities and heavy precipitation it is the main contributor to water pollution (Fig 1.1). This type of pollution cannot be regulated and monitored as their point source counterparts (municipal sewage treatment facilities) [28]. By establishing sufficient measures for quality control as well as strategies for water quality monitoring, nonpoint source discharge pollution could be minimized and the cost of cleaning up polluted rivers and streams could be significantly decreased. However, the task of controlling pollution is complex mainly due to these nonpoint sources of pollution.

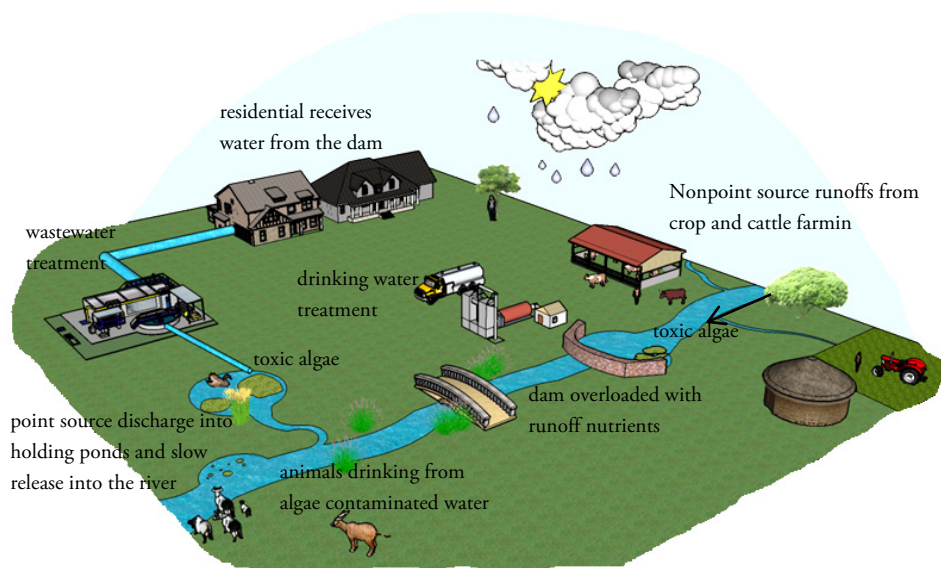


Fig 1.1: Sketchup pictorial illustrating ways of surface water pollution. Illustration by Refilwe Lebogang.

1.3.3 Drinking water

Cyanobacterial blooms and their release of toxins into source water seriously affect the drinking water quality and mostly threaten human health. Surface waters that experience episodes of cyanobacterial blooms are thus subject to contamination by cyanotoxins, i.e. microcystins, which is the commonly encountered class in drinking water. Large quantities of these toxins are released into the water during cyanobacteria bloom senescence [26]. Depending on the timing and duration of the bloom, they can persist for months in temperate climate to almost continuous in subtropical regions [9]. Microcystins dissolved in water are extremely stable, they can remain potent even after boiling. Since humans most often come into contact with microcystins through the consumption of contaminated drinking water, these toxins present a greater danger to people who rely solely on the boiling of water as a way of treatment. An example is when a sugar industry in Sweden got contaminated with untreated river water containing microcystin-producing *P. agardhii*. People who consumed the water exhibited signs consistent with microcystin intoxication even after boiled water was used for making tea [24].

Exposure to high levels of microcystins especially intravenous exposure (usually experienced by kidney dialysis patients) is acute and deadly e.g. the Caruaru, Brazil case discussed earlier. Even at low levels microcystins can be damaging after extended period of exposure. Effects of chronic exposure was evidenced by a case in Southern China, where people utilizing surface water for drinking and cooking suffered more from liver

cancer compared to people using deep wells. It was observed that surface waters in the region experienced abundant cyanobacteria growth and hence the attribution of this variation to microcystins in their drinking water [15, 29, 30]. Another example relates to the Zimbabwean capital, Harare, where children from one specific area suffered from gastroenteritis each year. It was later found that the reservoir supplying that district contained decaying microcystin blooms [26]. In addition, livestock watering points are also threatened by microcystin presence in many parts of the world. In Botswana, microcystins have been isolated from a river pond that receives sewage effluent and were suspected to cause livestock deaths which occurred around the area [31].

These few examples highlight that the presence of microcystins in drinking water is an alarming global issue, and it is of vital importance that the levels are monitored to preserve the safety of drinking water. Globally, surface water impaired by the presence of microcystins is a challenge to water authorities as they struggle to provide the expanding populations with clean drinking water. Therefore, sufficient protection from microcystin exposure requires sensitive and efficient detection and quantification techniques for these cyanobacterial cyclic peptide toxins. It is also required that the methods should be sensitive to the whole spectrum of microcystins at concentrations well below the regulatory limits.

2. Microcystin structure, biosynthesis and toxicity

Cyanobacteria growth is a natural phenomenon, however recently both a severe increase and a geographical expansion of these algae have been recorded. Their prevalence is associated primarily with eutrophic water caused by nutrient overloads. These toxin-producing blooms need monitoring due to their increase in abundance and high toxicity risk [32].

2.1 General structure and description

Microcystins are among the most commonly occurring and ubiquitous cyanotoxins. They are hepatotoxins produced by cyanobacterial genera such as *Microcystis*, *Planktothrix*, *Anabaena*, *Nostoc* and *Aphanizomenon* [31, 33-37]. Microcystins are cyclic heptapeptides containing a conserved and unusual β -amino acid tail called 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) at position 5 of their structure. The toxicity of microcystins is attributed to this Adda side chain and the stereochemistry of its conjugated double bond [22, 33, 38]. Major variations of amino acids are exhibited at positions 2 and 4 (X and Y). These variations are the basis for a two-letter denotation of the different microcystin variants as exemplified in Fig 2.1. The most commonly occurring as well as the most studied microcystin variant, which accounts for most of the reported poisonings (MCLR) contains the amino acids leucine (L) arginine (R) in position 2 and 4, respectively. Amino acids at positions 3 and 7 could either be methylated or demethylated [6, 15, 39, 40]. These variations bring about more than 80 structurally varied microcystins with an average size of 3 nm [1]. Different variants confer different toxicities attributed to their differing hydrophobicity and membrane permeability [33, 41].

Nodularins, which are produced in eight variants by a *Nodularia* sp. that lives in brackish water, are closely related cyclic pentapeptides showing the conservation of the Adda molecule [42, 43]. The adda tail, to date, has been found only in these cyclic hepatotoxins of cyanobacterial origin. The uniqueness of the Adda molecule to microcystins and nodularins provides the basis for their detection because whatever

structural modification occurs, the Adda remains conserved, i.e., is a marker characteristic for all cyclic cyanotoxins [22, 43]. Furthermore, owing to the presence of D-amino acids and the cyclic nature of their structure, microcystins and nodularins represent a chemically stable molecule group and can persist in water for up to 10 weeks at average temperature and alkalinity after release from the cells [15, 44-46].

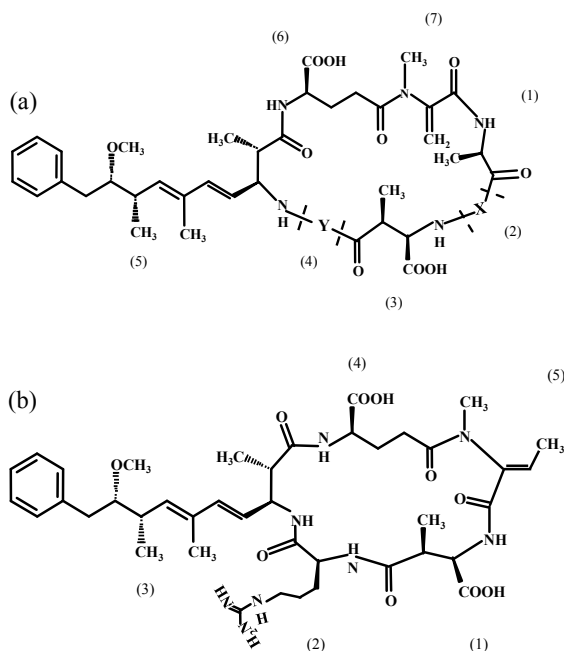


Fig 2.1: (a) General structure of microcystins; (D-Ala¹-X²-D-MeAsp³-Y⁴-Adda⁵-D-Glu⁶- Mdha⁷). X and Y indicate the variable amino acids at position 2 and 4 respectively. (b) Structure of nodularin; (D-MeAsp¹-L-Arg²-Adda³-D-Glu⁴- Mdha⁵)

Over the last two decades, there has been a rapid increase in the number of microcystin variants that have become isolated, characterized and identified (Table 2.1). The main reason for this escalation is the development of advanced analytical technologies, some of which will be discussed in the next chapter.

Table 2.1: Increasing number of reported microcystin variants over the last two decade.

Year	Variants	References
1994-1998	50	An and Carmichael,[39]; Bourne et al. [47]; Dawson, [40].
1999-2000	60	Sivonen and Jones, [33]); Hitzfeld et al. [26].
2001-2005	>60	Fischer et al. [22]; Newcombe et al. [48]; McElhiney and Lawton, [49]; Antoniou et al.[1].
2006-2007	>70	Zhao et al. [50]; Crush et al. [51]; Ho et al. [52]; Hiller et al. [43].
2008-2011	> 80	Welker et al. [53]; Kim et al. [30]; Campo and Ouahid,[36]; Tong et al. [54].
2011-2013	>89	de la Cruz et al. [55]; Srivastava et al. [56].

The continuous increase in the number of microcystin variants has rendered the earlier developed microcystin analysis methods inadequate and is still a major challenge to overcome.

2.2 Microcystins intoxication effects

Microcystin intoxications affect both humans and livestock mainly through consumption of contaminated water, skin exposure or inhalation. Microcystin poisonings can also occur through consumption of microcystin-contaminated fish since the toxin is known to accumulate in fish tissues especially in the liver and kidney [31, 57]. Moreover, cyanobacteria dietary supplements have been shown to contain traces of microcystins [58]. Although long-term exposure to microcystins affects mostly the liver, other organs such as kidney and colon can also be affected [24]. It is worth noting that these effects are time and concentration dependent, as such toxicological effects may differ from case to case [14]. In human populations that are highly exposed to microcystins, intoxication show marked effects such as hepatic haemorrhage, gastroenteritis and allergic reaction [16, 40, 59]. Microcystin toxicity expressed as LD₅₀ (intraperitoneal) ranges between 50 µg-1200 µg toxin per kg mouse body weight depending on the variant tested [15, 26].

2.3 Microcystin biosynthesis and toxicity

Microcystin-producing cyanobacteria species are unicellular and usually form floating colonies under conditions of intense sunlight, developing into dense scums at the water surface [26]. The role of microcystins in the cells is still not understood [26, 60, 61]. There are many hypotheses about the purpose of microcystin production ranging from being a siderophore [60] to a deterrent of cyanobacteria predators. The role of microcystin as a deterrent to predators has however been argued since the toxins remain inside the cells until cell lysis [61]. Some new postulates indicate that microcystins protect cyanobacterial cells from oxidative stress [62], which could explain why their biosynthetic genes have been retained and widely distributed across cyanobacteria genera irrespective of the geographical location [60, 62, 63].

2.3.1 Genes involved in biosynthesis of microcystins

Bioanalytical technology in combination with molecular biology has enabled the study of microcystin production at gene level by characterizing DNA responsible for its production. Tillett et al. [64] and Nishizawa et al. [65] identified and sequenced the gene cluster that is responsible for microcystin synthesis. The sequence analysis revealed that the biosynthetic region, called *mcy*, contains a 55kb DNA that consists of 10 open reading frames (ORFs) denoted *mcyA-J* (Fig 2.2), coding for 10 proteins responsible for sequentially incorporating amino acids constituting microcystin peptide and tailoring its functions. The region is arranged into two gene operons (*mcyA-C* and *mcyD-J*) that are transcribed in opposite directions. A larger operon (*mcyD-J*) containing 6 gene clusters encodes for polyketide synthase (PKS) and non-ribosomal protein synthases (NRPS) domains. PKS and NRPS are multifunctional enzymes that catalyze the formation of Adda and its linkage to D-glutamate, whereas the smaller operon (*mcyA-C*) encodes the NRPS modules for extending of the molecule intermediates to a heptapeptidyl stage and subsequent peptide cyclization [36, 63-65].

Aside the fact that the reported sequences may represent only a fraction of microcystin biosynthetic genes, they revealed a highly conserved structural organization of multi-enzyme domains (NRPS/PKS), which account for the same basic reaction steps in different cyanobacterial genera; *Microcystis* (Chroococcales), *Planktothrix* (Oscillatoriales) and *Anabaena* (Nostocales) [64-67]. However, some differences have been found regarding the gene arrangement, which appeared to be shuffled between the genera (Fig 2.2). Differently, the non-producing strains lacked the complete *mcy* gene cluster, although exceptions may exist. While comparison with nodularin biosynthetic genes showed lack of some modules especially the *mcyA* and *mcyB*, still there was enough evidence of a close relation and that the nodularin biosynthetic cluster may have evolved from the microcystin cluster by domain deletion [37, 60, 63].

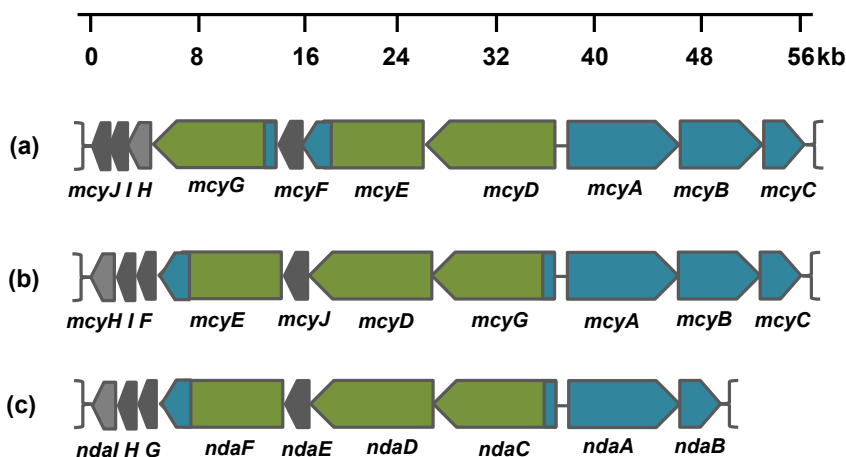


Fig 2.2: Organization of *mcy* gene cluster involved in microcystin synthesis (a) *Microcystis* (Tillett et al. [64]), (b) *Anabaena* (Rouhiainen et al. [67]), and (c) nodularin synthesis by *Nodularia* (Moffit and Neilan, [68]). The figure is redrawn with modification from Dittman and Börner, [37].

The information regarding the gene conservation exhibited among microcystin-producing cyanobacteria are now being used to establish molecular bioanalytical techniques for specific screening of toxic blooms [37]. This would be useful in the early phase of bloom growth to tell whether it is microcystin producing or not.

2.3.2 Mode of intoxication

Microcystins are nonribosomal peptides that manifest liver toxicity by irreversible inhibition of serine/threonine protein phosphatase types 1 and 2A, denoted PP1 and PP2A, respectively [36, 62, 69]. These enzymes are the major components of the cell signaling pathways and upon contact with microcystin through the Adda chain, a covalent bond is formed with a cysteine moiety of their catalytic subunits [26, 40]. Microcystin interaction with the enzymes occurs in two steps, first by binding to the enzyme and subsequent formation of covalent adducts [36].

The protein phosphorylation-dephosphorylation process is an important but very complex route for the regulation of protein activity in the cells, catalyzed by phosphatases. As such, uncontrolled PP1 and PP2A inhibition would lead to hyperphosphorylation of proteins, destruction of cytoskeletons and liver cells damage with consequent haemorrhage. Unregulated phosphatases also display tumor-promoting activities by causing excessive oxidative stress in cells, eventually triggering apoptotic processes [36]. These enzymes should be active to suppress tumor proliferation.

2.4 Removal of microcystins from water

Removal of microcystins has been a challenge using current and conventional water purification methods because of their low molecular weight in the range of 800-1100 Da [26, 33]. It is often possible for toxins to pass through the filters because of their small size. Additionally, microcystins exhibit remarkable stability and persistence, making their presence in the municipal water a widespread risk [70].

Cyanobacteria cells containing microcystins can however be removed safely using coagulation [71], flotation and flocculation [26]. Sedimentation does not work since the colony-forming cells are buoyant and tend to float on the surface [15]. Algaecides, e.g. based on copper sulphate removes cyanobacteria blooms, however the resulting lysis of the cells release the toxins into the waters, increasing their concentrations even more. Algaecides and other chemical means of toxin elimination (chlorination, ozonation, permanganate oxidation) and their by-products may be toxic to other aquatic organisms when used since high doses are required for an effective destruction [1, 15]. Several other algal removal methods have been developed included degradation (bio- and photo-), adsorption and electrochemical oxidation.

2.4.1 Adsorption and membrane filtration

Adsorption is the most investigated method for removal of dissolved microcystins from the water. Activated carbon has become an increasingly useful toxin removal technique since it contains different pore sizes, making it effective in trapping all types of proteinaceous toxins. Different types of low-cost wood-based activated carbon have been used for adsorbing microcystins, mainly in batch adsorption experiments [72]. To date, activated carbon (powdered and granulated) has been the most effective adsorbent in removing microcystins with 99 % removal reported [15]. However, the use of activated carbon suffers from competition with natural organic matter. The non-regenerable usage and disposal of spent carbon are also a challenge. Furthermore, high toxin loads need to be tested in order to establish saturation levels [1, 12, 26].

Generally, traditional filtration is used in conventional treatment plants to remove intact algal cells, but it sometimes exacerbate the problem by breaking the cells to release the toxins, which then pass through the filters [26, 73]. Nanofiltration and ultrafiltration are other options for removing microcystins where up to 98 % removal has been reported [26]. However being a pressure driven technique, much energy is invested in the nanofiltration process, hence becoming non-viable treatment method for resource-disadvantaged regions of the world [12]. Slow sand and bank filters, although biologically aided have been suggested as good options for microcystins removal [52].

2.4.2 Degradation

It is generally known that the cyclic microcystin peptides are resistant to breakdown, i.e., they can remain intact and toxic in the water even after the death of the cyanobacterial blooms. Their stable chemical structure gives them the edge to exist in both warm and cold water as well as tolerating changes in pH [1, 33, 44, 48]. However, as natural organic molecules these toxins have shown susceptibility to photo- and biological degradation [47, 74, 75].

2.4.2.1 Biodegradation

As mentioned earlier, the cyclic structure of microcystins is responsible for their stability against factors that could degrade them. However, studies suggest that some bacterial genera, especially *Sphingopyxis* and *Pseudomonas* are able to cleave the toxin ring, linearise and make it non-toxic. The genes encoding the hydrolytic enzymes have been isolated and the biodegradation pathway has been suggested [47, 52]. The most studied microcystin variants in microbial degradation are MCLR and MCRR and it is proposed that the ring is cleaved at a peptide bond between Adda and arginine, followed by hydrolysis at Ala-Leu to yield a tetrapeptide, then Adda as the prominent final product (Fig 2.3). The enzymes encoding the genes involved in degradation process have been characterized. These genes have shown to be similar in all bacterial isolates currently reported to degrade microcystins [1, 47, 52].

A microbial biodegradation pathway for microcystin by *Sphingomonas sp.* has been characterized with the *mlr* gene cluster playing a crucial role in the sequential enzymatic hydrolyses of the peptide bonds. This cluster consists of four genes; *mlrA* (microcystinase), *mlrB*, *mlrC*, and *mlrD*, and codes for at least three intracellular enzymes [47, 74].

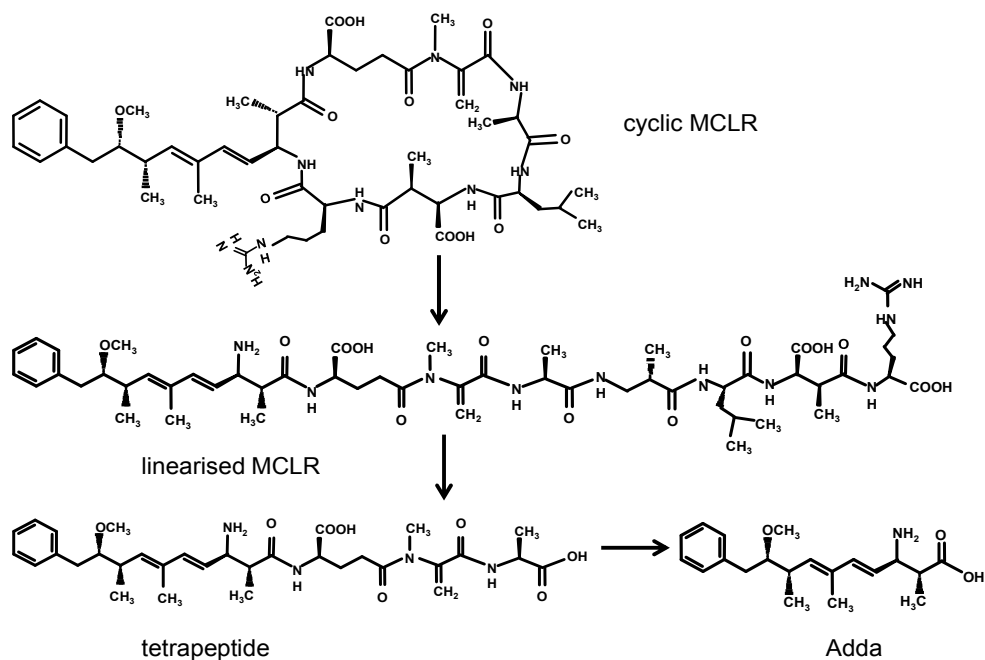


Fig 2.3: Microbial degradation pathway of MCLR. Modified from Bourne et al. (1999).

- *mlrA* - most important gene of this cluster because it encodes the enzyme that initiates hydrolysis of cyclic microcystin structure by cleaving the Adda-Arg peptide bond in MCLR and opening the cyclic structure, resulting in a substantial reduction in molecular toxicity, 200 times compared to the initial cyclic MCLR.
- *mlrB* - encodes a serine peptidase which degrades the linear MCLR to the tetrapeptide (H-Adda-Glu-Mdha-Ala-OH).
- *mlrC* - degrades the tetrapeptide to Adda or other small amino acids.
- *mlrD* - has not shown to express any hydrolytic activity but suggested to code for a transporter protein.

Nodularins can also be degraded by microorganisms, possibly due to the similar mode of action of *mlrA* that hydrolytically cleaves the cyclic structure at the Adda-Arg peptide bond [75] although exhibiting relative resistance [52]. Consequently, biodegradation seems to be the potential way of detoxifying microcystin and needs more evaluation.

2.4.1.2 Photocatalysis

Detoxification of the chemically persistent microcystins is also achievable by photodegradation using ultra-violet (UV) irradiation. Most importantly, degradation is efficient in the presence of pigments and humic acids. However, high intensities of

UV are needed for substantial decomposition of microcystins [15, 76]. Under natural conditions pigments and humic acids have shown to enhance the UV degradation process by absorbing the light near the absorption maximum of microcystins (238 nm) and acting as photosensitizers for the formation of highly oxidizing species (free radicals) [77]. Photosensitized reactions effect rapid degradation of microcystins through isomerization of the molecule [76]. Photocatalysis can also be enhanced by combination with titanium oxide (TiO₂) oxidation. In contrast to microbial decomposition, the degradation mechanism is through isomerization and cleavage of the conjugated dienes of the Adda amino acid [78].

Literature concerning microcystin treatment efforts is overwhelming but none of the method alone seems efficient for application in large scale. Due to these challenges on microcystin treatment/removal, once microcystins are present in the water intervention is the ideal way to prevent exposure. The best prevention approach will be to solve the root of the problem of water pollution, but with the increasing activities and effects of global climate change it seems impossible to control the conditions that promote proliferation of the toxin-producing blooms. Hence the next logic step for prevention of public exposure to microcystin is the qualitative determination and detection of these toxins, despite the claims of methods being effective in the removal of microcystins. A number of existing detection methods will be discussed in the following chapter as well as methods developed in this thesis in chapter 4.

3. Conventional methods for detection of microcystins

It is of crucial importance to know about the presence of cyanotoxins in the source water for remedial interventions to be instituted and prevent health risk exposures. A variety of detection approaches have been developed for quantification of microcystins that include bioassays, immunoassays and several chromatographic techniques combined with UV, photodiode array (PDA) or mass spectrometry (MS) detection [79]. Eventhough they all have the ability to quantify and identify microcystin variants, they are expensive to perform, require high technical expertise as well as pretreatment steps [79]. The most recent technology to be applied in microcystin detection is biosensor technology using different biorecognition and signal producing formats. The biosensor concept will be discussed in detail in the next chapter.

3.1 Bioassays and immunoassays

3.1.1 Bioassays

Bioassays of microcystins are qualitative and have been used primarily to determine the toxicity of microcystins in the sample. The use of both invertebrate and vertebrate animals has been reported in literature [80, 81]. Plants bioassays are also reported in which bioaccumulation and growth inhibition studies were carried out [49, 82, 83]. Despite their usefulness in screening, bioassays were found to be non-specific and very slow [50, 84].

Table 3: Some of the bioassays developed for microcystin detection and toxicity testing.

Bioassay type	Specific bio-	Comments	References
Plants	Mustard seedlings (<i>Synapis alba</i> L), watercress (<i>Lepidium sativum</i>)	uptake and growth inhibition	McElhiney et al. [49]; Gehring et al. [82].
Vertebrate	mouse /rats	bioethics issues	Siame et al. [31]; Sheng, [81].
Invertebrate	Brine shrimp (<i>Artemia salina</i>)	more time needed than mouse assay	Lee et al. [80]; Vezie et al. [85].

3.1.2. Enzyme bioassay

The concept of protein phosphatase inhibition (PPI) is already discussed in section 2.3.2. In the light of many reports that microcystin inhibits protein phosphatase types 1 and 2A, the inhibition capability has led to development of sensitive bioassay methods for screening of microcystins and their related nodularins in the affected waters. An and Carmichael, [39] introduced the colorimetric PPI assay (PPIA), which has been applied successfully for quantification of microcystins in environmental samples. The bioassay was suitable in detecting microcystins and nodularins at nanograms levels. Different toxin variants exhibit different intoxication modes on animals and therefore toxicity can either be masked or enhanced in some bioassay methods when variants act together. Fortunately, the PPIA is useful when estimates on total toxicity contributions of different variants are required [16, 84] because either of the two enzymes binds equally well to microcystins.

Being sensitive enough and extremely helpful in confirming toxicity of microcystins in samples, this method is however not widely used due to the non-specificity of the enzymes to microcystins and nodularins. The enzymes are also sensitive to other environmental protein phosphatase inhibitors such okadaic acids and tautomycin [35, 84]. In order to distinguish microcystins and nodularins from other protein phosphatase inhibitors, Metcalf et al. [35] refined the PPIA into a novel assay by incorporating antibodies into the assay and called it colorimetric immuno-protein phosphatase inhibition assay (CIPPIA). The CIPPIA was highly specific and successful in discriminating between microcystin and non-microcystin related protein phosphatase inhibitors.

The major problems that limit the use of bioassay for microcystins screening in the water include interfering compounds such as disinfectants and their byproducts, various metals present in the water. Lack of sensitivity to reach low microcystin levels

for drinking water guidelines and lack of speed to allow real-time monitoring of water supplies [16, 86] are also some limiting factors. Therefore, bioassays are not practicable in monitoring cyanotoxins when aiming for public protection.

3.1.3 Immunoassay

Immunoassays are based on the binding properties between the (contaminant) antigen and the target-specific antibodies. Among several immunoassays techniques, enzyme-linked immunosorbent assay (ELISA) combined with colorimetric detection is the most common. ELISA-based analysis for microcystins with high specificity and sensitivity has been developed using either polyclonal antibodies (pAbs) or monoclonal antibodies (mAbs) [14, 87]. A lot of antibodies have been generated against these cyanotoxins since the early development of microcystin antibodies. For instance, pAbs against microcystins were developed by some research groups [88, 89]. However, most of them were directed against the MCLR variant [81]. In parallel to the production of pAbs, mAbs against microcystins were also produced, early examples being mAbs for MC-LA by Kfir et al. [90]. Most of these antibodies showed variable cross-reactivities with different toxic peptide variants [14, 16, 50]. The variation in cross-reactivity created a big problem of either under- or overestimation of total microcystin content resulting from different affinities due to structural variations [50, 91].

To solve the problem of many variants and unknown microcystins, next generation of mAbs was introduced in 2001 by Zeck et al. [38] and Fischer et al. [22] when both groups developed mAbs that targeted the special Adda amino acid. They proved that all tested variants showed similar cross-reactivities and could give reliable total microcystin content [14]. The Adda-specific mAbs are now commercially available and have been used in many applications including development of a novel ELISA kit and in biosensors (**papers I and II**), as well as immuno-chromatographic columns (**paper III**). Although, validated ELISA kits are at present widely used for microcystin analysis and are highly recommended by legislators, they are not sustainable methods for routine monitoring. These immunoassay kits are extremely expensive and this factor has proven to be the limiting factor in analytical methods when mAbs are used [14]. The Adda-specific ELISA was used as a confirmatory test in **paper II**.

3.2 Chromatography

The bioassay methods discussed in section 3.1.1 give very useful toxicity information, they are however not good when it comes to routine surveillance since they cannot identify the different variants present in the sample [92]. Therefore, there is need for development of integrated analytical techniques that can provide reliable information

on both identity and quantity of microcystins. To fill the void, liquid chromatographic methods, especially HPLC with either UV, photodiode detection methods or mass spectrometry were developed [58]. The principle of chromatography is that a mixture of a sample is separated through a separation column. In order to identify compounds in a sample, retention times of individual molecules appearing as peaks are matched with known standards or structural information is obtained by MS.

3.2.1 Liquid chromatography

The most applied analysis for microcystins and nodularins is HPLC [16, 93]. When coupled with other detection techniques such as UV/Vis and PDA [92, 94], it has the ability to provide both quantitative and qualitative information, even though the sensitivity of LC-UV is limited [95]. For HPLC-UV, the absorption profile between 200 and 300 nm is usually used for identification of microcystins and nodularins. Most of these cyclic peptide toxins have UV absorption maximum at 238 nm, which is attributed to the presence of a chromophore resulting from a diene in the Adda residue. However, those containing amino acids with aromatic rings such as MCLW, which contain tryptophan, have their maximum absorption at 222 nm [16, 84, 93].

Octadecylsilica (C18) HPLC columns and solid-phase extraction cartridges are the typically used stationary phase for microcystin separation [30, 58, 84, 96] and retentions are based on hydrophobic interactions. But because the variations in the structure confer variable hydrophobicities [15, 41], C18 matrix may be not suitable for all variants. Immunoaffinity columns have been used for separation or capture in immunochromatographic assays. Immunocapture column modified with Adda-specific antibodies on CNBr activated Sepharose followed by amperometric detection was used in **paper III**, and will be discussed further in chapter 4.

3.2.2 Mass spectrometry

In the light that total toxicity is usually contributed by more than one microcystin variant, using the LC-UV/PDA would thus become impossible to identify all the present variants considering the large number of microcystin variants (plus unknown variants) and that the standards of microcystin variants are limited. In this case, LC-MS/MS offers a breakthrough in variants detection in that it obtains structural information and quantification of microcystins when no analytical standards are available or when new variants appear in the sample [31, 50, 92].

Moreover, to accurately determine the molecular masses of proteins and peptides, tandem-MS with the ability to elucidate structure and identify molecules by determining amino acids sequence is normally employed to produce both molecular ions and characteristic fragment ions. MS offers highly sensitive analysis of molecules,

eventhough the knowledge about the compound is needed in order to apply product ion scan and/or multiple-reaction monitoring (MRM) techniques [43]. Several MS setups using different ionization techniques/configurations for microcystin analysis have been reported, such as MALDI-TOF [9, 53], electrospray ionization (ESI) [30, 43, 75, 94, 96, 97].

In this thesis, a triple-quadrupole ion trap ESI-MS coupled to a C18 column was used for identification of microcystins. It became easy because microcystins possess a conserved and unique amino acid (Adda) that can be used as a fingerprint. The Adda fragment has a mass over charge (m/z) of 135, which always dominate the MS/MS profile on CID fragmentation when microcystins are present. This fragment can be used to detect unknown or microcystins when standards are not easily available [58, 94, 95, 97].

In **Paper II**, LC-MS/MS was used to separate and identify variants that were produced in a batch cultivation of *Microcystis aeruginosa*, which was isolated from oxidation ponds in Botswana. MS analysis of the samples was performed using positive mode electrospray ionization. Full-scan mass and CID MS/MS product ion spectra were acquired in the ranges m/z 500-1100 and m/z 100-1000, respectively, and were used to obtain the molecular weight and information of the resulting fragment ions.

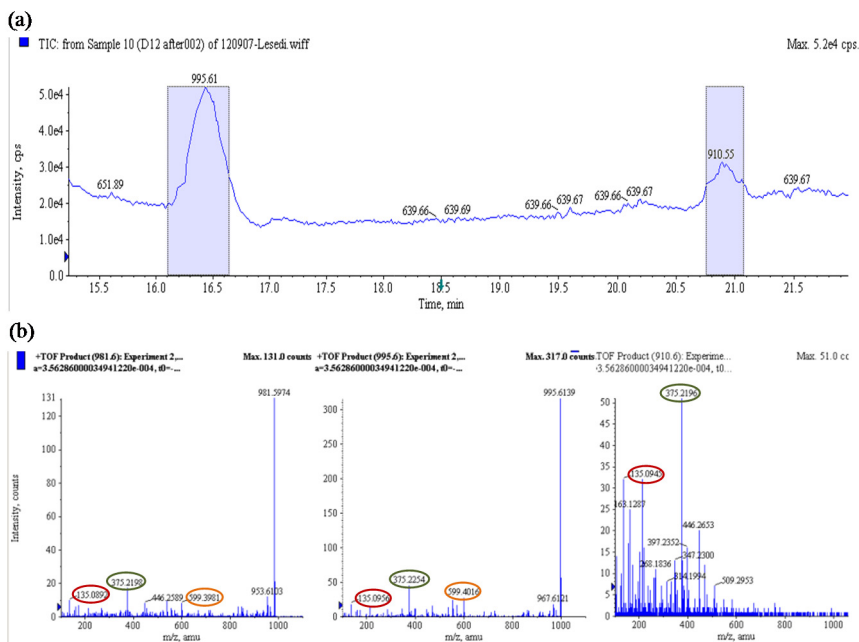


Fig 3.1: LC-MS/MS analysis of microcystin variants from a laboratory culture of *M. aeruginosa*. (a) total ion chromatogram obtained from ODS-C18 column in RP-HPLC showing retention peaks of microcystin variants, peak 1 combined two microcystin variants. (b) Full scan spectra of extracted precursor ions and their CID spectra in positive ion mode MS extracted from **paper II**.

Fig 3.1 (a) shows the LC peaks and their retention time and (b) CID spectra following the precursor ion that was used as fingerprint for microcystin. It shows that three microcystin variants were produced (viz MCLR, dmMCLR and MCLA with m/z of 995.6, 981.2 and 910.6 respectively). Characteristic ions most importantly m/z 135 were produced by the CID of the protonated microcystin molecules. This ion correlates to the Adda fragment. Other ions are m/z 213, 375, 553 and 599 [47, 53, 95, 97, 98]. The importance of MS was revealed when two microcystin variants with close molecular weight were co-eluted from the LC column within a few seconds and differentiated in MS analysis (Fig 3.1(a)) the first peak.

LC-MS/MS methods come out as powerful for separation and multi-toxin identification. Apart from advantages they present, HPLC-UV or -PDA methods are confined to a narrow microcystin spectrum analysis because of the limited availability of microcystin standards, or very costly if available [49]. This makes it even difficult to analyze unknown samples that may contain different variants. In view of this and the implication that the concentration of the present variants are expected to be additive [22], it is thus expected that antibodies that target a common moiety in microcystin structure will confer cumulative determination [14] and be applicable in antibody-based biosensors. The biosensors technique will be introduced in the next chapter.

4. Biosensing

Awareness and knowledge about the emerging or earlier unknown contaminants have created an urgent demand for highly sensitive and selective analytical techniques capable of detecting such molecules even at trace levels with high precision. Normally both selectivity and sensitivity are difficult to accomplish in one assay, however with the advent of biosensors this was made possible through the use of biomolecular recognition events and electronic detection. Whilst the biorecognition element gives the biosensor specificity, sensitivity is attained through the transducer [99].

The ability of biologically active compounds to bind to their receptors can be directly measured when an analyzer integrates a biorecognition molecule with a physicochemical transducer to produce an electrochemical signal, which is proportional to a given target molecule (analyte) [17, 99-101]. The signal is then conveyed to a detector device as a readable output. The design and configuration of an instrument with such properties is called a biosensor (Fig 4.1). By IUPAC definition a biosensor is a 'self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is retained in direct spatial contact with a transducer element [102].

There has been a steady progress since Clark and Lyon [103] pioneered the biosensor concept in 1962 when they developed an oxygen biosensor [100]. Yet, in recent years biosensors have established a niche in the biotechnological applications of ultrasensitive analytical techniques. Biosensors achieve this status by (1) combining the potential of automation and integration for portable devices, (2) mobile communication technology to design and develop software chips to control parameters and operations of analytical system and (3) exploiting the newly researched area of nanotechnology to design sensitive and selective biosensing platforms [100, 104] aimed at detecting biomarkers in various fields such as medical diagnostics, food production, environmental monitoring, bioprocess control as well as security and welfare applications [105]. The versatile nature of biosensors has enabled design of many sensing platforms for any analyte of concern.

4.1 Principle of a biosensor

Constructing a biosensor involves attachment of a biomolecule onto a support that makes contact with a transducer. The analyte then binds to the attached biomaterial to form a biomolecule-analyte complex, which in turn produces an electrical response that can be measured. Alternatively the analyte is converted to a product before being translated into an electrical signal [99].

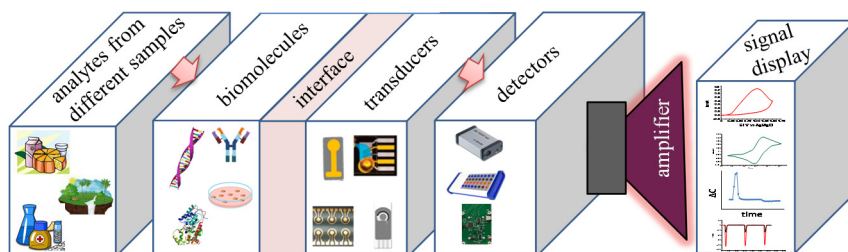


Fig 4.1: A schematic illustration of a biosensor configuration.

As schematically demonstrated in Fig 4.1, the main components of a biosensor configuration are thus (i) the biomolecule for specific and selective recognition of the analyte, (ii) the interface architecture dictating immobilization strategy on the support system, (iii) the transducer for signal processing and amplification. All these parts influence the ultimate sensitivity of the biosensor [100]. The choice of each component will be discussed in the next sections of this chapter.

4.1.1 Biosensor categorization

Biosensors can be classified based on either the type of biorecognition molecule or detection modes used. The former will have two groups namely catalytic and affinity recognition-based biosensors whereas the latter is divided according to the transducing mechanisms employed (section 4.4).

Catalytic biosensors involve a catalytic reaction between biorecognition molecules, in most cases using enzymes or whole cells to convert the analyte into a new measurable product [100]. Affinity biosensors involve binding between biorecognition molecule (antibodies, receptor proteins, DNA, aptamers) and the analyte. Subsequently, the binding event is evaluated for analyte quantification either by determination of change in mass, light path, layer thickness, potential shift in case of complex formation or electrical charges exposed to the transducer due to the binding reaction that yield measurable conformational changes. After the analyte detection, the initial state is

reinstated by dissociating of the affinity complex formed with some form of regeneration solution [99, 100].

4.2 The biorecognition platform

The first step in the construction of a biosensor is the demonstration of the biorecognition event between the biomolecule (ligand) and the targeted analyte, normally in the form of an assay. Once this is confirmed, the biomolecule can be attached to an appropriate transducer via a support, at this point the whole configuration is now called a biosensor. The choice of a biorecognition molecule is dependent upon desirable properties such as intrinsic biological selectivity and specificity towards the target analyte. The most popularly used biorecognition molecules are antibodies and antibody-based biosensors are referred to as immunosensors. Other molecules of biological origin; cells, enzymes, receptor proteins, DNA and some artificial receptors (MIPs) are also commonly used [106]. The next sections will focus only on antibodies and MIPs since they were used in this thesis.

4.2.1 Antibodies

The ability of antibodies to specifically recognize target analytes has been widely exploited in bioanalytical systems. Antibodies are capable of providing real-time information on biomolecular interactions upon binding to the analyte [104, 107, 108] in immunoanalysis. Immunosensors are based on immobilized antibodies on some kind of support material or surface for the detection of specific antigens using different transduction modes. The immobilization strategy of antibodies on a sensor surface is very crucial in terms of analytical performance of the immunosensor, since it influences the affinity, orientation and stability of the antibodies. Antibodies in aqueous solutions maintain their distinct three-dimensional structure and are free to move. However, their movement could be restricted and/or experience conformational change when they are immobilized at an interface affecting their orientation and structural functional [100, 109]. Moreover, the surface distribution of antibodies and the size of the analyte are crucial factors in avoiding steric hindrances during the binding process [108]. Therefore, mild interactions between antibodies and surfaces should be considered in order for antibodies to maintain their biological function. Adda-specific monoclonal antibodies were immobilized on functionalized gold electrode in **papers I and II** and on CNBr activated Sepharose beads (**paper III**).

4.2.2 Molecularly imprinted polymers

Although antibodies are highly selective to their molecular targets, they are however expensive and face challenges of instability. When natural receptors for particular molecules are difficult to obtain, or does not exist, artificially constructed receptors with high affinity and selectivity towards their target molecules can be used instead. One way to produce such constructs is by designing molecularly imprinted polymers (MIPs) [110, 111]. MIPs are formed by polymerization of monomers in the presence of an analyte, (in this context called a template). A monomer-template solution is deposited on an electrode surface and allowed to polymerize under for example, UV light [112, 113], and form a thin polymer mould around the template. After extraction of the template molecule, cavities complementary to the analyte are produced. The analyte can then rebind to these cavities with high affinity and specificity [114].

Traditionally, MIPs were synthesized for separation purposes but have since evolved to find their application in sensors [115] either as small MIP particles and powder, or imprinting at surfaces mimicking immobilization of biomolecules [104, 110]. Biosensors based on surface molecular imprinting either by thin film or microcontact imprinting have been used for detection of various analytes including cancer biomarkers [116], mostly using optical measurements, for instance Surface Plasmon Resonance [112]. Recently from our group at the Department of Biotechnology, Lund University, a microcontact imprinted capacitive sensor for a model protein, BSA was developed [117]. A microcontact imprinting concept (Fig 4.2) for small molecules was developed and used for trace-determination of microcystin-LR variant in a capacitive sensor in **paper IV**.

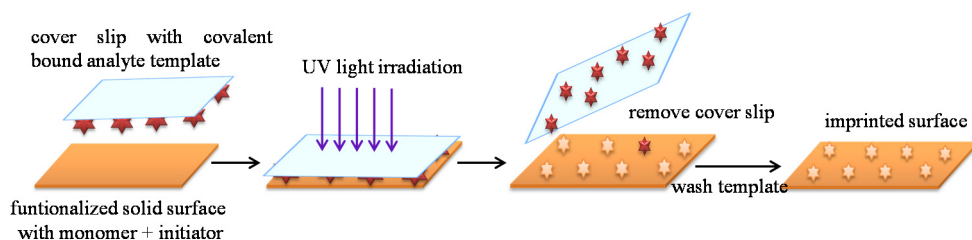


Fig 4.2: Schematic illustration of microcontact imprinting concept.

The main advantage of MIPs over their natural counterparts is the rigidity and stability they present [115, 118]. Whilst the usefulness of antibodies is restricted mainly by their inherent instability, application of highly selective MIPs in biosensors represents an alternative to the use of antibodies [110, 114, 115].

4.3 Surface architecture and signal amplification

As mentioned earlier, the overall performance of biosensors is generally determined by the design of the biointerfaces that connect the biological sensing element to the transducer at a nanoscale level. As such, immobilization of a biorecognition molecule is of crucial importance with regard to orientation of receptor sites, aiding strong binding between the ligand and its target analyte.

4.3.1 Surface functionalization

The molecular characteristics of transducer surfaces prepared for biomolecule binding are highly important in order to create dynamic interfaces such as exposed functional groups and/or redox state [119]. One most important aspect of biomolecule immobilization is to maintain its inherent affinity for the target. Moreover, the interface between the anchor surface and the biomolecule should be uniformly created and be stable for a reliable and reproducible results [120]. The choice of material for surface functionalization in biosensor application depends on the transduction technique used. Most widely used materials are gold, graphite, glass, glassy carbon and some metal oxides [100]. The next sections will focus on a few most commonly used surface designs.

4.3.1.1 *Self-assembled monolayers*

Organization of molecules to form highly ordered organic films on novel metal surfaces occurs through strong chemisorption between the surface and the head group of the available organic molecule. This phenomenon is referred to as self-assembly of monolayers [54, 121, 122]. Self-assembled monolayers (SAMs) are used to functionalize electrode surfaces prior to bioligand attachment as well as providing a highly packed and ordered surface for electrode insulation. Gold surfaces can be readily reacted with the sulfur head of thiolated molecules followed by activation to yield the desired functional groups for subsequent immobilization of biorecognition molecules [122-124]. Depending on the length of SAM desired on the electrode surface, different thiolated molecules can be used. Limbut et al. [123] carried out capacitance measurement experiments to compare immobilizations of different SAMs of thiourea, thiotic acid and 3-mercaptopropionic acid on a gold electrode, and concluded that thiourea was best SAM immobilization for their purpose.

Advantages offered by SAMs are that the highly ordered structure mimics the cellular microenvironment, thus providing a favourable environment for immobilizing biomolecules. Their compatibility with metal surfaces is beneficial for electrochemical measurements in biosensor applications [122, 125]. Nevertheless, SAMs are prone to defects created either by pinholes, low-density or uneven packing of monolayer chains,

thus making the surface irreproducible [126]. As it has proven difficult to create a defect-free SAMs, Losic et al. [127] integrated polytyramine into the SAM monolayer. Other groups used long chain alkylthiol such as dodecanethiol to block defective monolayer [106, 128, 129].

4.3.1.2 *Matrix immobilization*

A wide range of matrices have been used as support for bioreceptor immobilization including Sepharose, agarose and silica beads as well as the cross-linked polystyrene-divinylbenzene (POROS). Modifications of these materials are easily attained and can be modified into different surface functionalization suitable for covalent binding of different biomolecules, thereby giving enhanced stability to the biomolecule [130, 131]. Application of CNBr activated Sepharose beads for antibody immobilization for use in flow-ELISA experiments is demonstrated in **paper III**.

4.3.1.3 *Electrodeposition of thin film polymers*

For the successful immobilization of a biorecognition ligand in an electrochemical biosensor, the anchor layer should be electrically insulating and extremely thin, particularly for capacitance measurements [105, 123, 126, 132] as will be discussed later in the chapter. An alternative to SAMs is coating the electrode surface with thin films of polymer, of particular non-conducting polymers by electrochemical deposition. Electropolymerization of thin films directly onto the electrode surface has advanced rapidly in the recent years of biosensor research. Polymers of *o*-phenylenediamine, pyrrole and tyramine have been used [96, 106, 120, 126, 127, 129, 133]. Owing to the ease of their preparation and possibility of precise and reproducible mass production, electropolymerized films are preferred in the fabrication of biosensing platforms.

In contrast to SAMs, by controlling and adjusting electrochemical conditions of electropolymerization, the desired thickness of the film can be easily tuned. Due to their self-limiting in growth, non-conducting polymer provide ultrathin films and because of the film permselectivity the sensor will present improved sensitivity [120, 127, 134, 135]. Wu et al. [126] obtained a 50 nm polytyramine layer under the electropolymerization conditions used. Besides acting as insulators, these polymers also provide functional groups for coupling to the biomolecules, and are therefore suitable for use in capacitive biosensor studies [106, 126, 127]. An electropolymerized tyramine layer on a gold electrode was used for immobilization of antibodies after activation of the free amino acids with glutaraldehyde in **papers I and II**, and activation using acryloyl chloride in **paper IV**.

4.3.2 Nanomaterials and nanoenvironments as signal enhancement

Small biomolecules, e.g. peptides may not evoke significant changes upon binding to their receptors and thus signal enhancement should be considered [136]. Microcystins are relatively small molecules (around 1000 Da) and at times require augmentation to induce pronounced changes in biosensor configurations. A conventional way to amplify signal in biosensors has been the use of enzyme as labels by exploiting their ability to catalyze specific reactions or be inhibited by certain molecules [136, 137]. Recently, the use of nanoscale configurations has greatly improved sensitivities of biosensor systems [104, 136, 138].

Rather than using enzymes as amplification labels to improve the performance of biosensing surface, nanostructures can be incorporated into the biosensing platforms [100, 136, 137]. Incorporation of nanomaterials to enhance and improve sensitivity has been extensively discussed [124, 139, 140]. Nanomaterials play several important roles in signal improvement such as labelling and increasing surface area. Interactions between nanomaterials, biomolecules and modified surfaces have to be compatible to establish a nanoparticle-biological interface (nano-biointerface) for desired reactions to occur in the suspending medium [141]. Examples of nanomaterials used as immobilization support and sensitivity enhancement for microcystins biosensors include carbon nanotubes and nanowires [137, 142, 143], silver and gold nanoparticles [54, 144], magnetic nanoparticles and quantum dots [145, 146]. In addition, these nanomaterials can be synthesized into different shapes and sizes depending on the purpose and can be functionalized in many different ways [147]. Most notably, gold nanoparticles have been highly acclaimed for the good properties they present. Besides being easy to synthesize, gold nanoparticles are biocompatible, increase surface-to-volume ratio of the sensing surface resulting in high loading capacity of the biorecognition molecules to be immobilized. Also, gold nanoparticles have good electrical properties which can create charge effects that facilitate electron tunneling to the electrode surface, thereby increasing the sensitivity of the biosensors [109, 124, 136, 140, 147].

Nanostructures and biomolecules are both nanoscale objects and interact at a nanoscale level, within a few nanometers from the surface [141, 148], consequently a nanoenvironment is created. The shrinking scale of reaction space means that traditional methods used in conventional bioanalytical techniques are no longer sensitive enough to analyze biomolecules at trace or single molecule levels [107, 149]. For highly sensitive biosensors, the interactions between the nanoscale materials must occur at an appropriate small space, which is intimate with the transducer. Achieving nanospace interactions provides advantages such as (a) extremely small space that allows for fast analysis due to short distance that the analyte has to diffuse (b) single molecular analysis due to confinement of molecules in very small volumes of the nanodevice, as well as high surface area to volume ratio [138, 141, 149].

Paper V discusses nanoenvironmental factors that affect sensitivity of capacitive immunosensors; (1) antibody-antigen binding kinetics are influenced by the local concentration of antibodies at the electrode surface, (2) the thickness of the sensing layer plays a major role on the sensitivity of the capacitive detection.

Some of the surface architectures and signal enhancement strategies discussed in this subchapter have been applied in the development of sensitive biosensors for the work presented in this thesis (Fig 4.3) and will be discussed later in the thesis. Electropolymerized films of polytyramine, with subsequent functionalization were used as an insulating and anchor layer in **papers I, II and IV**. Gold nanoparticles were used as signal enhancement in the detection of microcystins (3 nm) in **papers I and II**. CNBr activated Sepharose beads were used for antibody immobilization in **paper III**.

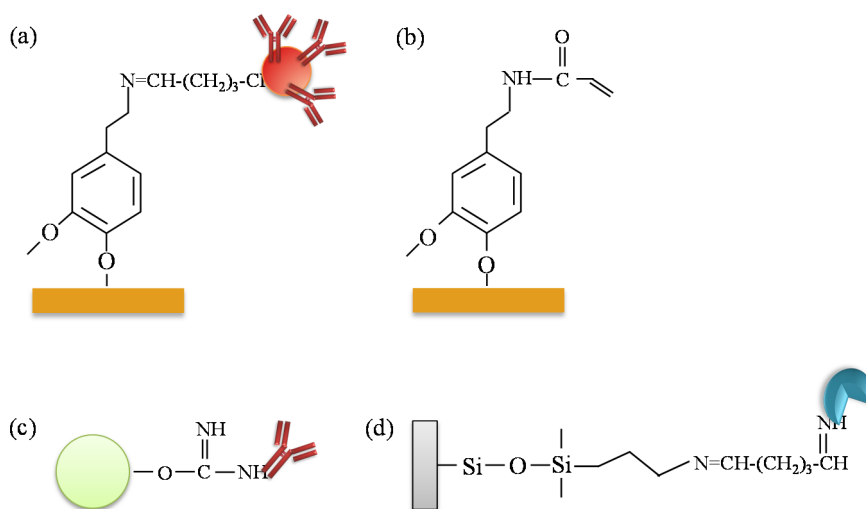


Fig 4.3: Schemes of different formats of surface functionalization and biomolecules immobilization used in this thesis; electropolymerization of a tyramine on a gold electrode (a) with subsequent glutaraldehyde activation and gold nanoparticle sorption, (b) functionalization with acryloyl chloride before UV polymerization, (c) covalent immobilization of antibody on CNBr activated Sepharose and amide reduction; (d) preparation of MIP stamp by silanization of glass substrate and subsequent covalent binding of MCLR.

4.4 Transductions methods

The transducer is a fundamental component of a biosensor because it translates the physiochemical changes resulting from the biorecognition/analyte interactive events into measurable electronic signal [99, 100]. Biosensors categorization based on transduction type can be electrochemical, optical, thermal, piezoelectric and acoustic

waves [105, 114, 120, 150], and the type of transduction strongly influence the general performance and sensitivity of the different types of sensors. Among other transductions, electrochemical biosensors are the most popular type and have proved superior with regards to sensitivity and low detection limits owing to the combination of their inherent transduction sensitivity and high affinity bio-interactions [124].

Electrochemically transducing biosensors operate by directly converting the biological information to an electronic signal, and usually aim for measurements such as potential, conductance, current or capacitance/impedance. Therefore, it is important for electrochemical biosensors that the transducer material is electrical conductive [100, 101]. Concerning detection of microcystins, there has been over the recent years advances in the development, integration and automation of biosensors using a wide range of transduction modes [17, 23, 114, 151-155], although most of the biosensors targeted one or a few variants. Amperometric and capacitive biosensors will be given a special focus in sections 4.4.1 and 4.4.2, respectively since they were used for the research described in this thesis, aiming for whole spectrum detection using the Adda-specific mAbs and MIPs.

4.4.1 Amperometric biosensors

Amperometric sensors are based on electron transfer reactions (oxidation or reduction of electroactive species) at the electrode surface. They are the preferred transducers for biocatalytic biosensors, whereby specific binding and catalytic activity of enzymes (oxidoreductases) are used to generate electroactive species that can be measured based on the oxidative and reductive current change produced when a constant potential is applied during a biochemical reaction. The current response produced is taken as proportional to the electroactive species present in the solution, which is then correlated to analyte concentration [100, 119]. This mode of operation results in indirect measurement of the analyte, which often is criticized for the reduced sensitivities related to these amperometric biosensors [124].

In addition, amperometric detection for biosensors can be performed in two ways. The first and common way is whereby the working electrode is modified with the biorecognition element and biochemical reactions take place at the surface, to produce redox species [100]. In this case the electrode works as both the transducer and reaction surface [155]. The second way is when the electrode is unmodified and just receives already generated electroactive product from the enzymatic reaction column or surface that is interfaced with the electrochemical flow cell for amperometric measurements [124].

For electrochemical surface characterization, cyclic voltammetry (CV) was used to obtain information about the electrodeposition of polytyramine on a clean gold surface (methanolic tyramine solution) and investigate the degree of insulation of the modified

working electrode using an electroactive solution ($K_3[Fe(CN)_6]$) in this thesis (Fig 4.4a).

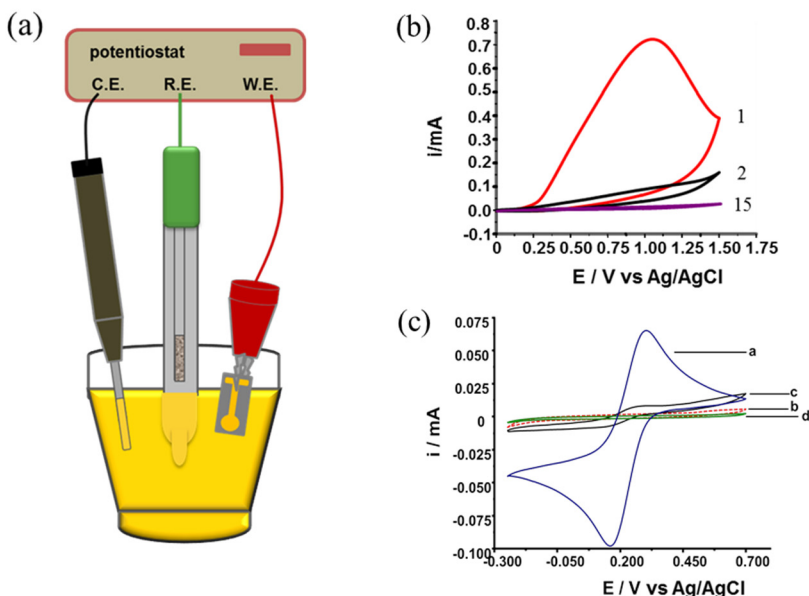


Fig 4.4 (a): Schematic representation of a 3-electrode electrochemical cell used for; (b) Surface coating: cyclic voltammograms for the electropolymerization of 100 mM tyramine in 0.3 M NaOH methanol solution with potential sweep from 0-1.5 V at scan rate 50 mV/s, showing scans 1, 2, and 15 of the 15 scans performed. Modified from **paper I**. (c) Surface characterization: cyclic voltammograms in 50mM $K_3[Fe(CN)_6]$ dissolved in 0.1 M KCl with potential range 0.25-0.70V at 50 mV/s; a. bare gold electrode, b. polytyramine modified gold electrode, c. after gold nanoparticle incorporation and d. with subsequent antibody immobilization. Reproduced from **paper II**.

Electropolymerization discussed in section 4.3.1.3 was performed in a 3-electrode cell as illustrated in Fig 4.4 (a). Irreversible oxidation of tyramine is depicted in Fig 4.4 (b) as indicated by the typical oxidation peak of electropolymerization of tyramine on gold surface. Tyramine oxidation was prominent at 1.0 V during the first circle, rapidly decreased with each scan and completely disappeared on the last scans with very minimal current conduction. The disappearance of the oxidation peak showed that there was hindrance of electron transfer between tyramine solution and the electrode surface when a non-conducting polymer layer (polytyramine) was formed on the electrode surface [126].

Fig 4.4 (c) depicts cyclic voltammograms of the modified gold electrode obtained in 50 mM ($K_3[Fe(CN)_6]$). The redox potential between $[Fe(CN)_6]^{3-/4-}$ and the gold working electrode demonstrates reversible oxidation and reduction of the redox couple shown by high oxidation and reduction peaks on a bare gold electrode (curve a) which means

that the electrode is highly electroactive. Upon coating with polytyramine layer, there was a decrease of the current peak, which suggests blockage of electrons transfer to the electrode surface (curve b). However, AuNPs deposition on top of the polytyramine layer led to a slight increase in peak current (curve c) suggesting electron transmission by AuNPs. Subsequently, the current peak decreased on antibody immobilization.

4.4.1.1 Application of amperometric detection for microcystins

Enzymes (oxidoreductases) are widely used biorecognition elements in amperometric biosensors since they can be easily coupled to other biomolecules. Peroxidases are commonly used in amperometric detection ([156, 157]. Horseradish peroxidase HRP was used in **paper III** to catalyse the oxidation of 2,2'-azinobis (3-ethylbenzothiazolinesulfonic acid) (ABTS).

Amperometric (also voltammetry) measurements utilize a three-electrode cell, comprising of a working electrode (gold or carbon), platinum counter electrode and a reference silver/silver chloride electrode as represented in Fig 4.5; (a) shows a picture of the set-up and (b) is a scheme of reaction and sensorgram for the work in **paper III**. A schematic drawing of the automated sequential injection system used in the whole thesis is shown in Fig 4.10.

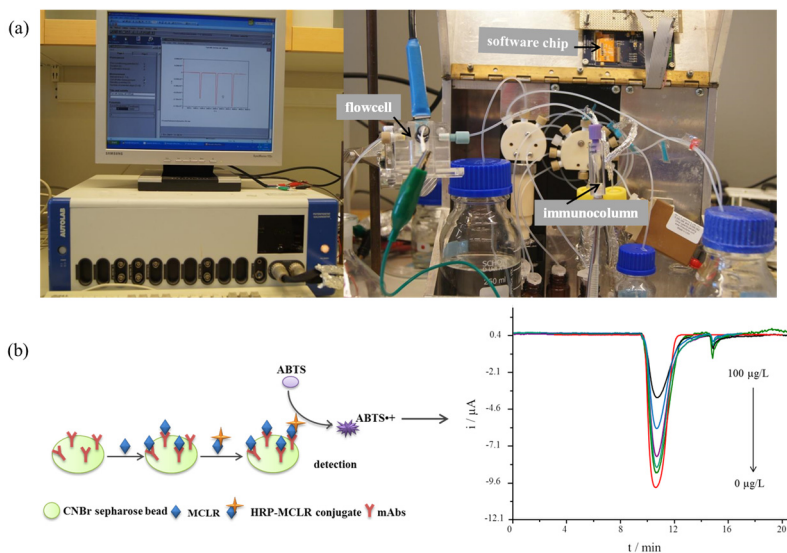


Fig 4.5: (a) A picture of the VersAFlow-amperometric system configuration and (b) schematic representation of a two-step competitive immunoassay and the resulting electrochemical responses.

In **paper III**, a competitive flow-ELISA amperometric biosensor was developed based on redox state of ABTS after oxidation by HRP catalyzed reaction. The assay involved immobilizing the Adda-specific antibodies on a CNBr activated Sepharose and were

reacted with the native and HRP-labelled MCLR. The assay was performed on two physically separated reaction compartments; (1) the biochemical reaction (where immuno-competitive assay and enzyme reaction take place) and (2) the detection cell for evaluation of amperometric measurements at a carbon electrode for the reduction of ABTS^{•+} generated in the reaction chamber. In this way, matrix effects are minimized by the quick washing steps after each reagent injection.

Since the electrode potential is a deciding factor for the selectivity of the measurements, the working potential was kept at 0.40 V, before electrochemical oxidation of ABTS starts at 0.48 V (**paper III**, **Fig 1**). The 0.40 V potential was set to eliminate the possibility of ABTS being electrochemically oxidized. Regeneration of the micro immunocolumn was achieved using a low pH glycine-HCl buffer. In summary, microcystin determination using the developed Flow-ELISA with amperometric biosensor demonstrated high sensitivity of 0.01 µg/L MCLR. The rapid generation of ABTS^{•+} by HRP (less than 2 min within tracer injection in **paper III**) makes the VersaAFlow-amperometric biosensor suitable for use in online situations when the need for immediate response is required.

4.4.2 Capacitive biosensors

Capacitive biosensors have been reported as highly sensitive bioanalytical tools [129, 132, 136, 144, 150, 158-162], which make them very useful when contaminant regulations become stringent. Capacitive biosensing was used for microcystin investigation under different immobilization strategies (**papers I, II and IV**).

Capacitive biosensors pass low conduction current, making other electrochemical biosensors including amperometric and conductimetric devices less sensitive for signal transduction [136]. Moreover, they are label-free and give binding information in real-time [107, 144]. The inherent sensitivity of capacitive biosensing is owed to the ability of the system to measure displacement of counter ions at the electrode-electrolyte solution interface, hence the power to measure trace analyte concentrations. When a working electrode is charged with either potential [129, 132, 160] or current [163], and is immersed in an electrolyte solution, an interface region called electrical double layer (EDL) is created. The EDL is very important in capacitance measurements since it is where charge transfer happens, it therefore influences the electrochemical activities of the system. The EDL theory has been used in capacitive biosensor construction [119, 132, 150, 164].

The capture of the analyte by the immunoelectrode means that solvated ions and water molecules are pushed away from the electrode surface, thereby increasing the thickness of the dielectric layer and a consequent decrease in capacitance [126]. More target analyte molecules binding to the affinity layer will cause more decrease in the relative permittivity of the interface as well as increased distance over which charge is distributed.

Hence greater ion displacement and decrease in capacitance signal [105, 107, 162, 165] as according to the equation (1). Therefore, for successful immobilization of biorecognition ligand dictates that the anchor layer must be both electrically insulating and as thin as possible [126, 132].

$$C = \frac{\epsilon \epsilon_0 A}{d} \quad (1)$$

where ϵ and ϵ_0 is the dielectric constant of the medium and permittivity of free space, respectively. A is the area of the plates and d the distance between them. This equation depicts that electron tunneling exponentially decays with distance, thus an increase in distance away from the electrode surface will result in a capacitance decrease.

$$\frac{1}{C_{\text{tot}}} = \frac{1}{C_{\text{ins}}} + \frac{1}{C_{\text{rec}}} + \frac{1}{C_{\text{Ag}}} + \frac{1}{C_{\text{DL}}} \quad (2)$$

Where C_{tot} represents total capacitance, C_{ins} , C_{bio} and C_{dl} are capacitances contributed by the insulating, biorecognition and diffuse layers respectively. According to equation 2 capacitance contribution by all layers that make the baseline should be high such that the binding of the analyte induces a significant change [159]. The capacitance level before introduction of analyte is considered as a baseline, and upon analyte introduction and binding, a capacitance decrease is obtained. The capacitance change (ΔC) is then related to the analyte concentration [144, 160, 162]. The whole capacitance measuring concept is demonstrated in Fig 4.6 below.

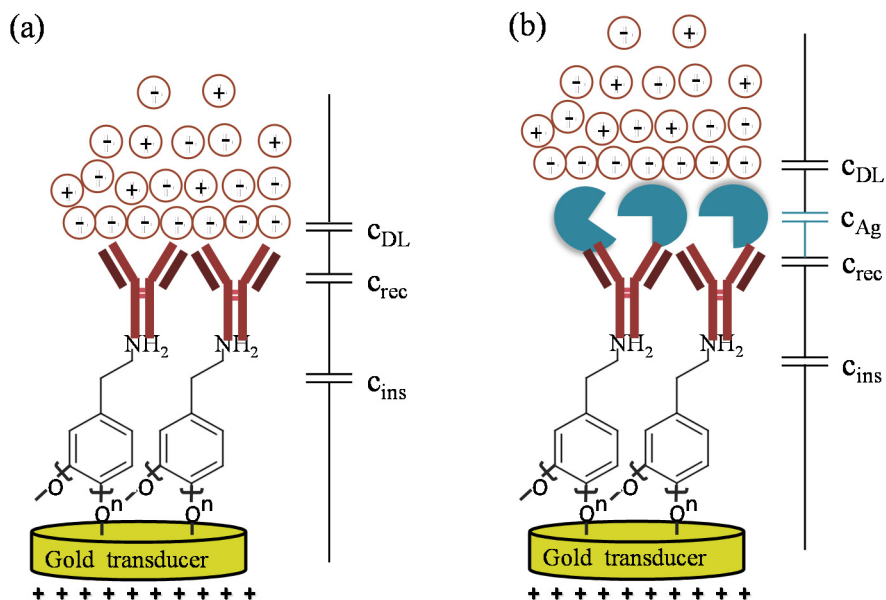


Fig 4.6: Schematic representation of a capacitive surface showing (a) contribution of capacitive by different layers of a modified gold electrode and (b) additional layer introduced upon analyte binding.

Consequently, when constructing a capacitive biosensor, it is important to sufficiently insulate the electrode before immobilization of biorecognition molecule. The defects in the electrode surface are not desirable since it allows contact of the electrolyte solution with the electrode surface, resulting in increased capacitance due to high water polarity [127, 166].

4.4.2.1 Capacitive biosensors; development and application

Deviant from potential perturbation systems [128, 132, 144, 162, 164, 167], but still based on the electric double layer theory, current pulse capacitance measurements were employed in papers I, II and IV. The measurements were performed using a software integrated capacitance system where constant 10 μ A pulses were applied and the resulting potential was measured [163]. The capacitance is automatically calculated according to the slope of the potential curve, which is created across the sensor when a constant current is applied using equation 3

$$C = \frac{It}{U} \quad (3)$$

where C is the total capacitance, I is the current applied to the working electrode, t is the current pulse period and U is the slope of the voltage built across the capacitor of the RC circuit multiplied with time [163, 168].

Capacitance measurements were performed using an automated sequential injection analysis system presented in Fig 4.10. The same immobilization protocol was employed in both **papers I and II** as illustrated in Fig 4.7 inset below.

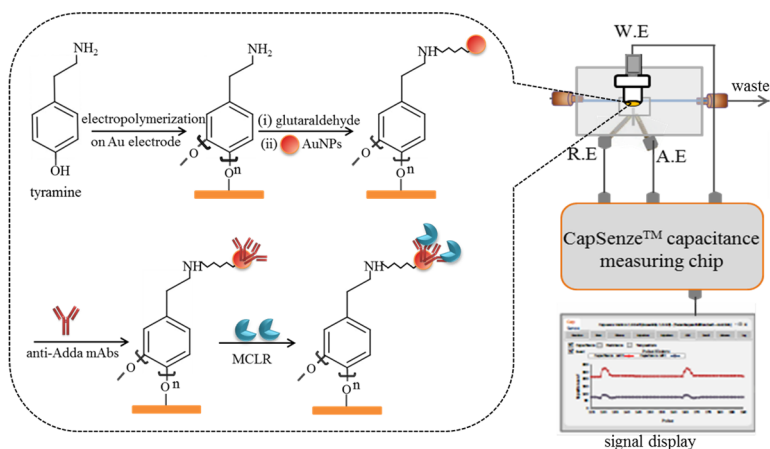


Fig 4.7: An electrochemical-capacitance flow-cell; inset: stepwise preparation of the immunoelectrode (**papers I and II**).

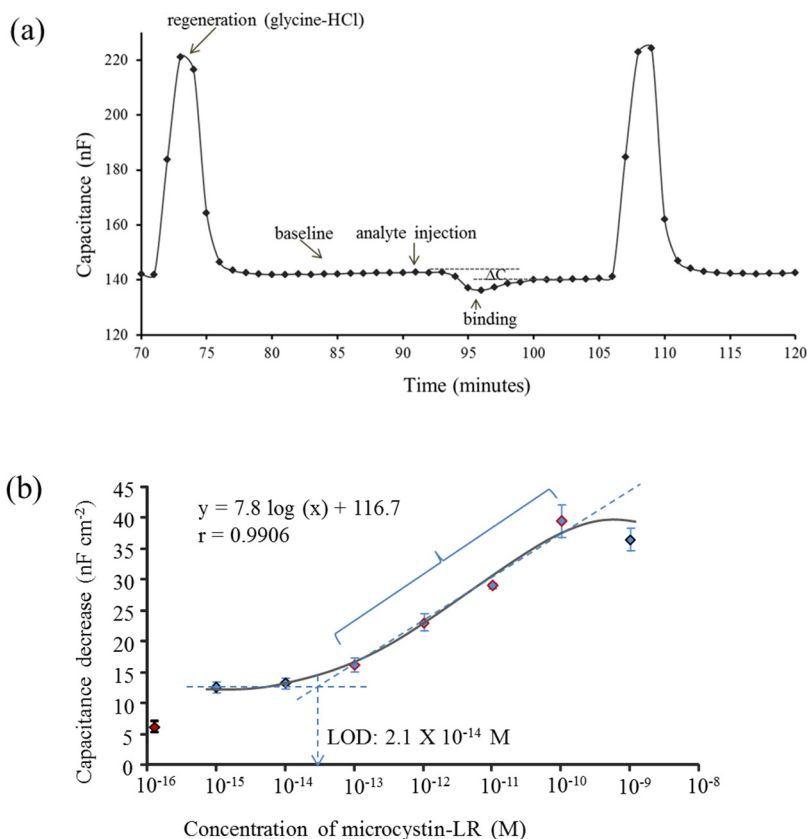


Fig 4.8: (a) Time course diagram showing the decrease in capacitance after standard analyte injection. Capacitance change (ΔC) is calculated from the difference of baseline capacitance and after analyte injection capacitance, 25 mM glycine-HCl, pH 2.5 was used for the regeneration of the surface, and (b) standard curve created from **paper I**.

Fig 4.8 (a) and (b) depict the actual sensorgrams of the time course of assaying and the standard curve from known concentrations of the microcystin standard, respectively. A comparison of the developed capacitive biosensor with the commercial ELISA revealed a good correlation with a regression equation of $y = 0.873x + 0.164$ ($r = 0.992$) (**paper II**, Fig 6). This correlation suggests that with further improvements the capacitive biosensor will provide simple and highly sensitive detection methods for microcystins. However, the biosensor system displayed high disturbance when using urine to test matrix effects (**paper I**, Fig 7). The capacitance system susceptibility to high ionic strength matrix has been reported before [166, 167] and thus simple pre-treatment is required.

Regarding MIPs for microcystins, Chianella group successfully computer modelled, developed and applied MIP particle for MCLR in biosensors [114, 169]. As of yet, no

studies have reported on microcontact technique for these very small toxins for example microcystins together with capacitive biosensor. Therefore, in **paper IV** microcontact imprinting technique was studied as surface modification strategy and used in capacitance biosensor. Rebinding of toxin to the formed cavities, was performed at different concentrations (from 10^{-13} to 10^{-8} M MCLR). Preliminary results indicated the lowest detection of 10^{-12} M MCLR. The signal change was low compared to gold nanoparticle enhanced immunosensor. Non-imprinted surfaces gave much lower capacitance change the MIP surfaces (**paper IV**, Fig 4).

4.4.2.2 *Nanoenvironmental effects on capacitive immunosensors*

The remarkable sensitivity of capacitive immunosensor has been discussed and demonstrated earlier in section 4.4.2 and subsection 4.4.2.1. Capacitive detection is highly sensitive and results in fast and real time antibody-antigen binding kinetics information using simple instrumentation [162, 164]. When pursuing sensitivity as is the goal of the methods developed in this thesis, factors that influence capacitive measurements at nanoenvironmental levels should be reflected. Sensitivity of capacitive measurements is influenced by factors such as the density of antibodies at the electrode surface given that antigen-binding sites are increased in favour of a pronounced response. Binding kinetics according to **equation 1**, **paper V**, suggests that when the concentration of the antibodies at the surface is exceedingly increased the reaction is pulled towards equilibrium and allows more antigen binding resulting in high antibody-antigen complex. The complex formation is the ideal situation as sensitivity of the assay is increased.

Fig 4.9 exhibits that capacitive immunosensor was 1000 times more sensitive than the ELISA using the same antibodies. This is related to the fact that capacitive measurements occur in the close proximity to the electrode surface (transducer) in a very confined space (**paper V**, Fig. 1), which shows that the only available space is estimated around 2000nm, while in the optically measured ELISA the measurement is made through the solution. Therefore the ELISA would give less sensitivity even if the amount of antibodies is the same in both cases.

Increasing antibody density at the electrode surface boosts more antigen binding, which in turn leads to high capacitance response due to increased surface thickness. According to equation 4, **paper V**, the more the thickness is increased, the further the diffuse layer is pushed away, resulting in marked signal. However, **paper V**, Fig. 5, demonstrates that this rule is surpassed when the diffuse layer is pushed away further beyond the appropriate distance. The additional antibody-AuNP layer resulted in rather decreased capacitance response. This is because the diffuse layer has a characteristic thickness that can maintain the electric field force (voltage between the plates).

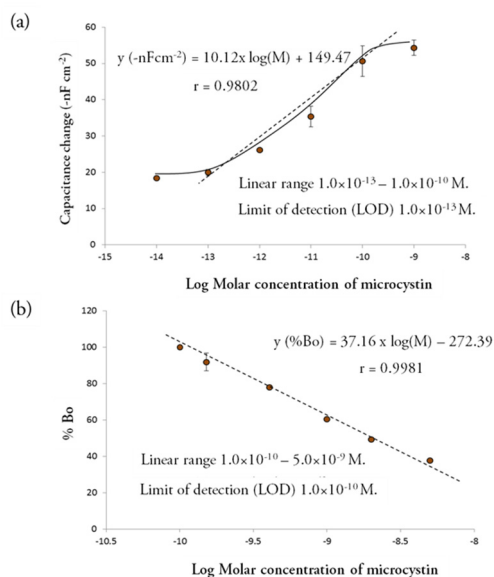


Fig. 4.9: Comparative study of a microcystin immunoassay using the capacitive biosensor and an ELISA technique.

4.4.3 Sequential injection and automation of electrochemical biosensors

The increased interest in electrochemical biosensor devices results from their major potential for automation, miniaturization and hence portability, as well as their suitability for *in-situ* monitoring of environmental toxins [124]. Additionally, automation allows for sequential injection modes, which can reduce sample and reagents volume used [101, 170]. The integration of the flow-injection systems with software-tailored electronics and ultimate automation of the biosensor systems has made a remarkable progress in the design and application of biosensors. One successful example where integrated and automated biosensor system is achieved is the CapSenzeTM capacitive biosystem [163, 171, 172]. Moreover, its user-friendly software simplifies its operations, thereby aiding fast and reliable preliminary screening of contaminants.

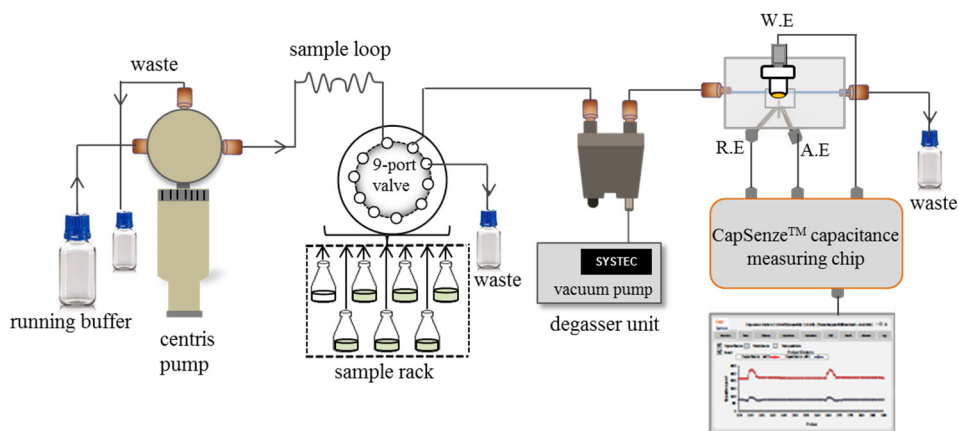


Fig 4.10: Illustration of the component of the Capsenze™ capacitive biosystem.

The biorecognition-based experimental work in this thesis was performed using the sequential-injection automated system illustrated in Fig 4.10. All the components presented are integrated into a box-format unit as previously shown in the subsection 4.4.1.1. Although the integration of biosensors into complete automated devices has been a success, there still remain some challenging issues into their portability, e.g. the battery space that now makes the devices bulky.

5. Challenges in microcystin analysis and biosensor development

- Toxicity evaluations for microcystins is still a challenge due to the large number of variants, lack of consolidated data and regulations for for all variants not just MCLR.
- Microcystin-producing blooms are no longer predictable due to changing climate conditions. Therefore insufficient removal techniques necessitate routing monitoring which could be costly.
- Application of environmental biosensor to real situations is far more limited due to lack of standardized and validated methods for biosensors in general, for example the rule for the background-signal ratio is not consolidated.
- Microcystin analysis is more complicated when dealing with electrochemical biosensors. Complex matrix effects becomes a limiting factor for these biosensors for real situation applications since electroactive molecules may be introduced to the electrode surface and interfere with the measurements.

5.1 Conclusions and future perspectives

Since poor health has been associated with deteriorated water quality, the supply of clean water can significantly improve people lives. Provision of toxin-free water can be achieved by the development of simple and affordable technologies. Most importantly, these technologies should not create other environmental problems such as disposal and fate of spent materials. This can be achieved through the use of sequential-injection automated biosensors, where less reagents are used.

- The application of biosensors in monitoring environmental contaminants, most notably immunosensing has been recognized and the field is expanding. A variety of biosensors for microcystins have been developed from many techniques and viably employed in real samples at laboratory scale.
- Scientific efforts made into developing the principle of biosensors and their requirements toward real applicability have been successfully demonstrated.

The next phase is then to focus on the transition from research scenarios to real application and commercialization by making them customer user-friendly.

- As molecular recognition elements are produced from various biomolecules ranging from cells to oligonucleotides, MIPs and aptamers are quickly gaining popularity to be next new molecular recognition candidates. The use of MIPs and aptamers will circumvent some of the problem encountered from the use of antibodies and will also help in non-discriminatory detection of microcystin variants.
- In the future, nanomaterials will undoubtedly benefit the field of biosensor technology through signal amplification of biological recognition events and promoting electrochemical reactions. Very small immunoreactors will play an important role in the trend of miniaturization of analytical systems, where the whole system is made of very small components and made adaptable for rapid immunoassays without compromising the sensitivity of the assay.

Despite the remarkable progress made on the current biosensor platforms and devices ranging from optical to electrochemical, and a wide range of biomolecules that have been investigated, room for improvement is still anticipated, particularly in the aspects of producing commercial tools.

- Miniaturization and integration into automatic electronic data analysis platforms will produce more sensitive and faster methods which are less expensive. These should be made into small and portable devices that can be implemented into monitoring programs for real-time data collection and processing. Also these should be easy to use by non-specialist (semi-skilled) personnel.
- Studies and biosensors involving the special genes (fingerprint) for microcystin biosynthesis should be intensified and could perhaps solve the multivariant differences, and discriminate between toxic and non-toxic blooms.

The capacitive biosensor developed at Lund University has made progress towards the commercialization of the device. In his PhD thesis conclusion, in 2009 Mahmoud Labib envisioned the evolution of the capacitive system towards sensitive and regular component routine detection but highlighted the need for automation of the system for portability. Five years later his aspirations has been fulfilled. The new version of capacitive device is not only automated for sequential injection but has gone into market under the name CapSense BiosystemTM.

Acknowledgements

The journey that I have embarked on since 2010 was an emotional rollercoaster; from excitement of getting interesting results to long nights of despair when things did not seem to work. Nevertheless, it was a journey worth undertaking. Not only did I become a better researcher, I have also grown in some other aspects of life. The Department of Biotechnology was one big multinational family and I have learnt a lot about different cultures. The journey is now coming to an end and I would like to acknowledge all the great people who became part of this exciting experience.

I would like to express my gratitude to my supervisors:

My sincere gratitude is extended to Professor (Emeritus) Bo Mattiasson. Thank you for giving me the opportunity to study for my PhD at the Department of Biotechnology. You believed that a rusty brain can be polished after my many years away from academics. Thank you for all the guidance and support. Also for keeping the promise. I will be there, behind the podium.

Assoc. Professor Martin Hedström, thank you for being there every step of the way and for all the encouragement. I couldn't have made it without you on the work involving the intimidating Mass Spectrometer.

To the former and present members of the Bioanalysis (Biosensors) group; most importantly the colleague who introduced me to the biosensor concept, Kinga, you had all the patience. Kumar, Mohammad, Alvaro, Ally, Dmitri, Maru, Yanling, Gaurav (kido), the group was small but never short of laughter. You made the study environment exciting and shared great moments. To my co-authors and Thai friends Kosin and Jit, Thank you for all the help and the fun moments we shared in and outside the lab. I have already developed some Thai taste buds.

Special thanks to the environmental group, you were my second group, you took me as one of your own and always invited me to the excursions of your group. That was very kind of you.

To all the past and present members of the Biotechnology department, you are all equally appreciated and you all helped me in one way or another. From the lunch discussions, defense parties to spring excursions, it was all fun. The whole environment was filled with love. To the great staff in the department, I got enlightened from the lectures on all aspects of Biotechnology.

Siv and Emma P., Thank you for helping with all the administration work. Frans Peder, you have all the theories that could turn the world round, thanks for the technical assistance.

Noche de chicas, 'let's go dancing', howelse can you release stress. All I can say...Bolivians know how to party. Thank you for making my stay in Lund so memorable. Gracias Tania por organizarlo.

I would also like to extended my gratitude to the two great mentors I have met in Botswana, Dr Joane E. Taylor and Mrs Nomusa Mhlanga. You always believed in me even when I doubted myself. Jo, I haven't got any bike accident yet ☺.

Special thanks to all the '*sons of the soil*' I've met in and around Lund. Thank you for the cultural gatherings. Ivo, ke a leboga rra. To all Batswana that I have met in Sweden....le ka moso. To my dear friends in Botswana, I appreciate all the calls and chats.

I am indebted to all members of my extended family who supported me and understood that I have to be away. Special Thanks to my mother for raising the person I am today and for passing that on to my daughter. My husband who took care of our home while I was away, you are one of a kind. To my daughter for making life worth living, it is a very nice picture you drew for my thesis☺.

Last but not least, I am especially thankful to the organization that made this work possible, Botswana International University of Science and Technology (BIUST). My appreciation also goes to the Government for the Republic of Botswana for educating its people.

References

1. Antoniou, M., de la Cruz, A., Dionysiou, D., Cyanotoxins: New generation of water contaminants. *J. Environ. Eng.*, 2005. **131**(9): p. 1239-1243.
2. Xagorarakis, I., Kuo, D., Water pollution: Emerging contaminants associated with drinking water. In: *International Encyclopedia of Public Health*, (H.K. Heggenhougen, Ed.), 2008, Academic Press: Oxford. p. 539-550.
3. Sauvé, S., Desrosiers, M., A review of what is an emerging contaminant. *Chemistry Central Journal*, 2014. **8**(15): p. 1-7.
4. Richardson, S.D., Ternes, T.A., Water analysis: emerging contaminants and current issues. *Anal. Chem.*, 2005. **77**: p. 3807-3838.
5. Francis, G., Poisonous Australian Lake. *Nature*, 1878. **18**: p. 12.
6. Carmichael, W.W., Cyanobacteria secondary metabolites-the cyanotoxins. *J. Appl. Bacteriol*, 1992. **72**(6): p. 445-459.
7. Turner, P.C., Gammie, A.J., Hollinrake, K., Codd, G.A., Pneumonia associated with contact with cyanobacteria. Vol. 300. 1990: *British Med. Journal*. 1440-1441.
8. Jochimsen, E.M., Carmichael, W.W., An, J.S., Cardo, D.M., Cookson, S.T., Holmes, C.E., Antunes, M.B., de Melo Filho, D.A., Lyra, T.M., Barreto, V.S., Azevedo, S.M., Jarvis, W.R., Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N. Engl. J. Med.*, 1998. **338**(13): p. 873-878.
9. Carmichael, W.W., Azevedo, S.M., An, J.S., Molica, R.J., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environ Health Perspect.*, 2001. **109**(7): p. 663-668.
10. Azevedo, S.M., Carmichael, W.W., Jochimsen, E.M., Rinehart, K.L., Lau, S., Shaw, G.R., Eaglesham, G.K., Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. *Toxicology*, 2002. **181-182**(0): p. 441-446.
11. Federal Register, Announcement of the drinking water contaminant candidate. 1998. **63**(40): p. 10273-10287.
12. Banicki, J.J., Something in the water: Research finds new methods to extract toxins from drinking Water. *Twineline Spring Edition*, 2006. **28**(2): p. 1-12.
13. World Health Organization, Guidelines for drinking water quality, 2nd ed. Addendum to vol. 2. Health criteria and other supporting information. World Health Organization, Geneva. 1998. **2**: p. 95-110.

14. Weller, M.G., Immunoassays and biosensors for the detection of cyanobacterial toxins in water. *Sensors* 2013. **13**(11): p. 15112.
15. Roegner, A.F., Brena, B., González-Sapienza, G., Puschner, B., Microcystins in potable surface waters: toxic effects and removal strategies. *J. Appl. Toxicol.*, 2014. **34**(5): p. 441-457.
16. Msagati, T.A.M., Siame, B.A., Shushu, D.D., Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. *Aquat. Toxicol.*, 2006. **78**(4): p. 382-397.
17. Campàs, M., Marty, J-L., Highly sensitive amperometric immunosensors for microcystin detection in algae. *Biosens. Bioelectron.*, 2007. **22**(6): p. 1034-1040.
18. International Agency for Research on Cancer, 2006.
19. Grosse, Y., Baan, R., Straif, K., Secretan, B., El Ghissassi, F., Cogliano, V., Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. *Lancet Oncol.*, 2006. **7**(8): p. 628-629.
20. Li, Y., Sheng, J., Sha, J., Han, X., The toxic effects of microcystin-LR on the reproductive system of male rats in vivo and in vitro. *Reprod. Toxicol.*, 2008. **26**(3-4): p. 239-245.
21. Chen, Y., Xu, J., Li, Y., Han, X., Decline of sperm quality and testicular function in male mice during chronic low-dose exposure to microcystin-LR. *Reprod. Toxicol.*, 2011. **31**(4): p. 551-557.
22. Fischer, W.J., Garthwaite, I., Miles, C.O., Ross, K.M., Aggen, J.B., Chamberlin, A.R., Towers, N.R., Dietrich, D.R., Congener-independent immunoassay for microcystins and nodularins. *Environ. Sci. Technol.*, 2001. **35**(24): p. 4849-4856.
23. Lebogang, L., Hedström, M., Mattiasson, B., Development of a real-time capacitive biosensor for cyclic cyanotoxic peptides based on Adda-specific antibodies. *Anal. Chim. Acta*, 2014. **826**: p. 69-76.
24. Dietrich, D., Hoeger, S., Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicol. Appl. Pharmacol.*, 2005. **203**(3): p. 273-289.
25. Stone, D., Bress, W., Addressing public health risks for cyanobacteria in recreational freshwaters: The Oregon and Vermont Framework. *Integrated Environmental Assessment and Management*, 2007. **3**(1): p. 137-143.
26. Hitzfeld, B.C., Höger, S.J., Dietrich, D.R., Cyanobacterial toxins: Removal during drinking water treatment, and human risk assessment. *Environ. Health Perspect.*, 2000. **108**: p. 113-122.
27. Wilson, M.A., Carpenter, S.R., Economic valuation of freshwater ecosystem services in the United States. *Ecol. Appl.*, 1999. **9**(3): p. 1971-1997.
28. Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., Nonpoint pollution of surface waters with phosphorus and nitrogen: Technical report. *Ecol. Appl.*, 1998. **8**(3): p. 559-568.

29. Zhou, L., Yu, H., Chen, K., Relationship between microcystins in drinking water and colorectal cancer. *Biomed. Environ. Sci.*, 2002. **15**(2): p. 166-171.
30. Kim, I.S., Nguyen, G.H., Kim, S., Lee, J., Yu, H-W., Evaluation of methods for cyanobacterial cell lysis and toxin (Microcystin-LR) extraction using chromatographic and mass spectrometric analyses. *Environ. Eng. Res*, 2009. **14**(4): p. 250-254.
31. Siame, B.A., Shushu, D.D., Masundire, H.M., Occurrence of highly toxic *microcystis* species in Botswana. In: W.J. de Koe, R.A. Samson, H.P. van Egmond, J. Gilbert, M. Sabino (Eds.), *Mycotoxins and Phycotoxins in perspective at the turn of the millenium*, Ponsen & Looyen: Wageningen, The Netherlands. 2001: p. 445-451.
32. Johnson, B.N., Mutharasan, R., A Cantilever biosensor-based assay for toxin-producing cyanobacteria *Microcystis aeruginosa* using 16S rRNA. *Environ. Sci. Technol.*, 2013. **47**(21): p. 12333-12341.
33. Sivonen, K., Jones, G., Cyanobacterial toxins. In: *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. World Health Organization (Eds.), E& SPON, London. 1999: p. 41-111.
34. Carmichael, W.W., Health effects of toxin-producing cyanobacteria: "The CyanoHABs". *Human and ecological risk assessment: An International Journal*, 2001. **7**(5): p. 1393-1407.
35. Metcalf, J.S., Bell, S.G., Codd, G.A., Colorimetric immuno-protein phosphatase inhibition assay for specific detection of microcystins and nodularins of cyanobacteria. *Appl. Environ. Microbiol.*, 2001. **67**(2): p. 904-909.
36. Campos, A., Vasconcelos, V., Molecular mechanisms of microcystin toxicity in animal cells. *Int. J. Mol. Sci.*, 2010. **11**(1): p. 268-287.
37. Dittmann, E., Börner, T., Genetic contributions to the risk assessment of microcystin in the environment. *Toxicol. Appl. Pharmacol.*, 2005. **203**(3): p. 192-200.
38. Zeck, A., Weller, M.G., Bursill, D., Niessner, R., Generic microcystin immunoassay based on monoclonal antibodies against Adda. *Analyst*, 2001. **126** p. 2002-2007.
39. An, J., Carmichael, W.W., Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. *Toxicon*, 1994. **32**(12): p. 1495-1507.
40. Dawson, R.M., The toxicology of microcystins. *Toxicon*, 1998. **36**(7): p. 953-962.
41. Vesterkvist, P.S.M., Meriluoto, J.A.O., Interaction between microcystins of different hydrophobicities and lipid monolayers. *Toxicon*, 2003. **41**(3): p. 349-355.
42. Sivonen, K., Kononen, K., Esala, A. L., Niemelä, S. I., Toxicity and isolation of the cyanobacterium *Nodularia spumigena* from the southern Baltic Sea in 1986. *Hydrobiologia*, 1989. **185**(1): p. 3-8.
43. Hiller, S., Krock, B., Cembella, A., Luckas, B., Rapid detection of cyanobacterial toxins in precursor ion mode by liquid chromatography tandem mass spectrometry. *J Mass Spectrom.*, 2007. **42**(9): p. 1238-1250.

44. Jones, G.J., Orr, P.T., Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Res.*, 1994. **28**(4): p. 871-876.
45. Lahti, K., Rapala, J., Färdig, M., Niemelä, M., Sivonen, K., Persistence of cyanobacterial hepatotoxin, microcystin-LR in particulate material and dissolved in lake water. *Water Res.*, 1997. **31**(5): p. 1005-1012.
46. Tsuji, K., Naito, S., Kondo, F., Ishikawa, N., Watanabe, M.F., Suzuki, M., Harada, K-I., Stability of microcystins from cyanobacteria: effect of light on decomposition and isomerization. *Environ. Sci. Technol.*, 1994. **28**(1): p. 173-177.
47. Bourne, D.G., Jones, G.J., Blakeley, R.L., Jones, A., Negri, A.P., Riddles, P., Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin-LR. *Appl. Environ. Microbiol.*, 1996. **62**(11): p. 4086-4094.
48. Newcombe, G., Cook, D., Brooke, S., Ho, L., Slyman, N., Treatment options for microcystin toxins: Similarities and differences between variants. *Environ. Technol. Lett.*, 2003. **24**(3): p. 299-308.
49. McElhiney, J., Lawton, L.A., Detection of the cyanobacterial hepatotoxins microcystins. *Toxicol. Appl. Pharmacol.*, 2005. **203**(3): p. 219-230.
50. Zhao, Y.-Y., Hrudey, S., Li, X-F., Determination of microcystins in water using integrated solid-phase microextraction with microbore high-performance liquid chromatography-electrospray quadruple Time-of-Flight mass spectrometry. *J Chromatogr Sci.*, 2006. **44**(6): p. 359-365.
51. Crush, J.R., Briggs, L.R., Sprosen, J.M., Nichols, S.N., Effect of irrigation with lake water containing microcystins on microcystin content and growth of rye grass, clover, rape and lettuce. *Environ. Toxicol.*, 2008. **23** (2): p. 246-252.
52. Ho, L., Hoefel, D., Saint, C.P., Newcombe, G., Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. *Water Res.*, 2007. **41**(20): p. 4685-4695.
53. Welker, M., Fastner, J., Erhard, M., von Döhren, H., Applications of MALDI-TOF MS analysis in cyanotoxin research. *Environ. Toxicol.*, 2002. **17**: p. 367-374.
54. Tong, P., Tang, S., He, Y., Shao, Y., Zhang, L., Chen, G., Label-free immunosensing of microcystin-LR using a gold electrode modified with gold nanoparticles. *Microchim. Acta*, 2011. **173**(3-4): p. 299-305.
55. de la Cruz, A.A., Antoniou, M.G., Hiskia, A., Pelaez, M., Song, W., O'Shea, K.E., He, X., Dionysiou D.D., Can we effectively degrade microcystins?-Implications on human health. *Anti-Cancer Agents in Med. Chem.*, 2011. **11**: p. 19-37.
56. Srivastava, A., Singh, S., Ahn, C-Y., Oh, H-M., Asthana, R.K., Monitoring approaches for a toxic cyanobacterial bloom. *Environ. Sci. Technol.*, 2013. **47**(16): p. 8999-9013.

57. Bogialli, S., Bruno, M., Curini, R., Di Corcia, A., Laganá, A., Mari, B., Simple assay for analyzing five microcystins and modularin in fish muscle tissue: hot water extraction followed by liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.*, 2005. **53**(17): p. 6586-6592.
58. Lawrence, J.F., Niedzwiedek, B., Menard, C., Lau, B.P., Lewis, D., Kuper-Goodman, T., Carbone S., Holmes C., Comparison of liquid chromatography/mass spectrometry, ELISA, and phosphatase assay for the determination of microcystins in blue-green algae products. *Journal of AOAC int.*, 2001. **84**(4): p. 1035-1044.
59. Nagata, S., Soutome, H., Tsutsumi, T., Hasegawa, A., Sekijima, M., Sugamata, M., Harada, K-I., Sukanuma, M., Ueno, Y., Novel monoclonal antibodies against microcystin and their protective activity for hepatotoxicity. *Nat. Toxins*, 1995. **3**(2): p. 78-86.
60. Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T., Sivonen K., Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc. Natl. Acad. Sci. USA.*, 2004. **101**(2): p. 568-573.
61. Schatz, D., Keren, Y., Vardi, A., Sukenik, A., Carmeli, S., Börner, T., Dittmann, E., Kaplan, A., Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. *Environ. Microbiol.*, 2007. **9**(4): p. 965-970.
62. Zilliges, Y., Kehr, J-C., Meissner, S., Ishida, K., Mikkat, S., Hagemann, M., Kaplan, A., Börner, T., Dittmann, E., The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of microcystis under oxidative stress conditions. *PLoS ONE*, 2011. **6**(3): p. doi:10.1371/journal.pone.0017615.
63. Welker, M., von Döhren, H., Cyanobacterial peptides-nature's own combinatorial biosynthesis. *FEMS Microbiol. Rev.*, 2006. **30**: p. 530-563.
64. Tillett, D., Dittmann, E., Erhard, M., von Döhren, H., Börner, T., Neilan, B.A., Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. *Chemistry & Biology*, 2000. **7**(10): p. 753-764.
65. Nishizawa, T., Ueda, A., Asayama, M., Fujii, E., Harada, K-I., Ochi, K., Shirai, M., Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. *J. Biochem.*, 2000. **127**: p. 779-789.
66. Christiansen, G., Fastner, J., Erhard, M., Börner, T., Dittmann, E. and Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. *J. Bacteriol.*, 2003. **185**(2): p. 654-572.
67. Rouhiainen, L., Vakkilainen, T., Siemer, B.L., Buikema, W., Haselkorn, R., Sivonen, K., Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. *Appl. Environ. Microbiol.*, 2004. **70**(2): p. 686-692
68. Moffitt, M.C., Neilan, B.A., Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. *Appl. Environ. Microbiol.*, 2004. **70**(11): p. 6353-6362.

69. Metcalf, J.S., Beattie, K.A., Pflugmacher, S., Codd, G.A., Immuno-cross reactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR. *FEMS Microbiol. Lett.*, 2000. **189**(2): p. 155-158.
70. Edwards, C., Graham, D., Fowler, N., Lawton, L.A., Biodegradation of microcystins and nodularin in freshwaters. *Chemosphere*, 2008. **73**(8): p. 1315-1321.
71. Chow, C.W.K., Drikas, M., House, J., Burch, M.D., Velzeboer, R.M.A., The impact of conventional water treatment processes on cells of the cyanobacterium *Microcystis aeruginosa*. *Water Res.*, 1999. **33**(15): p. 3253-3262.
72. Pendleton, P., Schumann, R., Wong, S.H., Microcystin-LR adsorption by activated carbon. *J Colloid Interface Sci.*, 2001. **240**(1): p. 1-8.
73. Hrudey, S., Burch, M., Drikas, M., Gregory, R., Remedial measures. In: Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. World Health Organization (Eds.), E&SPON, London. 1999.
74. Saito, T., Okano, K., Park, H.D., Itayama, T., Inamori, Y., Neilan, B.A., Burns, B.P., Sugiura, N., Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes. *FEMS Microbiol Lett.*, 2003. **229**(2): p. 271-276.
75. Imanishi, S., Kato, H., Mizuno, M., Tsuji, K., Harada, K-I., Bacterial degradation of microcystins and nodularin. *Chem. Res. Toxicol.*, 2005. **18**(3): p. 59-598.
76. Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M.F., Suzuki, S., Nakazawa, H., Suzuki, M., Uchida, H., Harada, K-I., Stability of microcystins from cyanobacteria-II. Effect of UV light on decomposition and isomerization. *Toxicon*, 1995. **33**(12): p. 1619-1631.
77. Welker, M., Steinberg, C., Indirect photolysis of cyanotoxins: one possible mechanism for their low persistence. *Water Res.*, 1999. **33**(5): p. 1159-1164.
78. Lawton, L.A., Robertson, P.K.J., Cornish, B.J.P.A., Marr, I.L., Jaspars, M., Processes influencing surface interaction and photocatalytic destruction of microcystins on titanium dioxide photocatalysts. *J. Catal.*, 2003. **213**(1): p. 109-113.
79. Silva-Stenico, M.E., Cantúcio Neto, R., Alves, I.R., Moraes, L.A.B., Shishido, T.K., Fiore, M.F., Hepatotoxin microcystin-LR extraction optimization. *J. Brazilian Chem. Soc.*, 2009. **20**: p. 535-542.
80. Lee, T-H., Chen, Y-M., Chou, H-N., Toxicity assay of cyanobacterial strains using *Artemia salina* in comparison with the mouse bioassay. *Acta Zoologica Taiwanica*, 1999. **10**(1).
81. Sheng, J-W., He, M., Shi, H-C., Qian, Y., A comprehensive immunoassay for the detection of microcystins in waters based on polyclonal antibodies. *Anal. Chim. Acta*, 2006. **572**(2): p. 309-315.
82. Gehringer, M.M., Kewada, V., Coates, N., Downing, T.G., The use of *Lepidium sativum* in a plant bioassay system for the detection of microcystin-LR. *Toxicon*, 2003. **41**(7): p. 871-876.

83. Dao, T-S., Thai-Hang Le, T-H., Pham, T-L., Do-Hong, L-C., Nguyen, P-D., Influences of cyanobacterial toxins microcystins on the seedling of plants. *Journal of Environmental Protection*, 2014. 5: p. 35-41.
84. Sangolkar, L.N., Maske, S.S., Chakrabarti, T., Methods for determining microcystins (peptide hepatotoxins) and microcystin-producing cyanobacteria. *Water Res.*, 2006. 40(19): p. 3485-3496.
85. Vezie, C., Benoufella, F., Sivonen, K., Bertru, G., Laplanche, A., Detection of toxicity of cyanobacterial strains using *Artemia salina* and MicrotoxR assays compared with mouse bioassay results. *Phycologia*, 1996. 35(6S): p. 198-202.
86. Nagata, S., Tsutsumi, T., Hasegawa, A., Yoshida, F., Ueno, Y., Watanabe, M.F., Enzyme immunoassay for direct determination of microcystins in environmental water. *Journal of AOAC Int.*, 1997: p. 408-417
87. Pyo, D., Choi, E., Cha, G.S., Lee, J., Jung, S., Kim, M.S., Production and characterization of monoclonal antibodies against microcystin-LR. *Bull. Korean Chem. Soc.*, 2003. 24(1): p. 126-128.
88. Brooks, W.P., Codd, G. A., Immunoassay of hepatotoxic cultures and water blooms of cyanobacteria using *Microcystis aeruginosa* peptide toxin polyclonal antibodies. *Environ. Technol. Lett.*, 1988. 9: p. 1343-1348.
89. Chu, F.S., Huang, X., Wei R.D., Carmichael, W.W., Production and characterization of antibodies against microcystins. *Appl. Environ. Microbiol.*, 1989. 55: p. 1928-1933.
90. Kfir, R., Johannsen, E., Botes, D.P., Monoclonal antibody specific for cyanoginosin-LA: Preparation and characterization. *Toxicon*, 1986. 24(6): p. 543-552.
91. Weller, M.G., Zeck, A., Eikenberg, A., Nagata, S., Ueno, Y. Niessner, R., Development of a direct competitive microcystin immunoassay of broad specificity. *Anal. Sci.*, 2001. 17(12): p. 1445-1448.
92. Gambaro, A., Barbaro, E., Zangrando, R., Barbante, C., Simultaneous quantification of microcystins and nodularin in aerosol samples using high-performance liquid chromatography/negative electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Sp.*, 2012. 26(12): p. 1497-1506.
93. Lawton, L.A., Edwards, C., Codd, G. A., Extraction and high performance liquid chromatographic method for the determination of microcystins in raw and treated waters. . *Analyst* 1994. 119: p. 1525-1530.
94. Allis, O., Dauphard, J., Hamilton, B., Ni Shuilleabhain, A., Lehan, M., James, K.J., Furey, A., Liquid Chromatography-tandem mass spectrometry application, for the determination of extracellular hepatotoxins in Irish Lake and drinking waters. *Anal. Chem.*, 2007. 79(9): p. 3436-3447.
95. Wang, J., Pang, X., Ge, F., Ma, Z., An ultra-performance liquid chromatography-tandem mass spectrometry method for determination of microcystins occurrence in surface water in Zhejiang Province, China. *Toxicon*, 2007. 49(8): p. 1120-1128.

96. Lebogang, L., Mattiasson, B., Hedström, M., Capacitive sensing of microcystin variants of *Microcystis aeruginosa* using a gold immunoelectrode modified with antibodies, gold nanoparticles and polytyramine. *Microchim Acta*, 2014. **181**(9-10): p. 1009-1017.
97. Yuan, M., Namikoshi, M., Otsuki, A. Sivonen, K., Effect of amino acid side-chain on fragmentation of cyclic peptide ions: differences of electrospray ionization/collision-induced decomposition mass spectra of toxic heptapeptide microcystins containing ADMAdda instead of Adda. *Eur. Mass Spectrom.*, 1998. **4**: p. 287-298.
98. Mayumi, T., Kato, H., Imanishi, S., Kawasaki, Y., Hasegawa, M., Harada, K-I., Structural Characterization of Microcystins by LC/MS/MS under Ion Trap Conditions. *J. Antibiot.*, 2006. **59**(11): p. 710-719.
99. Scheller, F., Schubert, F., Biosensors. Elsevier Science Publishing Company Inc. New York, USA., 1992.
100. Grieshaber, D., Mackenzie, R., Vörös, J., Reimhul, T.E., Electrochemical biosensors-sensor principles and architectures. *Sensors* 2008. **8**(3): p. 1400-1458.
101. Byrne, B., Stack, E., Gilmartin, N., O'Kennedy, R., Antibody-based Sensors: principles, problems and potential for detection of pathogens and associated toxins. *Sensors*, 2009. **9**(6): p. 4407-4445.
102. Thévenot, D.R., Toth, K., Durst, R.A., Wilson, G.S., Electrochemical biosensors: recommended definitions and classification. *Pure Appl. Chem.*, 1999. **71**: p. 2333-2348.
103. Clark, I.C., Lyons, C., Electrode systems for continuous monitoring in cardiovascular surgery. *Annals of the New York Academy of Sciences.*, 1962. **102** (1): p. 29.
104. Turner, A.P.F., Biosensors: sense and sensibility. *Chem. Soc. Rev.*, 2013. **42**: p. 3184-3196.
105. Berney, H., Alderman, J., Lane, W., Collins, J.K., A differential capacitive biosensor using polyethylene glycol to overlay the biolayer. *Sens. Actuators B Chem.*, 1997. **44**(1-3): p. 578-584.
106. Cheng, Z., Wang, E., Yang, X., Capacitive detection of glucose using molecularly imprinted polymers. *Biosens. Bioelectron.*, 2001. **16**(3): p. 179-185.
107. Mattiasson, B., Teeparuksapun, K., Hedström, M., Immunochemical binding assays for detection and quantification of trace impurities in biotechnological production. *Trends Biotechnol.*, 2010. **28**(1): p. 20-27.
108. Vashist, S.K., Dixit, C.K., MacCraith, B.D., O'Kennedy, R., Effects of antibody immobilization strategies on the analytical performance of a surface plasmon resonance-based immunoassay. *Analyst*, 2011. **136**: p. 4431-4436.
109. Petkova, G.A., Záruba, K., Žvátora, P., Král, V., Gold and silver nanoparticles for biomolecule immobilization and enzymatic catalysis. *Nanoscale Research Letters*, 2012. **7**(1): p. 1-10.
110. Haupt, K., Mosbach, K., Molecularly imprinted polymers and their use in biomimetic sensors. *Chem. Rev.*, 2000. **100**(7): p. 2495-2504.

111. Pichon, V., Chapuis-Hugon, F., Role of molecularly imprinted polymers for selective determination of environmental pollutants-A review. *Anal. Chim. Acta*, 2008. **622**(1-2): p. 48-61.
112. Sener, G., Ozgur, E., Rad, A.Y., Uzun, L., Say, R., Denizli, A., Rapid real-time detection of procalcitonin using a microcontact imprinted surface plasmon resonance biosensor. *Analyst*, 2013. **138**(21): p. 6422-6428.
113. Chou, T-C., Rick, J., Weng, Y-C., Nanocavity protein biosensor-fabricated by molecular imprinting. *Proceedings of the 7th IEEE International Conference on Nanotechnology*, 2007: p. 19-20.
114. Chianella, I., Piletsky, S.A., Tothill, I.E., Chen, B., Turner, A.P.F., MIP-based solid phase extraction cartridges combined with MIP-based sensors for the detection of microcystin-LR. *Biosens. Bioelectron.*, 2003. **18**(2-3): p. 119-127.
115. Fuchiwaki, Y., Kubo, I., Electrochemical sensor based on biomimetic recognition utilizing molecularly imprinted polymer receptor. *Biomimetics Learning from Nature*. (Ed: A. Mukherjee), 2010. Pp.385-398.
116. Wang, Y., Zhang, Z., Jain, V., Yi, J., Mueller, S., Sokolov, J., Liu, Z., Levon, K., Rigas, B., Rafailovich, M.H., Potentiometric sensors based on surface molecular imprinting: Detection of cancer biomarkers and viruses. *Sens. Actuators B*, 2010. **146**(1): p. 381-387.
117. Ertürk, G., Berillo, D., Hedström, M., Mattiasson, B., Microcontact-BSA imprinted capacitive biosensor for real-time, sensitive and selective detection of BSA. *Biotechnology Reports*, 2014. **3**: p. 65-72.
118. Udomsap, D., Branger, C., Culioli, G., Dollet, P., Brisset, H., A versatile electrochemical sensing receptor based on a molecularly imprinted polymer. *Chem. Commun.*, 2014. **50**(56): p. 7488-7491.
119. Gebala, M., Schuhmann, W., Understanding properties of electrified interfaces as a prerequisite for label-free DNA hybridization detection. *Phys. Chem. Chem. Phys.*, 2012. **14**(43): p. 14933-14942.
120. Cosnier, S., Biosensors based on electropolymerized films: New trends. *Anal. Bioanal. Chem.*, 2003. **377**(3): p. 507-520.
121. Varatharajan, S., Berchmans, S., Yegnaraman, V., Tailoring self-assembled monolayers at the electrochemical interface. *Journal of Chemical Sciences*, 2009. **121**(5): p. 665-674.
122. Gooding, J.J., Hibbert, D.B., The application of alkanethiol self-assembled monolayers to enzyme electrodes. *TrAC, Trends Anal. Chem.*, 1999. **18**(8): p. 525-533.
123. Limbut, W., Kanatharana, P., Mattiasson, B., Asawatreratanakul, P., Thavarungkul, P., A comparative study of capacitive immunosensors based on self-assembled monolayers formed from thiourea, thioctic acid, and 3-mercaptopropionic acid. *Biosens. Bioelectron.*, 2006. **22**(2): p. 233-240.

124. Campas, M., Garibo, D., Prieto-Simon, B., Novel nanobiotechnological concepts in electrochemical biosensors for the analysis of toxins. *Analyst*, 2012. **137**(5): p. 1055-1067.
125. Chaki, N.K., Vijayamohanan, K., Self-assembled monolayers as a tunable platform for biosensor applications. *Biosens. Bioelectron.*, 2002. **17**(1-2): p. 1-12.
126. Wu, Z-S., Li, J-S., Deng, T., Luo, M-H., Shen, G-L., Yu, R-Q., A sensitive immunoassay based on electropolymerized films by capacitance measurements for direct detection of immunospecies. *Anal. Biochem.*, 2005. **337**(2): p. 308-315.
127. Losic, D., Shapter, J.G., Gooding, J.J., Integrating polymers with alkanethiol self-assembled monolayers (SAMs): blocking SAM defects with electrochemical polymerisation of tyramine. *Electrochem. Commun.*, 2002. **4**(12): p. 953-958.
128. Jantra, J., Kanatharana, P., Asawatreratanakul, P., Wongkittisuksa, B., Limsakul, C., Thavarungkul, P., Detection of staphylococcal enterotoxin A (SEA) at picogram level by a capacitive immunosensor. *J. Environ. Sci. Health, Part A*, 2011. **46**(6): p. 560-568.
129. Teeparuksapun, K., Hedström, M., Wong, E.Y., Tang, S., Hewlett, I.K., Mattiasson, B., Ultrasensitive detection of HIV-1 p24 antigen using nanofunctionalized surfaces in a capacitive immunosensor. *Anal. Chem.*, 2010. **82**(20): p. 8406-8411.
130. Mattiasson, B., Danielsson, B., Flow injection analysis in combination with biosensors. In: *Handbook of chemical and biological sensors* (Eds: Taylor R.F. and Schultz J.S.). Elsevier Science Publishers B.V. New York, USA, 1996: p. 533-552.
131. Beyer N.H., H.M.Z., Schou C., Höjrup P., Heegaard N.H.H., Optimization of antibody immobilization for on-line or off-line immunoaffinity chromatography. *J. Sep.Sci.*, 2009. **32**(10): p. 1592-1604.
132. Berggren, C., Johansson, G., Capacitance measurements of antibody-antigen interactions in a flow system. *Anal. Chem.*, 1997. **69**(18): p. 3651-3657.
133. Ramanavičius, A., Ramanavičienė, A., Malinauskas, A., Electrochemical sensors based on conducting polymer-polypyrrole. *Electrochim. Acta*, 2006. **51**(27): p. 6025-6037.
134. Panasyuk, T.L., Mirsky, V.M., Piletsky, S.A., Wolfbeis, O.S., Electropolymerized molecularly imprinted polymers as receptor layers in capacitive chemical sensors. *Anal. Chem.*, 1999. **71**(20): p. 4609-4613.
135. Tierney, M.J., Practical examples of polymer-based chemical sensors. In: *handbook of chemical and biological sensors* (Eds: R.F. Taylor and J.S. Schultz). Elsevier Science Publishers B.V. New York, USA, 1996: p. 349-370.
136. Wang, J., Profitt, J.A., Pugia, M.J., Suni, I.I., Au nanoparticle conjugation for impedance and capacitive signal amplification in biosensors. *Anal. Chem.*, 2006. **78**(6): p. 1769-1773.
137. Zhao, H., Tian, J., Quan, X., A graphene and multienzyme functionalized carbon nanosphere-based electrochemical immunosensor for microcystin-LR detection. *Colloids Surf., B*, 2013. **103**(0): p. 38-44.

138. Collier, C.P., Simpson, M.L., Micro/nanofabricated environments for synthetic biology. *Curr. Opin. Biotechnol.*, 2011. **22**(4): p. 516-526.
139. Pingarrón, J.M., Yáñez-Sedeño, P., González-Cortés, A., Gold nanoparticle-based electrochemical biosensors. *Electrochim. Acta*, 2008. **53**(19): p. 5848-5866.
140. Saha, K., Agasti, S.S., Kim, C., Li, X., Rotello, V.M., Gold nanoparticles in Chemical and Biological Sensing. *Chem. Rev.*, 2012. **112**(5): p. 2739-2779.
141. Nel, A.E., Madler, L., Velegol, D., Xia, T., Hoek, E.M.V., Somasundaran, P., Klaessig, F., Castranova, V., Thompson, M., Understanding biophysicochemical interactions at the nano-bio interface. *Nat. Mater.*, 2009. **8**(7): p. 543-557.
142. Zhang, J., Lei, J., Xu, C., Ding, L., Ju, H., Carbon nanohorn sensitized electrochemical immunosensor for rapid detection of Microcystin-LR. *Anal. Chem.*, 2010. **82**(3): p. 1117-1122.
143. Chen, K., Liu, M., Zhao, G., Shi, H., Fan, L., Zhao, S., Fabrication of a novel and simple Microcystin-LR photoelectrochemical sensor with high sensitivity and selectivity. *Environ. Sci. Technol.*, 2012. **46**(21): p. 11955-11961.
144. Loyprasert, S., Thavarungkul, P., Asawatreratanakul, P., Wongkittisuksa, B., Limsakul, C., Kanatharana, P., Label-free capacitive immunosensor for microcystin-LR using self-assembled thiourea monolayer incorporated with Ag nanoparticles on gold electrode. *Biosens. Bioelectron.*, 2008. **24**(1): p. 78-86.
145. Ma, W., Chen, W., Qiao, R., Liu, C., Yang, C., Li, Z., Xu, D., Peng, C., Jin, Z., Xu, C., Zhu, S., Wang, L., Rapid and sensitive detection of microcystin by immunosensor based on nuclear magnetic resonance. *Biosens. Bioelectron.*, 2009. **25**(1): p. 240-243.
146. Yi, D.K., Selvan, S.T., Lee, S.S., Papaefthymiou, G.C., Kundaliya, D., Ying, J.Y., Silica-Coated Nanocomposites of magnetic nanoparticles and quantum dots. *J. Am. Chem. Soc.*, 2005. **127**(14): p. 4990-4991.
147. Zhao, L., Jiang, D., Cai, Y., Ji, X., Xie, R., Yang, W., Tuning the size of gold nanoparticles in the citrate reduction by chloride ions. *Nanoscale*, 2012. **4**(16): p. 5071-5076.
148. Thomas, J.B., Peppas, N.A., Sato, M., Webster, T.J., Nanotechnology and Biomaterials. In: *Nanomaterials Handbook* (Ed: Gogotsi Y.). Taylor and Francis CRC Press, 2006: p. 605-630.
149. Yasui, T., Kaji, N., Baba, Y., Nanobiodevices for biomolecule analysis and imaging. *Annu. Rev. Anal. Chem.*, 2013. **6**: p. 96.
150. Jiang, D., Tang, J., Liu, B., Yang, P., Shen, X., Kong, J., Covalently coupling the antibody on an amine-self-assembled gold surface to probe hyaluronan-binding protein with capacitance measurement. *Biosens. Bioelectron.*, 2003. **18**(9): p. 1183-1191.
151. Long, F., He, M., Zhu, A., Song, B., Sheng, J., Shi, H., Compact quantitative optic fiber-based immunoarray biosensor for rapid detection of small analytes. *Biosens. Bioelectron.*, 2010. **26**(1): p. 16-22.

152. Long, F., He, M., Zhu, A., Shi, H., Portable optical immunosensor for highly sensitive detection of microcystin-LR in water samples. *Biosens. Bioelectron.*, 2009. **24**(8): p. 2346-2351.
153. Herranz, S., Marazuela, M.D., Moreno-Bondi, M.C., Automated portable array biosensor for multisample microcystin analysis in freshwater samples. *Biosens. Bioelectron.*, 2012. **33**(1): p. 50-55.
154. Herranz, S., Bocková, M., Marazuela, M.D., Homola, J., Moreno-Bondi, M.C., An SPR biosensor for the detection of microcystins in drinking water. *Anal. Bioanal. Chem.*, 2010. **398**(6): p. 2625-2634.
155. Lotierzo M., A.R., Davis F., Tothill I.E., A membrane-based ELISA assay and electrochemical immunosensor for microcystin-LR in water samples. *Environ. Sci. Technol.*, 2012. **46**: p. 5504-5510.
156. Zór, K., Castellarnau, M., Pascual, D., Pich, S., Plasencia, C., Bardsley, R., Nistor, M., Design, development and application of a bioelectrochemical detection system for meat tenderness prediction. *Biosens. Bioelectron.*, 2011. **26**(11): p. 4283-4288.
157. Castillo, J., Gáspár, S., Sakharov, I., Csöregi, E., Bienzyme biosensors for glucose, ethanol and putrescine built on oxidase and sweet potato peroxidase. *Biosens. Bioelectron.*, 2003. **18**(5-6): p. 705-714.
158. Mirsky, V.M., Riepl, M., Wolfbeis, O.S., Capacitive monitoring of protein immobilization and antigen-antibody reactions on monomolecular alkylthiol films on gold electrodes. *Biosens. Bioelectron.*, 1997. **12**(9-10): p. 977-989.
159. Gebbert, A., Alvarez-Icaza, M., Stoecklein, W., Schmid, R.D., Real-time monitoring of immunochemical interactions with a tantalum capacitance flow-through cell. *Anal. Chem.*, 1992. **64**(9): p. 997-1003.
160. Bontidean, I., Berggren, C., Johansson, G., Csöregi, E., Mattiasson, B., Lloyd, J.R., Jakeman, K.J., Brown, N.L., Detection of heavy metal ions at femtomolar levels using protein-based biosensors. *Anal. Chem.*, 1998. **70**(19): p. 4162-4169.
161. Li, J., Wu, Z., Wang, H., Shen, G., Yu, R., A reusable capacitive immunosensor with a novel immobilization procedure based on 1,6-hexanedithiol and nano-Au self-assembled layers. *Sens. Actuators, B*, 2005. **110**(2): p. 327-334.
162. Hedström, M., Galaev, I.Y., Mattiasson, B., Continuous measurements of a binding reaction using a capacitive biosensor. *Biosens. Bioelectron.*, 2005. **21**(1): p. 41-48.
163. Erlandsson, D., Teeparuksapun, K., Mattiasson, B., Hedström, M., Automated flow-injection immunosensor based on current pulse capacitive measurements. *Sens. Actuators, B* 2014. **190**: p. 295-304.
164. Labib, M., Hedström, M., Amin, M., Mattiasson, B., A novel competitive capacitive glucose biosensor based on concanavalin A-labeled nanogold colloids assembled on a polytyramine-modified gold electrode. *Anal. Chim. Acta*, 2010. **659**(1-2): p. 194-200.

165. Laczka, O., Baldrich, E., Munoz, F.X., del, Campo F.J., Detection of *Escherichia coli* and *Salmonella typhimurium* using interdigitated microelectrode capacitive immunosensors: The importance of transducer geometry. *Anal.Chem.*, 2008. **80**(19): p. 7239-7247.
166. Berggren, C., Bjarnason, B., Johansson, G., An immunological Interleukine-6 capacitive biosensor using perturbation with a potentiostatic step. *Biosens. Bioelectron.*, 1998. **13**(10): p. 1061-1068.
167. Zór, K., Ortiz, R., Saatci, E., Bardsley, R., Parr, T., Csöregi, E., Nistor, M., Label free capacitive immunosensor for detecting calpastatin-A meat tenderness biomarker. *Bioelectrochemistry*, 2009. **76**(1-2): p. 93-99.
168. Snook, G.A., Urban, A.J., Lanyon, M.R., McGregor, K., Current pulse method for in situ measurement of electrochemical capacitance. *J. Electroanal. Chem.*, 2008. **622**(2): p. 225-232.
169. Chianella, I., Lotierzo, M., Piletsky, S.A., Tothill, I.E., Chen, B., Turner, A.P.F., Rational design of a polymer specific for microcystin-LR using a computational approach. *Anal. Chem.*, 2002. **74**(6): p. 1288-1293.
170. Hartwell, S., Grudpan, K., Flow based immuno/bioassay and trends in micro-immuno/biosensors. *Microchim Acta*, 2010. **169**(3-4): p. 201-220.
171. Kumar, M.A., Mazlomi, M.A., Hedström, M., Mattiasson, B., Versatile automated continuous flow system (VersAFlo) for bioanalysis and bioprocess control. *Sens. Actuators, B*, 2012. **161**(1): p. 855-861.
172. <http://www.capsenze.se>.